ENZYMES IN FARM ANIMAL NUTRITION
ENZYMES IN FARM ANIMAL NUTRITION

Edited by

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and

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The rearing and feeding of domestic animals and the use of enzymes in processes such as brewing have been distinct parts of human life for many thousands of years, but it is only recently that these two disciplines have crossed paths. The first commercial use of feed enzymes dates back to 1984 in Finland, where opportunities existed to improve significantly the nutritional quality of barley-based rations by inclusion of enzymes derived from the brewing industry (A. Haarisilta, Suomen Rehu). The years since then have seen an exponential increase in the usage of many enzyme types in rations for poultry and, to a lesser extent, swine. Significant recent interest has also been shown by the ruminant sector. Scientific studies describing the use of exogenous enzymes in animal nutrition dates back to the mid 1920s and they now number in excess of 1300 papers for broilers alone (Rosen, 2000, personal communication). This rapidly expanding field is becoming increasingly multi-disciplinary as more is understood of the mode of action of feed enzymes. Due to the complexity of this field it is timely to produce a single source from where the uninitiated and well versed alike can ground themselves with the basics required to grasp the subject. Furthermore, it is the intention of this book to provide sufficient detail to enable an understanding of the complexities of response to such products in all classes of farm animal livestock.

It is of interest that not only the usage of enzymes has increased but also the scope of their use. This is likely to continue as the price of enzyme application falls, with improvements in both enzyme efficacy and production costs making previously uneconomic solutions more attractive. Particular attention is drawn to the ruminant sector, where usage is in its infancy but research is demonstrating that significant gains can be made. The challenge is to find methods of predicting enzyme response so that enzyme application in all classes of livestock becomes increasingly a science rather than an art.
In memoriam

It is with great sadness that this book is produced after the death of Dr E.T. Kornegay, a significant contributor to this publication and to the field of phytase research in poultry and pigs. Dr Kornegay was particularly active and enthusiastic in his enzyme phytase research and will be sadly missed.
The Current Feed Enzyme Market and Likely Trends

C. SHEPPY

Finnfeeds, PO Box 777, Marlborough, Wiltshire, SN8 1XN, UK

Global Animal Feed Production

As with any publication dealing with the application of a particular technology to a certain industry, it is important to assess the commercial environment in which the technology is being applied.

Despite the general euphoria and enthusiasm surrounding the dawn of a new millennium, the challenges faced by the global animal feed industry have a familiar feel. Human health, environmental safety and animal welfare are all issues that continue to be at the fore of consumers’ minds and are at the top of the agenda of the media and politicians alike. Although tending to emerge in Europe and North America these issues are now of worldwide importance, a development that mirrors the increasing globalization of the food chain and feed industry. Recent controversies surrounding genetically modified crops, antibiotic growth promoters and dioxin-contaminated feedstuffs provide some very real examples of this. A direct result of these ‘health scares’ has been the adoption, and in some cases the imposition, of increased regulatory, testing and quality control procedures aimed at maintaining and improving the trust between animal producers and the ultimate consumers of their products.

Faced with these challenges, how is the industry currently faring? Although global compound feed production actually fell by 5% in 1998 to 575 Mt (the first such fall in over 50 years), 1999 experienced something of a recovery, albeit small, to 586 Mt (Table 1.1).

The Asian economic crisis of 1997–1998 saw the collapse of the feed industry in many previously booming markets and with the benefit of hindsight it is probably fair to say that the previous rates of growth were unsustainable. As demand for meat, milk and eggs tumbled, so did feed production: Indonesian feed output plummeted by 40%; Korean production fell by 30%; Thai output was down nearly 25%. Even the Chinese market appeared to stumble, with an approximate 2% decline in industrially manufactured feed to below 55 Mt. These Asian falls were followed by a general stagnation of the global feed market in 1998, with EU feed production falling by 6% and the non-EU European countries witnessing a 12% decline. Only North...
America (+1%), Latin America (+1%) and the Middle East and African countries (+10%) experienced any growth in their feed industries in 1998. As clearly seen in Table 1.1, the recovery in 1999 was global, with all major regions showing some growth as the Asian economies improved and demand picked up in other markets (Gill, 2000).

Despite the fall, or correction, in feed production seen in 1998, one trend within the feed industry continues unabated – the rationalization of capacity and the concentration and integration of production into the major food-producing companies. Driven by the need for cost reduction, increased regulatory requirements and hygiene and safety standards, fewer than 3800 feed mills now manufacture more than 80% of the world’s compound feed. However, there is still plenty of room for further consolidation and rationalization as the ten largest feed manufacturers still only account for approximately 9–10% of global output.

Although pioneered in the poultry industry, food company integration now extends to most of the major farmed species – poultry, swine, beef, dairy cattle and aquatic. Swine feed is still the largest proportion of feed produced but broiler feed production continues to grow at the expense of beef cattle feeds (Table 1.2), spurred on by increased consumer demand for cheap, safe and healthy meat products (Gill, 2000).

So what does the future hold for the feed industry? Most informed commentators believe that the drop in feed production seen in 1998 was a ‘one-off’ or correction caused by the dramatic collapse experienced by the Asian economies. Muller (1999) comments that:

> in the last 25 years compound feed production has always grown parallel with the increase in world population. The decline in per capita ‘consumption’ of compound feed from 104 kg to 97 kg in 1998 does not represent a reverse trend in global compound feed production, but instead must be categorized correctly in conjunction with the Asian crisis.

With the world population set to grow from the current 6 billion to approximately 7.8 billion over the coming two decades, the demand for food, including animal

<table>
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<th>1999 production (Mt)</th>
<th>Percentage difference (1999/1998)</th>
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<tr>
<td>North America</td>
<td>159.8</td>
<td>160.7</td>
<td>+0.6</td>
</tr>
<tr>
<td>Asia-Pacific</td>
<td>128.1</td>
<td>132.2</td>
<td>+3.2</td>
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<tr>
<td>European Union</td>
<td>114.9</td>
<td>116.4</td>
<td>+1.3</td>
</tr>
<tr>
<td>Latin America</td>
<td>63.3</td>
<td>65.4</td>
<td>+3.3</td>
</tr>
<tr>
<td>Non-EU Europe</td>
<td>47.4</td>
<td>48.6</td>
<td>+2.5</td>
</tr>
<tr>
<td>Middle East and Africa</td>
<td>21.6</td>
<td>24.0</td>
<td>+11.1</td>
</tr>
<tr>
<td>Other</td>
<td>39.9</td>
<td>38.7</td>
<td>–3.1</td>
</tr>
<tr>
<td>Total</td>
<td>575.0</td>
<td>586.0</td>
<td>+2.0</td>
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protein, is set to increase at an even greater rate as the growing populations in the developing economies alter their dietary habits. Set against this scenario, the future for compound feed production looks positive. However, those companies who wish to continue in the industry and expand their businesses will need to become more adept at operating in a market environment where many of the key influencing factors are beyond their control.

Background to Enzyme Technology

Enzymes exist practically everywhere. They are naturally occurring and are produced by all living organisms and, as nature’s catalysts, they speed up the chemical reactions that enable all living things, from simple single-celled organisms, through plants and insects to humans, to function. Without them food could not be digested.

So far over 3000 different enzymes have been discovered. Enzymes, as with all proteins, are made from chains of amino acids. They speed up or catalyse reactions by binding to their substrate and ‘stabilize’ the entire reaction process through to product formation, so that far less activation energy is required to move the reaction forwards. As a result, the rate of reaction progression is greatly increased for any given energy status. It is the three-dimensional shape and position of the reactive amino acids within the molecule that confer the catalytic properties of enzymes. Conditions that significantly alter the structure of the active enzyme often result in loss of activity. As a result, enzymes are very sensitive to the environment in which they function and many work best in mild temperatures and mid-range pH.

Humans have made use of enzymes, often unknowingly, throughout history. Cheese making and the use of malted barley in brewing are examples of the harnessing of the power of enzymes. Modern enzyme technology really started in 1874 following the first documented production of a refined enzyme that was prepared from the contents of calves’ stomachs. That enzyme, rennet, is still used today in cheese manufacture.

Since then the technology to identify, extract and produce enzymes on a commercial scale has progressed dramatically and they are now used in many industrial processes. Currently enzymes are used in detergents, paper production
and in leather and textile processing. In addition, they are widely used in the food and drink industry in products ranging from bread and cheese to fruit juice, wine and coffee. The global market value for enzymes in 1995 was estimated to be worth US$1 billion (Godfrey and West, 1996) and is forecast to rise to between US$1.7 billion and US$2 billion by 2005.

Why use enzymes in animal feeds?

The principal rationale for the use of enzyme technology is to improve the nutritive value of feedstuffs.

All animals use enzymes in the digestion of food, those produced either by the animal itself or by the microbes present in the digestive tract. However, the digestive process is nowhere near 100% efficient; for example, swine are unable to digest 15–25% of the food they eat. Therefore, the supplementation of animal feeds with enzymes to increase the efficiency of digestion can be seen as an extension of the animal’s own digestive process.

In many animal production systems feed is the biggest single cost and profitability can depend on the relative cost and nutritive value of the feeds available. Often, the limiting factor when formulating rations is the animal’s ability to digest different constituent parts of the feed raw materials, particularly fibre. Despite recent advances, the potential nutritional value of feedstuffs is not achieved at the animal level. This inefficiency in the utilization of nutrients can result in a cost to the farmer, the food company and the environment. Put simply, there are four main reasons for using enzymes in animal feed:

1. To break down anti-nutritional factors that are present in many feed ingredients. These substances, many of which are not susceptible to digestion by the animal’s endogenous enzymes, can interfere with normal digestion, causing poor performance and digestive upsets.
2. To increase the availability of starches, proteins and minerals that are either enclosed within fibre-rich cell walls and, therefore, not as accessible to the animal’s own digestive enzymes, or bound up in a chemical form that the animal is unable to digest (e.g. phosphorus as phytic acid).
3. To break down specific chemical bonds in raw materials that are not usually broken down by the animal’s own enzymes, thus releasing more nutrients.
4. To supplement the enzymes produced by young animals where, because of the immaturity of their own digestive system, endogenous enzyme production may be inadequate.

In addition to improving diet utilization, enzyme addition can reduce the variability in nutritive value between feedstuffs, improving the accuracy of feed formulations. Trials have shown that ensuring feed consistency in this way can increase the uniformity of groups of animals, thus aiding management and improving profitability. The general health status of animals can also be indirectly influenced, resulting in fewer of
the non-specific digestive upsets that are frequently provoked by fibre components in the feed.

Of increasing importance and relevance to the feed industry are the environmental benefits of harnessing enzyme technology. Since the animal better utilizes the feed, less is excreted. This results in manure volume being reduced by up to 20% and nitrogen excretion by up to 15% in pigs and 20% in poultry. As significant is the opportunity for enzymes to reduce phosphorus pollution.

How do they work and how are they used in animal feeds?

Broadly speaking, four types of enzymes currently dominate the animal feed market: enzymes to break down fibre, protein, starch and phytic acid.

Fibre-degrading enzymes

One of the main limitations to digestion is the fact that monogastrics (pigs and poultry) do not produce the enzymes to digest fibre. In diets containing ingredients such as wheat, barley, rye or triticale (the main ‘viscous’ cereals), a large proportion of this fibre is soluble and insoluble arabinoxylan and β-glucan (White et al., 1983; Bedford and Classen, 1992). The soluble fibre can increase the viscosity of the contents of the small intestine, impeding the digestion of nutrients and thereby reducing the growth of the animal. It has also been linked with the incidence of digestive disorders such as non-specific colitis in swine, and sticky litter and hock burns in poultry.

The fibre content of wheat and barley can vary considerably according to variety, growing location, climatic conditions, etc. This in turn means that there can be considerable variability in the nutritional value of these ingredients and hence diets containing them. In breaking down the fibre, enzymes (e.g. xylanase targeting arabinoxylans, β-glucanase targeting β-glucans) can reduce this variability in nutritional value, giving rise to improvements in the performance of the feed and the consistency of the response. An added benefit is the reduced incidence of certain digestive disorders.

Protein-degrading enzymes

Various raw materials contribute to the protein content in the diet and ultimately the amino acids that fuel lean meat deposition. There is considerable variability in the quality and availability of protein from the different raw materials typically found in monogastric diets. Within the primary vegetable protein sources such as soybean meal, certain anti-nutritional factors (ANFs), such as lectins and trypsin inhibitors, can lead to damage to the absorptive surface of the gut, impairing nutrient digestion. In addition, the underdeveloped digestive system of young animals may not be able to make optimal use of the large storage proteins found in the soybean meal (glycinin and β-conglycinin).

The addition of a protease can help to neutralize the negative effects of the proteinaceous ANFs in addition to breaking down the large storage protein molecules into smaller, absorbable fractions.
Starch-degrading enzymes

To many nutritionists maize is viewed as the ‘gold standard’ of raw materials. Most nutritionists do not consider maize digestion as being poor: in fact most would argue that it is better than 95% digested. However, recent evidence presented by Noy and Sklan (1994) suggests that, at the ileal level, starch digestibility rarely exceeds 85% in broilers between 4 and 21 days of age. The addition of an amylase to animal feed can help to expose the starch more rapidly to digestion in the small intestine, and in doing so lead to improved growth rates from enhanced nutrient uptake.

At weaning, piglets often suffer a growth check because of changes in their nutrition, environment and immune status. The addition of an amylase, usually in conjunction with other enzymes, to augment the animal’s endogenous enzyme production has been shown to improve nutrient digestibility and absorption and, hence, growth rate for a range of diets (Close, 1995).

Phytic acid-degrading enzymes

Phosphorus is required for bone mineralization, immunity, fertility and growth and is an essential mineral for all animals. Swine and poultry digest only about 30–40% of the phosphorus found in feedstuffs of vegetable origin, with the remainder being tied up in a form inaccessible to the animal – phytic acid. In many instances additional phosphorus must be added to the diet to meet the animal’s requirement. More than half of the phosphorus consumed from such feedstuffs is excreted in the faeces, which can result in major environmental pollution. By adding a phytase to the diet, the phytic acid is broken down, liberating more of the phosphorus for use by the animal.

The two main benefits of phytase supplementation are, firstly, the reduction in feed costs from the reduced additional supplementation of phosphorus to the diet and, secondly, environmental from reduced excretion of waste products and the threat of pollution.

The Current Feed Enzyme Market

It should come as no surprise that the adoption of enzyme technology by the animal feed industry has, to a large degree, been driven by the mode of action of these four particular types of enzymes. Taken in conjunction with the different structure of the various segments of the animal feed industry, the background to the adoption of the technology becomes even clearer.

The global poultry industry, particularly the broiler industry, is highly integrated and dominated by a relatively small group of large companies. For example, Tyson’s alone in the USA is estimated to produce some 2 billion broilers per year, considerably larger than the entire annual production of the UK poultry industry. The company controls every step of the production chain from the growing of the raw materials and the breeding of the birds through to the processing of the meat and ultimately the retailing of the finished product. This integration and concentration of the majority of industry capacity in a relatively small number of
The adoption of new technology is a rapid occurrence, particularly when the value of the opportunity is significant. The value of enzyme addition to broiler feeds will typically deliver a return on investment (ROI) well in excess of 2:1.

By contrast, the swine industry is considerably more fragmented, with many more producers and steps in the chain. In addition, the efficacy response to enzyme addition has been more variable and harder to measure commercially.

Although first considered in the 1950s, it was not until the 1980s that the animal feed industry really began to understand how to harness the power of enzyme technology properly.

As already discussed, feed grains such as wheat and barley contain relatively high levels of fibre that monogastrics are unable to digest. If the problem element of the fibre can be broken down, the animal has greater access to the available nutrients, so overcoming the negative impacts of incorporating the ingredient in the diet. Spurred on by a plentiful supply of cheap barley, European poultry nutritionists and enzymologists investigated the opportunity to reduce the negative impact of including barley in broiler diets by adding β-glucanase to the diet. This proved to be a success with the ‘rule of thumb’ being adopted that ‘barley + β-glucanase = wheat’.

Encouraged by the outstanding success of this approach, wheat became the next target for enzymatic enhancement via the use of xylanase. Work this time concentrated on the hypothesis that ‘wheat + xylanase = maize’.

Again this approach was successful and the mid-1990s saw the rapid acceptance of the application of enzyme technology to the animal feed industry. It is probably no exaggeration to say that by 1996 in excess of 80% of all European broilers diets that contained a viscous cereal (wheat, barley, etc.) would have also contained a fibre-degrading enzyme – certainly an impressive adoption rate for a new technology in the feed industry.

Taking a global perspective across all species and diet segments, current estimates of market penetration suggest that approximately 65% of all poultry feeds containing viscous cereals also contain a fibre-degrading enzyme. Given the very different structure of the industry and the difficulties in accurately measuring an efficacy response, it is no surprise that current penetration into the swine industry is considerably lower, approaching 10%.

In terms of geographical spread, the utilization of fibre-degrading enzymes is concentrated on those regions where viscous cereals are the predominant energy source, namely Europe, Canada and Australia/New Zealand. (This is not to discount completely the USA, South America and the rest of Asia-Pacific, where there are regular ‘window’ opportunities depending on the post-harvest price ratio between maize and, for example, wheat.) It is for this reason that fibre-degrading enzymes are largely seen as a ‘European’ niche product. To gain global acceptance it will be necessary for the enzyme manufacturers to break significantly into the North American and Asia-Pacific maize–soybean markets. Maize–soybean diets are historically regarded as the ‘gold standard’ in terms of consistency of animal response, though most nutritionists recognize that these raw materials are more variable than is often presumed.

There is increasing evidence and acceptance that this ‘gold standard’ can be improved upon and that there is a role for enzymes in these diets that do not
superficially present a problem related to fibre or viscosity. The focus of considerable research-and-development (R&D) dollars over the last 10 years has seen the first generation of maize–soybean enzymes for poultry diets, launched in 1996. Despite initial mixed results the industry is now beginning to understand more fully how best to apply this technology to reap an economic reward. Current estimates suggest that this market segment is worth approximately US$20 million and that 5% of broiler diets based upon maize–soybean now contain an enzyme. The combined market for these viscous and non-viscous (carbohydrase) enzymes is currently (1999/2000) estimated to be in excess of US$100 million.

One enzyme concept that already has both global acceptance and global application is phytase. The type of carbohydrate source present does not drive its mode of action, as phytic acid is present at varying levels in all vegetable feed raw materials. Estimates suggest that the phytase market is currently worth up to US$50 million, with approximately 8% of global swine and poultry feeds containing phytase. This again is an exceptional adoption rate for a new technology, given the short period since the concept was launched.

The driving force behind this success has been increasing concern about the impact of phosphorus on environmental pollution. In a number of countries legislation has either been imposed (e.g. The Netherlands) or is being imposed (e.g. the Delmarva Peninsula in the USA) on pig and poultry farmers. The historical application of phytase is to replace a proportion of the supplementary calcium and phosphorus added to the diet, but now phytase suppliers are increasingly concentrating on the apparent potential of the product to improve the availability of certain other nutrients (e.g. amino acids and trace minerals) in the diet. The aim is to improve the ROI and so the uptake of this new technology.

The largest applications for phytase are currently into laying hens and swine, given the scope to remove larger quantities of supplementary phosphorus and the ease of application (note that many layer and swine diets are not heat treated). There remains significant scope to increase phytase application and application uptake in the broiler segment as the value equation improves and the enzyme industry overcomes the issues surrounding liquid application and the thermostability of enzymes.

**Future Needs and Opportunities**

The adoption of enzyme technology has forced the animal feed industry to challenge traditional assumptions about diet formulation, ingredient selection, nutrient requirements and the desired productive response. Ten years ago many European nutritionists viewed wheat in the same light as North Americans view maize – basically as an ingredient that could not be improved. As enzyme technology becomes more accepted and widespread throughout the global animal feed industry, so the enzyme producers must continue to develop new products and applications to overcome the existing obstacles to growth, as well as to offer new solutions and opportunities.
The thermal stability of enzymes and their ability to survive the heat processing steps in the manufacture of pelleted animal feed is of major concern to the industry. This, along with concerns over enzyme assays and the need for full traceability throughout the food chain, will force the enzyme manufacturers to increase their efforts to discover new enzyme products that can survive the increasingly high processing temperatures, deliver \textit{in vivo} efficacy and be recovered in standard quality control procedures. Alternatively, improved post heat treatment liquid application systems will offer the industry short- to medium-term solutions.

The adoption of existing enzyme technology in the animal feed sector has increased as knowledge of the interaction between the enzyme, its substrate and the environment of the animal’s gut has evolved and improved. It is very likely that the increased uptake of enzymes in the maize–soybean type diets will be driven by an enhanced understanding of which ingredients will respond to enzyme treatment and the amount of enzyme required to deliver the most economic and cost-effective response. The ultimate scenario would see a computerized and automated ‘on-line’ process that tests the raw material as it enters the feed mill and then doses the economically optimal amount of enzyme into the mixer as the feed is produced.

The decision by the EU authorities to ban the use of certain antibiotic growth promoters, and the knock-on effect in other countries around the world, has forced feed manufacturers and meat producers to explore alternatives. Enzymes are regularly put forward as a technology that will allow producers to continue to produce safe, nutritious and cheap meat, milk and eggs whilst meeting the ever increasing consumer demand for these products. It is important to note here that there are many products being promoted opportunistically on the back of the antibiotic growth promoter ban. When searching out realistic and sustainable solutions to fill this gap, it is important that the feed industry is rigorous and consistent in its demand for scientifically credible technologies and products.

Despite the impressive uptake of the technology, only approximately 10% of monogastric feeds today contain a feed enzyme, giving a total market value in excess of US$150 million. As such, perhaps the enzyme supply industry needs to ask some searching questions of itself as to why uptake has not been faster, particularly in those applications where there appears to be a sound commercial case. Standardized and more transparent quality control procedures, improved heat stability, more accurate liquid application systems, clearer presentation of technical information and products that deliver an even more consistent productive response are just some of the factors raised by the feed industry to explain lack of use. It is clear that there remains huge untapped potential for enzyme technology in the animal feed industry.

References

Introduction

Cellulose and hemicellulose are the major plant structural polysaccharides and account for approximately 70% of plant biomass (Ladisch et al., 1983). It has been estimated that the amount of carbon fixed by plants during photosynthesis is over 100 billion tons per annum (Ryu and Mandels, 1980). In addition to their pivotal role in maintaining the structural integrity of plants, cellulose and hemicellulose serve as a major source of nutrients for herbivores and as renewable substrates for the production of food, animal feed, paper and pulp as well as textiles (Ryu and Mandels, 1980; Gilbert and Hazlewood, 1993; Beguin and Aubert, 1994).

It has been unambiguously established that cellulose and hemicellulose can be converted to soluble sugars by enzymes of mainly microbial origin collectively termed cellulases and hemicellulases (Mandels, 1985; Viikari et al., 1993). Microorganisms including fungi, bacteria and actinomycetes produce mainly three types of cellulase components, namely endoglucanase, exoglucanase and β-glucosidase, either as separate entities or in the form of an aggregated complex, for cellulose hydrolysis (Wood, 1985; Lamed and Bayer, 1987, 1988; Bhat and Bhat, 1997). The hemicellulose fraction of the cell walls of most species of land plants contains mainly xylan and mannan and requires a more extensive repertoire of enzymes to effect complete hydrolysis to soluble sugars (Biely et al., 1992; Hazlewood and Gilbert, 1998a). The two main enzymes involved are endoxylanases (xylanases) and endomannanases (mannanases), which attack the backbone structure (Viikari et al., 1993). Other hemicellulases, including β-xylanase, β-mannosidase, α-L-arabinofuranosidase, α-D-glucuronidase, α-galactosidase, acetyl and phenyl esterases, remove side-chains and substituents (Biely et al., 1992; Coughlan, 1992; Coughlan and Hazlewood, 1993a).

The interesting biochemical and catalytic properties of cellulases and hemicellulases, coupled with their key role in the natural world and their tremendous
potential for biotechnological applications, have stimulated research aimed at understanding the detailed biochemistry, molecular biology and structure–function relationships of these enzymes. This chapter summarizes current knowledge of the enzymology and other characteristics of cellulases and xylanases from both fundamental and applied viewpoints. Further background information can be found in Ryu and Mandels (1980), Wood (1985, 1992a,b), Lamed and Bayer (1988), Coughlan (1992), Coughlan and Hazlewood (1993a,b), Gilbert and Hazlewood (1993), Bayer et al. (1994), Beguin and Aubert (1994), Bhat and Bhat (1997, 1998) and Hazlewood and Gilbert (1998a).

Structure of Cellulose and Hemicellulose

Plant cell walls consist mainly of cellulose (40–45%), hemicellulose (30–35%) and lignin (20–23%) (Ladisch et al., 1983). Cellulose is a linear polymer of glucose linked by β-1,4-glycosidic bonds, having a simple primary and complex tertiary structures. The repeating unit of cellulose is cellobiose (Fig. 2.1a).

The degree of polymerization (DP; number of glucose residues) per cellulose chain varies from 500 to 14,000 (Marx-Figini and Schultz, 1966). In plant cell walls, the cellulose chains are oriented in parallel with varying degrees of order. In some regions the cellulose chains are highly ordered and strongly hydrogen bonded to form crystallites, whereas loosely arranged cellulose molecules form the amorphous regions (Fig. 2.1b). Native crystalline cellulose has a structure specified as type I, which can be converted to type II by alkali treatment (Beguin and Aubert, 1994). These two types of cellulose differ in their intrachain hydrogen bonding. In addition, native cellulose may be composed of two slightly different forms of type I cellulose, called Iα and Iβ, which differ in their intermolecular hydrogen bonding (Atalla and Vander Hart, 1984).

The degree of crystallinity of cellulose varies with its origin and its treatment after isolation (Hoshino et al., 1992). It ranges from 0% for amorphous and acid-swollen cellulose to nearly 100% for cellulose from Valonia macrophysa (Beguin and Aubert, 1994). Cotton cellulose is approximately 70% crystalline, while the degree of crystallinity of commercial celluloses varies from 30 to 70% (Fan et al., 1980; Wood, 1988). The crystalline regions of cellulose are rigid and not easily accessible to endo-acting cellulases, while the amorphous regions are easily attacked by either dilute acid, endoglucanases or exoglucanases (Sinitsyn et al., 1990). Thus, for the complete hydrolysis of cellulose, either concentrated acid or a complete cellulase system capable of attacking both amorphous and crystalline regions is necessary.

Hemicellulose, the second most abundant plant structural polysaccharide, is present in association with cellulose in the walls of most plant species, and can be extracted by alkali. Based on the main sugar residues present in the polymer backbone, hemicelluloses can be termed xylans, glucomannans, galactans or arabinans. The two main types of hemicelluloses are generally considered to be xylans and
Characteristics of Cellulases and Xylanases


glucomannans (Timell, 1967; Whistler and Richards, 1970; Stephen, 1983; Puls and Schusel, 1993; Viikari et al., 1993).

Xylans from annual plants are more heterogeneous than xylans from perennial plants and are designated as arabinoxylans. The two main types of arabinoxylans are: (i) highly branched and without uronic acid substitution – found in cereal endosperms; and (ii) much less branched and substituted with uronic acid and/or with 4-O-methyl ether and galactose – present in lignified tissues. Arabinoxylans of graminaceous plants contain acetic and phenolic acids (ferulic, p-coumaric) which are esterified to the backbone xylose units and the arabinose side groups, respectively (Fig. 2.1c) (Hartley and Ford, 1989). In addition to enzymes (endoxyylanase and
β-xyllosidase), which cleave the backbone structure, accessory enzymes like α-L-arabinofuranosidase, acetyl and phenyl esterases and α-D-glucuronidase are necessary for the removal of side-chains and the complete hydrolysis of arabinoxylans (Biely et al., 1992).

Enzymology of Cellulases and Xylanases

Source

Cellulases and xylanases are produced by a wide range of bacteria and fungi, including aerobes, anaerobes, mesophiles, thermophiles and extremophiles. Aerobic fungi and bacteria generally produce extracellular cellulases and hemicellulases. Interestingly, anaerobic bacteria (Clostridium thermocellum, C. cellulovorans, Ruminococcus albus, R. flavefaciens, Fibrobacter succinogenes, Acetivibrio cellulolyticus) and anaerobic fungi (Neocallimastix frontalis, N. patriciarum, Pirormyces equi) produce cellulases in the form of a multienzyme aggregated complex (Groleau and Forsberg, 1981; Lamed et al., 1987; Wood, 1992a; Gilbert and Hazlewood, 1993; Beguin and Lemaire, 1996; Bhat and Bhat, 1997).

Most of the early studies were carried out on the biochemistry and enzymology of cellulases from aerobically mesophilic fungi, Trichoderma viride, T. reesei, Penicillium pinophilum, Sporotrichum pulverulentum, Fusarium solani, Talaromyces emersonii and Trichoderma koningii (Coughlan and Ljungdahl, 1988). In the past two decades, it has been recognized that other microorganisms such as thermophilic fungi (Sporotrichum thermophile, Thermoascus aurantiacus, Chaetomium thermophile, Humicola insolens), mesophilic anaerobic fungi (N. frontalis, N. patriciarum, P. communis, Sphaeromonas communis, P. equi, Orpinomyces sp.), mesophilic and thermophilic aerobic bacteria (e.g. Cellulomonas fimi, Pseudomonas fluorescens subsp. cellulosa, Cellvibrio sp., Microbiflora bispora, Clostridium cellulolyticum and C. cellulovorans), mesophilic and thermophilic anaerobic bacteria (A. cellulolyticus, Bacteroides cellulosolvens, F. succinogenes, R. albus, R. flavefaciens, C. thermocellum and C. stercorarium), as well as actinomycetes (Thermomonaspora fusca), produce highly active cellulase and hemicellulase systems (Bhat and Maheswari, 1987; Aubert et al., 1988; Beguin and Lemaire, 1996; Claeyssens et al., 1998). In addition, hyperthermophilic microorganisms – namely, Thermotoga sp., Pyrococcus furiosus and Thermofilum sp., which grow between 85 and 110°C – produce extremely stable cellulolytic and hemicellulolytic enzymes (Simpon et al., 1991; Antranikian, 1994; Winterhalter and Liebl, 1995).

In addition to many of those listed above, other microorganisms such as Aspergillus and Cryptococcus produce xylanases and xylan debranching enzymes (Coughlan and Hazlewood, 1993a,b; Viikari et al., 1993). The evidence currently available suggests that the xylan- and mannan-degrading enzymes of anaerobic microorganisms may be produced as integral components of the aggregated multienzyme cellulase complexes that are characteristic of these species (Beguin and Lemaire, 1996; Hazlewood and Gilbert, 1998b).
Characteristics of Cellulases and Xylanases

Enzyme assays

The measurement of cellulase and xylanase activities is hampered by the nature of substrates used and the complexity of the enzyme systems produced by different microorganisms. In order to overcome these problems, numerous assays have been developed (Wood and Bhat, 1988; Biely et al., 1992). The substrates and the methods used to measure cellulase and xylanase activities are summarized in Table 2.1. For more detailed information on assays for accessory enzymes involved in the degradation of substituted xylans, the reader is referred to Coughlan and Hazlewood (1993a).

Quantitative assays for cellulases and xylanases

The cellulase system contains mainly endoglucanase (EC 3.2.1.4, 1,4-β-D-glucan glucanohydrolase), exoglucanase or cellobiohydrolase (EC 3.2.1.91, 1,4-β-D-glucan cellobiohydrolase) and β-glucosidase or cellobiase (EC 3.2.1.21, β-D-glucoside glucohydrolase). Endoglucanase activity is generally determined by measuring the reducing sugars released from either carboxymethyl (CM-) or hydroxyethyl (HE-) cellulose (Wood and Bhat, 1988). This activity can also be measured by determining either the decrease in viscosity of CM-cellulose, the swelling of cotton fibre in alkali or the decrease in turbidity of amorphous cellulose. In addition, substituted, unsubstituted, radio- and reduced end-labelled cello-oligosaccharides have been used to characterize endoglucanases (Bhat et al., 1990). Interestingly, some endoglucanases catalyse transferase reactions and act synergistically with cellobiohydrolase during the solubilization of crystalline cellulose (Wood et al., 1988; Claeyssens et al., 1990a).

Cellobiohydrolase (CBH; exoglucanase) activity is determined by measuring the reducing sugars released from either Avicel or H₃PO₄-swollen cellulose (Wood and Bhat, 1988). Besides, CBH activity can be measured by determining either the release of dyed cellobiose from dyed Avicel or the decrease in turbidity of amorphous cellulose. Substituted and unsubstituted cello-oligosaccharides have been used to characterize CBHs ( Claeyssens et al., 1989).

β-Glucosidase activity is generally determined by measuring the release of glucose and o-/p-nitrophenol from cellobiose and o-/p-nitrophenyl β-D-glucoside, respectively (Wood and Bhat, 1988). Also, the increase in reducing power of cello-oligosaccharides can be used as a measure of β-glucosidase activity.

Total cellulase activity, comprising endoglucanase, exoglucanase and β-glucosidase, is measured by determining the solubilization of either cotton fibre, filter paper or Avicel. The release of dyed soluble fragments from dyed Avicel can also be used to measure total cellulase activity. It is generally accepted that either cotton fibre or filter paper is the best substrate, but some consider Avicel is ideal for measuring total cellulase activity. All three substrates contain a high proportion of crystalline cellulose and are therefore useful for determining total cellulase activity (Wood and Bhat, 1988).

Xylanase (EC 3.2.1.8) activity is generally determined by measuring the reducing sugars released from xylan by either the Somogyi–Nelson or the dinitrosalicylic...
Table 2.1. Substrates and assays used for determining the cellulase and xylanase activities.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Substrate</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoglucanase</td>
<td>CM-cellulose; hydroxyethylcellulose</td>
<td>Release of reducing sugars (Somogyi, 1952); decrease in viscosity (Wood and Bhat, 1988)</td>
</tr>
<tr>
<td>(CM-cellulase,</td>
<td>Cotton; amorphous cellulose (Wood, 1988)</td>
<td>Swelling in alkali (Marsh et al., 1953); release of reducing sugars (Somogyi, 1952); decrease in turbidity (Nummi et al., 1981)</td>
</tr>
<tr>
<td>endocellulase)</td>
<td>Substituted and unsubstituted</td>
<td>Increase in reducing sugars (Somogyi, 1952); analysis of products by HPLC (Bhat et al., 1990)</td>
</tr>
<tr>
<td>Cellobiohydrolase</td>
<td>Avicel; hydrocellulose and dyed Avicel</td>
<td>Release of reducing sugars (Somogyi, 1952); dyed cellobiose (Wood and Bhat, 1988)</td>
</tr>
<tr>
<td>(exoglucanase,</td>
<td>Amorphous cellulose</td>
<td>Release of reducing sugars (Somogyi, 1952); decrease in turbidity (Nummi et al., 1981)</td>
</tr>
<tr>
<td>exocellulase,</td>
<td>Substituted and unsubstituted</td>
<td>Increase in reducing sugars (Somogyi, 1952); release of ( p )-nitrophenol from ( p )-nitrophenyl-( \beta )-o-cellobioside (Deshpande et al., 1984); analysis of products by HPLC (Bhat et al., 1990)</td>
</tr>
<tr>
<td>Avicelase)</td>
<td>cello-oligosaccharides</td>
<td></td>
</tr>
<tr>
<td>( \beta )-Glucosidase</td>
<td>Cellobiose</td>
<td>Release of glucose (Wood and Bhat, 1988)</td>
</tr>
<tr>
<td>(cellobiase)</td>
<td>Cello-oligosaccharides</td>
<td>Increase in reducing sugars (Somogyi, 1952)</td>
</tr>
<tr>
<td></td>
<td>( \alpha )- or ( \beta )-Nitrophenyl-( \beta )-o-glucosides</td>
<td>Release of ( \alpha )- or ( \beta )-nitrophenol (Wood and Bhat, 1988)</td>
</tr>
<tr>
<td>Total cellulase</td>
<td>Cotton</td>
<td>Cellulose residue (Wood, 1969); release of reducing sugars, weight loss and loss in tensile strength (Wood and McCrae, 1972)</td>
</tr>
<tr>
<td></td>
<td>Filter paper; hydro-cellulose; Avicel; Solka Floc</td>
<td>Release of reducing sugars (Somogyi, 1952; Miller, 1959)</td>
</tr>
<tr>
<td></td>
<td>Dyed Avicel</td>
<td>Release of soluble dyed fragments (Wood and Bhat, 1988)</td>
</tr>
<tr>
<td>Xylanase</td>
<td>Birchwood xylan; oat spelt xylan</td>
<td>Release of reducing sugars (Somogyi, 1952; Miller, 1959; Bailey et al., 1992)</td>
</tr>
<tr>
<td>(endoxyanlase)</td>
<td>Insoluble xylan</td>
<td>Decrease in turbidity (Nummi et al., 1985)</td>
</tr>
<tr>
<td></td>
<td>Arabinoxylan; CM-xylan</td>
<td>Decrease in viscosity (Sengupta et al., 1987)</td>
</tr>
<tr>
<td></td>
<td>RBB-xylan; Ostazine Brilliant Red (OBR)-xylan</td>
<td>Release of RBB- or OBR-dyed fragments (Biely et al., 1985)</td>
</tr>
<tr>
<td></td>
<td>([1-\text{H}])-labelled, unsubstituted and substituted xylo-oligosaccharides</td>
<td>Analysis of products by HPLC and subsequent radioactive counting, if necessary (Bhat et al., 1990; Biely et al., 1992)</td>
</tr>
</tbody>
</table>
2. Qualitative assays

Endoglucanase  CM-cellulose; RBB-CM-cellulose; OBR-hydroxyethyl cellulose; methylumbelliferyl-β-D-celllobioside (MeUmbGlc₂); methylumbelliferyl-β-D-cellotrioside (MeUmbGlc₃)  Detection by using Congo Red and zone clearing (Coughlan, 1988)

Cellobiohydrolase  RBB-Avicel; p-Nitrophenyl-β-D-celllobioside  Zone clearing (Coughlan, 1988)  Release of p-nitrophenol (van Tilbeurgh et al., 1988)

β-Glucosidase  p-Nitrophenyl-β-D-glucoside; methylumbelliferyl-β-D-glucoside (MeUmbGlc)  Detection of p-nitrophenol (Bhat et al., 1993) or MeUmb by UV (van Tilbeurgh et al., 1988)

Xylanase  Soluble xylan; RBB-xylan; OBR-xylan; methylumbelliferyl-β-D-xylobioside (MeUmbXyl₂); methylumbelliferyl-β-D-xylotrioside (MeUmbXyl₃)  Detection by using Congo Red and zone clearing (Biely et al., 1992)  Detection of MeUmb by UV (Biely et al., 1992; Kalogiannis et al., 1996)
acid (DNS) method (Somogyi, 1952; Miller, 1959; Bailey et al., 1992). The DNS method tends to give a higher value than the Somogyi–Nelson method. Nevertheless, xylanase activity determined by the DNS method, standardized with respect to a particular xylan, is very reproducible (Biely et al., 1992). Based on a round-robin test that involved 20 laboratories, Bailey et al. (1992) proposed a suitable procedure for preparing xylan and measuring xylanase activity. In addition, a viscometric method using a soluble xylan (arabinoxylan or carboxymethyl xylan) has been used for the determination of xylanase activity in samples containing high background sugar values (Sengupta et al., 1987). Although the above methods detect the ability of an enzyme sample to hydrolyse xylan, they do not reveal the type of products released, which may be important when evaluating the ability of an enzyme to degrade xylan. Hence, the hydrolysis products should be analysed to obtain detailed information on the mode of action of the xylanase in question.

Another specific assay, which can be used to determine xylanase activity in the presence of either large amounts of reducing sugars or viable cells utilizing xylan fragments (Biely, 1985), involves the use of soluble and covalently dyed xylan (Remazol Brilliant Blue) (RBB-xylan). Although this assay is highly sensitive to temperature and ionic strength, interlaboratory tests revealed that the release of low molecular weight dyed fragments is a useful alternative for measuring xylanase activity (Biely et al., 1992). A nephelometric (turbidometric) assay using insoluble xylan has also been found to be suitable for determining xylanase activity (Nummi et al., 1985). Furthermore, [1-3H]-labelled and substituted xylo-oligosaccharides have been used to characterize xylanases (Biely et al., 1992; Bennett et al., 1998).

Qualitative assays for cellulases and xylanases

Qualitative assays have been developed either to select microbial strains producing high levels of cellulases and xylanases, or to identify and/or characterize these enzymes in a given sample. Insoluble xylan and RBB-xylan are ideal substrates to select microorganisms producing xylanase on solid agar medium (Sprey and Lambert, 1983; Farkas et al., 1985). Similarly, methods using either RBB-CM-cellulose or CM-cellulose stained with Congo Red can be used to select microorganisms producing endoglucanase activity (Kluepfel, 1988).

The capacity of Congo Red to complex with either polymeric xylan or CM-cellulose, but not with small oligosaccharide products, is conveniently used to detect xylanase and endoglucanase activities in zymograms after the fractionation of protein mixtures by SDS/IEF-PAGE (Coughlan, 1988). RBB-xylan and RBB-CM-cellulose can be used for similar purposes (Biely et al., 1985). The advantages of the latter method are: (i) that the dyed fragments released from RBB-xylan/CM-cellulose diffuse from the detection gel into the separating gel and further help to identify the position of a xylanase or endoglucanase, which can subsequently be eluted; and (ii) the hydrolysis of the dyed substrate can be visually followed and the reaction terminated when necessary. The use of these substrates facilitates the identification of multiple forms of xylanase or endoglucanase produced by different microorganisms. Likewise, chromogenic and fluorogenic cello- and xylo-oligosaccharides have been successfully used to identify multiple forms of cellulases and xylanases after the
separation of crude enzyme mixtures by SDS/IEF-PAGE (Biely et al., 1992; Kalogiannis et al., 1996).

**Substrate specificity**

*Endoglucanases*

Endoglucanases specifically cleave the internal β-1,4-glycosidic bonds of amorphous, swollen and substituted celluloses as well as cello-oligosaccharides. These enzymes are generally inactive towards crystalline cellulose and cellobiose. Some endoglucanases attack barley glucan with mixed β-1,3 and β-1,4 linkages (Petre et al., 1986). Using substituted, unsubstituted, [1-3H]-labelled and reduced cello-oligosaccharides, Bhat et al. (1990) demonstrated a marked difference in the substrate specificities of endoglucanases purified from *P. pinophilum*. Thus, endoglucanases III and IV were active on cellotriose and higher cello-oligosaccharides, whereas endoglucanases II, V and I required at least four, five and six glucose residues, respectively. Although such variation in substrate specificity of endoglucanases was unexpected, it was speculated that microorganisms secrete multiple endoglucanases with a wide range of substrate specificities to effect efficient hydrolysis of complex cellulosic substrates.

*CBHs (cellobiohydrolases; exoglucanases)*

Like endoglucanases, the CBHs are highly active on amorphous and swollen celluloses, but degrade crystalline cellulose and cello-oligosaccharides rather poorly (Wood and Bhat, 1988). These enzymes are specific for β-1,4 linkages of the cellulose chain, but are inactive on cellobiose, CM- and hydroxyethyl-celluloses. In general, CBHs attack cellulose chains from the non-reducing end and release cellobiose (Wood et al., 1988). Recent kinetic studies and high-resolution structural data confirmed that there are two classes of CBHs (Barr et al., 1996). The class one enzymes (e.g. CBH I from *T. reesei* and two exoglucanases E4 and E6 from *T. fusca*) hydrolyse the cellulose chain preferentially from the reducing end, while class two CBHs (e.g. CBH II from *T. reesei* and E3 from *T. fusca*) release cellobiose specifically from the non-reducing end (Barr et al., 1996; Teeri, 1997).

*β-Glucosidases*

β-Glucosidases can be classified as either aryl β-D-glucosidases (hydrolysing exclusively aryl-β-D-glycosides), cellobiases (hydrolysing diglucosides and cello-oligosaccharides) or β-glucosidases with broad substrate specificities. Most β-glucosidases characterized so far show broad substrate specificities and hydrolyse aryl and alkyl β-D-glycosides and β-1,1-, β-1,2-, β-1,3-, β-1,4- and β-1,6-linked diglucosides, as well as substituted and unsubstituted cello-oligosaccharides (Bhat et al., 1993; Christakopoulos et al., 1994a). Interestingly, an intracellular β-glucosidase from *S. thermophile* was found to be an aryl-β-glucosidase, whereas two extracellular β-glucosidases from the same organism hydrolysed only cellobiose (Bhat et al., 1993). Some β-glucosidases showed activity towards H$_3$PO$_4$-swollen and CM-celluloses (Sadana et al., 1988), but most β-glucosidases are inactive...
towards these and other polymeric substrates such as Avicel, filter paper and cotton (Woodward and Wiseman, 1982).

**Xylanases**

In general, xylanases are specific for the internal 1,4 linkages of polymeric xylan and are designated as endoxylanases. Based on their action on different polysaccharides, endoxylanases have been classified as either specific or non-specific (Coughlan, 1992; Coughlan et al., 1993). The specific endoxylanases are active on xylans with only 1,4 linkages, whereas non-specific endoxylanases hydrolyse 1,4-linked xylans, 1,4 linkages of mixed xylans and other 1,4-linked polymers such as CM-cellulose. In fact, the determination of the kinetic constants ratio $k_{cat}/K_m$ for xylan and CM-cellulose, for a particular non-specific xylanase, should confirm whether the enzyme in question is a xylanase or a cellulase. Generally, endoxylanase activity and affinity for xyloligosaccharides decrease with decreasing DP (Coughlan et al., 1993). Most endoxylanases are specific for unsubstituted xylosidic linkages of xylans and release both substituted and unsubstituted xyloligosaccharides. In contrast, some endoxylanases are specific for xylosidic linkages adjacent to substituted groups in the main chain xylan. For example, two endoxylanases from Aspergillus niger (pI 8.0 and 9.6) showed little or no action on xylolignans or xylans from which the arabinose substituents were removed (Frederick et al., 1985). Furthermore, the endoxylanase with pI 9.6 cleaved the xylan chain only in the vicinity of 4-O-methylglucuronic acid, and this substitution was reported to be an absolute requirement for the action of the enzyme (Nishitani and Nevins, 1991). Nevertheless, both enzymes failed to release any substituent as a free product, which indicated that these substitutions are necessary for the proper orientation of the substrate in the active site.

Most endoxylanases, active on mixed xylan (rhodymenan with 1,3 and 1,4 linkages), are specific for the 1,4 linkages (Coughlan, 1992). Also, endoxylanases have been grouped either as debranching or non-debranching based on their ability to release arabinose in addition to hydrolysing the main chain (Coughlan et al., 1993). Although some endoxylanases purified to homogeneity hydrolysed both main-chain xylan and arabinose side-chains (Matte and Forsberg, 1992), there is always a question as to whether the release of arabinose is due to an intrinsic property of the enzyme or is due to the presence of a trace contaminant.

**Kinetics and subsite mapping**

Kinetic constants for the hydrolysis of CM-cellulose and xylan by purified endoglucanases and xylanases have been determined (Bhat et al., 1989; Coughlan et al., 1993). It is often difficult to determine the kinetic constants of these enzymes using polymeric substrates. Therefore, substituted (chromogenic or fluorogenic) and [1-3H] labelled cello- and xyloligosaccharides have been prepared and conveniently used for this purpose (Bhat et al., 1990; Claeyssens et al., 1990a,b; Biely et al., 1992). When an enzyme attacks more than one glycosidic bond in a particular
oligosaccharide, the kinetic constants ($K_m$ and $k_{cat}$) for the cleavage of all glycosidic bonds must be determined in order to understand the mode of action of the enzyme, as well as to identify the preferred site of attack. Using this approach, the number of glycosyl-binding sites present in different endoglucanases, CBHs and xylanases have been determined biochemically (Claeyssens et al., 1989; Coughlan et al., 1993; Bhat et al., 1994). Detailed information on glycosyl-binding sites and active site architecture of the above enzymes has also been derived by determining their three-dimensional structures with bound substrates or substrate analogues in the active site (Davies et al., 1995; Sakon et al., 1997). In contrast, the kinetic constants of $\beta$-glucosidases have been determined using either cellobiose or pNPC (Bhat et al., 1993; Christakopoulos et al., 1994a), and have been shown to depend on enzyme source and preference for alkyl or aryl-glycosides (Woodward and Wiseman, 1982; Bhat et al., 1993).

Inhibition

Cellulases and xylanases are often inhibited by the presence of high concentrations of their hydrolysis products. For example, most CBHs are inhibited by cellobiose (Wood and McCrae, 1986), even though cellobiose (> 10 mM) stimulates the activity of CBH II from $P$. pinophilum towards $H_3PO_4$-swollen cellulose. Similarly, endoglucanases are inhibited by cellobiose at or above 100 mM (Bhat et al., 1989). However, glucose (up to 100 mM) showed little effect on many CBHs and endoglucanases (Wood et al., 1988; Bhat et al., 1989). In contrast, $\beta$-glucosidases are inhibited by glucose and other mono- and disaccharides, such as xylose, fucose, galactose, maltose, lactose and melibiose (Bhat et al., 1993). Besides, nojirimycin and gluconolactone are known to be potent inhibitors of $\beta$-glucosidases (Bhat et al., 1993).

Like cellulases, endoxylanases are believed to be inhibited by high concentrations of xylobiose but not by xylose. In general, the activity of cellulases and xylanases is neither activated nor inhibited by metal ions and reducing agents, though the aggregated cellulase systems of anaerobic bacteria may require divalent cations and reducing agents for their activity towards crystalline cellulose, but not towards swollen and substituted celluloses (Lamed and Bayer, 1988).

Transferase activity

Many cellulases and endoxylanases catalyse both hydrolysis and transferase (or transglycosylase) reactions, especially in the presence of high concentrations of oligomeric substrates or alcohol (Bhat et al., 1990, 1993; Christakopoulos et al., 1994a–c). The essential difference between hydrolysis and transferase reactions is that, in the former, water acts as an acceptor of the glycosyl moiety, whereas in the latter the acceptor is an alcohol or a sugar molecule.

Transferase reactions have considerable significance from both fundamental and applied viewpoints. It is generally accepted that the transferase products of cellobiose
and xylobiose could be the true inducers of microbial cellulases and xylanases (Biely, 1993; Bhat and Bhat, 1997). In addition, enzymatic transglycosylation can be used to synthesize high value alkylglycosides and oligosaccharides with desired linkages, which have uses both for research and for commercial applications (Christakopoulos et al., 1994a–c).

Mode of action

Fungal cellulase

All endoglucanases attack swollen and substituted celluloses and the amorphous regions of cellulose randomly and release glucose, cellobiose and cello-oligosaccharides (Table 2.2) (Wood, 1985; Coughlan and Ljungdahl, 1988). The

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mode of action</th>
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<tr>
<td>Endoglucanase or endocellulase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.4)</td>
<td>( -G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G ) ↑ ↑ Cleaves 1,4-β linkages at random (Wood and Bhat, 1988)</td>
</tr>
<tr>
<td>Cellobiohydrolase (CBH) or exocellulase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91)</td>
<td>( G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G ) ↑ (type I) ↑ (type II) Releases cellobiose from both reducing (type II) and non-reducing ends (type I) (Wood et al., 1988; Vrsanska and Biely, 1992; Barr et al., 1996; Gilkes et al., 1997)</td>
</tr>
<tr>
<td>Exoglucanase or glucohydrolase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.74)</td>
<td>( G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G ) ↑ Releases glucose from the non-reducing end (McHale and Coughlan, 1980; Wood and McCrae, 1982)</td>
</tr>
<tr>
<td>β-Glucosidase or cellobiase (β-D-glucoside glucohydrolase; EC 3.2.1.21)</td>
<td>( G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G\backslash G ) ↑ ↑ ↑ ↑ ↑ Releases glucose from cellobiose and hydrolyses short-chain cello-oligosaccharides from both reducing and non-reducing ends by releasing one glucose unit at a time (Wood and Bhat, 1988; Christakopoulos et al., 1994a)</td>
</tr>
<tr>
<td>Xylanase or endoxylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8)</td>
<td>( S ) ( S ) ( X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X\backslash X ) ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ → S Cleaves 1,4-β linkages of xylan at random with preference to unsubstituted regions (Biely et al., 1992; Coughlan et al., 1993; Coughlan and Hazlewood, 1993a)</td>
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CBHs hydrolyse H₃PO₄-swollen cellulose and Avicel sequentially by removing cellobiose units from either reducing or non-reducing ends of the cellulose chain (Wood et al., 1988; Vrsanska and Biely, 1992; Barr et al., 1996). Endoglucanase and CBH act synergistically to effect extensive hydrolysis of crystalline cellulose. Subsequently β-glucosidase completes the hydrolysis by converting the resultant cello-oligosaccharides and cellobiose to glucose (Wood, 1985).

The modes of action of endoglucanase, CBH and β-glucosidase from several sources have been extensively studied using substituted, unsubstituted and labelled cello-oligosaccharides (van Tilbeurgh et al., 1988; Bhat et al., 1990; Claeyssens and Henrissat, 1992). Based on these studies, it was concluded that most endoglucanases attack internal glycosidic bonds of cello-oligosaccharides and release mainly cellobiose and cellotriose, while CBHs hydrolyse the second glycosidic bond from either the reducing or non-reducing end of the cello-oligosaccharides (Table 2.2). However, β-glucosidase sequentially removes one glucose unit from either the reducing end, the non-reducing end or both ends (Table 2.2).

The viscosity of a cellulose solution is directly related to the DP of individual cellulose chains. An endoglucanase that attacks the cellulose chain randomly produces the largest decrease in the viscosity of a cellulose solution per unit increase in reducing power. Based on the relationship between viscosity reduction and the increase in reducing end groups, the multiple endoglucanases from T. koningii and P. pinophilum were classified as more or less randomly acting enzymes (Wood and McCrae, 1978; Bhat et al., 1989). Using a similar approach, the mode of action of endoglucanases and CBHs (exoglucanases) from C. fimi, P. pinophilum and C. thermocellum has been clarified (Gilkes et al., 1984; Bhat et al., 1989, 1994; Wood et al., 1989).

Some fungi, such as P. pinophilum and T. emersonii, produce an exoglucanase that catalyses the removal of glucose from the non-reducing end of cellobextrins, but does not interact cooperatively with endoglucanases during the hydrolysis of crystalline cellulose (McHale and Coughlan, 1980; Wood and McCrae, 1982).

Anaerobic fungi such as N. frontalis, N. patriciarum and P. equi use a different mechanism to degrade cellulose when compared with aerobic fungi (Wood, 1992a). It has been reported that N. frontalis produces a multicomponent enzyme complex called crystalline cellulose-solubilizing factor (CCSF), which has a molecular mass of 700 kDa and consists of a number of subunits with molecular mass ranging from 68 to 135 kDa (Wood, 1992a). Functionally, CCSF resembles the cellulases from the aerobic fungi in solubilizing crystalline cellulose, since it acts synergistically with an endoglucanase and a β-glucosidase of N. frontalis which are not part of CCSF (Wood, 1992a). Furthermore, it was demonstrated that CCSF of N. frontalis consists of endoglucanase, β-glucosidase and perhaps also CBH. Although the mode of action of CCSF on cellulose is not clearly understood, it is believed that all subunits of CCSF act cooperatively to degrade cellulose (Wood et al., 1994). More recent molecular biology studies of individual cellulases and hemicellulases from anaerobic fungi support the view that these organisms produce an aggregated cellulase complex analogous to the cellulosome of C. thermocellum (Hazlewood and Gilbert, 1998b).
Bacterial cellulase

Bacterial cellulases are known to adopt different mechanisms for the hydrolysis of cellulose. For example, the aerobes *Cellulomonas*, *Pseudomonas*, *Thermoactinomyces*, *T. fusca* and *Microbispora* and the anaerobe *C. stercorarium* produce a cellulase system similar to that of aerobic fungi, and degrade cellulose by the cooperative interaction of the different cellulase components (Beguin *et al*., 1992; Wood, 1992b; Gilbert and Hazlewood, 1993). In contrast, the anaerobic thermophilic bacterium *C. thermocellum* degrades crystalline cellulose very effectively by means of a high molecular mass multi-enzyme complex called the cellulosome (Lamed and Bayer, 1988; Beguin and Lemaire, 1996). The cellulosome of *C. thermocellum* has been extensively studied and used as a model system for understanding how anaerobic bacteria, including rumen bacteria, degrade cellulose (Wood, 1992a). The production of cellulosomes is not restricted to *C. thermocellum* alone. Various other anaerobic bacteria, such as *R. albus*, *R. flavefaciens*, *F. succinogenes*, *A. cellulolyticus* and *C. cellulovorans* also produce cellulosomes on their cell surface which possess similar properties to the cellulosome from *C. thermocellum* (Groleau and Forsberg, 1981; Lamed *et al*., 1987; Beguin and Lemaire, 1996).

Cellulosomes of *C. thermocellum* degrade crystalline cellulose extensively in the presence of Ca$^{2+}$ and DTT (Lamed and Bayer, 1988). According to current understanding, the cellulosome contains a large molecular mass, non-catalytic subunit termed scaffoldin (CipA, S1 or S1) which possesses a cellulose-binding domain (CBD) and numerous duplicated attachment sites called cohesins (Bayer *et al*., 1994; Beguin and Lemaire, 1996; Beguin and Alzari, 1998; Bhat and Bhat, 1998). In addition to catalytic domains, the enzymatic subunits of the cellulosome each contain a highly conserved duplicated docking domain termed a dockerin, which interacts with the cohesins of the scaffoldin polypeptide (Bayer *et al*., 1994). Some of the enzymatic subunits also contain a CBD. The exact mechanism by which the cellulosome achieves cellulolysis is unclear, but it is evident that the efficiency of the multi-enzyme complex is a function of its quaternary structure, and is dependent on both endoglucanase/CBH synergism and clustering of the complex on to the substrate surface (Beguin and Lemaire, 1996).

Fungal and bacterial endoxylanases

The mode of action of endoxylanases has been extensively studied (Coughlan, 1992; Coughlan and Hazlewood, 1993a,b; Biely *et al*., 1997). In general, these enzymes cleave the internal β-1,4 linkages of the xylan backbone and release mainly xylobiose, xylotriose and substituted oligomers having two to four residues (Table 2.2). It has been reported that most endoxylanases cleave the xylan backbone leaving the substituent at the non-reducing end of the xylosyl chain or the oligosaccharide (Dekker, 1985). Moreover, the presence of glucopyranosyl uronic acid sterically hinders the hydrolysis of the second and third xylosidic linkages to the right of the branch points, by the xylanases from *S. dimorphosporum* and *Trametes hirsuta* (Kubackova *et al*., 1978; Comtat and Joseleau, 1981). Nevertheless, the xylanases from *A. niger* and *C. sacchari* were found to cleave the substituent on the reducing end and in the middle of the oligosaccharide chain, respectively (Dekker, 1985).
By analysing the structures of the substituted products released during the extensive hydrolysis of different xylans by purified endoxylanases, it was possible to draw the following conclusions about their modes of action.

1. An endoxylanase from *Streptomyces* sp. yielded arabinoxylotrioside (IV, Fig. 2.2) as the smallest product from wheat arabinoxylan. This revealed that the minimal spatial requirement for the attack of β-1,4 linkage is two adjacent unsubstituted xylose units.

2. An endoxylanase from *M. verrucaria* liberated arabinoxylobiose (I, Fig. 2.2) from wheat straw xylan, indicating that this enzyme attacks the glycosidic linkage adjacent to the substitution.

3. Hydrolysis of spear grass hemicellulose by an endoxylanase from *Ceratocystis paradoxa* yielded products I and III (Fig. 2.2), while a xylanase from *A. niger* liberated products II and V (Fig. 2.2).

4. An extensive hydrolysis of larchwood glucuronoxylan by an endoxylanase from *T. aurantiacus* yielded xylose, xylobiose and aldotetrauronic acid (VII, Fig. 2.2). This enzyme did not cleave main-chain β-1,4 linkages between the residues carrying the substituent and the adjacent xylose on either side.

5. Action of endoxylanase from *S. dimorphosporum* on redwood arabinoglucuronoxylan released products I, III, VI and VIII (Fig. 2.2). This endoxylanase appeared to require at least a xylotriose group (IX) with an unsubstituted (at O-2 position) xylosyl residue at the non-reducing end and an unsubstituted (at O-2 and O-3 positions) central xylosyl residue, while the reducing end could be substituted or unsubstituted (Fig. 2.2).

Based on amino acid sequence similarities and hydrophobic cluster analysis, Henrissat and Bairoch (1993, 1996) grouped endoxylanases in glycosyl hydrolase families 10 and 11. Family 10 includes mainly acidic and high molecular mass endoxylanases, while basic and low molecular mass endoxylanases belong to family 11. Studies on the mode of action of endoxylanases from these two families revealed that: (i) endoxylanases of family 10 hydrolyse heteroxylans (substituted xylan) and homoxylans (rhodymenan with β-1,4 and β-1,3 linkages) to a higher degree than those of family 11; (ii) only endoxylanases of family 10 are capable of cleaving the glycosidic linkages of xylan either closer or adjacent to substituents, such as methylglucuronic acid (MeGlcA) and acetic acid; and (iii) family 10 endoxylanases release smaller oligosaccharides from glucuronoxylan, acetylxylan and rhodymenan than family 11 (Biely et al., 1997). In addition, it was reported that the endoxylanases of family 10 require two unsubstituted xylose residues between the branch points, whereas family 11 endoxylanases require at least three unsubstituted xylose residues in a sequence (Fig. 2.3a–c) (Biely et al. 1997). The predicted mode of action of endoxylanases from families 10 and 11 on rhodymenan is shown in Fig. 2.3b.

Studies of the mode of action and biochemical characteristics of endoxylanases I and III from *Aspergillus awamori* indicated that they belong to families 10 and 11, respectively (Kormelink et al., 1993). Thus, endoxylanase I (family 10) cleaves β-1,4 linkages adjacent to arabinose substitution as well as internal β-1,4 linkages, whereas endoxylanase III (family 11) cleaves only the internal β-1,4 linkages of cereal
arabinoxylan (Fig. 2.3c) (Kormelink et al., 1993). The isolation and identification of methylglucuronic acid and arabinose-linked oligosaccharides released from methylglucuronoxylan and arabinoxylan by endoxylanases of family 10 revealed that the substitution at position 3 could cause greater steric hindrance of enzyme attack.
than at position 2. Furthermore, only endoxylanases of family 10 released monoacetylated xylobiose from acetylxylan, with the exception of a family 11 endoxylanase from *T. reesei* (Biely et al., 1997).

Endoxylanases from families 10 and 11 can be distinguished further, based on their mode of action on substituted and unsubstituted oligosaccharides. In general, the family 10 endoxylanases hydrolyse xylotriose and xylotetraose more rapidly than those from family 11. Also, endoxylanases of family 10 hydrolyse pNPC, pNPXyl₂, pNPXylGlc, MeUmbXyl₂, and MeUmbXylGlc, while endoxylanases of family 11 hydrolyse only pNPXyl₂ and MeUmbXyl₂. Thus, the ability to cleave β-1,4 linkages between xylose and glucose residues is one of the major differences between endoxylanases from families 10 and 11. Based on this information, Biely et al. (1997) concluded that if a model β-1,4-glucoxylan is available, it is most likely that the endoxylanases of family 10 will cleave certain β-1,4-glucopyranosyl linkages, but those from family 11 will not.

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**Fig. 2.3.** Mode of action of endoxylanases from families 10 and 11 on (a) 4-O-methyl-D-glucurono-D-xylan, (b) rhodymenan, and (c) cereal l-arabino-D-xylan. The arrows indicate the sites of attack on different substrates. Reprinted from *Journal of Biotechnology*, Vol. 57, Biely, P., Vrsanska, M., Tenkanen, M. and Kluepfel, D., Endo-β-1,4-xylanase families: differences in catalytic properties, pp. 151–166, 1997, with permission from Elsevier Science.
Synergism

Between cellulases

Synergism is an enhanced effect of two or more enzymes when acting cooperatively, compared with their additive effect. Giligan and Reese (1954) first demonstrated synergism between cellulase components during the hydrolysis of cellulose. Subsequently several research groups have demonstrated synergism between endo- and exoglucanases during the solubilization of crystalline cellulose (Wood et al., 1988, 1989; Klyosov, 1990; Bhat et al., 1994). Five different types of synergism have been reported between fungal cellulase components: (i) a non-hydrolytic protein called C1 and endoglucanase (Reese et al., 1950); (ii) β-glucosidase and either endoglucanase or CBH (Eriksson and Wood, 1985); (iii) two immunologically related and distinct CBHs (Wood and McCrae, 1986); (iv) endoglucanase and CBH from either the same or different microorganisms (Wood et al., 1989); and (v) between two endoglucanases (Klyosov, 1990). In addition, synergism between bacterial and fungal cellulases, as well as between the subunits of the C. thermocellum cellulosome, has been reported (Bhat et al., 1994; Wood et al., 1994).

Synergism between fungal cellulase components has been studied most extensively (Coughlan and Ljungdahl, 1988; Wood et al., 1988, 1989; Klyosov, 1990). The most interesting types of synergism are between: (i) endoglucanase/exoglucanase (CBH); (ii) exoglucanase (CBH)/exoglucanase (CBH); and (iii) endoglucanase/endoglucanase. An early model postulated by Wood and McCrae (1972) suggested that cellulose chains cleaved by endoglucanase become the substrate for exoglucanase, and that these two enzymes cooperatively degrade the cellulose. However, this model did not explain the synergism seen between two different CBHs, or the inability of CBH to synergize with endoglucanases from different microorganisms. Subsequently, using highly purified endoglucanases and CBHs from P. pinophilum, it was demonstrated that only two endoglucanases (EGIII and EGV), which were strongly adsorbed on to cellulose, best synergized with CBHs I and II (Fig. 2.4; Wood et al., 1989). The authors explained the observed synergism between CBHs I and II and endoglucanases in terms of the different stereospecificities of these two enzymes.

The attack of a cellulose chain by a stereospecific endoglucanase will generate only one of two possible types of non-reducing ends, which will be hydrolysed by a stereospecific CBH. The successive removal of cellobiose by CBH will expose another chain-end of a different configuration, which will be attacked by the other stereospecific CBH. Hydrolysis of the two chain-ends by two CBHs acting randomly, together with attack of the cellulose chains by another stereospecific endoglucanase to generate a reducing end of a different configuration, would facilitate the synergism observed between these enzymes. Nevertheless, it was argued that the above synergism could be due to the strong adsorption of CBHs and endoglucanases on to cellulose (Klyosov, 1990). Furthermore, Klyosov (1990) reported that two endoglucanases which adsorbed strongly on to cellulose degraded the cellulose synergistically. Although the adsorption appeared to be important, it is difficult to explain the contribution of adsorption towards synergistic interaction...
between endoglucanases and CBHs. Recent studies (Barr et al., 1996; Teeri, 1997) revealed that there are two classes of CBHs (exoglucanases) which attack cellulose chains from both reducing and non-reducing ends. Based on these results, it was speculated that the synergism observed between CBH/CBH (exo/exo) was due to their ability to expose new hydrolysis sites to each other, as well as their ability to act from reducing and non-reducing ends (Barr et al., 1996). Thus, two CBHs acting from reducing and non-reducing ends and an endoglucanase appeared to be essential for the effective hydrolysis of crystalline cellulose. The presence of another endoglucanase with different substrate specificity would increase the synergistic efficiency further, as was observed in the case of the *P. pinophilum* cellulase system (Wood et al., 1989).

Recent studies of the *C. thermocellum* cellulase system revealed that two cellulosomal exoglucanases (S₅ and S₈ subunits) and an endoglucanase (S₁₁ subunit), together with the S₁ (scaffoldin) subunit, are essential for maximum synergism during the hydrolysis of crystalline cellulose (Bhat et al., 1994). Furthermore, the S₁ subunit mediated the formation of an enzyme complex with the S₅, S₈ and S₁₁ subunits.

**Fig. 2.4.** Synergism between *P. pinophilum* CBHs (I and II) and endoglucanases (EI to EV) in solubilizing cotton fibre. Reproduced from *Biochemical Journal*, Vol. 260, Wood, T.M., McCrae, S.I. and Bhat, K.M., The mechanism of fungal cellulase action. Synergism between enzyme components of *P. pinophilum* cellulase in solubilizing hydrogen bond-ordered cellulose, pp. 37–43, 1989, with permission from Portland Press, London.
subunits (Bhat et al., 1994). Using recombinant scaffoldin (CipA) polypeptides and the endoglucanase CelD, it was demonstrated that a truncated scaffoldin polypeptide with a CBD and a single cohesin domain was adequate for the maximum activity of CelD towards Avicel (Kataeva et al., 1997; Beguin et al., 1998). It was also reported that linkage of the CelD–CipA complex with the CBD was critical for maximum synergism during Avicel solubilization, rather than the clustering of catalytic domains (Kataeva et al., 1997; Beguin et al., 1998). These results strongly indicate that, in the case of the aggregated C. thermocellum cellulase system, assembly of an enzyme complex is crucial for the maximum synergistic interaction of subunits during the solubilization of crystalline cellulose. Other studies using recombinant cellulosomal components have confirmed the important role of the CipA CBD in efficient cellulolysis (Ciruela et al., 1998).

Between xylan-degrading enzymes

Efficient and complete hydrolysis of xylan requires the synergistic action of main- and side-chain-cleaving enzymes with different specificities (Coughlan et al., 1993; Coughlan and Hazlewood, 1993a). For xylan-degrading enzymes, it is difficult to demonstrate the synergism by measuring only the reducing sugars produced, because of the heterogeneous nature of the substrate. Hence, the hydrolysis products must be separated, identified and quantified in order to obtain a detailed picture of the synergistic action of xylan-degrading enzymes. In fact, three types of synergy between such enzymes have been described: (i) homeosynergy; (ii) heterosynergy; and (iii) antisynergy (Coughlan et al., 1993). Homeo- and heterosynergies could be either uni- or biproduct. Homeosynergy is the synergistic interaction between two or more different types of side-chain-cleaving enzymes or between two or more types of main-chain-cleaving enzymes (Coughlan et al., 1993). Homeosynergy is best demonstrated for Neurospora crassa (Deshpande et al., 1986), Talaromyces byssochlamydoides (Yoshioka et al., 1981) and Trichoderma harzianum (Wong et al., 1986), where endoxylanases of different specificities, or mixtures of endoxylanases and β-xylosidases, cooperate in the hydrolysis of xylan. Another example of homeosynergy is reported between ferulic acid esterase from A. oryzae and α-L-arabinofuranosidase from P. capsulatum, where the former enzyme facilitates the release of arabinose from feruloylated arabinoxylan by the latter (Coughlan et al., 1993).

Heterosynergy is the synergistic interaction between side-chain- and main-chain-cleaving enzymes (Coughlan et al., 1993). Heterosynergy is uniproduct if the action of the main-chain-cleaving enzyme facilitates the release of a substituent by the side-chain-cleaving enzyme, or vice versa. Heterosynergy is biproduct if the extent of liberation of substituent, and the hydrolysis of the main chain, by the action of combined enzymes exceeds the sum of the actions of the individual enzymes. Heterosynergy has been reported between ferulic acid esterases and endoxylanases (Faulds and Williamson, 1991; Tenkanen et al., 1991), α-L-arabinofuranosidases and endoxylanases (Tuohy et al., 1992), acetyl xylan esterases and endoxylanases (Biely et al., 1986; Lee et al., 1987), as well as between α-glucuronidas and endoxylanases (Puls et al., 1987). A good example of heterosynergy is shown in Fig. 2.5.
Antisynergy occurs when the action of one type of enzyme prevents the action of a second enzyme (Coughlan et al., 1993). This can be observed in the case of either arabinoxylan xylanohydrolase (Frederick et al., 1985) or glucuronoxylan glucuronohydrolase (Nishitani and Nevins, 1991), an enzyme that cleaves the main chain linkages only in the vicinity of a particular substituent. For example, in the case of glucuronoxylan glucuronohydrolase, the presence of substituents is essential for its action on the main-chain and the removal of the substituent by the relevant side-chain-cleaving enzyme would preclude its action on the main chain. Although antisynergy is unlikely to occur in vivo, it may be observed in in vitro experiments where only one or two enzymes are used in a specific application.

### Catalytic mechanism

Cellulases and xylanases catalyse stereoselective hydrolysis of the glycosidic bond with either retention or inversion of configuration around the anomic centre (Sinnott, 1990; Davies and Henrissat, 1995). Inversion occurs by way of a single displacement reaction, while retention is via double displacement (Sinnott, 1990). Both reaction mechanisms are assisted by general acid/base catalysis (Fig. 2.6).

During inversion, the protonation of the glycosidic oxygen and aglycone departure are accompanied by a concomitant attack of a water molecule that is activated by the catalytic base. This single nucleophilic substitution yields a product with opposite stereochemistry to the substrate. In contrast, the retention mechanism involves two steps: glycosylation and deglycosylation. In the glycosylation step, the catalytic residue, acting as a general acid, protonates the glycosidic oxygen with
concomitant C-O breaking of the scissile glycosidic bond. A deprotonated carboxylate, functioning as a nucleophile, attacks the anomeric centre to give a covalent glycosyl–enzyme intermediate. The deglycosylation step involves the attack of a water molecule, assisted by the conjugate base of the general acid, to release the free sugar with overall retention of configuration. Both steps proceed via transition states with considerable oxocarbonium ion character. In both retention and inversion mechanisms, the position of the proton donor is within hydrogen-bonding distance of the glycosidic oxygen. In retaining enzymes, the nucleophilic catalytic base is in close vicinity to the sugar anomeric carbon, whereas in inverting enzymes this base is more distant, since it must accommodate a water molecule between the base and the sugar. This difference results in an average distance between the two catalytic residues of 5.5 Å in retaining enzymes, as opposed to 10 Å in inverting enzymes (Davies and Henrissat, 1995).

Fig. 2.6. (a) Retention and (b) inversion mechanisms of glycosyl hydrolases. Reprinted from Structure, Vol. 3, Davies, G. and Henrissat, B. Structures and mechanisms of glycosyl hydrolases, pp. 853–859, 1995, with permission from Current Biology Ltd, London.
The stereochemical course of hydrolysis has been determined for glycosyl hydrolases belonging to 42 of the 64 known families, of which families 1, 3, 5–9, 12, 26, 43–45, 48, 60 and 61 represent cellulases, and 10 and 11 represent xylanases (Henrissat and Davies, 1997; Davies, 1998; Henrissat, 1998). Enzymes of families 1, 3, 5, 7, 10–12 and 26 proceed with retention of configuration, while enzymes from families 6, 8, 9, 43, 45 and 48 proceed with inversion of configuration (Table 2.3). Interestingly, all representatives of a particular family follow the same catalytic mechanism (Gebler et al., 1992). Thus, it is possible to predict the catalytic mechanism of a given cellulase or xylanase accurately once its family is known.

Other Characteristics of Cellulases and Xylanases

Induction and regulation

Induction and regulation of microbial cellulases and xylanases have been the subject of detailed investigation (Ryu and Mandels, 1980; Kubicek, 1992; Biely, 1993; Kubicek et al., 1993; Saloheimo et al., 1998). Both cellulases and xylanases are inducible enzymes. It has been reported that cellulose or cellulose-rich material and xylan or xylan-rich material are the best carbon sources for the production of high levels of cellulases and xylanases by many microorganisms (Ryu and Mandels, 1980; Biely, 1993). Since these polysaccharides cannot enter the microbial cell, it is generally accepted that the low levels of cellulases and xylanases constitutively produced by the microorganisms hydrolyse the corresponding polysaccharides to soluble sugars, which enter the microbial cell and either act as, or get converted into, true inducers and stimulate the transcription of cellulase and xylanase genes (Biely, 1993; Kubicek et al., 1993). In fact, it has been suggested that the conidial-bound CBH of T. reesei hydrolyses cellulose to cellobiose and cellobiono-δ-1,5-lactone (CBL), which promotes cellulase synthesis (Kubicek et al., 1988). Although cellobiose and xylobiose are assumed to be the natural inducers of cellulases and xylanases, the rapid utilization of these disaccharides suggested that the transglycosylated products of these disaccharides or their positional isomers could in fact be the true inducers (Ryu and Mandels, 1980; Biely, 1993). Sophorose (β-1,2-glucobiose), a positional isomer of cellobiose, is reported to be an effective inducer of cellulases in T. reesei and other species of Trichoderma, even at low concentrations (Sternberg and Mandels, 1979). However, the inability of sophorose to induce cellulase synthesis in other fungi (Moloney et al., 1983), and the ability of cellobiose to induce the production of cellulase by T. reesei, in the presence of either a β-glucosidase inhibitor or in a β-glucosidase defective mutant (Fritscher et al., 1990), strongly indicates that cellobiose could be the true inducer of the cellulase system. Also, the positional isomers of xylobiose (Xylβ,1-2Xyl; Xylβ,1-3Xyl) were found to be poor inducers of xylanase in C. albidus compared with xylobiose (Xylβ,1-4Xyl), which suggests that the latter is probably the true inducer of xylanase (Biely, 1993).

Initial studies using protein synthesis inhibitors suggested that the production of cellulase is regulated at the translational level (Nishizawa et al., 1972). However, the
Table 2.3. List of cellulases and xylanases belonging to different families of glycosyl hydrolases and their catalytic mechanism.

<table>
<thead>
<tr>
<th>Family</th>
<th>Enzyme</th>
<th>Organism</th>
<th>EC number</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>β-Glucosidase</td>
<td><em>Trifolium repens</em></td>
<td>3.2.1.21</td>
<td>Retention</td>
<td>Barrett et al., 1995</td>
</tr>
<tr>
<td>3</td>
<td>β-Glucosidase</td>
<td>Fungi and bacteria</td>
<td>3.2.1.21</td>
<td>Retention</td>
<td>Warren, 1998</td>
</tr>
<tr>
<td>5</td>
<td>Endo A</td>
<td><em>C. cellulolyticum</em></td>
<td>3.2.1.4</td>
<td>Retention</td>
<td>Gebler et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Cel B</td>
<td><em>C. thermocellum</em></td>
<td>3.2.1.4</td>
<td>Retention</td>
<td>Gebler et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Cel C</td>
<td><em>C. thermocellum</em></td>
<td>3.2.1.4</td>
<td>Retention</td>
<td>Gebler et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Cel H</td>
<td><em>C. thermocellum</em></td>
<td>3.2.1.4</td>
<td>Retention</td>
<td>Gebler et al., 1992</td>
</tr>
<tr>
<td></td>
<td>EG III</td>
<td><em>T. reesei</em></td>
<td>3.2.1.4</td>
<td>Retention</td>
<td>Gebler et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Endo 5</td>
<td><em>T. fusca</em></td>
<td>3.2.1.4</td>
<td>Retention</td>
<td>Gebler et al., 1992</td>
</tr>
<tr>
<td></td>
<td>EG Z</td>
<td><em>E. chrysanthemi</em></td>
<td>3.2.1.4</td>
<td>Retention</td>
<td>Barras et al., 1992</td>
</tr>
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<td>CBH II</td>
<td><em>T. reesei</em></td>
<td>3.2.1.91</td>
<td>Inversion</td>
<td>Rouvènè et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Exoglucanase</td>
<td><em>T. fusca</em></td>
<td>3.2.1.91</td>
<td>Inversion</td>
<td>Barr et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Endoglucanase</td>
<td><em>T. fusca</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Spezio et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Cbh A</td>
<td><em>C. timi</em></td>
<td>3.2.1.91</td>
<td>Inversion</td>
<td>Meinke et al., 1994</td>
</tr>
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<td></td>
<td>Cen A</td>
<td><em>C. timi</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Withers et al., 1988</td>
</tr>
<tr>
<td>7</td>
<td>CBH I</td>
<td><em>T. reesei</em></td>
<td>3.2.1.91</td>
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<td>Divne et al., 1994</td>
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<td>Endoglucanase</td>
<td><em>H. insolens</em></td>
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<td>Retention</td>
<td>Davies and Henrissat, 1995</td>
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<td>Retention</td>
<td>Claeyssens et al., 1990b</td>
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<td>3.2.1.4</td>
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<td>Sulzenbacher et al., 1996; Mackenzie et al., 1997</td>
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<td><em>C. thermocellum</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Alzari et al., 1996</td>
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<td>Cel C</td>
<td><em>C. cellulolyticum</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Fierobe et al., 1993</td>
</tr>
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<td>Species</td>
<td>EC Number</td>
<td>Activity</td>
<td>Source(s)</td>
</tr>
<tr>
<td>-----</td>
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<tr>
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<td><em>C. thermocellum</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Juy et al., 1992</td>
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<tr>
<td></td>
<td>Cen B</td>
<td><em>C. fimi</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Gebler et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Endo I</td>
<td><em>T. fusca</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Gebler et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Endo 4</td>
<td><em>T. fusca</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Sakon et al., 1997</td>
</tr>
<tr>
<td>10</td>
<td>Xylanase A</td>
<td><em>S. lividans</em></td>
<td>3.2.1.8</td>
<td>Retention</td>
<td>Derewenda et al., 1994</td>
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<td>CeX</td>
<td><em>C. fimi</em></td>
<td>3.2.1.91</td>
<td>Retention</td>
<td>Withers et al., 1988</td>
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<td>Xyn Z</td>
<td><em>C. thermocellum</em></td>
<td>3.2.1.8</td>
<td>Retention</td>
<td>Gebler et al., 1992</td>
</tr>
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<td>Xylanase A</td>
<td><em>P. fluorescens</em></td>
<td>3.2.1.8</td>
<td>Retention</td>
<td>Harris et al., 1994</td>
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<td>11</td>
<td>Xylanase</td>
<td><em>B. circulans</em></td>
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<td>Xylanase</td>
<td><em>S. commune</em></td>
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<td>Retention</td>
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<td><em>T. reesei</em></td>
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<td>Retention</td>
<td>Havukainen et al., 1996</td>
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<td>12</td>
<td>Endoglucanase (Cel B)</td>
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<td>Retention</td>
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<td>43</td>
<td>Xylanases</td>
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<td>Inversion</td>
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<td>45</td>
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<td>Inversion</td>
<td>Davies et al., 1995</td>
</tr>
<tr>
<td>48</td>
<td>CBH b</td>
<td><em>C. fimi</em></td>
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<td>Inversion</td>
<td>Shen et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Endoglucanase – E6</td>
<td><em>T. fusca</em></td>
<td>3.2.1.4</td>
<td>Inversion</td>
<td>Barr et al., 1996</td>
</tr>
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</table>
The presence of high levels of cellulase mRNA in *T. reesei* grown on solka floc and the complete absence of cellulase mRNA in *T. reesei* grown on glucose revealed that the cellulase expression is regulated at transcriptional level (El-Gogary *et al.*, 1989; Saloheimo *et al.*, 1998). The addition of low levels (1–2 mM) of sophorose resulted in the accumulation of very high levels of mRNA in *T. reesei* (Ilmen *et al.*, 1997). Nevertheless, no cellulase mRNA was detected when sophorose was added to glucose-grown culture or glucose was added to cellulose-grown culture of *T. reesei* (Ilmen *et al.*, 1997). These results clearly indicated that both induction and glucose repression are operating at the transcriptional level during the regulation of cellulase synthesis. Furthermore, all cellulase inducers promoted the synthesis of a constant ratio of three major cellulases such as CBH I, CBH II and endoglucanase I in *T. reesei*. This indicates that the expression of cellulase genes is coordinately regulated, at least in *T. reesei* (Kubicek, 1992; Saloheimo *et al.*, 1998).

In contrast, the induction pattern of hemicellulase genes appeared to be variable even in *T. reesei*. For example, the expression pattern of *xyn I* and *II* genes was somewhat different (Saloheimo *et al.*, 1998). Also, genes coding for debranching enzymes were efficiently induced by xylan having the appropriate substitutions. Thus, regulation of hemicellulase genes appears to be more complex than cellulase genes, even though both sets of genes are regulated through induction and catabolite repression. Interestingly, two activator genes corresponding to proteins called ACE I and II, responsible for the activation of cellulase expression, were isolated from *T. reesei* (Saloheimo *et al.*, 1998).

Recent studies on induction of cellulases and xylanases in *Schizophilum commune* revealed that cellulose or cellulose-rich substrates greatly stimulated the formation of both cellulases and xylanases by this fungus. Also, other carbon sources such as cellobiose, lactose, sophorose and L-sorbose induced production of both cellulase and xylanase (Haltrich *et al.*, 1995). Although the production of xylanases along with cellulases on media containing cellulose has been reported in the case of *Aspergillus* sp., *S. rolsii*, *T. aurantiacus*, *P. pinophilum*, etc., there are distinct differences between these strains and *S. commune* (Brown *et al.*, 1987; Yu *et al.*, 1987; Lachke and Deshpande, 1988; Bailey and Poutanen, 1989). Thus, these fungi produced both cellulase and xylanase when xylan was present as a contaminant with cellulose, and its removal from cellulose reduced their ability to produce xylanase. In contrast, the removal of xylan from Avicel induced higher levels of both cellulase and xylanase production by *S. commune*. In addition, cellulose from *Acetobacter pasteurianus*, free of xylan, strongly induced the production of both cellulase and xylanase in *S. commune*. Furthermore, cellobiose, the repeating unit of cellulose, induced the production of both cellulase and xylanase by *S. commune*, which strongly suggests that this fungus appears to possess a common regulatory mechanism for the synthesis of cellulases and hemicellulases (Haltrich *et al.*, 1995). A similar regulatory system appears to operate in another wood-rotting fungus, *Polyporus adustus* (Eriksson and Goodell, 1974). Thus, the mechanism governing induction and regulation of cellulases and xylanases appears to be quite complicated, and markedly different from one microorganism to another.
Physicochemical properties

Most fungal endoglucanases and CBHs (exoglucanases) have molecular masses ranging from 20 to 100 kDa, whereas the molecular mass of β-glucosidase ranges from 50 to 300 kDa (Coughlan and Ljungdahl, 1988; Bhat et al., 1993). In general, fungal cellulases are optimally active between pH 4.0–6.0, whereas bacterial enzymes show a pH optimum of 6.0–7.0 (Wood, 1985). Many fungal endoglucanases and CBHs are highly glycosylated while bacterial enzymes are less glycosylated (Wood et al., 1988; Bhat et al., 1989). Endoglucanases, CBHs and β-glucosidases from mesophilic fungi and bacteria are optimally active between 40 and 55°C (Wood et al., 1988; Bhat et al., 1989; Christakopoulos et al., 1994a). Cellulases from thermophilic and hyperthermophilic microorganisms are optimally active between 60–80 and 90–110°C, respectively (Khandke et al., 1989; Bhat et al., 1993; Antranikian, 1994).

Fungal and bacterial xylanases are almost exclusively single subunit proteins with molecular masses ranging from 8.5 to 85 kDa, and with pI values of 4.0–10.3 (Coughlan et al., 1993). Based on their physicochemical properties, most xylanases may be assigned to two classes: proteins with molecular mass < 30 kDa are usually basic, whereas those with molecular mass > 30 kDa are usually acidic (Wong et al., 1988). Also, most xylanases have acidic pH optima (Wong et al., 1988), with the exception of xylanases from an alkalophilic thermophilic Bacillus sp., which are optimally active at alkaline pH and are particularly suitable for paper and pulp applications. In addition, xylanases from thermophiles are optimally active at 60–80°C, while those from mesophiles show optimal activity from 40–55°C (Coughlan et al., 1993; Bennett et al., 1998).

Multiplicity of cellulases and xylanases

Most microorganisms capable of degrading cellulose and xylan produce multiple forms of cellulases and xylanases with either very similar substrate specificities or varying substrate specificities (Wong et al., 1988; Bhat et al., 1989). Based on biochemical studies, it has been reported that T. reesei and P. pinophilum produce two forms of CBHs and four to seven forms of endoglucanases (Gong et al., 1979; Wood et al., 1988; Bhat and Wood, 1989), whereas A. niger, T. viride and T. emersonii produce 15, 13 and 11–13 forms of xylanases, respectively (Biely, 1985; Coughlan et al., 1993). Nevertheless, it is not certain whether the above multiple forms of cellulases and xylanases are the products of multiple genes or the result of post-translational modification of a single gene product by differential glycosylation or partial proteolysis. Also, it is often difficult to distinguish the multiple forms of cellulases and xylanases based on substrate specificity, because of the lack of defined and structurally characterized substrates, and the preponderance of enzymes with broad substrate specificity. It has been speculated that the apparent broad substrate specificity of some cellulases and xylanases could be due to traces of contaminating protein(s) present in purified samples. However, it has been clearly shown that P. pinophilum produces seven to eight forms of endoglucanase, which differ with
respect to their pI values, during the early logarithmic growth phase, and further incubation only increases their concentration with minor variations in relative proportions (Bhat and Wood, 1989).

The above results suggest that multiple endoglucanases must be the result of expression of different genes, rather than post-translational modification. This fact has been confirmed by the isolation and characterization of many cellulase and xylanase genes from a number of bacteria and fungi (Gilbert and Hazlewood, 1993). It has been shown conclusively that the isomeric forms of cellulases and xylanases, observed in several microorganisms, are the consequence of large multigene families and are not the result of processing of a single gene product. For example, at least 21 endoglucanase, three exoglucanase, six xylanase, and two β-glucosidase genes, and one xylan esterase gene, have been isolated from *C. thermocellum* (Hazlewood *et al*., 1988, Sakka *et al*., 1989; Hayashi *et al*., 1997). Likewise, multiple cellulase and xylanase genes have been isolated and characterized from strains of *Ruminococcus, Pseudomonas, Cellulomonas, Bacillus, Fibrobacter* and *Butyrivibrio*, etc. (Gilkes *et al*., 1991a; Flint and Zhang, 1992; Gilbert and Hazlewood, 1993; Hazlewood and Gilbert, 1998b–d). Furthermore, multiple cellulase and xylanase gene products have been characterized and their structural and functional differences have been demonstrated. Even though it is clear that multiple forms of cellulases and xylanases are the products of different genes, a significant role for post-translational modification in the production of some cellulase and xylanase isoforms cannot be completely ruled out.

**Adsorption**

Adsorption of cellulases on to cellulose not only facilitates physical contact between enzyme and substrate, but also plays an important role in the efficiency of the enzymatic hydrolysis of crystalline cellulose (Klyosov, 1990). Cellulases that adsorbed to a greater extent showed an increase in the rate and extent of crystalline cellulose hydrolysis when compared with cellulases that adsorbed less strongly (Klyosov, 1990). Moreover, the ability of endoglucanases to adsorb on to crystalline cellulose varies significantly. For example, an endoglucanase from *Aspergillus foetidus* showed weak adsorption on to cellulose, whereas that from *C. thermocellum* adsorbed very strongly on to cellulose (Klyosov, 1990). The discovery of CBDs in some cellulolytic enzymes explains in part the differences in adsorption ability of cellulases (Gilkes *et al*., 1991b). However, a correlation between the hydrophobicity of endoglucanases and their ability to adsorb on to cellulose has also been reported (Klyosov, 1990). A study of the adsorption of 12 cellulase preparations and their ability to hydrolyse amorphous and crystalline cellulose revealed that a 20-fold increase in the adsorption capability of endoglucanases does not change the rate of hydrolysis of CM-cellulose, but increases the rate of hydrolysis of amorphous and crystalline celluloses by twofold and 50-fold, respectively (Klyosov, 1990). More convincingly, Wood and co-workers (1989) demonstrated that a mixture of CBHs I and II from *P. pinophilum* significantly synergized and effected extensive hydrolysis
of crystalline cellulose when mixed with strongly adsorbed endoglucanases III and V from the same source, but not in the presence of weakly adsorbed endoglucanases I, II and IV.

Architecture and classification

The presence of separate catalytic and cellulose-binding domains in cellulases was first demonstrated by proteolytic cleavage of cellulases from *C. fimii* and *T. reesei* (Tomme *et al*., 1988; Gilkes *et al*., 1991b). Typically the two domains are linked by a highly glycosylated amino acid sequence (six to 59 residues), rich in proline and hydroxyamino acids. Some linkers are relatively rich in either Asp or Glu, or both. The occurrence of this type of cellulase architecture has been extensively reviewed by Tomme *et al*. (1995b). It was subsequently shown that many xylan-degrading enzymes are also modular in structure. For example, in *Pseudomonas fluorescens* subsp. *cellulosa*, four xylanases, an arabinofuranosidase and an acetylxylan esterase all have modular architecture and, like the cellulases from this organism, all contain both catalytic and non-catalytic CBDs, joined together by serine-rich linkers or hinges (Hazlewood and Gilbert, 1998a,c,d). Moreover, a similar pattern of architecture has been reported for xylanolytic enzymes from *R. flavefaciens*, *C. fimii* and *Cellvibrio mixtus* (Flint *et al*., 1993; Millward-Sadler *et al*., 1994, 1995). Although the majority of polysaccharide-binding domains mediate specific binding to cellulose, binding domains with specificity for xylan have been described in xylanases from *C. fimii* (Black *et al*., 1995) and *T. fusca* (Irwin *et al*., 1994). Other interesting non-catalytic domains described in modular xylanases include thermostabilizing domains and the so-called Nod B domains. Thermostabilizing domains have been found in xylanases from thermophilic bacteria and appear to confer stability in a broad sense on the enzymes that contain them. Nod B domains occur in xylanases from *P. fluorescens*, *C. mixtus* and *C. fimii* and have the capacity to release acetyl groups from acetylated xylan (Hazlewood and Gilbert, 1998a).

Cellulose-binding domains

The CBDs of cellulases and xylanases from a wide range of microorganisms have been extensively characterized (Tomme *et al*., 1995b; Linder and Teeri, 1997). Based on their amino acid sequences, CBDs have been classified into more than 10 families (Tomme *et al*., 1995b). Most of the CBDs characterized so far belong to families I, II and III, while the remaining families have a few or only one member in each family. Family I contains fungal CBDs of 30–40 amino acid residues; the best characterized are from CBH I, CBH II, and endoglucanases II and III from *T. reesei*. All other families contain bacterial CBDs, which are much larger in size than family I CBDs. Family II CBDs are found almost exclusively at either the N or C terminus of the enzyme and consist of typically 95–108 amino acids. The well-characterized CBDs of this family are from Cex, CenA and CenB from *C. fimii*, CelA, CelB, CelC, XylA, XylB, XylC and XylD from *P. fluorescens* subsp. *cellulosa* (Tomme *et al.*,...
1995c). Family III CBDs are found in cellulosome-producing bacteria, namely *C. thermocellum* and *C. cellulovorans*, and contain 132–172 amino acids (Tomme et al., 1995b). Based on amino acid sequence differences and other properties of family III CBDs, Bayer et al. (1998) proposed three subfamilies called IIIa, b and c. Family IIIa includes scaffoldin CBDs from *C. thermocellum*, *C. cellulovorans* and *C. cellulolyticum*; family IIIb includes the CBDs of free cellulases listed in family III, while family IIIc includes CBDs that lack cellulose-binding and anchoring residues.

Family IV CBDs are found in enzymes from *C. fimi*, *C. cellulolyticum* and other bacteria, and contain 125–168 amino acids. CBDs of families V (*E. chrysanthemi*, EgZ, 63 amino acids), VII (*C. thermocellum*, CelE, 240 amino acids) and VIII (*D. discoideum*, CelA, 152 amino acids) are the sole members of their families, while families VI, IX and X contain three to seven members with 85–90, 170–189 and 51–55 amino acids, respectively (Tomme et al., 1995b).

The three-dimensional structures of CBDs of families I (CBD of *T. reesei* CBH; Kraulis et al., 1989), II (CBD of *C. fimi Cex*; Xu et al., 1995) and IV (CBD of *C. fimi* β-1,4-glucanase; Johnson et al., 1996) have been determined by nuclear magnetic resonance (NMR), while those of families IIIa (CBD of *C. thermocellum* scaffoldin; Tormo et al., 1996) and IIIc (CBD of *T. fusca* cellulase E4; Sakon et al., 1997) were determined by X-ray crystallography.

Although there is an overall functional similarity between CBDs from different families, it is clear that they differ with respect to their affinity for different types of cellulose (Linder et al., 1995, 1996; Tomme et al., 1995c, 1996). Also, the presence of two successive CBDs in an enzyme is believed to result in synergistic binding and an increase in affinity for the ligand (Linder et al., 1996; Tomme et al., 1996). A number of approaches has been used to investigate the role of CBDs in cellulose and xylan hydrolysis, including truncation of enzymes, domain swapping and site-directed mutagenesis (Tomme et al., 1988, 1995b; Reinikainen et al., 1992; Black et al., 1997; Srisodsuk et al., 1997), but a unifying role for CBDs has yet to be established and their biological function continues to be a matter for conjecture. Although the removal of CBDs from endoglucanases and CBHs does not affect the activity of the respective enzymes against soluble substrates, there is evidence that CBDs play a major role in potentiating the activity of cellulases against the more resistant forms of insoluble cellulose (Tomme et al., 1988; Coutinho et al., 1993; Hall et al., 1995). Indeed, an isolated family II CBD from *C. fimi* CenA was shown to open the structure of crystalline cellulose, making it more accessible to enzyme attack (Din et al., 1991). The fact that CBDs occur with high frequency in xylan-degrading enzymes which have no activity against cellulose suggests that these domains potentiate catalytic activity of hemicellulases by promoting close contact between the enzymes and their native substrates in plant cell walls. Support for this view has come from a number of recent studies (Hazlewood and Gilbert, 1998b,c,d), X, including one which showed that the activities of a xylanase and an arabinofuranosidase from *P. fluorescens* subsp. *cellulosa*, against plant cell walls, were significantly enhanced by the presence of their CBDs (Black et al., 1997).
Catalytic domains

The catalytic domains of cellulases and xylanases are relatively large and represent more than 70% of the total protein. Sequence analysis of these domains from various cellulases and xylanases revealed extensive conservation of sequence and, using hydrophobic cluster analysis, the catalytic domains of cellulases and xylanases were initially grouped into 11 structurally related families (Henrissat et al., 1989). According to this classification, the members of a single family possess the same protein fold and display the same stereoselectivity (Gebler et al., 1992). This suggested that the enzymes of a given family would follow either retaining or inverting mechanisms (Table 2.3). Subsequently, the catalytic domains of some 1000 glycosyl hydrolases have been classified into 64 families (Henrissat and Bairoch, 1993, 1996; Henrissat, 1998). The cellulases and xylanases are contained in families 1, 3, 5–12, 26, 43–45, 60 and 61 (Henrissat and Davies, 1997; Davies, 1998; Henrissat, 1998). This sequence-based classification has been enormously beneficial in reflecting common structural features and evolutionary relationships, and as a tool for deducing mechanistic information.

Structure/function relationships

Crystal structures have been determined for more than 20 glycosyl hydrolases, including β-glucosidases, endoglucanases, exoglucanases and xylanases from families 1, 5–12 and 45. The main structural and functional properties of cellulases and xylanases studied so far are summarized in Table 2.4. Interestingly, members of families 1, 5 and 10 show a typical eightfold α/β barrel structure, commonly known as a TIM barrel, but these families include β-glucosidase, endoglucanase and xylanase, respectively (Table 2.4). Families 5, 8, 9, 12, 26 and 45 contain mainly endoglucanases, families 10 and 11 include only xylanases, while families 6 and 7 contain both endoglucanases and CBHs.

The catalytic domains of *T. reesei* CBH II and endoglucanase (E2) from *T. fusca* belong to family 6 and show a distorted α/β barrel structure (Rouvinen et al., 1990; Spezio et al., 1993). Although both these enzymes belong to the same family and possess a similar three-dimensional structure, there is a clear difference in their active site architecture, with an enclosed tunnel consisting of four glucose-binding sites in the case of CBH II, and an open active site in the case of the endoglucanase. The catalytic domains of CBH I from *T. reesei* and endoglucanase I from *H. insolens* belong to family 7 and show a similar β-jelly roll structure (Divne et al., 1994; Sulzenbacher et al., 1996, 1997). However, CBH I has an extended active site tunnel with seven glycosidic binding sites, while endoglucanase I from *H. insolens* shows a more open structure suitable for endo-action.

CelA from *C. thermocellum* belongs to family 8 and folds into an α6/α6 barrel consisting of six internal and six external helices (Alzari et al., 1996). A strictly conserved residue, Glu-95, at the centre of the substrate-binding cleft, serves as a proton donor. Three glucose-binding sites are identified on either side of the scissile glycosidic linkage. Furthermore, the subsites do not lie in a straight line, which forces
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Family</th>
<th>Organism</th>
<th>Structure</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Glucosidase</td>
<td>1</td>
<td><em>T. repens</em></td>
<td>Eightfold α/β-barrel with pocket type active site</td>
<td>Cleaves disaccharides from the non-reducing end</td>
<td>Barrett et al., 1995</td>
</tr>
<tr>
<td>Endoglucanase A</td>
<td>5</td>
<td><em>C. cellulolyticum</em></td>
<td>Eightfold α/β-barrel with groove type active site</td>
<td>Cleaves cellulose chain randomly</td>
<td>Ducros et al., 1995</td>
</tr>
<tr>
<td>Cel C</td>
<td>5</td>
<td><em>C. thermocellum</em></td>
<td>As above with an additional segment</td>
<td>As above</td>
<td>Dominguez et al., 1995</td>
</tr>
<tr>
<td>CBH II</td>
<td>6</td>
<td><em>T. reesei</em></td>
<td>Distorted (α/β) barrel with tunnel shape active site</td>
<td>Releases cellobiose from the non-reducing end and facilitates threading of cellulose chain through it</td>
<td>Rouvinen et al., 1990</td>
</tr>
<tr>
<td>Endoglucanase-E2</td>
<td>6</td>
<td><em>T. fusca</em></td>
<td>As above with an open active site</td>
<td>Randomly acting enzyme</td>
<td>Spezio et al., 1993</td>
</tr>
<tr>
<td>CBH I</td>
<td>7</td>
<td><em>T. reesei</em></td>
<td>β-Jelly roll with a long active site tunnel</td>
<td>Releases cellobiose from the reducing end of cellulose chain</td>
<td>Divne et al., 1994</td>
</tr>
<tr>
<td>Endoglucanase I</td>
<td>7</td>
<td><em>H. insolens</em></td>
<td>β-Jelly roll with a long substrate binding groove</td>
<td>Randomly acting enzyme</td>
<td>Sulzenbacher et al., 1996, 1997</td>
</tr>
<tr>
<td>Cel A</td>
<td>8</td>
<td><em>C. thermocellum</em></td>
<td>α/α Barrel – six internal and six external helices with groove type active site</td>
<td>Randomly acting enzyme with six glycosyl binding sites</td>
<td>Alzari et al., 1996</td>
</tr>
<tr>
<td>Cel D</td>
<td>9</td>
<td><em>C. thermocellum</em></td>
<td>α/α Barrel with two distinct domains, and extended active site</td>
<td>Randomly acting enzyme with six glycosyl binding sites</td>
<td>Juy et al., 1992</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td><em>T. fusca</em></td>
<td>Possess both family 9 catalytic domain with an (α/α) barrel fold, and a family III CBD, having an anti-parallel β-sandwich fold</td>
<td>Shows both endo- and exoglucanase activities with six glycosyl binding sites</td>
<td>Sakon et al., 1997</td>
</tr>
<tr>
<td>Enzyme Type</td>
<td>Organism</td>
<td>Characteristics</td>
<td>Function</td>
<td>References</td>
<td></td>
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<tr>
<td>Exoglucanase - CeX</td>
<td>C. fimi</td>
<td>Eight-parallel stranded α/β protein with an open cleft active site, and belongs to 4/7 superfamily</td>
<td>Releases cellobiose from the non-reducing end of cellulose chain and hydrolyses xylosidic and glucosidic linkages</td>
<td>White et al., 1994</td>
<td></td>
</tr>
<tr>
<td>Xylanase A</td>
<td>S. lividans</td>
<td>(α/β)8 Barrel with pocket type active site and belongs to 4/7 superfamily</td>
<td>Hydrolyses xylan and pNPC preferentially, and possesses six glycosyl binding sites</td>
<td>Derewenda et al., 1994</td>
<td></td>
</tr>
<tr>
<td>Xylanase A</td>
<td>P. fluorescens</td>
<td>(α/β)8 Barrel, belongs to 4/7 superfamily and with an unexpected Ca²⁺ binding site which stabilizes the catalytic core</td>
<td>Randomly cleaves xylan chain and possesses five glycosyl binding sites</td>
<td>Harris et al., 1994</td>
<td></td>
</tr>
<tr>
<td>Xylanase</td>
<td>B. circulans</td>
<td>β-Jelly roll with an overall structure of partially closed right hand</td>
<td>Randomly acting enzyme with five glycosyl binding sites</td>
<td>Wakarchuck et al., 1994</td>
<td></td>
</tr>
<tr>
<td>Xylanase</td>
<td>T. harzianum</td>
<td>As above</td>
<td>As above</td>
<td>Campbell et al., 1993</td>
<td></td>
</tr>
<tr>
<td>Xylanase I</td>
<td>T. reesei</td>
<td>As above</td>
<td>As above</td>
<td>Torronen and Rouvinen, 1997</td>
<td></td>
</tr>
<tr>
<td>Xylanase II</td>
<td>T. reesei</td>
<td>As above</td>
<td>As above but with four glycosyl binding sites</td>
<td>Torronen et al., 1994; Havukainen et al., 1996</td>
<td></td>
</tr>
<tr>
<td>Xylanase</td>
<td>T. lanuginosus</td>
<td>As above</td>
<td>As above but with seven glycosyl binding sites</td>
<td>Gruber et al., 1998</td>
<td></td>
</tr>
<tr>
<td>Endoglucanase (Cel B)</td>
<td>S. lividans</td>
<td>β-Jelly roll with anti-parallel β-sheets arranged in a sandwich fashion, and open active site</td>
<td>Randomly acting enzyme with five glycosyl binding sites</td>
<td>Sulzenbacher et al., 1997</td>
<td></td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>H. insolens</td>
<td>Six-stranded β-barrel with an open cleft active site</td>
<td>Releases cellobiose from the reducing end and possesses six glycosyl binding sites</td>
<td>Davies et al., 1993</td>
<td></td>
</tr>
</tbody>
</table>
the substrate to bend at the active site and thus requires lower energy for cleavage of
the glycosidic bond. CelD of *C. thermocellum* belongs to family 9 and has a globular
and slightly elongated shape with a small N-terminal β-barrel closely packed against a
large, mostly α-helical domain (Juy et al., 1992). An extended active site with
six glycosyl-binding sites has been suggested since it was possible to fit cellohexaose
into the active site. Recently, the three-dimensional structure of E4 from *T. fusca,*
belonging to the same family, has been determined (Sakon et al., 1997). This enzyme
contains both a family 9 catalytic domain with an α/α6 barrel fold, and a family III
CBD, having an antiparallel β-sandwich fold. In addition, this enzyme functions as
both endo- and exoglucanase and possesses an extended glycosyl-binding site, similar
to that of *C. thermocellum* CelD.

Xylanases from families 10 and 11 follow the retaining mechanism and possess
essential glutamates in their active sites. Family 10 xylanases show a typical eightfold
α/β barrel structure (Harris et al., 1994), while family 11 xylanases possess a β-jelly
roll architecture with two parallel β-sheets and an α-helix (Torronen and Rouvinen,
1997; Pickersgill et al., 1998). Family 12 endoglucanase from *Streptomyces lividans*
shows a β-jelly roll topology with antiparallel β-sheets arranged in a sandwich
manner, whereas the family 45 endoglucanase from *H. insolens* has a six-stranded
β-barrel structure (Davies et al., 1993; Sulzenbacher et al., 1997). The family 12
enzyme possesses five glycosyl-binding sites and cleaves the glucan chain randomly,
while the family 45 enzyme has six glycosyl-binding sites and attacks between the −1
and +1 subsites at the reducing end of the cellulose chain.

Applications

In addition to their established use as animal feed additives, cellulases and xylanases
have a wide range of applications in food, paper and pulp, textile, fuel and chemical
industries (Table 2.5) (Mandels, 1985; Gilbert and Hazlewood, 1993; Beguin and
Aubert, 1994; Bhat and Bhat, 1997). In a wider context, these enzymes have
potential uses in waste management, pollution treatment, protoplast production,
genetic and protein engineering as well as other research and development
programmes (Table 2.5) (Beguin and Aubert, 1994; Bayer et al., 1995; Bhat and
Bhat, 1997). Thus, cellulases and xylanases have a wide range of potential
applications, but further research is necessary to exploit the commercial potential
of these enzymes to the fullest extent.

Conclusions

Basic and applied research carried out during the past decade has shown that
cellulases and xylanases are of great intrinsic interest, and have a range of novel
and valuable uses in biotechnology. Early biochemical studies of purified enzymes,
coupled with more recent molecular studies utilizing recombinant DNA tech-
nologies, have provided detailed information on the biochemistry, architecture,
### Table 2.5. Applications of cellulases and xylanases.

<table>
<thead>
<tr>
<th>Industry and research</th>
<th>Enzyme</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Food, brewery and wine</td>
<td>Cellulases</td>
<td>Extraction and clarification of fruit juices and oil from seeds; increasing the soaking efficiency and homogeneous water adsorption of cereals; improving the digestion of ball-milled lignocellulose and the nutritive quality of fermented foods; production of cello-oligosaccharides and sugar derivatives; releasing flavours, enzymes and proteins; improving the filtration of beer and increasing the aroma of wines (Coughlan, 1985; Mandels, 1985; Beguin and Aubert, 1994; Caldini et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Xylanases</td>
<td>Clarification of fruit juices and wines; production of food thickeners; improving the texture and quality of bakery products, production of xylo-oligosaccharides, xylitol (artificial sweetener) and sugar derivatives (Maat et al., 1992; Viikari et al., 1993)</td>
</tr>
<tr>
<td>2. Animal feed</td>
<td>Cellulases and</td>
<td>As a feed supplement for poultry, pigs and ruminants; pretreatment of forages; dehulling of cereal grains; treatment of silage (Bedford and Classen, 1992; Selmer-Olsen et al., 1993; Viikari et al., 1993; Lewis et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>xylanases</td>
<td></td>
</tr>
<tr>
<td>3. Paper and pulp</td>
<td>Xylanases</td>
<td>Pre-bleaching of kraft pulps and debarking; refining pulp fibres and preparation of dissolving pulps (Wong and Saddler, 1992, 1993; Viikari et al., 1993; Ryan et al., 1998)</td>
</tr>
<tr>
<td>4. Textile</td>
<td>Cellulases</td>
<td>Biostoning (removing excess dye from the denim fabric in pre-faded jeans), use in washing powders for retaining the originality of cotton fabrics after several washing cycles by removing the cellulose microfibrils as well as restoring the softness and colour brightness of cotton fabrics (Mandels, 1985; Beguin and Aubert, 1994)</td>
</tr>
<tr>
<td>5. Waste management</td>
<td>Cellulases and</td>
<td>Treatment of agricultural and municipal wastes; composting and enzymatic processing of industrial pollutants (Rivart et al., 1994; Philippidis and Smith, 1995; Scott et al., 1995; Bhat and Bhat, 1997)</td>
</tr>
<tr>
<td></td>
<td>xylanases</td>
<td></td>
</tr>
<tr>
<td>6. Research and development</td>
<td>Cellulases and</td>
<td>Production of protoplasts and hybrid strain; genetic engineering; affinity purification; understanding the plant cell wall structure and chemistry (Beguin and Aubert, 1994; Bayer et al., 1995; Bhat and Bhat, 1997)</td>
</tr>
<tr>
<td></td>
<td>xylanases</td>
<td></td>
</tr>
</tbody>
</table>
catalytic mechanisms and structure–function relationships of these important enzymes. Two clear models have emerged for native cellulase–hemicellulase systems. In the first model, exemplified by aerobic fungi and bacteria, individual cellulases and hemicellulases are secreted freely by the host, without aggregating, and interact synergistically to degrade their target substrates in plant cell walls. Anaerobic bacteria, and apparently also anaerobic fungi, exhibit an alternative mechanism for cellulase/hemicellulase hydrolysis in which the individual enzymes involved form an ordered multienzyme complex or cellulosome. In both models, individual enzymes are typically modular in structure and, in addition to one or more catalytic domains, contain non-catalytic functional domains, the most common of which is a cellulose-binding domain that enables the enzyme to cluster on the surface of cellulosic substrates. Structural studies of single enzymes have resulted in high resolution crystal structures for representatives of more than 20 glycosyl hydrolase families, and the identification of key catalytic residues.

Notwithstanding the progress of recent research, there are still many unanswered questions regarding: (i) the biological significance and prevalence of multiple domains in cellulases and xylanases; (ii) the relative efficiencies of the multienzyme and disaggregated cellulase/hemicellulase systems; and (iii) the roles of individual enzymes during the solubilization of complex lignocellulosic substrates. Having the answers to such questions will not only enhance understanding of how cellulases and xylanases function, but should also enable these enzymes to be used more effectively as animal feed additives.

Acknowledgements

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References


**Characteristics of Cellulases and Xylanases**


Characteristics of Cellulases and Xylanases


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Enzymatic Characteristics of Phytases as they Relate to Their Use in Animal Feeds

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Introduction

Substantial quantities of phytic acid occur in plant-based diets fed to production animals throughout the world. Phytic acid is poorly digested by monogastric animals. Disposing of manure containing excess phytate-phosphorus can damage freshwater and other ecosystems. The poor digestibility of phytate-P increases the cost of production since additional sources of available P are needed in ration formulation to meet the nutritional requirements of the animal. Further, undigested phytate is known to have a negative impact on mineral and protein digestibility. In recent years considerable research has been directed toward understanding the process of phytate digestion and developing methods to improve phytate-P utilization by animals. The development of enzyme technology based on supplementing diets with sources of microbial phytase has proven to be a practical and effective method of improving phytate digestibility in animal diets.

Several excellent reviews have appeared covering the environmental and nutritional consequences of phytic acid (Cheryan, 1980; Torre et al., 1991; Ravindran et al., 1995; Rickard and Thompson, 1997), and the application, structure and kinetic properties of phytase enzymes (Dvorakova, 1998; Liu et al., 1998). The purpose of this chapter will not be to revisit material that has been extensively covered in other publications. Rather, the intentions are to provide a general overview of the subject area in combination with a specific focus on factors that affect the efficacy of phytate hydrolysis and methods to reduce further or eliminate indigestible phytate in plant-based diets fed to production animals.
Phytic Acid in Animal Diets

Structure of phytic acid

Structurally, phytic acid consists of a fully phosphorylated myo-inositol ring (Fig. 3.1) that exists in a chair conformation in dilute solution (Johnson and Tete, 1969). The molecule contains a total of 12 proton dissociation sites. Six of these sites are strongly acidic, with pKa values approximating 1.5; three sites are weakly acidic, with pKa values of 5.7, 6.8 and 7.6; and the remaining three sites are very weakly acidic, with pKa values > 10 (Costello et al., 1976). The structure of the molecule is consistent with a high chelation capacity for multivalent cations. In solution, the stability of phytate–mineral complexes can be measured by the pH-drop method whereby metals displace H+ for binding to phosphate groups on the phytate molecule (Maddaiah et al., 1964; Vohra and Kratzer, 1965). In summarizing the results of several studies using the pH-drop method, Cheryan (1980) concluded that phytic acid readily forms complexes with multivalent cations, with Zn2+ forming the most stable complexes followed by Cu2+, Ni2+, Co2+, Mn2+, Ca2+ and Fe2+ in decreasing order of stability. The phytate–mineral complex can exist as either soluble chelates or as insoluble complexes that precipitate out of solution, depending on the concentrations of phytic acid and mineral and the pH of the solution (Cheryan, 1980).

Some information is available on the structure of soluble mineral–phytate complexes. Champagne et al. (1990) and Champagne and Fisher (1990) studied shifts in 31P NMR spectra of phytic acid in solution at pH 7 with a relatively low molar ratio of minerals to phytate. These workers proposed that, under the conditions used in their experiment, a single Zn2+ ion binds to the P5 phosphate groups and thereby forms a bridge between two molecules of phytate. In comparison, Ca2+ and Cu2+ showed more of a tendency to associate with the P4 and P6 phosphate groups and thus was likely to form soluble complexes by electrostatic binding within a single phytate molecule. Mineral–phytate precipitants formed as the molar ratio of mineral to phytate increased at neutral pH (Champagne et al., 1990).

Fig. 3.1. Structure of the fully deprotonated form of phytic acid (myo-inositol hexakis phosphate).
Insoluble phytate–mineral complexes tend to form at neutral and basic pH when the concentration of mineral exceeds the concentration of phytate. At medium pH < 5, phytate does not precipitate out of solution in the presence of Ca\(^{2+}\) (Grynspan and Cheryan, 1983) or Mg\(^{2+}\) (Cheryan et al., 1983) even at mineral : phytate ratios of 12 : 1. These results imply that one or more of the weak acid phosphate groups on the molecule has a higher affinity for protons than Ca\(^{2+}\) or Mg\(^{2+}\). In both of these studies, as the pH of the medium was increased, the mineral : phytate molar ratio required for phytate precipitation decreased. Under conditions of excess mineral at neutral pH, it was suggested that precipitates consisted of pentacalcium (Grynspan and Cheryan, 1983) or pentamagnesium (Cheryan et al., 1983) salts of phytate. At acidic pH, partial protonation of phytic acid will decrease net association of minerals to the molecule and thus prevent the formation of insoluble precipitates. As described later, the structure of phytic acid as affected by minerals and pH has profound effects on the process of phytate hydrolysis.

**Occurrence of phytic acid in plants**

Phytic acid serves as the storage form of P in plant seeds. Ravindran et al. (1995) provided an extensive review of the occurrence of phytic acid in a wide variety of plants and plant materials. Cereals (maize, barley, wheat, oats) and grain legumes (field peas, chickpeas) that are commonly used as feed ingredients all have similar phytate levels, approximating 0.25% of dry matter. In general, oilseed meals tend to have higher levels of phytate-P as indicated by the percentage of dry matter composition for soybean (0.39%), rapeseed meal (0.70%), cottonseed meal (0.84%) and sunflower meal (0.89%). On average, 70% of the total P in the feed ingredient is found as phytate-P.

In plants, phytic acid complexes K\(^{+}\) and Mg\(^{2+}\), and to a lesser extent Ca\(^{2+}\), to form phytin. Phytin is deposited within the proteinaceous matrix of membrane-bound protein vacuoles or protein bodies. Under the electron microscope, phytin is often visible within the protein bodies as insoluble globoïd crystal structures. In rice grains, globoïd crystals are composed of 67% phytic acid, 19% K, 11% Mg and 1% Ca on a dry weight basis (Ogawa et al., 1975). The size of the globoïd crystals is dependent upon the ratio of divalent cations/K in the protein bodies. Ratios that favour divalent cations result in large insoluble crystals (Lott et al., 1985). Legumes such as soybeans and peas have higher levels of K and thus smaller globoïd crystal structures and a proportionately greater amount of phytin as predominantly K-bound soluble forms of phytic acid (Lott and Ockenden, 1986). This soluble phytin tends to exist as phytin–protein complexes that are evenly dispersed within the protein bodies.

The location of phytin-containing protein bodies in the seed varies considerably between plants. In wheat and rice phytin is found primarily in the aleurone layer and the bran, while in maize phytin is concentrated within the endosperm (O’Dell et al., 1972; de Boland et al., 1975). Phytin tends to be distributed evenly throughout the
Effects of phytic acid in animal diets

Environmental effects
In the United States, agriculture is the leading source of contaminants that have a negative impact on water quality (Parry, 1998). An estimated 60% of river miles and 50% of lakes that are classified as impaired are negatively affected by agriculture (Daniel et al., 1998). Animal wastes are the primary source of P pollution from agricultural practices (Daniel et al., 1998). In areas of intensive livestock production, P added to land from animal waste can readily exceed the capacity of the crop to incorporate the mineral. This, in turn, can lead to excess P accumulation in the soil and the potential for runoff into lakes and rivers. Problems associated with agriculture and P pollution are greatest in areas of intensive livestock production with close proximity to freshwater systems. In The Netherlands there is a N, P and K imbalance and livestock production contributes over 80% of the excess minerals accumulating in the environment (de Boer et al., 1997). The manure problem in The Netherlands has led to legislation that limits mineral application to soils from animal wastes (de Boer et al., 1997).

Undigested phytate-P is the primary source of P in the manure. Any measures that decrease unavailable phytate-P in animal diets will be effective in decreasing the P load on the environment arising from animal production. Further, for mixed farming operations, decreasing the P content of swine manure may benefit net economic returns in areas that are generally not classified as environmentally sensitive. Bosch et al. (1998) determined that reducing manure P decreases the land area required for application based on crop P requirements which, in turn, decreases crop production costs.

Effects on mineral utilization
Undigested phytic acid is known to decrease mineral bioavailability. The reader is referred to the reviews of Cheryan (1980) and Torre et al. (1991) on the formation of mineral–phytate complexes and the effects of phytate on mineral digestibility. In general an inverse relationship exists between the phytate content of the diet and digestibility of multivalent cations. As previously described, phytate can complex with multivalent cations, forming insoluble complexes at neutral pH. These complexes appear to be resistant to the digestive–absorptive process and the net result is that a portion of the dietary mineral pool is unavailable to the animal.

In rats, Zn bioavailability is markedly affected by the phytate content of the diet (Forbes et al., 1984; Zhou et al., 1992; Saha et al., 1994). These results are consistent with in vitro work indicating a high stability of Zn–phytate complexes (Cheryan,
Other minerals, such as Ca, when added at increasing levels in the presence of a constant level of phytate, further reduced Zn bioavailability in rats (Ellis et al., 1982; Forbes et al., 1984). The mechanism of this effect is likely to involve increased formation of insoluble phytate–mineral complexes containing both Ca and Zn that are resistant to the digestive–absorptive process.

In humans, dephytinization of preparations of soybean protein isolates doubled the mean fractional Fe incorporation into red cells (Davidsson et al., 1994) and resulted in 2.3-fold increase in Mn absorption (Davidsson et al., 1995). Fe solubility under simulated physiological conditions in vitro was increased from 3 to 53% with dephytinization of wheat bran and from 5 to 21% with dephytinization of rye flour (Sandberg and Svanberg, 1991).

Han et al. (1994) compared the effects of IP₆ (phytic acid), IP₅, IP₄ and IP₃ on Fe and Zn uptake by the human adenocarcinoma cell line Caco-2 and found that mineral solubility and uptake rates decreased with the degree of phosphorylation of the inositol ring. Lower forms of inositol phosphates that are formed during the hydrolysis of the parent phytate have fewer mineral-binding sites, a diminished chelation capacity, and thus less of an effect on mineral bioavailability than is the case for phytic acid (Tao et al., 1986).

Interestingly, certain compounds with chelation capacity have been shown to increase mineral availability when included in plant-based diets fed to animals. Supplementation of Zn-deficient diets with various chelators such as EDTA promoted growth and prevented symptoms of Zn deficiency in chicks (Nielsen et al., 1966) and turkey poults (Vohra and Kratzer, 1964). Pectins characterized by a high degree of esterification and a low molecular weight are known to complex with minerals and have been shown to improve Fe solubility and absorption in rats (Kim and Atallah, 1993). Ascorbic acid and citric acid were shown to enhance Fe bioavailability in vegetarian diets (Anand and Seshadri, 1995) and ascorbic acid increased Fe bioavailability in soy-based infant formulas (Davidsson et al., 1994). A mechanism of competitive chelation could account for the effect of chelators on improving mineral bioavailability. In this model the chelator binds minerals and thereby decreases the pool of minerals forming insoluble phytate complexes. The mineral–chelator complex exists in a soluble form and thus could be absorbed intact or could release minerals to binding sites on the brush border membrane of the intestinal epithelium. The concept of competitive chelation as a mechanism for improved phytate hydrolysis will be developed in a later section of this review.

Effects on protein digestibility
The charged phosphate groups on phytic acid can form electrostatic associations with the terminal amino group on proteins or with the free amino group on lysine and arginine residues within protein molecules (Cheryan, 1980). In addition, phytate–mineral–protein complexes can form with multivalent cations acting as a bridge between phosphate groups on the phytate molecule and the terminal carboxyl group of proteins or free carboxyl group on aspartate and glutamate residues within protein molecules (Cheryan, 1980). In theory, protein in the phytate-bound form may be less susceptible to protease activity during intestinal passage. In addition, phytate
binding to proteins and minerals in the digesta has the potential to impair the activity of digestive enzymes. Phytate has been shown to inhibit trypsin activity (Singh and Krikorian, 1982; Caldwell, 1992) and in vitro starch digestion with human saliva (Yoon et al., 1983). The mechanism may involve mineral chelation and thus removal of cofactors required for optimum enzyme activity and/or the formation of less reactive phytate–enzyme complexes.

With a few notable exceptions, a review of the literature generally supports the concept that phytic acid does impair in vivo protein digestibility. Thompson and Serraino (1986) reported that phytic acid did not affect rapeseed protein digestibility and amino acid absorption. Chitra et al. (1995) found a negative correlation between phytic acid content and in vitro protein digestibility in genotypes of mung bean, urd bean and soybean. Phytic acid–protein interactions have been reported to decrease the availability of protein in legumes (Camovale et al., 1988). Complete dephytinization of a diet based on maize and soybean meal increased dialysable protein by 12–29% (Zyla et al., 1995). Dephytinization of soy-protein concentrate improved protein digestibility in fish (Storebakken et al., 1998). One could hypothesize that the effect of phytate on protein and amino acid digestibility might vary considerably between diets based on plant ingredients and the mineral status of the diet. Phytate that is largely in the form of insoluble mineral-bound complexes may be less active as an inhibitor of protein digestion. Clearly, interactions between phytate, minerals and proteins and effects of these interactions on protein digestibility are a complex issue that deserves further research.

Digestion and Utilization of Phytate

In diet formulation, plant P is often classified as available and unavailable and a figure of 30% is routinely assigned as an estimate of available plant P. This exercise is useful in that it permits a simple calculation of supplemental P that must be added to the diet to meet the nutritional requirements of the animal. However, an assumption that 30% of total plant P is available and thus 70% is unavailable is simplistic and, in many circumstances, may be grossly inaccurate. As described in detail by Ravindran et al. (1995), a review of the literature shows considerable variation in the digestibility of dietary P of plant origin. As an example, Nelson (1976) reported 0% phytate-P digestibility in chicks fed maize-based diets and 8% phytate-P digestibility with wheat-based diets. In comparison, other studies have shown that chicks can retain up to 60% of dietary phytate-P (Temperton and Cassidy, 1964a,b). Ballam et al. (1984) found that phytate hydrolysis ranged from 3 to 42% in the chick and was dependent upon the level of dietary Ca. Phytate-P retention values of 37–56% were reported for chicks fed suboptimal levels of non-phytate-P (Edwards, 1983).

Interestingly, phytate-P when added to the diets in acidic form or as a sodium salt is readily available to chicks and will support growth and bone mineralization to the same extent as P originating from dicalcium phosphate (Harms et al., 1962; Waldroup et al., 1964a). P originating from supplemental calcium phytate could support growth and bone mineralization at levels up to 0.2% dietary P and at a
Ca : P ratio of 0.8 : 1 (Waldroup et al., 1964b). In this study, increasing the dietary Ca : P ratio to 1.4 : 1 or 2 : 1 decreased the apparent utilization of P from calcium phytate (Waldroup et al., 1964). These studies provide strong support for the concept that the form and location of phytate within the plant material is an important factor defining phytate-P utilization.

Phytate digestibility is ultimately dependent upon the efficacy of hydrolysis of phytate prior to feeding and during digestive passage. Hydrolysis is dependent upon phytase activity originating from plant, animal or microbial sources. The efficiency of this hydrolytic process is dependent upon the total phytase activity in combination with other factors such as the form and location of phytin within the plant ingredient and the physical conditions of the reaction.

Sources of phytase activity

Dvorakova (1998) and Liu et al. (1998) provided extensive reviews of the structure and kinetic characteristics of phytase from microbial, plant and animal sources. There will be no attempt to elaborate further or to assemble an extensive review of this area here. The discussion in this chapter will be restricted to key points on what is known of phytase structure and kinetic function.

Phytases can be broadly categorized into 6-phytase and 3-phytase classes. This designation refers to the site of initial hydrolysis on the phytate molecule. The 6-phytases are commonly found in plants, while 3-phytases are produced by fungi (Dvorakova, 1998). The full sequence of hydrolysis of phytic acid down to myo-inositol has yet to be determined. However, it would appear that no single enzyme is capable of fully dephosphorylating phytic acid and thus a combination of phytase and non-specific phosphatase activities are involved in the process. No information is available as to the mechanism of phytate hydrolysis by the small intestinal phytase activity in animals.

Animal phytase

Often it is assumed, and frequently stated, that animals do not possess the capacity to hydrolyse phytic acid. However, a specific phytase activity has been demonstrated in the brush border membrane of the chick small intestine (Maenz and Classen, 1998). Earlier studies demonstrated phytate hydrolysis with small intestinal mucosal preparations from chick (Davies et al., 1970; Bitar and Reinhold, 1972; Davies and Motzok, 1972), human (Bitar and Reinhold, 1972; Iqbal et al., 1994), calf (Bitar and Reinhold, 1972) and rat (Bitar and Reinhold, 1972; Yang et al., 1991; Iqbal et al., 1994). The contribution of mucosal phytase to phytate-P utilization by animals is not known, but animals are known to utilize a portion of the total phytate-P with no supplemental phytase in the diet. Further, a substantial body of literature indicates that dietary supplementation with 1,25-dihydroxycholecalciferol improves phytate-P digestibility in poultry (Ravindran et al., 1995). Rats (Yang et al., 1991) and chickens (Davies et al., 1970) fed phosphorus-deficient diets show an increase in intestinal phytase activity and in the case of the rat this correlated with an increase in a 90 kDa
protein isolated from the intestinal mucosa (Yang et al., 1991). Taken together, these studies indicate that an intestinal phytase probably does contribute to the utilization of phytate-P and the activity of the enzyme may well be regulated by the mineral and vitamin status of the animal.

**Plant phytase**

Almost all plants contain some phytase activity, though the level of phytase and the significance of the enzyme in hydrolysing phytate within the seed varies considerably between plants. Eeckhout and De Paepe (1994) evaluated phytase levels in 51 feedstuffs used in Belgian feed mills and concluded that substantial phytase activity occurs in cereal grains such as rye, triticale, wheat and barley while all other feedstuffs, including soybean meal, contained marginal levels of phytase. Phosphorus availability in wheat fed to poultry ranged from 45 to 70% (Barrier-Guillot et al., 1996b). Barrier-Guillot et al. (1996a) measured phytase activity in 56 wheat samples grown in France in 1992 and found a substantial variation that ranged from 206 to 775 mU g\(^{-1}\). In a follow-up study (Barrier-Guillot et al., 1996b), the same group found a linear correlation between wheat phytase activity and P retention in broilers. Pointillart (1994) pooled results on plant P availability in pigs fed cereal-based diets. Availability ranged from less than 20% for diets with little phytase up to 60% for diets rich in plant phytase activity and a clear linear relationship was found between the plant phytase activity in the diet and P digestibility. Kemme et al. (1998) measured gastric degradation of phytic acid in pigs as 3% when fed a maize-based diet (91 phytase units kg\(^{-1}\)), 31% when fed a maize–wheat-based diet (342 phytase units kg\(^{-1}\)) and 47% when fed a wheat–barley-based diet (1005 phytase units kg\(^{-1}\)). Clearly, the high phytase activity found in wheat and barley is significant and does contribute to phytate digestibility. These studies argue against a simple value for available P based solely on the total and phytate-P content of the diet. As such, the potential exists to refine diet formulation to meet the P requirements of the animal based on a realistic estimate of available P in a given plant-based diet.

**Microbial phytase**

Enzymes with hydrolytic activity directed toward phytic acid are produced by a variety of microorganisms. Dvorakova (1998) listed 29 fungal, bacterial and yeast species known to produce a phytase activity. Of the 29 species listed, 21 produced an extracellular phytase. Strains of the filamentous fungi *Aspergillus niger* produce a high-activity extracellular phytase (Volfova et al., 1994). Currently, commercial phytase products are based on the phytase encoding gene originating from *A. niger*.

The enzymes produced by *A. niger* var. *ficusum* are by far the most extensively studied phytase. Originally a single activity was described with an optimum pH of 5.0 plus a secondary optimum pH at 2.5 (Van Hartingsveldt et al., 1993). Subsequently, a second distinct phytase from *A. niger* var. *ficusum*, with a pH optimum of 2.5 and no activity at pH 5.0, was described (Ullah and Phillippy, 1994). The pH 5 optimum enzyme has been termed PhyA and the pH 2.5 optimum enzyme has been termed PhyB. The amino acid composition of the unglycosylated *A. niger* var. *ficusum* PhyA indicates a molecular weight of 62 kDa (Ullah, 1988).
The protein is 27.3% glycosylated with a molecular weight of 84 kDa in the native form (Ullah and Gibson, 1987). PhyA has a high affinity for phytic acid with a $K_m$ that approximates 40 $\mu$M (Ullah and Gibson, 1987). Variations in temperature and pH were found to have little effect on the affinity of PhyA for phytic acid (Ullah, 1988). PhyA has a very low turnover number of 216 mol P formed per mol phytase sec$^{-1}$ at optimal conditions of pH 5.0 and 58°C (Ullah and Gibson, 1987). A comparison of amino acid sequences for the protein products of expression of cloned phyA (Van Hartingsveldt et al., 1993) and phyB (Ehrlich et al., 1993) genes revealed a 23.5% homology with four proposed catalytic sites on phyA and a single proposed catalytic site on phyB. These differences are likely to account for the multiple pH optimum reported for phyA and the single pH optimum of phyB.

As reviewed by Dvorakova (1998), the industrial importance of phytase from A. niger has fostered intensive investigation and numerous patent applications to develop cost-effective microbial expression systems for production of the enzyme. With simple gene amplification, overexpression is repressed by inorganic P (Van Hartingsveldt et al., 1993) and significant overexpression requires strong P-resistant promoters (Piddington et al., 1993). Effective expression systems for phytase cDNA originating from A. niger var. ficuum are currently described in a variety of microorganisms (Brocades, 1991, 1993; Berka et al., 1995a,b). Efficient hydrolysis of phytic acid down to myo-inositol and P requires phytase plus non-specific phosphatase activities. Expression systems for DNA sequences encoding for both phytase and acid phosphatase have been described (Alko, 1994; Panlabs, Alko, 1994).

Extracellular phytase enzymes from microbial sources are moderately heat stable. A step-wise increase in the temperature of the reaction medium from room temperature to 58°C resulted in a corresponding increase in the rate of phytate hydrolysis by A. ficuum phytase (Ullah and Gibson, 1987). Further increases in medium temperature resulted in a dramatic drop in activity, with no detectable phytate hydrolysis at 68°C (Ullah and Gibson, 1987). The temperature sensitivity of the enzyme must be considered in any processing situation that involves heat after enzyme application to the feed ingredient.

Thermostable phytase activities have been described from Bacillus sp. DS11 (Kim et al., 1998) and Aspergillus fumigatus (Pasamontes et al., 1997). The A. fumigatus phytase has a broad pH activity range and can withstand extreme temperatures of 100°C for 20 min or 90°C for 120 min (Pasamontes et al., 1997). The gene for the enzyme has been cloned and overexpressed in A. niger. Phytase from A. fumigatus has commercial potential in that it can withstand conditions of feed pelleting without significant loss of activity.

Factors affecting phytate hydrolysis during intestinal passage

Minerals

Several studies have shown that phytate-P digestibility varies with the mineral content of the diet. Increasing the level of dietary calcium results in a decreased phytate-P digestibility in rats (Nelson and Kirby, 1979), poultry (Scheideler and Sell,
1987) and pigs (Sandberg et al., 1993). Presumably, increasing the concentration of a multivalent cation such as calcium will increase the formation of insoluble mineral-bound phytin crystals, which may be resistant to hydrolysis by phytase activities. Mohammed et al. (1991) found that decreasing the calcium level in diets low in P from 1% down to 0.5% resulted in a 15% increase in phytate-P digestibility in chicks. Ballam et al. (1984) found similar results in that phytate-P digestibility in chicks was increased when dietary calcium levels were reduced from 1.0% to 0.85% of the diet.

Mineral binding may well have negative effects on phytate hydrolysis by endogenous phytase of animal or plant origin in the diet. In general, Ca : phytate ratios of greater than 2 : 1 are thought to reduce intrinsic phytate digestibility (Wise, 1983). In pigs, supplementing a diet based on barley, rapeseed meal and peas with 1.25% calcium carbonate decreased phytate digestibility (Skoglund et al., 1997). In a separate study with pigs, total gastrointestinal hydrolysis of phytate decreased with increasing levels of dietary calcium carbonate (Sandberg et al., 1993).

In vitro studies have demonstrated that minerals impair the efficiency of phytate hydrolysis by phytase of microbial origin. At neutral pH, Maenz et al. (1999) ranked Zn^{2+} >> Fe^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+} > Mg^{2+} in terms of potency as inhibitors of phytate hydrolysis by microbial phytase. Decreasing the pH resulted in a substantial drop in the inhibitory effects of minerals. These results are consistent with the model of pH-dependent mineral–phytate interaction whereby protonation of weak acid phosphate groups displaces minerals and thereby converts phytate from phytase-resistant mineral-bound forms to phytase-susceptible forms.

Other in vitro studies have demonstrated that minerals have varying effects on the rate of phytic acid hydrolysis by animal phytase. Maenz and Classen (1998) found that inclusion of 25 mM MgCl₂ doubled the rate of phytate hydrolysis by chick small intestinal brush border phytase. Rat mucosal phytase activity is increased 40% by low concentrations of Zn (Bitar and Reinhold, 1972). At Zn concentrations greater than 1 mM, hydrolysis of phytic acid by chick small intestinal brush border phytase was markedly decreased (Maenz and Classen, 1998). Animal brush border phytase is likely to have a requirement for minerals as cofactors for optimal activity. The activation of activity may be confounded by mineral binding to phytate and a decrease in the susceptibility of the substrate to hydrolysis.

**pH**

Very little information exists describing phytate hydrolysis during intestinal passage. In comparing pH conditions down the length of the gut one could speculate that the majority of phytate hydrolysis by dietary plant or microbial phytase will occur during gastric retention. The low pH in the glandular stomach of monogastric animals will favour protonation of the weak-acids phytate groups. As such, after consumption of a meal, one would anticipate substantial formation of partially protonated forms of phytate that are susceptible to hydrolysis during residence in the stomach or crop. The optimum pH value for phytase activity approximates 5, which is below the effective pH for the formation of phytase-resistant mineral-bound complexes. In the upper regions of the small intestine the pH of the digesta will increase and thus
favour the reformation of mineral-bound phytase-resistant forms of phytate. *In vitro*,
0.5 M Ca was ineffective as an inhibitor of microbial phytase at pH 5, while 0.005 M
Ca caused complete inhibition when the pH value of the medium was increased to
7.5 (Maenz *et al.*, 1999). The extent of hydrolysis during gastric residence may well
be limited by the amount of enzyme and the physical conditions of retention time,
pH, mineral concentration, temperature, moisture, mixing, etc., that influence reac-
tion rates. Kemme *et al.* (1998) determined average gastric phytate-P degradability in
pigs as 47% for wheat–barley-based diets (1005 plant phytase units kg\(^{-1}\)), 3% for
maize-based diets (91 plant phytase units kg\(^{-1}\)) and 47% for microbial phytase-
supplemented maize-based diets (1565 microbial phytase units kg\(^{-1}\)). The authors
evaluated the effect of diurnal variations in gastric retention time and pH of the
stomach. At optimum conditions of prolonged retention time, phytate degradation
approached 90% in the stomach of pigs fed diets with high levels of plant phytase.
They concluded that the efficacy of phytase degradation by plant and microbial
sources of phytase is to a large extent determined by conditions of pH and retention
time in the stomach.

A model of phytate hydrolysis by microbial and plant phytase occurring
primarily in the stomach is largely hypothetical. Research quantifying phytate
hydrolysis down the full length of the gut with various dietary constituents and levels
of dietary phytase is needed for a better understanding of the physiology of lumenal
phytate hydrolysis. In the caecum, bacterial populations may provide an additional
source of phytase activity, but the significance of gut bacteria in the process of
phytate hydrolysis is unknown.

The process of phytate hydrolysis by mucosal phytase found on the brush border
membrane of the small intestine of the animal is distinct from lumenal hydrolysis by
plant and microbial phytases. The mucosal enzyme is fixed on the membrane and
the microenvironment in the unstirred water layer immediately adjacent to the
brush border is maintained at a moderately acidic pH of 6 (Lucas, 1983) down the
length of the gut. The pH of the unstirred water layer approximates the pH optimum
of intestinal phytase activity and will be less than optimal for the formation of
phytase-resistant mineral complexes. As such, brush border phytase hydrolysis will
depend upon a combination of the degree of enzyme expression and substrate access
to the binding site on the enzyme within the unstirred water layer. In broiler chicks
and laying hens the specific and total brush border phytase activity was highest in the
duodenum and decreased progressively down the length of the small intestine
(Maenz and Classen, 1998). The contribution of brush border phytase to the process
of phytate hydrolysis may well be limited by poor mixing of insoluble mineral-
phytate complexes relative to soluble nutrients in the digesta.

**Temperature, duration, moisture and mixing**
The rate of any enzymatic reaction is affected by the physical conditions of the reac-
tion mixture. *In vitro*, the activity of microbial phytase near the body temperature of
the animal is less than half of that obtained under optimal temperatures conditions of
55°C (Ullah and Gibson, 1987). Insoluble mineral-bound phytin associated with the
fibrous portions of the seed is likely to require prolonged extensive mixing in a slurry
with excess water to optimize enzyme access to the substrate. Physical conditions of the digesta, especially in the small and large intestine, may well limit the efficacy of lumenal phytate hydrolysis.

Improving Phytate Digestibility

Decreasing plant phytate levels

Selecting for low-phytate plants
A viable low-phytate mutant of maize (lpa1-1) has been described by Ertl et al. (1998). When compared with conventional maize, this mutant is characterized by a similar total P content, a 60% reduction in phytate, and a corresponding molar increase in the inorganic P content of the seed. The inorganic P in the lpa1-1 mutant maize supports growth and bone mineralization in broiler chicks. The lpa1-1 mutant has the potential to provide genetic stock for production of low-phytate maize with acceptable agronomic traits. Given the success obtained with low-phytate maize, development of other low-phytate plants with higher levels of available P for animal feeding can be envisaged.

Germination of plants to induce phytase activity
The function of plant phytase is to convert phosphorus from the storage form as phytate-P to readily available inorganic phosphate during development and growth of the plant. Germination is associated with increased phytase activity, decreased phytate and increased inorganic phosphate in canola (Lu et al., 1987), rapeseed (Mahajan and Dua, 1997), triticale (Niziolek, 1995) and soybean (Bau et al., 1997). For some feed ingredients a short period of controlled germination prior to further processing has potential as a practical and effective method of improving plant P availability.

Maugenest et al. (1997) cloned the DNA encoding for the phytase expressed in germinating maize seedlings. Further isolation and characterization of associated regulatory genes may eventually allow for controlled expression of phytase in plants.

Steeping to reduce phytate levels
Steeping (water soaking) a barley–rapeseed cake–pea-based diet for 9 h caused a 50% reduction in phytate levels and a corresponding increase in lower-level inositol phosphates and free inorganic phosphate (Skoglund et al., 1997). The authors speculate that prolonged soaking of the meal provided conditions whereby a substantial portion of the phytate was susceptible to hydrolysis by plant phytase.

Enzyme pretreatment of plant meals
Pretreatment of plant meals with microbial phytase under controlled conditions has potential as an alternative method of achieving efficient and measurable hydrolysis of phytase. Thus far, published work on controlled dephytinization during meal
pretreatment has been restricted to the laboratory investigation of solid-state fermentation of plant meals with phytase-producing organisms and enzyme cocktail application under controlled conditions in vitro.

Solid-state fermentation involves inoculation of the plant meal with an extracellular phytase-producing organism, followed by batch incubation under controlled conditions to maximize growth of the organism, production of phytase and subsequent hydrolysis of phytic acid. Inoculation of canola meal with *A. ficuum* resulted in a massive increase in phytase, a linear decrease in phytic acid and complete elimination of phytate after 144 h of incubation (Nair *et al*., 1991). The same research group further characterized the effects of phosphate, glucose and surfactants on the efficacy of solid-state fermentation of canola meal with *Aspergillus carbonarius* (Al-Ashed and Duvnjak, 1994a,b, 1995a,b) and *A. ficuum* (Ebune *et al*., 1995a,b). From this work, predictive models that relate biomass, enzyme production and reduction of phytate content to reaction conditions have been developed. Refinement of fermentation conditions has increased the efficiency of enzyme production such that under some conditions complete dephytinization of canola meal occurs within 48 h (Al-Ashed and Duvnjak, 1994a, 1995a; Ebune *et al*., 1995a,b). The economics and practicality of solid-state fermentation as a method for the commercial production of dephytinized plant meals has yet to be determined.

Batch dephytinization of plant meals and mixed feed using phytase-containing enzyme cocktails has the advantage over in-feed enzyme application in that the physical conditions of the reaction can be set to optimize the process of phytate hydrolysis. Zhu *et al.* (1990) achieved 81% phytate hydrolysis of a maize–soybean meal–wheat bran mixture using a two-step procedure that involved a 2 h initial conditioning of the maize–soybean meal with citric acid at pH 3.1 and a 2 h treatment with wheat bran at pH 5.1. Presumably citric acid acted as a competitive chelator to increase phytate susceptibility to hydrolysis by the wheat phytase during the second stage. Zyla *et al.* (1995a,b) developed a cocktail of commercial fungal phytase, acid phosphatase, citric acid and pectinase which, under simulated intestinal conditions of the turkey, resulted in complete dephytinization of a maize–soybean meal-based feed. Citric acid and cofactor enzymes probably functioned to improve substrate susceptibility to hydrolysis by the phytase and non-specific phosphatases in the cocktail. Dephytinization resulted in a 12–29% increase in dialysable protein and utilization of plant P for growth and bone mineralization in turkey poults (Zyla *et al*., 1995a, 1996). Zyla and Koreleski (1993) found that rapeseed meal could be dephytinized during a 4 h incubation at 40°C with fungal phytase and acid phosphatase in a 3 : 1 water : meal slurry at acidic pH. The practicalities of commercial batch dephytinization of plant feed ingredients are not known at present.

The cost of drying the meal after dephytinization of a high-moisture slurry presents a considerable obstacle to the application of the technology. However, if practical and cost-effective, batch dephytinization of plant meals has a distinct advantage over conventional in-feed enzyme application in that, by controlling reaction conditions and measuring inorganic P, it should be possible to produce a consistent, highly dephytinized feed ingredient.
Transgenic plants expressing foreign phytase activity

Transgenic tobacco (Verwoerd et al., 1995) and soybeans (Li et al., 1997) expressing phytase originating from *A. niger* have been developed. At present, transgenic plants expressing phytase are viewed not as a feed ingredient but as another potential method for manufacturing the enzyme. However, the potential does exist for developing transgenic plants with controlled expression of a foreign phytase that could be fed directly to animals. Cereal grains that do not undergo extensive heat treatment prior to diet formulation are the more likely targets for developing such transgenic plants.

Increasing phytate digestibility through in-feed supplementation

**Vitamin D**

Numerous studies have indicated that vitamin D (Mohammed et al., 1991), the active vitamin D metabolite 1,25-dihydroxycholecalciferol (Edwards, 1993; Mitchell and Edwards, 1996) and various active analogues of vitamin D (Biehl and Baker, 1997; Biehl et al., 1998) are effective in increasing phytate-P availability when added to diets fed to poultry. The magnitude of the effect of vitamin D on phytate-P digestibility varies considerably between studies and appears to depend upon the mineral content of the diet. Mohammed et al. (1991) measured phytate-P availability in broiler chicks as 50.1% for control diets, 58.5% for normal Ca, high vitamin D diets, and 76.5% for low Ca, high vitamin D diets.

The mechanism of phytate-P digestibility enhanced by vitamin D is poorly resolved. One of the physiological functions of the vitamin is to enhance Ca and P absorption. Increased Ca absorption could decrease Ca–phytate formation in the digesta, which could increase the efficacy of phytate hydrolysis (Ravindran et al., 1995). Vitamin D has been shown to induce chick intestinal mucosal phytase activity (Davies et al., 1970). In the same study, gut phytase activity was threefold greater in birds on P-deficient diets (Davies et al., 1970). As such, a part of the mechanism of vitamin D action in promoting absorption of dietary P may well involve activation of intestinal mucosal phytase. Mitchell and Edwards (1996) found that supplementation with vitamin D$_3$ and microbial phytase had an additive effect on increasing phytate utilization in broiler chicks and they suggested a separate mechanism of action. With both microbial phytase and vitamin D$_3$ in the diet, one could hypothesize increased lumenal and brush border hydrolysis of the substrate.

**Supplementation with microbial phytase**

Dietary supplementation with sources of microbial phytase is well established as an effective and practical method of improving phytate digestibility in production animals. Ravindran et al. (1995) summarized a large body of literature in stating that microbial phytase supplementation generally results in a 20–45% improvement in phytate-P utilization in poultry. A similar range for the effect of phytase supplementation on P availability has been reported for plant-based diets fed to pigs. Phytase supplementation of a barley–soybean meal diet resulted in a 40%
improvement in P absorption in comparison with a control diet and a 23% improvement in P absorption in comparison with a diet supplemented with dicalcium phosphate (Nasi and Helander, 1994). Harper et al. (1997) found a linear improvement in P digestibility with increasing phytase in maize–soybean meal-based diets with low dicalcium phosphate fed to growing pigs. In the same study, faecal P excretion was decreased by 21.5% with optimal phytase and low dicalcium phosphate (Harper et al., 1997). In weanling pigs, phytase supplementation is associated with a 50% increase in P retention (Lei et al., 1993; Pallauf et al., 1994) and a 42% decrease in faecal P (Lei et al., 1993). Similar improvements in P digestibility were reported for sows (Kemme et al., 1997a) and growing–finishing pigs (Kemme et al., 1997b) fed phytase supplemented tapioca–soybean-based diets. Bruce and Sundstol (1995) found that ileal P digestibility increased from 19.4% to 26.8% with phytase supplementation of an oat-based diet fed to growing pigs. In piglets, Mroz et al. (1994) reported that apparent ileal phytate digestibility increased from 31% to 79% with 800 units phytase kg⁻¹ of a maize–tapioca–soybean meal-based diet.

The efficacy of lumenal hydrolysis of phytate is affected by the mineral content of the diet. In turkeys, an increase in the dietary Ca : P ratio from 1.1 : 1 to 2.0 : 1 decreased the efficacy of microbial phytase as an enhancer of P digestibility (Qian et al., 1996). Increasing Ca levels from low (0.4%) to normal (0.8%) levels in low P maize–soybean meal-based diets markedly reduced the efficacy of microbial phytase in improving the performance of weanling pigs (Lei et al., 1994).

Not surprisingly, the negative effect of phytic acid on mineral digestibility is ameliorated by dietary supplementation with microbial phytase. Dietary supplementation with microbial phytase improved Zn utilization in rats (Rimbach and Pallauフ, 1993) and broiler chicks (Sebastian et al., 1996a). In pigs, phytase supplementation increased the apparent absorption of Mg, Zn, Cu and Fe (Adeola, 1995). Microbial phytase supplementation of maize–soybean meal-based diets improved Ca availability in poultry (Sebastian et al., 1996a,b). Increasing the Ca : P ratio while maintaining a constant level of phytase supplementation in maize–soybean meal-based diets correlated with a decrease in Ca retention (Yi et al., 1996a). This result is consistent with the formation of phytase-resistant insoluble mineral–phytate complexes as the mineral content the diet increases.

Overall, studies on phytase supplementation of diets fed to production animals tend to indicate improvements in protein and amino acid digestibility (Ravindran et al., 1995). Microbial phytase supplementation of maize–tapioca–soybean meal-based diets increased the ileal digestibility of crude protein, and most amino acids in pigs (Mroz et al., 1994). In turkey poult’s fed maize–soybean meal-based diets, microbial phytase tended to increase the ileal digestibility of N and most amino acids (Yi et al., 1996b). Phytase supplementation of maize–soybean-based diets had little effect on amino acid digestibilities in male broiler chicks but improved amino acid digestibility in females, with an optimum effect on low P/low Ca diets (Sebastian et al., 1997).

The improvement of plant P and Ca digestibility with microbial phytase supplementation of diets fed to production animals has led to the concept of defining microbial phytase as a dietary ingredient that contributes available P and Ca. Qian
et al. (1996) determined that 652 units of phytase kg\(^{-1}\) of maize–soybean meal-based diet was equivalent to 1 g of non-phytate P to turkey poults when the diet contained 0.27% non-phytate P, while 963 units where required to supply 1 g of non-phytate P when the diet contained 0.36% non-phytate P. With pigs, Yi et al. (1996a) found that 1146 units kg\(^{-1}\) of phytase provided 1 g of available P when added to soybean meal-based semipurified diets, while 785 units kg\(^{-1}\) provided 1 g of available P in maize–soybean meal-based diets. Harper et al. (1997) estimated that 500 units of phytase kg\(^{-1}\) of maize–soybean-based diets provided 0.87–0.96 g of available P to grower–finisher pigs.

Based on the efficacy of the enzyme in improving plant P and Ca availability in production animals, suppliers of commercially available sources of microbial phytase have developed a set of recommendations for the P and Ca feed equivalence of the enzyme. These recommendations are listed as levels of phytase required for a given age and species of animal that allow for a 0.1% reduction in available P and Ca levels in the diet. Recommendation for starter diets are in the order of 500 units kg\(^{-1}\) and considerably lower for grower and finisher diets. These guidelines are useful and provide some general indication of the sparing effect of phytase on the need for dietary supplementation with P and Ca. However, caution should be exercised in adapting these recommendations to a wide range of diets. As indicated in this chapter, the efficacy of microbial phytase will vary considerably with the ingredient composition of the diet. Maximum efficacy of phytase would be expected for a diet characterized by a high level of plant phytate in combination with low intrinsic (plant) phytase activity and a low multivalent mineral content. One could hypothesize that supplemental phytase is likely to be largely ineffective in a meat meal-based diet and of lower efficacy in a wheat-based diet with high intrinsic phytase. Clearly, research is needed to establish accurate recommendations for effective levels of phytase for specific types of diets.

**Supplementation with mineral chelators**

Chelating agents such as EDTA and EDTA-like compounds are known to increase Zn availability to chicks (Nielsen et al., 1966) and turkey poults (Vohra and Kratzer, 1964). Citrate and ascorbate improve Fe availability in vegetarian meals (Anand and Seshadri, 1995) and certain pectins improve Fe absorption in rats (Kim and Atallah, 1993). The mechanism of the effect of chelators on mineral absorption may involve competitive chelation whereby soluble mineral–chelates form in the digesta and thereby decrease mineral binding to phytic acid. In this model, minerals bound to chelators are readily absorbed either as intact complexes or after dissociation and re-binding to mineral-binding transport sites on the intestinal brush border membrane. In theory, chelators may well be effective in converting phytate from mineral-bound phytase-resistant forms to phytase-susceptible forms in the digesta. Indeed Zyla et al. (1995) found that citrate, when added to a phytase-containing cocktail, increased phytate hydrolysis of maize–soybean meal under simulated intestinal conditions. With pigs fed a maize–soybean meal-based diet, citrate tended to enhance the efficacy of phytase in promoting P and Ca digestibility (Li et al.,
Further investigation is required to determine the potential for chelators as dietary supplements to promote phytate digestion.

Summary and Conclusions

Application of commercial products containing microbial phytase activity to feed ingredients has proved to be a practical and effective method for improving phytate-P utilization by monogastric animals and thereby reducing P output in the manure. However, the process of phytate-P digestion is a complex issue and varies considerably with the ingredient composition of the diet and the physical conditions in the digesta. Feed ingredients such as wheat with high levels of intrinsic phytase are characterized by relatively high utilization of phytate-P in comparison with feed ingredients such as oilseed meals that contain low levels of intrinsic phytase. Multivalent minerals can complex with phytate at neutral pH and form phytase-resistant complexes. At present, oversimplistic guidelines are in place defining the available P content for a broad spectrum of feed ingredients. The opportunity exists to measure actual phytate-P availability for specific feed ingredients and diet formulations and to measure the true efficacy of microbial phytase with different feeding scenarios. This information can then be used for more precise diet formulation based on a true reflection of available P requirements. More precise diet formulation will improve the overall efficiency of dietary P utilization and thereby reduce P levels in the manure. Finally, methods of reducing the phytate content of the feed ingredient, such as developing low-phytate plants, controlled germination, or enzyme pretreatment of feed ingredients under controlled conditions in vitro, have the potential to produce feed ingredients characterized by low levels of phytate and high levels of readily available inorganic P.

References


Characteristics of Phytases


Introduction

Enzymes are added to animal feed to increase its digestibility, to remove anti-nutritional factors, to improve the availability of components, and for environmental reasons (Campbell and Bedford, 1992; Walsh et al., 1993). A wide variety of carbohydrase, protease, phytase and lipase enzymes find use in animal feeds. In monogastric diets, enzyme activity must be sufficiently high to allow for the relatively short transit time. Also, the enzyme employed must be able to resist unfavourable conditions that may be experienced in feed preparation (e.g. extrusion and pelleting) and that exist in the gastrointestinal tract. Measurement of trace levels of enzymes in animal feed mixtures is difficult. Independent of the enzyme studied, many of the problems experienced are similar; namely, low levels of activity, extraction problems, inactivation during feed preparation, non-specific binding to other feed components and inhibition by specific feed-derived inhibitors, e.g. specific xylanase inhibitors in wheat flour (Debyser et al., 1999).

In this chapter, some of the procedures used to assay for β-glucanase, β-xylanase, α-amylase, α-galactosidase, phytase and endo-protease enzymes will be described. In particular, some of the problems and limitations of current assay procedures will be discussed.

β-Glucanase

The anti-nutritional properties of β-glucans (1,3:1,4-β-D-glucans, mixed-linkage β-glucans) have been known for many years (Aastrup, 1979). In animal feeds the major source of β-glucan is barley grain. Although levels are also high in oats, these are rarely fed to chickens and pigs. The anti-nutritional properties of β-glucan are attributed to their viscosity-inducing properties, which significantly affect the rate of movement of barley-based diets through the digestive tract of chickens, and reduce the rate of nutrient absorption. This problem is effectively ‘dissolved’ by the judicious use of β-glucan-degrading enzymes.
A wide range of enzymes are active on β-glucan and these include the endo-acting fungal cellulases [endo-1,4-β-D-glucanase (EC 3.2.1.4)] and the bacterial β-glucanase [lichenase; endo-1,3 :1,4-β-D-glucanase (EC 3.2.1.73)]. Both groups of enzymes cleave within the main chain of mixed-linkage β-glucan, although their point of action is different (Fig. 4.1). Fungal cellulases also hydrolyse cellulose (1,4-β-D-glucan) in an endo-hydrolytic pattern, but the bacterial 1,3 : 1,4-β-glucanases have no action on cellulose. This point of difference is important in designing specific substrates for the assay of these groups of enzymes.

A range of substrates and assay procedures is available for the measurement of bacterial β-glucanase and cellulase enzymes, some of which are listed in Table 4.1. Bacterial 1,3 : 1,4-β-D-glucanase is assayed using substrates based on a 1,3 : 1,4-β-D-glucan polysaccharide, i.e. oat or barley β-glucan or lichenan. Procedures for the purification of barley and oat β-glucan have been developed and are reported in the literature (McCleary, 1988), and high purity polysaccharides are commercially available. However, the purification of lichenan is much more difficult. Lichenan is purified from Icelandic moss, but it occurs together with isolichenan (Chandra et al., 1957), from which it is hard to separate. Other glucose-containing polysaccharides (starch and cellulose) are absent from purified lichenan.

![Fig. 4.1. Schematic representation of the mode of action of cellulase and 1,3 : 1,4-β-glucanase on mixed-linkage β-glucan.](image)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nature</th>
<th>Assay procedure</th>
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</thead>
<tbody>
<tr>
<td><strong>Cellulase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley β-glucan</td>
<td>Soluble</td>
<td>Reducing-sugar or viscometric</td>
</tr>
<tr>
<td>Lichenan</td>
<td>Soluble</td>
<td>Reducing-sugar or gel plate</td>
</tr>
<tr>
<td>CMC-7M</td>
<td>Soluble</td>
<td>Reducing-sugar or viscometric</td>
</tr>
<tr>
<td>CMC-4M</td>
<td>Soluble/gel</td>
<td>Reducing-sugar</td>
</tr>
<tr>
<td>Azo-CMC</td>
<td>Soluble</td>
<td>Chromogenic substrate</td>
</tr>
<tr>
<td>Azo-barley glucan</td>
<td>Soluble</td>
<td>Chromogenic substrate</td>
</tr>
<tr>
<td>Cellazyme C tablets</td>
<td>Gel particles</td>
<td>Chromogenic substrate</td>
</tr>
<tr>
<td>Cellazyme T tablets</td>
<td>Gel particles</td>
<td>Chromogenic substrate</td>
</tr>
<tr>
<td>(tamarind xyloglucan)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-Glucazyme tablets</td>
<td>Gel particles</td>
<td>Chromogenic substrate</td>
</tr>
<tr>
<td><strong>1,3 : 1,4-β-Glucanase</strong></td>
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<tr>
<td>Barley β-glucan</td>
<td>Soluble</td>
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<td>Azo-barley glucan</td>
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</tr>
<tr>
<td>Beta-Glucazyme tablets</td>
<td>Gel particles</td>
<td>Chromogenic substrate</td>
</tr>
</tbody>
</table>
Reducing-sugar methods

Pure β-glucan (barley or oat) can be used directly to assay cellulase or β-glucanase employing either a reducing-sugar or viscometric assay procedure. Of the reducing-sugar procedures available, the only commonly used method that gives a stoichiometric colour response with homologous oligosaccharides of varying degrees of polymerization (DP) is the Nelson/Somogyi procedure (Somogyi, 1960). Consequently, it is the only method that gives a true measure of glycosidic bonds cleaved, and thus of enzyme activity. For this reason, this is the method of choice for use as a reference method against which all other assay procedures can be compared and standardized. Details of a suggested format for the assay of β-glucanase using barley β-glucan and the Nelson/Somogyi reducing-sugar procedure are given in Appendix 4.1.

Several cellulose-based substrates are available for the assay of endo-1, 4-β-glucanase (cellulase). These substrates range from highly crystalline cotton cellulose to amorphous cellulose and lightly or heavily substituted chemical derivatives such as carboxymethyl-cellulose 4M (CMC-4M) and CMC-7M. CMC-4M is a medium viscosity (300–600 cp at 2%, 25°C) cellulose with a degree of substitution with carboxymethyl groups of 0.4 (i.e. over ten sugar residues, only four of the 30 available hydroxyl groups are substituted by carboxymethyl groups). CMC-7M is completely soluble, whereas CMC-4M at 1% concentration in water is somewhere between a solution and a colloidal suspension. Many cellulase enzymes hydrolyse CMC-7M at the same rate as CMC-4M, but for some the rate is substantially lower. Consequentially, CMC-4M is the substrate of choice. Cellulase enzymes act on other polysaccharide substrates, such as mixed-linkage β-glucan and tamarind-seed xyloglucan (which is composed of a 1,4-β-D-glucan backbone, to which D-xylose is attached in a relatively regular manner, some of which is further substituted by D-galactose). Both of these polysaccharides serve as the base for excellent dyed substrates for the assay of cellulases (Table 4.1).

Traditionally, cellulase activity has been standardized using a reducing-sugar assay with either CMC-7M or barley β-glucan as substrate. In most cases the dinitrosalicylic acid (DNSA) reducing-sugar method (Bailey, 1988) was used with cellobiose as the standard. The DNSA method does not give a stoichiometric colour response with homologous oligosaccharides of varying degrees of polymerization; thus it is not ideal as a reference method. The Nelson/Somogyi reducing-sugar method (Somogyi, 1960) is preferable. Also, since some cellulase enzymes hydrolyse CMC-4M more rapidly than CMC-7M, then CMC-4M or barley β-glucan are the substrates of choice.

Viscometric methods

The Nelson/Somogyi reducing-sugar procedure (Somogyi, 1960) is an excellent method for the assay and standardization of relatively pure β-glucanase preparations, i.e. preparations essentially devoid of other enzyme activities active on the substrate,
and for preparations containing low levels of reducing-sugars (which interfere with the assay). However, the method cannot be applied to the measurement of β-glucanase in animal feed mixtures. For such materials, other procedures need to be employed, such as viscometric methods and methods based on the use of dyed polysaccharide substrates. Both procedures specifically measure endo-hydrolase activity and can be used in the presence of high levels of reducing sugars.

In industry, the most commonly used viscometric assay for polysaccharide endo-hydrolase activity is the Institute of Brewing procedure for the measurement of β-glucanase in malt (Bathgate, 1979). For reproducibility, accuracy and reliability, a β-glucan preparation of high purity and a defined viscosity range is essential. In principle, the enzyme preparation is mixed with buffered β-glucan solution (10 mg ml⁻¹) under controlled temperature conditions. The viscosity (as the time to flow between two marked points on a standardized type C U-tube viscometer) is measured at various time intervals after the addition of the enzyme to the substrate. The inverse reciprocal viscosity is calculated and plotted against time of incubation (see Appendix 4.2). The slope of the curve is a direct measure of enzyme activity. An alternative substrate is CMC-7M. This is completely soluble in water and is rapidly hydrolysed by some endo-cellulases. A procedure for the measurement of β-glucanase or xylanase in animal feeds, based on the Institute of Brewing IRVU method, is given in Appendix 4.2.

Agar plate diffusion procedures

Several semi-quantitative procedures have been developed for the assay of enzyme activity based on the rate and degree of diffusion of the enzyme through agar plates. The enzyme is applied in a central well and diffuses through agar containing a specific substrate. The level of activity is then monitored by staining the plate with a dye specific for the particular polysaccharide substrate. In one such procedure, Walsh et al. (1995) detected β-glucanase using an agar plate impregnated with lichenan, and activity was detected by staining with Congo Red. These procedures do work, but at best are semi-quantitative.

Soluble, dye-labelled substrates

The two general types of dye-labelled substrates available for the assay of cellulase and β-glucanase are soluble dye-labelled substrates and insoluble dyed substrates. The soluble dyed substrates include azo-CM-cellulose (azo-CMC) and azo-barley glucan (McCleary and Shameer, 1987). These substrates find widespread application in the assay of β-glucanase in feeds (Rotter et al., 1990; Cosson et al., 1999). In Fig. 4.2, a standard curve relating enzyme activity to colour released on hydrolysis of azo-CMC (CMC-4M dyed with Remazolbrilliant Blue R dye) is shown. The curves obtained for the two unfractionated commercial enzyme preparations and for a purified cellulase from a Trichoderma sp. are relatively similar. Differences can be attributed
to the range of activities in the crude preparations, as well as to subtle differences in the action patterns of the individual endo-cellulase enzyme components. This procedure has been adopted by the UK silage industry for the standardization of cellulase activity in silage additive preparations. The major limitation in the application of this procedure to measurement of cellulase activity in animal feed preparations is the lack of sensitivity. A second concern is the fact that the base substrate is CM-cellulose rather than \( \beta \)-glucan, which is the target substrate in animal feeds. The action of pure Trichoderma sp. cellulase on azo-barley glucan (barley glucan dyed with Remazolbrilliant Blue R dye) is shown in Fig. 4.3. The sensitivity with azo-barley glucan is about three times that with azo-CMC, but unfortunately is still not adequate for the measurement of trace levels of cellulase in animal feeds. However, it has been found that action on azo-barley glucan is a good predictor of in vivo response to cellulase supplementation of barley-based diets in young chickens (Rotter et al., 1990).

**Insoluble dye-labelled substrates**

Insoluble dyed substrates are prepared by dyeing and cross-linking soluble polysaccharides to give dyed cross-linked polysaccharide gel matrices. Such substrates are readily hydrolysed and include AZCL-HE-cellulose (Cellzyme C tablets), AZCL-\( \beta \)-glucan (Beta-Gluczyme tablets) and AZCL-xyloglucan (Cellzyme T tablets). Assays based on the use of these substrates are three to ten times more sensitive than assays using azo-barley glucan or azo-CMC. In Fig. 4.4, standard curves for

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**Fig. 4.2.** Standard curves for the action of cellulase preparations on azo-CMC (lot 60601): (A) a crude Trichoderma viride preparation; (B) a crude T. longibrachiatium preparation; (C) a pure cellulase from a T. longibrachiatium preparation.
Fig. 4.3. Standard curve relating the activity of a pure *T. longibrachiatium* cellulase on CMC-4M (Nelson/Somogyi assay) to action on: (A) azo-barley glucan (lot 60602) (using EGME/zinc acetate-based precipitant) and (B) azo-CMC (lot 60601) (using ethanol/zinc acetate-based precipitant).

Fig. 4.4. Standard curves relating the activity of a pure *T. longibrachiatium* cellulase on barley β-glucan (Nelson/Somogyi assay) to activity on: (A) Cellazyme T tablets (lot 70801); (B) Beta-Glucazyme tablets (lot 50901); and (C) Cellazyme C tablets (lot 80201).
Trichoderma sp. cellulase on dyed cross-linked HE-cellulose (Cellazyme C), dyed cross-linked β-glucan (Beta-Glucayzyme) and dyed cross-linked tamarind xyloglucan (Cellazyme T tablets) are compared. It is evident that, of the substrates compared, the Cellazyme T tablets give the greatest sensitivity, and thus are the substrate of choice for the detection and measurement of trace levels of cellulase in animal feed. The reason for this greater sensitivity must be due to the regular distribution of xylose residues along the cellulose backbone of the polysaccharide, compared with the irregular to random distribution of hydroxypropyl residues in HE-cellulose.

Measurement of cellulase and β-glucanase enzymes in feed is complicated by several factors, including inactivation of the enzyme during feed pelleting, adsorption of the enzymes to feed components, competition with endogenous substrate and possible enzyme inhibitors. These same problems are experienced in the assay of xylanase and protease enzymes in feed.

Endo-1,4-β-D-Xylanase (Xylanase)

The major endosperm cell-wall polysaccharide in wheat and rye grain is arabinoxylan, which represents about 2–5% of the grain weight. About two-thirds of wheat-flour arabinoxylan is water insoluble (Amado and Neukom, 1985). However, both the soluble and the insoluble components have high water-absorbing properties. Both can absorb about ten times their weight of water (Kulp, 1968). Consequently, arabinoxylans in feed cause problems similar to those experienced with β-glucans (Annison and Choct, 1991). The development and application of a range of xylanase enzymes have resolved these problems. Measurement of xylanase in feeds is complicated by the fact that the levels added are very low, and this enzyme can be lost through inactivation during pelleting, adsorption to feed components or inactivation by specific xylanase inhibitors (Debyser et al., 1999).

Several assay procedures have been developed for the assay of xylanase, and these include reducing-sugar assays, viscometric assays, and assays based on the use of chromogenic substrates (Table 4.2).

Reducing-sugar and viscometric assays

Pure wheat arabinoxylan forms the basis of both reducing-sugar and viscometric assays for xylanase. Reducing sugar assays are not specific and cannot be used for the measurement of xylanase in trace levels in feed mixtures. However, the Nelson/Somogyi reducing-sugar procedure (Somogyi, 1960) is the method of choice for the standardization of pure enzyme preparations. These enzyme preparations can then be used to standardize other assay procedures that use less defined substrates (e.g. the dyed xylan and arabinoxylan substrates) and assay procedures that are expressed in non-conventional units (e.g. viscometric assays). Viscometric assays are used in some laboratories, and the substrate generally employed is wheat arabinoxylan (20–30 cSt). Other xylan substrates (from oat and birchwood) are available, but the
viscosity of commercially available preparations is so low that they are of little use as substrates in viscometric assays. While viscometric assays allow specific measurement of endo-xylanase, the assay procedure is very tedious. With manual viscometric equipment (type C U-tube viscometer) a single operator can assay just five samples in 1 day. This compares with about five to ten samples by a single operator in 2 h with the newer chromogenic substrates.

### Soluble chromogenic substrates

Various dye-labelled substrates have been prepared for the assay of endo-1, 4-β-D-xylanase (xylanase), including dyed oat, birchwood and beechwood xylans and dyed wheat arabinoxylan (Table 4.2). In most cases, the dye used is Remazolbrilliant Blue. The reason for this is that it is possible to get good colour intensity with this dye; and because the dye is hydrophobic, it is easy to separate reaction products from unhydrolysed substrate, by alcohol precipitation. In the preparation of soluble dyed substrates for the measurement of xylanase there are several important considerations, including: (i) the final substrate must be readily hydrolysed by the enzyme, with good sensitivity and a good standard curve covering approximately one absorbance unit (and preferably linear); (ii) the substrate must be homogeneous and preferably completely soluble; (iii) the substrate must be specific for the measurement of xylanase, i.e. the base polysaccharide should have a xylan backbone and not be contaminated by other polysaccharides; and (iv) the substrate must form the base of a simple assay procedure.

The relative sensitivities of three soluble, chromogenic xylan substrates are compared in Fig. 4.5. Of the three, azo-wheat arabinoxylan is the most sensitive, and as such is the substrate of choice in the assay of trace levels of xylanase in animal feeds. This substrate is completely soluble and gives a good (essentially linear) standard curve over one absorbance unit.

In the measurement of xylanase in feeds, a further set of problems is experienced. The levels of enzyme added to the feed are usually low (just enough to depolymerize the endogenous arabinoxylan), which causes analytical problems, especially since some of this activity is lost due to pelleting or adsorption to feed components and

### Table 4.2. Substrates for the assay of β-xylanase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nature</th>
<th>Assay procedure</th>
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<tbody>
<tr>
<td>Wheat arabinoxylan</td>
<td>Soluble</td>
<td>Reducing-sugar</td>
</tr>
<tr>
<td>Wheat arabinoxylan</td>
<td>Soluble</td>
<td>Viscometric</td>
</tr>
<tr>
<td>Xylosezyme AX tablets</td>
<td>Gel particles</td>
<td>Chromogenic substrate</td>
</tr>
<tr>
<td>Azo-wheat arabinoxylan</td>
<td>Soluble</td>
<td>Chromogenic substrate</td>
</tr>
<tr>
<td>Azo-xylan (oat spelt)</td>
<td>Soluble</td>
<td>Chromogenic substrate</td>
</tr>
<tr>
<td>Azo-xylan (birchwood)</td>
<td>Soluble</td>
<td>Chromogenic substrate</td>
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</table>
some is inhibited by endogenous xylanase inhibitors. Assays are also complicated by the fact that the feed material contains arabinoxylan that acts as a competing substrate. Consequently, the analyst is left with a choice of either determining the directly measurable activity (i.e. a simple assay on feed extracts) or trying to estimate the level of active enzyme in the feed. In the latter case, it is essential to get some estimate of the degree of adsorption and inhibition of the enzyme. This can be achieved in one of two ways. In the first and preferable approach, if a sample of essentially identical non-enzyme-treated feed is available, a standard curve can be constructed by adding increasing quantities of enzyme to feed slurries to give final analytical values that cover the range of the activity extracted from the feed. Then, by reference to this standard curve, it is possible to calculate the actual amount of enzyme in the feed. Alternatively, and perhaps more realistically, recovery experiments can be performed. In this case, samples of non-enzyme-treated feed are not available. Instead, a sample of the feed is extracted with buffer, or with buffer to which set amounts of enzyme have been added. From the recovery of the added enzyme, it is possible to calculate the actual amount of enzyme in the original feed. Since this format can be used to estimate any enzyme in a feed mixture, it is described in detail in Appendix 4.3.
Insoluble chromogenic substrates

In an attempt to improve the sensitivity of chromogenic substrates for the assay of endo-xylanase, insoluble dyed substrates have been prepared by dyeing and cross-linking soluble xylan substrates, such as oat and birchwood xylans and wheat arabinoxylan (McCleary, 1995). Of these, the preferred starting material is wheat arabinoxylan, because it has a high molecular weight (which is preferable for cross-linking) and stable gel formation. Dyed, cross-linked arabinoxylan (AZCL-arabinoxylan) is available in tablet form (Xylazyme AX) (McCleary, 1992).

Assays for the measurement of xylanase using Xylazyme AX tablets are approximately three to four times more sensitive than assays employing azo-wheat arabinoxylan (Fig. 4.6). Thus, where enzyme activity is present in very low concentrations, Xylazyme AX tablets are the preferred substrate. However, in the use of these tablets, some problems are experienced that are not experienced in the use of azo-wheat arabinoxylan. The tablet substrate (AZCL-arabinoxylan) is quite sensitive to salt concentration. High salt concentrations affect the swelling of the gel particles which results in the substrate being less susceptible to hydrolysis. The effect of salt concentration on the rate of release of coloured products from Xylazyme AX tablets is shown in Fig. 4.7. To minimize this effect and maximize the sensitivity of the substrate, feed samples can be extracted with 0.1 M acetic acid (instead of 0.1 M sodium acetate buffer). The final pH in the extract must be in line with the pH activity profile of the enzyme under evaluation. The major limitation with this

Fig. 4.6. Standard curve relating the activity of pure A. niger xylanase on wheat arabinoxylan (Nelson/Somogyi assay) to action on Xylazyme AX tablets (lot 40602).
approach is that it is not possible to assay the pure enzyme in the extracting solution (0.1 M acetic acid), as this will inactivate the enzyme.

On the basis of the above findings, the preferred substrate for the measurement of xylanase in animal feeds is azo-wheat arabinxylan. However, if extra sensitivity is essential, then Xylazole AX tablets should be used.

**Enzyme-linked sorbent assays**

An enzyme-linked sorbent assay (ELSA) has been developed for the assay of β-glucanase and β-xylanase in animal feeds (Wong et al., 1999). This assay offers the advantage of greater sensitivity than any of the procedures previously described. Adoption of the method by industry will depend on numerous factors, including assay reproducibility, accuracy and reliability, which remain to be ascertained.

**α-Amylase**

Several methods are available for the measurement of α-amylase in pure enzyme preparations and in cereal flours or animal feeds. Procedures for assaying pure enzyme preparations usually employ soluble starch as substrate and measure the increase in reducing-sugar levels by either the DNSA (Bailey, 1988) or the Nelson/Somogyi (Somogyi, 1960) method. Traditional methods for the assay of α-amylase in cereal flours are based on the reaction of iodine with starch, or particularly the β-limit dextrin of starch (Sandsted et al., 1939; Farrand, 1964; European Brewing Convention, 1987). Hydrolysis of the starch results in a decrease in the purple-blue colour formed on reaction with iodine.

![Fig. 4.7. Effect of buffer salt concentration on the activity of *A. niger* xylanase on Xylazole AX tablets (lot 40602).](image-url)
Several newer and simpler procedures for the assay of \( \alpha \)-amylase are now available. Of these, the preferred method employs 'end-blocked' \( p \)-nitrophenyl maltoheptaoside as substrate (in the presence of a thermostable \( \alpha \)-glucosidase). The principle of this assay is shown in Fig. 4.8 (McCleary and Sheehan, 1987). The substrate is specific for endo-acting \( \alpha \)-amylase. On hydrolysis of the oligosaccharide chain by \( \alpha \)-amylase, the thermostable \( \alpha \)-glucosidase immediately hydrolyses the nitrophenyl-oligosaccharide fragment to glucose and \( p \)-nitrophenol. The thermostable \( \alpha \)-glucosidase (in place of an amyloglucosidase/yeast maltase mixture used in previous formulations) allows the reagent to be used in the pH range of 5.2–7.5, and at temperatures up to 60°C. The major advantage of this method over other methods is that the substrate is a defined oligosaccharide. With all other methods, the substrate is starch or modified starch, and thus is ill defined.

In situations where the levels of \( \alpha \)-amylase are very low, it may be necessary to employ a more sensitive substrate such as Amylazyme tablets (McCleary, 1991). This substrate is prepared by dyeing and cross-linking amylose to give gel particles that are dehydrated and incorporated into tablets. On hydrolysis by \( \alpha \)-amylase, soluble dyed amylase fragments are released into solution and the non-hydrolysed material is removed by filtration. Assays based on this substrate can be performed directly on feed slurries, allowing an increase in sensitivity of at least tenfold. These assays are

\[
\text{Blocked } p^{-}\text{nitrophenyl maltoheptaoside (BPNPG7)}
\]

\[
\begin{align*}
\text{ Blocked maltosaccharide} + p^{-}\text{nitrophenyl maltosaccharide} \rightarrow \\
\text{Thermostable } \alpha^{-}\text{glucosidase} \rightarrow \\
\text{Reaction stopped and yellow colour developed}
\end{align*}
\]

**Fig. 4.8.** Schematic representation of the assay of \( \alpha \)-amylase with Amylase HR reagent (Ceralpha method).
standardized in international units (U) of activity against either the Nelson/Somogyi reducing-sugar assay or the Ceralpha assay.

\textbf{α-Galactosidase}

α-Galactosidase enzymes act on galactosyl-sucrose oligosaccharides (raffinose, stachyose and verbascose) producing galactose and sucrose, which removes the anti-nutritional properties of these oligosaccharides (Gitzelmann and Auricchio, 1965; Liebmann, 1991). To be effective, these enzymes must rapidly hydrolyse galactosyl-sucrose oligosaccharides, and must be able to work under conditions of limited water availability.

α-Galactosidase is readily assayed with \( p \)-nitrophenyl \( α \)-D-galactoside (10 mM) as substrate. However, for a particular \( α \)-galactosidase, the relative rates of hydrolysis of \( p \)-nitrophenyl \( α \)-D-galactoside, raffinose, stachyose and verbascose should be determined to ensure that the enzyme being used is actually active on the component of interest. Activity of \( α \)-galactosidase on galactosyl-sucrose oligosaccharides can be determined using the particular oligosaccharide (10 mg ml\(^{-1}\)) and the Nelson/Somogyi reducing-sugar assay (Somogyi, 1960).

\textbf{Phytase}

Phytase is successfully used as a feed additive to improve phosphorus availability in feed. This enzyme is found in plant material and is produced by many microorganisms, especially moulds of the \textit{Aspergillus} type. Commercially available microbial phytases are non-specific phosphomonoesterases (EC 3.1.3.8) belonging to the group of acid phosphatases (Jongbloed \textit{et al.}, 1993). These enzymes catalyse dephosphorylation of myo-inositol hexakisphosphates in a step-wise manner, producing five classes of intermediate products (myo-inositol pentakis-, tetrakis-, tris-, bis- and monophosphates) of variable stereochemistry (Frolich \textit{et al.}, 1986). A schematic illustration of dephosphorylation of phytate is shown in Fig. 4.9.

Phytase activity in industrial enzyme preparations and animal feeds is usually assayed by measuring the amount of orthophosphates released from phytic acid.

\begin{center}
\textbf{Fig. 4.9.} Schematic representation of the hydrolysis of phytic acid by phytase.
\end{center}
within a period of linear release with time. One unit of phytase activity is defined as
the amount of enzyme required to release 1 \( \mu \text{mol} \) of orthophosphate from phytic acid
under standard assay conditions (pH 5.5, 37\(^\circ\)C) in 1 min. However, for general
acceptance of phytase enzyme as a feed additive, there is a need for validation of this
type of assay. To satisfy this need, a major international evaluation of an assay format
has been performed under the auspices of AOAC International. The results of these
studies are currently under evaluation (P. Randsdorp, personal communication,
1999).

**endo-Protease**

Several substrates and assay procedures are available for the measurement of protease.
Many of those based on the use of native proteins such as casein, albumin and
haemoglobin do not distinguish between *endo-* and *exo-*protease (peptidase) activity.
Assays based on trichloroacetic acid (TCA) precipitation of non-hydrolysed substrate
and measurement of the absorption (235 nm) of the supernatant solution on
centrifugation (Kunitz, 1947; AACC, 1985) are likely to be more specific than
procedures that measure the release of free amino groups (Lin *et al.*, 1969). However,
even TCA precipitation methods are not specific. A greater specificity can be
obtained with dye-labelled proteins (Charney and Tomarelli, 1947), or dye-labelled
and cross-linked proteins. Action of *exo*-acting peptidases on these substrates will be
hindered or stopped by the dye molecule, or at the point of cross-linking.

Several dyed protein substrates are commercially available, including albumin,
casein and collagen dyed with sulphanilic acid (azo-albumin, azo-casein and azo-
collagen), and collagen dyed with Remazolbrilliant Blue (Hide powder azure). These
are useful substrates and form the basis of simple assay procedures, but the quality of
commercially available substrates varies significantly, limiting their value as analytical
reagents. Standard curves for Subtilisin A on two commercially available azo-casein
substrates are shown in Fig. 4.10 and regression equations for several proteases on
one of the substrates is shown in Table 4.3 (McCleary and Monaghan, 1999). It is
evident that one of the preparations (ex. Megazyme) is more useful than the other,
with better sensitivity and linearity of the standard curve. This preparation is useful
in the assay of industrial enzyme preparations, but problems are experienced in the
measurement of enzymes in feed. Whether this is due to binding of the enzyme to the
feed, inhibition by endogenous protease inhibitors or competition by other protein
substrates is not clear.

An alternative *endo*-protease substrate, and one that finds considerable use, is
cowhide azure (basically collagen dyed with Remazolbrilliant Blue). This substrate
is very fibrous and difficult to weigh accurately. Consequently, an alternative
collagen-based substrate was prepared by dyeing and cross-linking collagen from the
swim bladders of fish. The structure of this collagen is very similar to that in cowhide.
The major difference in the final product is that the new product from fish collagen
(AZCL-collagen) can be milled to a fine powder and incorporated into tablets
(Protazyme OL). The suitability of this material as a general or selective substrate was
determined by comparing the activity of a wide range of proteases on this substrate to a similar material prepared from casein (AZCL-casein; Protazyme AK). The conclusion derived from this study was that a similar relative rate of hydrolysis of the two substrates was obtained for every protease tested. Consequently, AZCL-collagen had no particular advantage over AZCL-casein, and since the standard curves obtained with AZCL-casein (Protazyme AK tablets) were consistently more linear, we consider that this is the substrate of choice.

Assays with Protazyme AK substrate are four to five times more sensitive than those with Azo-casein, and the standard curves obtained for all of the proteases studied are linear (Table 4.4). However, the substrate tablets do not hydrate as rapidly as do tablets containing dyed and cross-linked polysaccharide substrates. Thus, for good reproducibility, the tube contents should be stirred during the

![Fig. 4.10. Standard curve relating the activity of Subtilisin A on casein to action on two commercially available azo-casein preparations: (A) Megazyme (lot 81001); (B) Sigma Chemical Co. (lot 74H7165).](image)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Protease (mU ml⁻¹)abc</th>
<th>Linear absorbance range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain (from Papaya latex)</td>
<td>270 × Abs. (440 nm) + 7</td>
<td>0.1–1.0</td>
</tr>
<tr>
<td>Bromelain (from pineapple stem)</td>
<td>460 × Abs. (440 nm) – 13</td>
<td>0.1–0.9</td>
</tr>
<tr>
<td>Ficin (from figs)</td>
<td>190 × Abs. (440 nm) + 3</td>
<td>0.1–1.1</td>
</tr>
<tr>
<td>Subtilisin A (from Bacillus licheniformis)</td>
<td>129 × Abs. (440 nm) + 4</td>
<td>0.1–1.0</td>
</tr>
<tr>
<td>Bacterial protease (from Bacillus subtilis)</td>
<td>250 × Abs. (440 nm) – 8</td>
<td>0.1–1.0</td>
</tr>
<tr>
<td>Proteinase K (from Tritirachium album)</td>
<td>140 × Abs. (440 nm) – 4</td>
<td>0.1–1.0</td>
</tr>
<tr>
<td>Fungal protease (A. niger; from Sigma Chemical Co.)</td>
<td>146 × Abs. (440 nm) – 4</td>
<td>0.1–1.0</td>
</tr>
</tbody>
</table>

*One protease unit is defined as the amount of enzyme that will produce the equivalent of 1 µmol tyrosine min⁻¹ from soluble casein at pH 7.0 and at 40°C.

*Abs. = absorbance.
reaction period. This can easily be achieved but it does make this assay a bit more complicated than that using Azo-casein.

Even with this greater sensitivity, it is still difficult to assay protease activity in feed mixtures accurately. Obviously, this area of analyses requires further input. The required sensitivity may be obtained with fluorimetric substrates but, even then, the apparent problems of adsorption of the enzyme to the feed and/or specific protease inhibitors will still need to be resolved.


Nelson/Somogyi reagents

1. **Reagent A.** Dissolve 25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartrate and 200 g of sodium sulphate in 800 ml of water and dilute to 1 l. Filter if necessary.
2. **Reagent B.** Dissolve 30 g of copper sulphate pentahydrate in 200 ml of water containing four drops of concentrated sulphuric acid.
3. **Solution C.** Dissolve 50 g of ammonium molybdate in 900 ml of water and carefully add 42 ml of concentrated sulphuric acid; separately, dissolve 6 g of sodium arsenate heptahydrate in 50 ml of water and add this to the above solution. Dilute the whole to 1 l. Warm to 55°C to dissolve completely (if necessary).
4. **Solution D.** Add 1 ml of reagent B to 25 ml of reagent A.
5. **Solution E.** Dilute solution C fivefold (50 ml to 250 ml) with distilled water before use (stable at 4°C for about 1 week).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Protease (mU ml⁻¹)ab (R = 0.99)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain (from Papaya latex)</td>
<td>76.2 × Abs. (590 nm) + 7.6</td>
</tr>
<tr>
<td>Bromelain (from pineapple stem)</td>
<td>304 × Abs. (590 nm) + 10</td>
</tr>
<tr>
<td>Ficin (from figs)</td>
<td>132 × Abs. (590 nm) + 3.5</td>
</tr>
<tr>
<td>Subtilisin A (from Bacillus licheniformis)</td>
<td>34.2 × Abs. (590 nm) + 0.6</td>
</tr>
<tr>
<td>Fungal protease (A. niger; from Quest International)</td>
<td>42.0 × Abs. (590 nm) + 2.9</td>
</tr>
</tbody>
</table>

aOne protease unit is defined as the amount of enzyme that will produce the equivalent of 1 µmol tyrosine min⁻¹ from soluble casein at pH 7.0 and at 40°C.

bAbs. = absorbance.
Substrates

Dissolve 1 g of pure barley β-glucan (20–30 cSt viscosity), CM-cellulose 4 M or wheat arabinoxylan (20–30 cSt) in 90 ml of hot deionized water. Cool to room temperature and add 5 ml of 2 M sodium acetate buffer (pH 4.6). Adjust the pH to 4.6 with 1 M sodium hydroxide or hydrochloric acid, and adjust the volume to 100 ml. Store in a well-sealed bottle at room temperature. Add two drops of toluene to prevent microbial contamination. If pH values other than pH 4.6 are required, use an appropriate buffer to obtain the correct pH at a final concentration in the substrate of 100 mM.

Procedure

1. Enzyme solution (0.2 ml) is incubated with pre-equilibrated substrate solution (0.5 ml) at 40°C for 0, 3, 6, 9 and 12 min. The reaction is terminated by adding 0.5 ml of solution D with vigorous stirring.
2. These tubes, along with reaction blank solutions (to which solution D is added before the enzyme) and glucose or xylose standard solutions (which contain substrate and glucose or xylose at 0–50 µg per tube) are incubated in a boiling-water bath for 20 min.
3. The tubes are cooled to room temperature and 3.0 ml of solution E is added. The tubes are stirred well (10 s on vortex mixer) and allowed to stand for 10 min before mixing again. Centrifuge (3000 rpm for 10 min) if necessary.
4. The absorbance of all solutions is measured at 520 nm against the reaction blank. A standard curve is prepared using appropriate amounts of glucose or xylose (0–50 µg).
5. Enzyme activity is calculated as µmol of glucose (or xylose) reducing-sugar equivalents released per minute ml⁻¹ or g⁻¹ of the original enzyme preparation.

Appendix 4.2 Measurement of β-Glucanase and Xylanase Activity by Viscometry

This method describes a viscometric assay for the measurement of endo-xylanase or β-glucanase in microbial preparations, animal feeds and bread improver mixtures.

Principle

The activity is measured by incubating suitably diluted enzyme extract with a solution of β-glucan or wheat-flour arabinoxylan (1% w/v, 20–30 cSt) in appropriate buffer (sodium acetate or sodium phosphate, 100 mM) and measuring the rate of decrease in viscosity at 40°C.
Unit definition

One inverse reciprocal viscosity unit (IRVU) is the increase in reciprocal viscosity \( h^{-1}\mathrm{ml}^{-1} \) (or g\(^{-1} \)) of enzyme (or plant material), under standard assay conditions.

Preparation of \( \beta \)-glucan or arabinoxylan substrate

1. Barley \( \beta \)-glucan (1 g, pure, 20–30 cSt) or wheat arabinoxylan (1 g, pure, 20–30 cSt) is accurately weighed into a 120 ml dry pyrex beaker.
2. The sample is wetted with 6 ml of 95% ethanol and then with 80 ml of cold water.
3. A magnetic stirrer bar is added to the beaker and the beaker is placed on a magnetic stirrer-hotplate and heated at a setting of 120°C with vigorous stirring. The beaker is loosely covered with aluminium foil and stirred and heated for about 15 min.
4. The polysaccharide should completely dissolve. If dissolution is not complete, continue stirring for a further 30 min with the heating turned off.
5. Add 10 ml of acetate buffer (1 M, pH 4.6) or 20 ml of phosphate buffer (0.5 M, pH 6.0) and adjust the pH to the desired value.
6. Adjust the volume to 100 ml and store in a well-sealed glass bottle. Prevent microbial contamination by adding a few drops of toluene. The solution can be stored at room temperature for several weeks.

Enzyme extraction and dilution

1. Plant samples are milled to pass through a 0.5 mm sieve on a Retsch centrifugal mill (or similar).
2. Accurately weigh 1.0 g of flour or powdered enzyme preparation into a 250 ml Erlemeyer flask and add 100 ml of appropriate extraction buffer; stir and extract over 15 min at room temperature.
3. Filter an aliquot of the slurry through a Whatman No. 1 filter circle, or centrifuge at 3000 rpm for 10 min. Store the filtrate in an ice bath.
4. Dilute the filtrate (or supernatant) with the appropriate buffer (by sequential dilution of 1 ml to 10 ml with dilution buffer) to give an enzyme concentration suitable for assay (i.e. a slope value ‘A’ of 0.06 to 0.60).
5. Prepare liquid enzyme samples by dilution of 1.0 ml to 100 ml with appropriate buffer. This solution is further diluted as for extracts of dry samples.

Viscometric assay of activity

1. Pre-equilibrate the enzyme preparation at 40°C for 5 min and then pipette 1 ml of this solution into 12 ml of pre-equilibrated, buffered substrate solution (wheat
arabinoxylan or β-glucan; 1% w/v) in a type C U-tube viscometer (in a water bath at 40°C), and mix the contents by blowing air into the viscometer tube. Immediately start a stop clock and leave this running throughout the entire assay to record incubation time (in minutes).

2. Using a second stop clock, take five falling time readings (in seconds) over a period of approximately 30 min. Take the time for each reading as the elapsed time from mixing the enzyme/substrate solutions to the mean of the falling time.

3. The viscosity ($n$) of the reaction digest is proportional to the falling number according to the following equation:

$$n_{\text{digest}} = \frac{(t_{\text{digest}} - t_{\text{solvent}})}{t_{\text{solvent}}}$$

where $n_{\text{digest}}$ = specific viscosity of the digest; $t_{\text{digest}}$ = falling time in seconds of the digest; and $t_{\text{solvent}}$ = falling time in seconds of the buffer (100 mM sodium acetate).

Calculations

The reciprocal viscosity ($\frac{1}{n}$) (Table 4.5) is calculated and plotted against incubation time (in minutes) (Fig. 4.11). The slope ($A$) is determined from the linear graph in terms of increase in reciprocal viscosity per hour.

$$\text{IRV units} = A \times 100 \times \text{Dilution}$$

where $A$ = slope from graph in terms of increase in reciprocal viscosity per hour; $100 = 1$ g of original enzyme preparation extracted with 100 ml of buffer (100 mM), or 1 ml of liquid enzyme preparation diluted to 100 ml with extraction/dilution buffer; and Dilution = further dilution of the extract or diluted liquid enzyme concentrate required to get an appropriate activity for assay.

Appendix 4.3 Extraction and Assay of Xylanase Enzymes in Animal Feeds

Example: *Trichoderma* sp. xylanase

<table>
<thead>
<tr>
<th>Incubation time (t) (min)</th>
<th>Time to flow (s)</th>
<th>Specific viscosity $n = (t - t_0)/t_0$</th>
<th>$1/n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>302.75</td>
<td>11.75</td>
<td>0.085</td>
</tr>
<tr>
<td>5</td>
<td>247.12</td>
<td>9.41</td>
<td>0.106</td>
</tr>
<tr>
<td>10</td>
<td>211.34</td>
<td>7.90</td>
<td>0.127</td>
</tr>
<tr>
<td>14</td>
<td>190.76</td>
<td>7.04</td>
<td>0.142</td>
</tr>
<tr>
<td>17</td>
<td>174.47</td>
<td>6.35</td>
<td>0.157</td>
</tr>
<tr>
<td>21</td>
<td>162.45</td>
<td>5.84</td>
<td>0.171</td>
</tr>
</tbody>
</table>
1. Mill a feed sample (approximately 100 g) to pass a 0.5 mm screen and mix thoroughly.
2. Weigh samples of the above feed (0.5 ± 0.01 g in quadruplicate) into glass test tubes (16 × 120 mm).
3. Treat each sample with 5 ml of 0.1 M sodium acetate buffer (pH 4.7) and stir on a vortex mixer. To two of these tubes, add water (0.2 ml) with stirring, and to the other two tubes add control *Trichoderma* sp. xylanase (0.2 ml, 1360 mU) with vigorous and immediate stirring on a vortex mixer.
4. Leave the slurries at room temperature with occasional stirring on a vortex mixer over the following 20 min.
5. Centrifuge tubes (3000 rpm, 10 min) in a bench centrifuge and use the supernatant directly. Assays should be initiated within 30 min of obtaining these extracts to minimize loss of enzyme activity.

**Assay**

1. Accurately transfer 0.5 ml aliquots of supernatant solutions (in duplicate) to glass test tubes (16 × 100 mm) at room temperature.
2. Add 0.5 ml of Azo-WAX to each tube and stir the tube vigorously. Immediately place the tube in a water bath set at 50°C ± 0.1°C and incubate for 30 min.
3. After exactly 15 or 30 min (depending on the level of enzyme activity), add 2.5 ml of industrial methylated spirits (IMS) and stir the tube vigorously on a vortex mixer. Store the tube at room temperature for 5 min. This treatment terminates the reaction and precipitates non-depolymerized dyed substrate.
4. Centrifuge the tubes at 3000 rpm for 10 min and measure the absorbance of the supernatant solutions at 590 nm against a reaction blank.

---

**Fig. 4.11.** Plot of reciprocal viscosity (1/\(n\)) against incubation time.
The reaction blank is prepared by adding 2.5 ml of IMS to a mixture of 0.5 ml of azo-wheat arabinoxylan and 0.5 ml of 0.1 M sodium acetate buffer (pH 4.6). The slurry is stirred and stored at room temperature for 5 min before centrifugation (3000 rpm, 10 min). A single reaction blank is required for each feed sample.

Calculation of activity

The level of xylanase in the flour sample is calculated as follows.

Activity in feed sample (0.5 g) = \( \frac{\text{Added activity} \times SA}{TA - SA} \)

where \( \text{Added activity} \) is the amount of xylanase added to the feed slurry at the time of assay (in the control xylanase solution; 0.2 ml) (e.g. 1360 mU); \( SA \) is the reaction absorbance obtained for extracts of the feed to which no control xylanase was added; \( TA \) is the total absorbance (i.e. the absorbance of extracts of the sample to which the control xylanase was added).

Example calculation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance (590 nm)/30 min incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Feed A</td>
<td>0.000</td>
</tr>
<tr>
<td>2. Feed A containing Trichoderma sp. xylanase (SA)</td>
<td>0.502</td>
</tr>
<tr>
<td>3. SA + 1360 mU xylanase (in the assay) (TA)</td>
<td>0.908</td>
</tr>
</tbody>
</table>

Activity in 0.5 g of feed A = \( \frac{\text{Added activity} \times SA}{TA - SA} \)

where \( SA \) = absorbance of extract of sample A assayed by the standard format (e.g. Abs = 0.502); \( TA \) = the total absorbance, i.e. the absorbance of extracts of sample A to which the additional xylanase (0.2 ml; 1360 mU) was added (e.g. Abs = 0.908).

Thus:

Activity in the feed = \( \frac{(1360 / 1000) \times 0.502}{(0.908 - 0.502)} \) (U per 0.5 g)

= 1.682 U.

U g\(^{-1}\) (or kU ton\(^{-1}\)) = 1.682 \times 2000 = 3363.

References


Introduction

On a world basis, maize is the most important cereal in livestock feeding. Having a high available energy content and a relatively low protein content, it has usually been looked on mainly as an energy source in a diet. However, since the levels used in poultry and swine diets can approach 70%, many diets have in excess of 20% of their protein content contributed by maize. Maize has always been considered to be a very uniform product with slight variations in protein and energy. Thus, feed compounders have spent little time monitoring its nutrient composition. There is growing evidence today that the feeding value of maize samples can vary significantly, based on animal performance. As there is little data available to suggest the reasons for these differences in nutritive value, it is of interest to review briefly some of the work reported on the composition of maize by the milling and food industries.

Composition and Physical Characteristics of Maize

Starch is a unique and omnipresent polysaccharide found in many foodstuffs. Most of the energy in maize is derived from the starch fraction that is found in the endosperm of the kernel. While many consider starch a homogeneous product it can in fact vary considerably in composition. Regular or normal maize (NM) has most of its starch present as amylopectin, while waxy maize (which is used mainly in the food industry) has little amylose present as starch sources. Normal maize, often referred to as dent maize, and waxy maize are the two most important maize varieties. Flint maize has similar composition to NM, but it has a hard, starchy layer entirely surrounding the outer part of the kernel. Other types of maize are important in small specialized markets: sweet maize (sweetcorn), for example, has a high level of sugars in its endosperm; pop maize (popcorn), used in the confectionery industry, has a high proportion of hard starch which, on heating, expands rapidly, resulting in a explosive rupture of the epidermis and starch granules. While these varieties differ in...
their composition compared with NM, there is little information available as to their exact make-up.

Starch granules can differ significantly in size and composition, depending on type and variety of cereal (South et al., 1991). Granule size and composition are also dependent on the age of the cells in the developing endosperm. Three distinct types of starch granules have been reported which appear to be developmentally regulated. These vary in their content of associated protein (Wasilick et al., 1994).

Not all starch is readily digested. Some starch and products of starch digestion have been shown not to be readily absorbed in the small intestine of healthy humans. Indeed, large amounts of starch compounds have been found in the large bowel contents of people who have succumbed to sudden death syndrome (Brown, 1996). Starch has been classified as three types: (i) starch gelatinized by cooking or heating, which is rapidly digested; (ii) native starch granules from many cereals that may be slowly but completely digested; and (iii) starch that is resistant to digestion. Three subcategories of resistant starch have been identified: (i) granules that are physically inaccessible, e.g. trapped in food matrix or in partially milled or whole grain, so that the size or composition of food particles prevents or delays the action of digestive enzymes; (ii) native starch granules where resistance is related to structure and conformation of granules, e.g. susceptibility to gelatinization – the gelatinization temperature of some starches is 70°C, while for high amylose maize the complete gelatinization temperature has to be around 170°C; and (iii) the formation of retrograde starch material during processing. Starch composed of amylose and amylopectin can be dispersed in water by heating beyond the gelatinization temperature. Upon cooling, the dispersed molecules of amylose and amylopectin spontaneously reassociate and can form crystallites that resist enzymatic hydrolysis. Retrograde and resistant starch can vary significantly with regard to enzyme susceptibility (Eerlingen et al., 1994). This type of starch usually passes undigested to the lower bowel (Brown, 1996).

Amylose content increases with age and size of granule. Some of the maize mutants differ in amylose concentration and it is this characteristic that is mainly responsible for the wide variations seen in some of the responses with these mutants (e.g. susceptibility to enzyme degradation, gelatinization, etc.). Maize varieties very high in amylose are high in resistant starch and fibre and are used in a number of novel and innovative foods.

While it is beyond the scope of this article to delve into the physical and biochemical properties of maize and its constituents, the above short review gives

<table>
<thead>
<tr>
<th>Grain</th>
<th>Gross energy (kcal g⁻¹)</th>
<th>Metabolizable energy (kcal g⁻¹)</th>
<th>Percentage available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>4.54</td>
<td>3.94</td>
<td>86.8</td>
</tr>
<tr>
<td>Wheat</td>
<td>4.46</td>
<td>3.52</td>
<td>78.9</td>
</tr>
<tr>
<td>Barley</td>
<td>4.39</td>
<td>3.04</td>
<td>69.2</td>
</tr>
<tr>
<td>Oats</td>
<td>4.64</td>
<td>2.74</td>
<td>59.1</td>
</tr>
</tbody>
</table>
some insight as to how maize quality, and thus its nutritive value, can be altered with different growing conditions, which could alter starch cell structure, and variations in drying conditions, especially time and temperature, which could affect the physical properties of the starch granules.

Nutrient availability of maize

Values of apparent metabolizable energy (AME) and true metabolizable energy (TME), determined for the most part by the faecal excretion method in adult roosters, have been used as a measure of the available energy in maize. The ratio of available energy to gross energy (AME : GE) is the highest for maize compared with other commonly used feedstuffs (Table 5.1). This could well be the reason that little effort has been expended in trying to improve the digestibility of the nutrients in maize.

A report from Indiana (Maier, 1995) shows the type of information usually available to the purchaser of maize. A number of samples were taken from different parts of the state and analysed for various nutrients. A wide range in these nutrients can be noted (Table 5.2). There is little in the nutrient composition data, except perhaps kernel density, to give an estimate of the available energy in the samples.

Several reported studies have looked at the composition of various samples of maize. Most of these studies have investigated maize samples that, due to the type of growing season, had a significant range in density or weight per bushel. Leeson and Summers (1976) compared maize samples of different moisture and bushel weights that were harvested during a wet, cold autumn and thus stage of maturity would be expected to differ. There was a marked range in bushel weights and also composition values. While bushel weights varied by approximately 40%, ME as determined with adult roosters varied by only 12%. Composition data for three of the highest, lowest and middle range bushel weight samples are shown in Table 5.3. The lower bushel weight samples had a higher level of protein than did the more normal heavier weight samples. Lilburn and Dale (1989) also reported higher levels of protein with lower bushel weight maize; however, levels of lysine and methionine were not increased in the same proportion as was total protein.

Whitacre (1987) had reported that as protein concentration in different maize samples increased, the relative increase in non-essential amino acids was greater than that of the essential amino acids (EAAs). A paper by Lilburn et al. (1991), in which they studied variation in the composition of maize harvested after a season of severe

<table>
<thead>
<tr>
<th>Table 5.2. Average composition of maize from nine areas of Indiana (values based on 15% moisture). (Selected data from Maier, 1995.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
</tr>
<tr>
<td>%</td>
</tr>
<tr>
<td>7.7</td>
</tr>
</tbody>
</table>
drought, again demonstrated the lower EAA values in the higher protein maize samples. In a further paper Leeson et al. (1993) studied 26 different maize samples produced in Ontario after a wet, cold growing season that resulted in a significant percentage of low bushel weight maize. A selected summary of their results is shown in Table 5.4. The composition values were quite variable and for the most part did not correlate with bushel weight of the maize. Indeed, little difference in energy or protein content could be traced to the bushel weight of the maize. These workers concluded, as did Dale (1994) with a similar study, that bushel weight is a poor estimator of the feeding value of maize. It is of interest that the low bushel weight maize of Dale (1994) had lower protein levels as compared with the higher protein levels reported for low bushel weight maize from a crop exposed to drought conditions or to a wet harvesting season. Hsu and Sell (1995) studied low and high bushel weight maize in feeding trials with young poults. They found little or no difference in the energy value of the different maize samples. The low bushel weight

<table>
<thead>
<tr>
<th>Bushel weight (lb)</th>
<th>Metabolizable energy (kcal kg(^{-1}))</th>
<th>Crude protein (%)</th>
<th>Fat (%)</th>
<th>Fibre (%)</th>
<th>Starch (%)</th>
<th>Sugar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>3300</td>
<td>7.6</td>
<td>3.4</td>
<td>2.7</td>
<td>55.3</td>
<td>5.1</td>
</tr>
<tr>
<td>55</td>
<td>3232</td>
<td>8.9</td>
<td>3.3</td>
<td>3.2</td>
<td>58.1</td>
<td>4.3</td>
</tr>
<tr>
<td>54</td>
<td>3410</td>
<td>8.9</td>
<td>4.0</td>
<td>3.1</td>
<td>57.6</td>
<td>4.3</td>
</tr>
<tr>
<td>49</td>
<td>3395</td>
<td>8.5</td>
<td>4.3</td>
<td>3.1</td>
<td>56.9</td>
<td>4.8</td>
</tr>
<tr>
<td>49</td>
<td>3247</td>
<td>9.1</td>
<td>3.7</td>
<td>2.2</td>
<td>57.9</td>
<td>6.8</td>
</tr>
<tr>
<td>48</td>
<td>3388</td>
<td>8.8</td>
<td>3.5</td>
<td>2.9</td>
<td>58.2</td>
<td>4.3</td>
</tr>
<tr>
<td>44</td>
<td>3054</td>
<td>9.1</td>
<td>3.2</td>
<td>3.8</td>
<td>56.3</td>
<td>6.2</td>
</tr>
<tr>
<td>42</td>
<td>3084</td>
<td>10.9</td>
<td>4.3</td>
<td>3.2</td>
<td>55.3</td>
<td>7.7</td>
</tr>
<tr>
<td>41</td>
<td>3051</td>
<td>10.0</td>
<td>4.3</td>
<td>2.5</td>
<td>56.6</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Table 5.3. Metabolizable energy and composition of maize samples (values expressed on an 85% moisture level). (Selected data from Leeson and Summers, 1976.)

<table>
<thead>
<tr>
<th>Bushel weight (lb)</th>
<th>Metabolizable energy (kcal kg(^{-1}))</th>
<th>Crude protein (%)</th>
<th>Crude fat (%)</th>
<th>Soluble fibre (%)</th>
<th>Insoluble fibre (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>58.5</td>
<td>3270</td>
<td>9.0</td>
<td>3.2</td>
<td>1.2</td>
<td>9.1</td>
</tr>
<tr>
<td>55.3</td>
<td>3306</td>
<td>8.9</td>
<td>2.7</td>
<td>1.4</td>
<td>10.6</td>
</tr>
<tr>
<td>58.5</td>
<td>3275</td>
<td>8.8</td>
<td>3.3</td>
<td>0.6</td>
<td>9.2</td>
</tr>
<tr>
<td>53.1</td>
<td>3163</td>
<td>7.5</td>
<td>2.5</td>
<td>1.1</td>
<td>8.3</td>
</tr>
<tr>
<td>53.1</td>
<td>2926</td>
<td>7.1</td>
<td>2.7</td>
<td>0.4</td>
<td>8.4</td>
</tr>
<tr>
<td>53.4</td>
<td>3363</td>
<td>7.7</td>
<td>2.9</td>
<td>0.7</td>
<td>8.7</td>
</tr>
<tr>
<td>50.2</td>
<td>3471</td>
<td>8.1</td>
<td>3.3</td>
<td>1.2</td>
<td>10.3</td>
</tr>
<tr>
<td>50.8</td>
<td>3065</td>
<td>7.5</td>
<td>2.6</td>
<td>1.2</td>
<td>8.7</td>
</tr>
<tr>
<td>45.2</td>
<td>2944</td>
<td>7.9</td>
<td>2.3</td>
<td>0.9</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Table 5.4. Bushel weight, metabolizable energy and proximate components of maize. (Selected data from Leeson et al., 1993.)
maize in their study also had a lower protein content at 6.63% versus 8.53% for maize of normal bushel weight.

Since maize quality can be reduced by the amount of broken kernels or presence of foreign matter, Dale and Jackson (1994) took samples from various shipments of maize and separated them into whole kernels, broken kernels and foreign matter (which ranged from 2 to 7% in the samples). The broken kernels averaged about 2.5% and the foreign matter contained 115 kcal kg$^{-1}$ less nitrogen corrected true metabolizable energy (TMEn) than did the whole grain maize.

Potential for Improving Maize Nutritive Value

It is estimated that 60–70% of the maize produced in the world ends up in livestock feed. Until very recently, seed companies were essentially interested in producing varieties that increase the yield per hectare. The last number of years have seen a shift in the goals of the plant breeders as they now are considering altering the composition of the maize in order to improve its nutritional level.

With gene transfer technology firmly established, the stage is set for a rapid advancement in nutrient quality improvements of cereal grains. While maize composition and quality, on a dry matter (DM) basis, has always been assumed to be fairly constant, marked differences in composition (as shown in previous tables) have been known for years. Because maize makes up such a large portion of swine and poultry diets, minor changes in composition can reflect on animal performance or carcass quality.

With maize being a major contributor of energy to diets, increasing the available energy in maize is an avenue being actively pursued. There are two strategies available to increase energy density of maize. One is to increase the gross energy value of the grain and, assuming that the ratio of GE : ME remains constant, an increase in available energy will take place. A second way is to increase the percentage of GE that can be utilized by the animal.

Oil

The approach that has received the most attention to date is to increase the GE of maize by increasing its oil content. This has proved to be successful and there are now maize types on the market with oil levels in excess of 8% on a dry matter basis, in contrast to around 3–4% for NM. The oil is found almost exclusively in the germ portion of the kernel and thus an increase in germ size brings about a corresponding decrease in endosperm and starch content. For every 1% increase in oil content, starch decreases by 1.3%. Since the germ is relatively high in protein, it also increases and will be higher by approximately 0.3% with a 1% increase in oil content. For every 0.1% increase in oil content, the available energy content of the maize is estimated to increase by approximately 4.5 kcal kg$^{-1}$. An increase in the ratio of ME : GE from 85 to 89% would have to see an increase in the oil content of the maize by three percentage points to equal the increased available energy produced.
A survey by Maier and Briggs (1997) also showed that high oil maize (HOM) can vary significantly in composition (Table 5.5). Han et al. (1987) investigated the feeding value of HOM for poultry in low protein diets in order to achieve diets containing high levels of the maize samples being tested. There was no difference in protein quality of the HOM or NM as measured by a net protein ratio test. In a test with broilers to 22 days of age, weight gain was equal while feed efficiency was significantly improved when the HOM replaced NM in the basal diet (Table 5.6). They concluded that the magnitude of the response was highly correlated with the oil content of the maize. In a 15-week test with laying hens, feed to egg mass output was significantly better when HOM replaced regular maize in a 17% protein diet when the maize was substituted on an isonitrogenous basis. There were no differences for other parameters measured between the two maize samples (Table 5.6).

Adams et al. (1994) evaluated HOM in a broiler study simulating commercial conditions, where male birds were reared to 42 days of age. The experiment was designed to demonstrate the ability of the HOM to increase the energy level in broiler diets without going to high levels of added dietary fat. A range of diets containing up to a level of 6% added poultry oil were compared with similar maize/soya diets where the ratio of energy to protein was kept constant. Thus the HOM diets contained a higher level of energy and protein. Broilers fed the HOM diets were

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oil</th>
<th>Starch</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.7</td>
<td>6.6–10.2</td>
<td>6.9</td>
<td>6.1–7.5</td>
</tr>
</tbody>
</table>

Table 5.5. Average composition of high oil maize from 44 samples produced in Indiana (values based on 15% moisture). (Selected data from Maier and Briggs, 1997.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Production (%)</th>
<th>Egg weight (g)</th>
<th>Feed : egg (g g⁻¹)</th>
<th>Body weight gain (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Regular maize (17% CP)</td>
<td>83.7</td>
<td>54.2</td>
<td>2.21</td>
<td>123.2</td>
</tr>
<tr>
<td>2. As (1), with HOM (18% CP)</td>
<td>84.4</td>
<td>54.1</td>
<td>2.14</td>
<td>183.9</td>
</tr>
<tr>
<td>3. As (2), diet kept isonitrogenous with (1)</td>
<td>86.8</td>
<td>54.7</td>
<td>2.08</td>
<td>180.0</td>
</tr>
</tbody>
</table>

Table 5.6. Comparison of regular maize and high oil maize for broilers and laying hens. (Selected data from Han et al., 1987.)

(a) Broilers (8–22 days)

(b) Layers (23–38 weeks)

*HOM added at same level as regular maize.
significantly heavier and had an improved feed : gain ratio than birds fed the NM diets. They also had less abdominal fat than the NM-fed birds (Table 5.7). These results show the possibility of formulating higher density diets with the use of HOM. In a further publication from Arkansas (Saleh et al., 1997) a HOM sample was compared with a NM variety. The HOM contained 2.32% more oil, 156 kcal kg\(^{-1}\) more TMEn and 0.38% more crude protein than the regular maize sample. These were fed in commercial-type diets, with equal levels of protein and energy, under commercial conditions, to 42 days of age. There were no differences in body weight, feed conversion or dressing percentage between the various diets. The HOM-fed birds had significantly less abdominal fat. The authors again pointed out the advantage of being able to use higher energy diets without reverting to higher levels of added dietary fat.

Bartov and Bar-Zur (1995) compared a HOM variety produced in Israel with local NM. The composition of the maize types, HOM versus regular, was: oil 6.7–2.8%; protein 9.8–7.2%; AMEn 3658–3437 kcal kg\(^{-1}\), respectively. One would expect to find improved performance of the HOM type based on its composition. However, a broiler feeding trial from 7 to 28 days resulted in no difference in bird performance when the HOM replaced an equal proportion of NM. Supplementing the NM diet with protein and fat to equal levels of protein and energy in the HOM diet resulted in a significant improvement in performance of the birds. The authors had no ready explanation for the failure of the HOM diets to give superior

Table 5.7. Effect of high oil (HOM) vs regular yellow dent maize (YDM) in diets with various levels of supplemental oil (male broilers to 42 days). (Selected data from Adams et al., 1994.)

(a) Body weight (g)

<table>
<thead>
<tr>
<th>% Supplemental oil</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOM</td>
<td>2124</td>
<td>2122</td>
<td>2257</td>
<td>2236</td>
<td>2185</td>
</tr>
<tr>
<td>YDM</td>
<td>2020</td>
<td>2056</td>
<td>2125</td>
<td>2158</td>
<td>2090</td>
</tr>
</tbody>
</table>

(b) Feed : gain

<table>
<thead>
<tr>
<th>% Supplemental oil</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOM</td>
<td>1.71</td>
<td>1.69</td>
<td>1.61</td>
<td>1.57</td>
<td>1.65</td>
</tr>
<tr>
<td>YDM</td>
<td>1.80</td>
<td>1.75</td>
<td>1.72</td>
<td>1.66</td>
<td>1.73</td>
</tr>
</tbody>
</table>

(c) Abdominal fat (% of body weight)

<table>
<thead>
<tr>
<th>% Supplemental oil</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HOM</td>
<td>2.08</td>
<td>1.96</td>
<td>2.09</td>
<td>2.16</td>
<td>2.07</td>
</tr>
<tr>
<td>YDM</td>
<td>2.26</td>
<td>2.48</td>
<td>2.35</td>
<td>2.24</td>
<td>2.33</td>
</tr>
</tbody>
</table>
performance, but they did point out that some of the reported responses to HOM have been variable.

Vieira et al. (1997) reported on a feeding trial with a HOM variety produced and grown in Brazil. Their HOM contained 5.7% oil compared with 3.8% in their NM sample. However interesting, unlike most other HOM samples reported, their sample contained slightly less protein than their NM (9.17% versus 9.39%, DM basis). Similar to the report from Bartov and Bar-Zur (1995), their data showed that the NM sample gave superior performance when supplemental oil was added to make the diets equal in energy. Rand et al. (1998) reported that under commercial conditions HOM was particularly advantageous in enhancing broiler performance during the hot summer months. This was probably due to the more efficient use of the higher oil diet.

Parsons et al. (1998) studied the availability of the amino acids in HOM as compared with an NM sample. Digestibility studies with adult cockerels suggested that digestibility for the amino acids in both samples of maize were the same. Using a chick feeding study they demonstrated that the bioavailability of the lysine in HOM was equal to or slightly better than that in NM. One would have to conclude that most of the HOM varieties on the market today are superior in feeding value to NM samples. Their greater nutrient density and superior nutritive value, along with their advantage in producing higher density diets without increased dietary fat inclusion, make them a potential competitor for the maize market of the future.

**Protein**

Several maize varieties developed in the 1960s showed promise due to their improved level of protein and the essential amino acids lysine and methionine. Opaque-2 maize had a higher level of protein and significantly more lysine than NM. When compared with NM, growth response of chicks was not as good until the opaque-2 maize diet was supplemented with methionine to balance the higher level of lysine. Floury-2 maize had increased levels of protein as well as lysine and methionine. This maize gave an improvement in performance when compared with NM without EAA supplementation. Cromwell et al. (1968) compared the feeding value of a sample of opaque-2 and floury-2 maize with that of NM. These were tested in a 21-day feeding trial with chicks fed 15% protein diets where equal amounts of maize protein were maintained by varying the amount of maize added. The results, shown in Table 5.8, demonstrate the superiority of the higher protein maize samples with proper EAA supplementation. These maize types never did gain favour with producers, due to poor storage problems as well as low yields.

An increase in the proportion of glutelin as compared with zein protein in the kernel is obviously the reason for the changes noted for the altered composition of the high protein maize varieties. Bond et al. (1991) tested two new high protein maize varieties that were reported to be free of the problems that plagued the earlier samples. These varieties
contained approximately 11% protein as against 9% for the NM that they used for comparison. In a 6-month test with layers, where the diets were formulated to be isocaloric but not isonitrogenous, production and egg size did not differ; however, feed per kilogram of egg mass produced was significantly lower with one of the new maize varieties (Table 5.9). In a test with broilers to 6 weeks of age, diets were formulated with the HPM-1 sample. The diets were formulated to be isonitrogenous.

### Table 5.8. Effect of adding methionine to normal, opaque-2 and floury-2 maize, and to normal maize plus lysine, in diets containing 15% protein. (From Cromwell et al., 1968.)

#### (a) 20 day weight (g)

<table>
<thead>
<tr>
<th>Maize type</th>
<th>0.45</th>
<th>0.60</th>
<th>0.80</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>377</td>
<td>332</td>
<td>330</td>
<td>333</td>
</tr>
<tr>
<td>Opaque-2</td>
<td>299</td>
<td>389</td>
<td>382</td>
<td>357</td>
</tr>
<tr>
<td>Floury-2</td>
<td>397</td>
<td>380</td>
<td>384</td>
<td>387</td>
</tr>
<tr>
<td>Normal + lysine</td>
<td>367</td>
<td>368</td>
<td>377</td>
<td>371</td>
</tr>
<tr>
<td>Average</td>
<td>351</td>
<td>368</td>
<td>367</td>
<td></td>
</tr>
</tbody>
</table>

#### (b) Feed : gain

<table>
<thead>
<tr>
<th>Maize type</th>
<th>0.45</th>
<th>0.60</th>
<th>0.80</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.74</td>
<td>1.77</td>
<td>1.74</td>
<td>1.75</td>
</tr>
<tr>
<td>Opaque-2</td>
<td>1.81</td>
<td>1.63</td>
<td>1.62</td>
<td>1.69</td>
</tr>
<tr>
<td>Floury-2</td>
<td>1.65</td>
<td>1.66</td>
<td>1.65</td>
<td>1.65</td>
</tr>
<tr>
<td>Normal + lysine</td>
<td>1.70</td>
<td>1.65</td>
<td>1.66</td>
<td>1.67</td>
</tr>
<tr>
<td>Average</td>
<td>1.72</td>
<td>1.68</td>
<td>1.67</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.9. Comparison of normal maize and high-protein maize. (Selected data from Bond et al., 1991.)

#### (a) Layers

<table>
<thead>
<tr>
<th></th>
<th>NM</th>
<th>HPM-1</th>
<th>HPM-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production (%)</td>
<td>69.1</td>
<td>70.7</td>
<td>68.5</td>
</tr>
<tr>
<td>Egg weight (g)</td>
<td>64.1</td>
<td>64.7</td>
<td>63.9</td>
</tr>
<tr>
<td>Feed intake (g per bird day)</td>
<td>109</td>
<td>106</td>
<td>109</td>
</tr>
<tr>
<td>Feed : egg mass</td>
<td>2.56</td>
<td>2.35</td>
<td>2.57</td>
</tr>
</tbody>
</table>

#### (b) Broilers

<table>
<thead>
<tr>
<th></th>
<th>NM</th>
<th>HPM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body-weight gain (g)</td>
<td>1633</td>
<td>1749</td>
</tr>
<tr>
<td>Feed : gain</td>
<td>2.00</td>
<td>1.96</td>
</tr>
</tbody>
</table>
as well as isocaloric. An improvement in weight gain and feed : gain ratio was noted with the higher protein maize sample. The authors suggested that higher levels of methionine, as well as several other EAA present, could account for the better performance of this particular sample.

Waxy maize, while not used to any extent in commercial feeding operations, at times does find its way into diets for poultry. Waxy maize contains amylpectin as its only source of starch, while NM contains approximately 73% amylpectin and 27% amylese as starch sources. Dinn et al. (1982) reported that the feeding value of the waxy maize was similar to that of NM when fed to broilers. Ertl and Dale (1997) compared the available energy content of normal and waxy maize of similar genetic backgrounds, and grown under similar conditions, using adult roosters. Their results showed that the TMEn values of the two maize varieties were quite similar, thus suggesting that they could be substituted in diets without any fear of reduced performance.

**Increasing the Available Energy of Maize by Improving Nutrient Digestibility**

The digestibility and availability of amino acids, starch and oil and the presence of poorly digested components of a grain determine the fraction of GE which shows up as energy available to an animal. Imbalances of EAA in relation to an animal’s needs obviously also reduce the nutritive value of a cereal. Most of the improved maize varieties in the past have resulted in increases or decreases in some of the major components such as protein, starch and oil with little or no change in the composition of these components.

Little headway has been achieved in increasing the nutritive value of maize by improving the digestibility or utilization of its various components. Recent work questions whether the AME and TMEn values used today accurately predict the energy derived by the bird, especially the young bird, from maize. Mahagna et al. (1995) reported that the AME of maize differed from the first week post-hatch to the third week by around 200–250 kcal kg\(^{-1}\). Collins et al. (1998) also reported that age of bird was an important consideration when assigning an available energy value to maize. It has been shown that measuring available energy with ileal rather than faecal contents indicates that starch digestion is not complete in the small intestine. Noy and Sklan (1995) reported low ileal digestibility of both starch and fat in young broiler chicks fed a maize/soybean-meal diet. It was shown that digestibility for starch in chicks from 4 to 21 days of age, up to the end of the small intestine, was as low as 82%, with no evidence of any increase as the birds got older. This suggests that a portion of the maize starch is reaching the hind gut, where it undergoes fermentation with poor energetic utilization. Microscopic examination of the ileal digesta supports this conclusion as large maize endosperm particles have been found to be undigested. Persia and Lilburn (1998), on studying the AME and starch digestibility in the small intestine of turkey poults, reported that there was a significant difference in AME for wheat and maize when measured with small intestine versus colon contents. Their
data showed 5.21% of undigested starch entering the colon from maize versus 2.67% from wheat. Pack et al. (1998) reviewed much of the recent work in the area of maize starch availability and reported that not only is starch not completely digested in the small intestine but also there can be a significant amount of protein – and in particular amino acids – entering the hind gut, depending on age of bird and maize sample. It has been reported that amino acid digestibility is significantly lower when measured with ileal as compared with faecal determinations. The reason for these differences is the contribution by the hind gut in digesting starch, which is not readily available to the animal, and also the error involved in determining amino acid digestibility values when a correction is made for endogenous losses. There is also a significant amount of data to indicate that overall digestion is low in the chick to around 10 days and then increases to a plateau around 4 weeks of age. Thus the values used for available energy for the young chick show significant room for improvement by way of altering maize composition or the use of specific enzymes. Noy and Sklan (1995) have shown that amylase, trypsin and lipase are low at 4 days of age and that by 21 days of age they increase by 100-, 50- and 20-fold, respectively. They suggest that only 82–89% of fatty acids and starch are digested in the small intestine. While nitrogen digestion is also not complete in the small intestine, it increased from around 78% at 4 days to 92% at 21 days of age.

**Enzyme Supplementation**

The action of enzymes on various starches and factors influencing starch degradation has been well documented (Knutson et al., 1982; Planchot et al., 1995). There is also a wealth of data reported on enhancing the feeding value of the coarse cereals by supplementing diets with various enzymes. These enzymes degrade the non-starch polysaccharides present in cereals such as wheat, barley, rye and oats, thus significantly improving their available energy content. Maize with a very low NSP level does not respond to such enzymes. However, in recent years it has been shown that a mixture of enzymes containing amylase, xylanase and protease is effective in enhancing the nutritive value of a maize/soybean-meal diet. Much of the recent work in this area is covered in the reports of Pack and Bedford (1997) and Pack et al. (1998). These reports, as well as others, suggests a 2–5% improvement in available energy with enzyme supplementation of a maize/soybean-meal diet. More uniform broiler weights at marketing and reduced mortality have also been noted with the enzyme-supplemented diets. These have been attributed to the possible removal of anti-nutritional factors present in the feed ingredients, along with more effective nutrient digestibility in the small intestine and hence an influence on gut microbial activity. It is of interest that Yan et al. (1998) also reported reduced mortality as one of their consistent observations when phytase was added to a maize/soybean diet for broilers.

Maize/soybean-meal diets have also been improved in nutritive value by the addition of phytase enzyme. While there is some information to indicate that the overall improvement of the diet has been enhanced with phytase supplementation, it
is mainly an improvement in phytate-phosphorus availability that has been noted. Simons et al. (1990) showed an improvement in dietary phosphorus availability for poultry of approximately 65% with microbial phytase supplementation of diets. Van Der Klis et al. (1997) reported a marked improvement in phytate degradation with laying hens, from 8 to 50%, with the addition of phytase to the laying diet of the hen (Table 5.10).

Recently, low phytic acid maize varieties have been identified and they hold promise in markedly enhancing the utilization of the phosphorus from maize as well as reducing faecal phosphorus excretion. Two papers from Arkansas at a recent poultry science meeting showed that the total phosphorus in the maize was similar to NM and that the non-phytic acid phosphorus was equal to the availability of inorganic phosphorus supplements (Kersey et al., 1998; Li et al., 1998; Yan et al., 1998).

### Physical Properties of Maize

Another factor to consider in looking at the response to diets containing maize is the type of grinding to which it is subjected. One of the major ways to improve the nutritive value of cereals for livestock feeding is to grind the material. Grinding increases the surface area and thus allows for better enzyme action in the gut. There has been renewed interest in looking at type and degree of grinding in order to enhance feeding value as well as to reduce energy costs for grinding. Deaton et al. (1995) reported that hammer-milling maize through a 9.53 mm screen gave as good performance with broilers as did maize processed through a 6.35 and a 3.18 mm screen (Table 5.11). Work reported by Washburn (1991) suggested that birds under heat stress increased the rate of food passage through the gut, thus altering digestion and subsequent feed utilization, but this was not confirmed in the study by Deaton et al. (1995). There are a number of papers looking at particle size and diet utilization. Nir et al. (1994) looked at particle size of maize and its influence on diet utilization (Table 5.12) and their work suggests that degree of grinding and particle size affect diet utilization.

<table>
<thead>
<tr>
<th>Inorganic phosphorus (g kg⁻¹)</th>
<th>Phytase (FTU kg⁻¹)</th>
<th>Ileal P absorption (g kg⁻¹ diet)</th>
<th>Phytate degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.72</td>
<td>21.7</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>1.25</td>
<td>21.6</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>1.41</td>
<td>20.9</td>
</tr>
<tr>
<td>0.5</td>
<td>250</td>
<td>1.72</td>
<td>54.2</td>
</tr>
<tr>
<td>0</td>
<td>250</td>
<td>1.64</td>
<td>59.0</td>
</tr>
<tr>
<td>0</td>
<td>500</td>
<td>1.71</td>
<td>71.7</td>
</tr>
</tbody>
</table>
An area that has received passing attention from time to time is the influence of fertilizer on composition of maize. Bird and Olsen (1972) reported that fertilizing with N, P and K resulted in increased crude protein content of the maize and an increase in some of the EAA. Diets formulated taking into account the higher level of protein in the maize, and without EAA supplementation, gave performance results for broilers equal to the control normal maize diet. However, use of the higher protein maize resulted in a 12% saving in soybean meal (Table 5.13).

Table 5.11. Effect of hammer-mill screen size of maize grind on male broiler weights to 49 days of age. (Selected and rearranged data from Deaton et al., 1995.)

<table>
<thead>
<tr>
<th>Screen size (mm)</th>
<th>Body weight (g)</th>
<th>Feed : gain</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.18</td>
<td>2442</td>
<td>1.94</td>
<td>3.2</td>
</tr>
<tr>
<td>6.35</td>
<td>2424</td>
<td>1.94</td>
<td>3.0</td>
</tr>
<tr>
<td>9.53</td>
<td>2434</td>
<td>1.93</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Average values for birds reared at 21 and 31°C.

Table 5.12. (a) Particle size of maize ground to various degrees of fineness.

<table>
<thead>
<tr>
<th>Particle sizea</th>
<th>Parameter</th>
<th>Coarse</th>
<th>Medium</th>
<th>Originalb</th>
<th>Fine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight : volume</td>
<td>0.642</td>
<td>0.545</td>
<td>0.630</td>
<td>0.482</td>
</tr>
<tr>
<td></td>
<td>Geometric mean diameter (mm)</td>
<td>2.01</td>
<td>0.897</td>
<td>1.102</td>
<td>0.525</td>
</tr>
<tr>
<td></td>
<td>Calculated specific surface (cm² g⁻¹)</td>
<td>24</td>
<td>54</td>
<td>54</td>
<td>102</td>
</tr>
</tbody>
</table>

aCoarse = 1.41 mm; fine = 0.64 mm; medium = mix of fine and coarse particles.
bRegular maize ground with an 8 mm screen size.

(b) Performance of chicks fed the above fractions in maize/soybean meal diets. (Selected data from Nir et al., 1994.)

<table>
<thead>
<tr>
<th>Particle sizea</th>
<th>Parameter</th>
<th>Coarse</th>
<th>Medium</th>
<th>Original + 18% fines</th>
<th>Original + 30% fines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight gain (g)</td>
<td>86</td>
<td>100</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Gain : feed</td>
<td>0.806</td>
<td>0.893</td>
<td>0.847</td>
<td>0.870</td>
</tr>
<tr>
<td></td>
<td>Weight gain (g)</td>
<td>473</td>
<td>522</td>
<td>463</td>
<td>474</td>
</tr>
<tr>
<td></td>
<td>Gain : feed</td>
<td>0.662</td>
<td>0.725</td>
<td>0.649</td>
<td>0.667</td>
</tr>
</tbody>
</table>

An area that has received passing attention from time to time is the influence of fertilizer on composition of maize. Bird and Olsen (1972) reported that fertilizing with N, P and K resulted in increased crude protein content of the maize and an increase in some of the EAA. Diets formulated taking into account the higher level of protein in the maize, and without EAA supplementation, gave performance results for broilers equal to the control normal maize diet. However, use of the higher protein maize resulted in a 12% saving in soybean meal (Table 5.13).
An interesting paper was published by Williams (1992), who investigated why birds fed on maize diets were much less susceptible to coccidiosis than birds fed on wheat diets. While no explanation could be given for the apparent immune response of the maize diet, differences in some of the vitamins in wheat versus maize were discussed as possible contributing factors.

In recent years the biotechnologists have produced several new plant varieties that offer protection against pests. The first commercial planting of insect-resistant maize hybrids, commonly referred to as ‘Bt’ maize, took place in 1996. These hybrids possess a gene that enables the plants to produce an insecticidal protein that is toxic to the larvae of the pests. Brake and Vlachos (1998) compared this type of maize with NM in a broiler feeding test. Their results suggest that the maize is equal to NM in its nutritive value.

There are several potential avenues for the plant breeders to investigate in their search for novel ways of improving the nutritive value of various cereals. With maize being such an important cereal for human as well as animal consumption, it will receive increased attention in the years ahead as energy becomes more of a limiting factor in producing food for an expanding world population.

### References


Poultry Science 73, 45–49.


Williams, R.B. (1992) Differences between the anticoccidial potencies of monensin in maize-based or wheat-based chicken diets. Veterinary Research Communications 16, 147–152.

Introduction

The cereal grains that constitute the bulk of animal feedstuffs also provide 30–60% of dietary amino acids (National Research Council (NRC), 1998). However, this protein is inadequate, not only in sufficiency but also in amino acid balance. To obtain optimum animal performance, whether it be growth, reproduction or lactation, protein must be added to the diet to provide both an adequate amount of total protein and a balance of amino acids that approaches the ideal amino acid pattern for the species and developmental stage of the animal. This chapter will concentrate on the use of vegetable protein meals (VPM) and exogenous enzymes in monogastric farm animals, as exogenous enzyme treatments are not in general use in ruminant diets.

On a global scale the majority of protein ingredients incorporated into animal feeds are supplied by vegetable proteins, with the oilseeds and legumes being the main providers. In the UK the demand for high quality VPMs has increased due to the ban on the use of meat and bone meal in animal feeds in the wake of the BSE crisis. The vegetable sources of protein vary both in crude protein concentration and in the amino acid composition of the protein. The proximate analyses of some of the protein supplements used in animal feeds are outlined in Table 6.1. Amino acid profiles for these meals can be obtained from various sources, for example Ewing (1997) and NRC (1998).

Anti-nutritional Factors Found in VPMs

The availability of nutrients in feedstuffs is often limited by the presence of anti-nutritional factors (ANFs), which may limit the use of these feedstuffs in animal diets. ANFs can be classified in different ways. Huisman and Tolman (1992) classified them on the basis of their effects on the nutritional value of feedstuffs, and

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the biological response in the animal as: (i) factors with a depressive effect on protein digestion and on the utilization of protein (protease inhibitors, lectins, phenolic compounds, saponins); (ii) factors with a negative effect on the digestion of carbohydrates (amylase inhibitors, phenolic compounds, flatulence factors); (iii) factors with a negative effect on the utilization of minerals (glucosinolates, phytic acid, oxalic acid, gossypol); (iv) factors that inactivate vitamins, or cause an increase in the animal’s vitamin requirement; (v) factors stimulating the immune system that may cause a damaging hypersensitivity reaction (antigenic proteins); and (vi) factors in feed that have a toxic effect (e.g. lectins, cyanide-containing compounds).

Table 6.2 shows the distribution and physiological effects of the various ANFs found in VPMs. The toxicity and effects of the different ANFs may vary considerably between VPMs and these toxic effects may also vary in potency between animal species. ANFs appear to play a role in the protection of plants against predation from moulds, bacteria, insects and birds by disturbing the digestive processes of these organisms, and presumably act in a similar manner in domestic animals (Birk, 1989).

Reduction and Elimination of ANFs in VPMs

The ANFs in VPMs can be reduced or eliminated by processing procedures. Maximum destruction of ANFs may require different processing treatments, due to differences in ANF structure and their biological effects. Processing can be supplied by physical, chemical, thermal or bacterial means. Currently, various methods can be applied in the reduction and elimination of ANFs in legume seeds (Table 6.3).

Processing technology is conventionally applied to soybean, the most common being some form of heat treatment, which has proved most effective at reducing levels of trypsin inhibitors and soybean lectin. Commercial heat processing is

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Table 6.1. Proximate analysis of some VPMs used in animal feeds. (From Ewing, 1997.)

<table>
<thead>
<tr>
<th>Feedstuff</th>
<th>DE MJ (kg⁻¹) pigs</th>
<th>ME MJ (kg⁻¹) poultry</th>
<th>DM (g kg⁻¹)</th>
<th>CP (g kg⁻¹)</th>
<th>Oil (s.e.) (g kg⁻¹)</th>
<th>CF (g kg⁻¹)</th>
<th>Ash (g kg⁻¹)</th>
<th>Starch and sugars (g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cottonseed meal (s.e.)</td>
<td>11.1</td>
<td>9.1</td>
<td>900</td>
<td>410</td>
<td>17</td>
<td>159</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>Faba beans</td>
<td>15.8</td>
<td>13.5</td>
<td>860</td>
<td>290</td>
<td>18</td>
<td>90</td>
<td>34</td>
<td>455</td>
</tr>
<tr>
<td>Lupin meal</td>
<td>13.2</td>
<td>11.0</td>
<td>860</td>
<td>320</td>
<td>53</td>
<td>135</td>
<td>37</td>
<td>130</td>
</tr>
<tr>
<td>Peas</td>
<td>15.4</td>
<td>13.0</td>
<td>860</td>
<td>260</td>
<td>16</td>
<td>70</td>
<td>35</td>
<td>495</td>
</tr>
<tr>
<td>Phaseolus beans*</td>
<td>–</td>
<td>–</td>
<td>870</td>
<td>238</td>
<td>16</td>
<td>35</td>
<td>39</td>
<td>541</td>
</tr>
<tr>
<td>Soybean meal (s.e.)</td>
<td>14.8</td>
<td>10.7</td>
<td>880</td>
<td>470</td>
<td>20</td>
<td>82</td>
<td>72</td>
<td>145</td>
</tr>
<tr>
<td>Soybeans (FF)</td>
<td>19.3</td>
<td>16.9</td>
<td>890</td>
<td>400</td>
<td>205</td>
<td>60</td>
<td>55</td>
<td>120</td>
</tr>
<tr>
<td>Sunflower meal (s.e.)</td>
<td>10.2</td>
<td>7.1</td>
<td>880</td>
<td>360</td>
<td>20</td>
<td>230</td>
<td>70</td>
<td>75</td>
</tr>
<tr>
<td>Rapeseed meal (s.e.)</td>
<td>12.0</td>
<td>10.5</td>
<td>880</td>
<td>385</td>
<td>32</td>
<td>11</td>
<td>70</td>
<td>145</td>
</tr>
</tbody>
</table>

*Adapted from van der Poel (1991).

s.e. = Solvent extracted.

FF = Full fat.
carefully controlled: underheating can result in inadequate inactivation of ANFs whilst overheating may reduce availability through the occurrence of Maillard reactions (reviewed by van der Poel, 1989, and Huisman and Tolman, 1992).

Little attention has been paid to the heat processing of other legumes and oilseeds (post oil extraction). The insufficiencies of some processing techniques have led to the search for new methods for the elimination of residual ANFs in VPMs. For example, new breeding technologies have led to the development of low glucosinolate varieties of rapeseed, and although this has made rapeseed a more

Table 6.2. Distribution and physiological effects of ANFs found in VPMs. (Adapted from Huisman and Tolman, 1992; Liener, 1994.)

<table>
<thead>
<tr>
<th>Anti-nutritional factor</th>
<th>Distribution</th>
<th>Physiological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>Most legumes</td>
<td>Reduction of (chymo)trypsin activity, impaired growth, pancreatic hypertrophy, pancreas carcinogen</td>
</tr>
<tr>
<td>Lectins</td>
<td>Most legumes</td>
<td>Gut wall damage, immune response, increased endogenous nitrogen loss</td>
</tr>
<tr>
<td>Amylase inhibitors</td>
<td>Kidney beans</td>
<td>Impaired digestion of starch</td>
</tr>
<tr>
<td>'Antigenic' proteins</td>
<td>Soybean, kidney beans</td>
<td>Immune response, interference with gut wall integrity</td>
</tr>
<tr>
<td>Polyphenols</td>
<td></td>
<td>Interference with protein and carbohydrate digestibility by formation of protein–carbohydrate complexes</td>
</tr>
<tr>
<td>Tannins</td>
<td>Most legumes</td>
<td></td>
</tr>
<tr>
<td>Glycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vicin / convicin</td>
<td>Faba beans</td>
<td>Haemolytic anaemia, adverse effect on egg production</td>
</tr>
<tr>
<td>Saponins</td>
<td>Soybean</td>
<td>Haemolysis, effects on intestinal permeability</td>
</tr>
<tr>
<td>Glucosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosinolates</td>
<td>Rapeseed</td>
<td>Impaired iodine utilization (goitrogenicity), reduced palatability and growth</td>
</tr>
<tr>
<td>Cyanogens</td>
<td>Linseed, traces in kidney beans and peas</td>
<td>Respiratory failure</td>
</tr>
<tr>
<td>Alkaloids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinolizidine</td>
<td>Lupin</td>
<td>Neural disturbances, depressed growth, reduced palatability</td>
</tr>
<tr>
<td>Other ANFs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytate</td>
<td>Most legumes</td>
<td>Formation of complexes with minerals and proteins, depresses mineral absorption</td>
</tr>
<tr>
<td>Gossypol</td>
<td>Cottonseed</td>
<td>Anaemia due to formation of iron complexes, reduced egg weight</td>
</tr>
<tr>
<td>Sinapins</td>
<td>Rapeseed</td>
<td>'Fish' taint in eggs</td>
</tr>
<tr>
<td>Oligosaccharides (NSP)</td>
<td>Soybeans, peas, faba beans, Phaseolus beans</td>
<td>Flatulence, diarrhoea, discomfort</td>
</tr>
</tbody>
</table>
attractive ingredient in animal feed it has not totally solved the ANF problem. Recent research has focused on the development of biotechnological approaches to this problem, mainly by the application of exogenous enzymes to feedstuffs containing ANFs.

**The Use of Exogenous Enzymes**

Until recently, the majority of feed enzyme research has been performed on cereal-based diets, for example barley and wheat, particularly for poultry diets (Dierick, 1989). The type of feed enzymes currently available are outlined in Table 6.4.

There are many factors evolving in the animal feed industry which suggest that the use of exogenous enzymes will become more important in the future (reviewed by Johnson *et al.*, 1993). These include: (i) an increasing shift in the use of ‘alternative’ feedstuffs in formulating diets; (ii) the use of enzymes known to be effective against particular dietary components; (iii) the production of novel by-products (for example, linseed meal derived after linseed oil production) that have a depressing effect on growth; (iv) the increased availability of free amino acids which may reduce the requirement for high quality protein supplements; (v) novel feed systems, such as liquid feed systems and those that take advantage of pretreatment, which support the use of in-feed enzymes; (vi) the introduction of pollution control, for example the reduction of sticky litter in poultry and post-weaning diarrhoea in pigs (Inborr and Ogle, 1988); and (vii) the possible reduction in animal performance due to the restricted use of growth-promoting antibiotics.

**Presentation of Exogenous Enzymes to the Animal**

The most common method of feeding enzymes is with dry feed, the enzymes usually being added to the feed during blending, often prior to processing treatments such as pelleting. However, most of the enzymes currently of interest to the animal feed

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**Table 6.3.** Processing techniques for reduction and elimination of ANFs in legume seeds (van der Poel, 1989).

<table>
<thead>
<tr>
<th>No.</th>
<th>Technology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Breeding and genetic manipulation</td>
</tr>
<tr>
<td>2</td>
<td>Feed formulation: selection of ingredients, supplementation with amino acids, other supplements, e.g. probiotics, enzymes, etc.</td>
</tr>
<tr>
<td>3</td>
<td>Primary processing: chemical treatments, enzymatic treatment, physical treatments (fractionation, heat treatments)</td>
</tr>
<tr>
<td></td>
<td>Secondary processing: conditioning/pelleting</td>
</tr>
</tbody>
</table>
industry, such as β-glucanase, amylases, proteases and phytase, suffer temperature-related reduction in activity due to heat treatments (Inborr, 1990; Cowan, 1992; Graham and Inborr, 1993; Bedford and Pack, 1998). This problem can be reduced by the application of the enzyme in a liquid base, post-pelleting, for example spraying or by use in liquid feed.

**Action of Feed Enzymes in the Animal**

The application of exogenous enzymes to dry diets presupposes that the enzyme will be active in the digestive tract of the animal, and it must therefore fulfil a number of criteria. The enzyme must be active under the physiological conditions prevailing in the animal’s digestive tract; it must be able to resist proteolysis by the animal’s endogenous proteases and supplement rather than antagonize the animal’s digestive enzymes. Species differences in the anatomy and physiology of the digestive tract are likely to affect exogenous enzyme activity in this respect. For example, between pigs and other animals.

### Table 6.4. Feed enzymes in use today. (From Ogden, 1995, modified from Rotter et al., 1989; Cowan, 1992.)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Action</th>
<th>Target substrate</th>
<th>Type of feed</th>
<th>Expected benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Glucanases</td>
<td>β-Glucans to oligosaccharides and glucose</td>
<td>Barley, oats and rye based diets</td>
<td>Poultry and pig diets</td>
<td>Reduction of sticky droppings, improved feed utilization</td>
</tr>
<tr>
<td>Amylases</td>
<td>Degrade cereal starch to dextrins and sugars</td>
<td>High starch cereal diets</td>
<td>Early pig/calf diets</td>
<td>Increased availability of cereals in weaner feeds</td>
</tr>
<tr>
<td>Cellulases</td>
<td>Cellulose to low molecular weight products and glucose</td>
<td>High fibre diets</td>
<td>Poor-grade forages</td>
<td>Improved energy availability</td>
</tr>
<tr>
<td>Pentosanases</td>
<td>Arabinofurans to low molecular weight products and sugars</td>
<td>Rye, barley and wheat</td>
<td>Pig and poultry diets</td>
<td>Improved litter quality, improved feed utilization</td>
</tr>
<tr>
<td>(Xylanases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>Degrades oligosaccharides and ANFs</td>
<td>Soybean and other legumes</td>
<td>Pig diets</td>
<td>Improved energy availability, reduced scours in piglets</td>
</tr>
<tr>
<td>Phytases</td>
<td>Increases availability of phosphorus from phytic acid</td>
<td>Many different diets</td>
<td>Pig and poultry diets</td>
<td>Reduces need for inorganic phosphorus</td>
</tr>
<tr>
<td>Proteases</td>
<td>Protein to peptides and amino acids</td>
<td>Wheat by-products, legume proteins</td>
<td>Milk replacer using soybean or soybean protein</td>
<td>Higher protein digestibility, lower nitrogen excretion</td>
</tr>
<tr>
<td>Lipases</td>
<td>Fats to fatty acids</td>
<td>Animal and vegetable fats</td>
<td>Pet diets/broiler diets</td>
<td>Improved digestibility of fat and enhanced energy retention as a result</td>
</tr>
</tbody>
</table>

Table 6.4. Feed enzymes in use today. (From Ogden, 1995, modified from Rotter et al., 1989; Cowan, 1992.)
and poultry these differences include the following (Partridge, 1993; Dierick and Decuyper, 1994).

1. Anatomical: in poultry, feed passes into the crop, where any added enzymes can act for several hours at a pH of approximately 6.0 before passing into the acid environment of the gizzard, whereas in the pig, feed passes directly into the acid environment of the stomach immediately after ingestion.

2. Digestive capacity: poultry have a shorter small intestine and thus reduced possibilities for enzyme inactivation by the microflora, a shorter mean retention time in the small intestine (1–2 h in poultry versus 4–5 h in the pig) and a lower water content in the upper part of the gastrointestinal tract.

3. Bacterial activity: the importance of the microflora in the gut of poultry is much less than in the pig.

4. Fiber fermentation: lower in poultry than in pigs, due to their widely different hind gut capacities.

Survivability of enzymes in the digestive tract varies widely; for example, Thacker and Baas (1996) and Baas and Thacker (1996) demonstrated that 84% of pentosanase and 26% of $\beta$-glucanase activity was recovered in the duodenal digesta of pigs 4 h after feeding diets supplemented with these enzymes. Approximately 75% of exogenous protease has been detected in the ileal digesta of young pigs fed protease-supplemented diets.

An alternative to the use of exogenous enzymes in dry diets is the pretreatment of the diet or its components prior to secondary processing. Adding enzymes to complete compound feeds may be appropriate when the enzyme is targeted at cereals, which make up the bulk of the diet. However, VPMs only constitute a small proportion of the total diet and therefore target substrates are considerably diluted. The dilution effect may be overcome by pretreating the VPM prior to inclusion in the diet. This approach has been adopted in much of the recent research into the effect of enzyme application to VPMs, much of which has focused on the effects of proteases on soybean meals.

**Pretreatment of Diets Containing VPMs**

**Pretreatment of soybean with proteases**

The prime target of proteases in soybean are the anti-nutritional factors, such as residual trypsin inhibitors, lectins and ‘antigenic’ protein. Pretreatment of specific feed components allows the effect of any enzyme addition to be ascertained prior to feeding the animal. This is a pertinent point as far as the inclusion of proteases in animal feeds is concerned, as it is impossible to assess exogenous protease activity in ileal digesta. Immunochemical techniques are being developed that will detect the presence of an exogenous protease in ileal digesta; however, these give no indication of functionality (Thorpe, 1999).
Pretreatment of soybeans with enzymes has been used, albeit inadvertently through the processes of fermentation, to improve the nutritional quality of legumes in human foods for many years (Campbell-Platt and Cook, 1991). It is not the intention of this chapter to review fermentation processes extensively as little work has been undertaken on the effect of fermenting protein meals for inclusion in animal feeds. However, Zamora and Veum (1979) fed heated soybeans fermented with *Aspergillus oryzae* or *Rhizopus oligosporus* to growing pigs. Table 6.5 summarizes their results. Fermentation significantly improved feed conversion ratio (FCR) with both organisms and average daily gain with *A. oryzae*, and showed an improvement (although not significant) in nitrogen retention with both organisms. This work indicated that fermentation improves the performance of growing pigs, possibly by the action of fungal enzymes on the soybean present in the feed.

In the 1950s, work by Lewis *et al.* (1955) and Baker *et al.* (1956) examined the effects of the addition of pepsin, pancreatin, a fungal protease, a diastatic protease and papain to various soybean diets in a number of trials using pigs from 6 to 67 days old. Pepsin and pancreatin supplementation showed some beneficial effects on average daily gain (ADG) and FCR, particularly in younger pigs. Papain and the diastatic protease were as effective as pepsin and pancreatin, but the fungal protease showed no beneficial effects. This work was aimed at supplementing the insufficiencies of proteolytic and amylolytic digestive enzymes in the baby pig, as opposed to the current work, which targets ANFs in protein meals.

More recent work has concentrated on the use of isolated enzymes produced from large-scale microbial fermentations. This research can be divided into either *in vitro* or *in vivo* studies. *In vitro* studies have been used by a number of workers in this field to determine the effect of exogenous enzymes on VPMs prior to expensive *in vivo* studies. Assessment of enzyme activity *in vitro* may indicate any potential benefits the enzyme may have *in vivo*. A number of studies have been undertaken utilizing proteases on soybean meals *in vitro*, analysing different parameters.

Huo *et al.* (1993) showed that one fungal and four bacterial protease enzymes could inactivate trypsin inhibitors and lectin in raw soybean (RSB) and low-temperature extruded soybean (LTES) *in vitro*, to various extents. The most effective of these reduced trypsin inhibitor levels in RSB and LTES by 96% after 12 h incubation at an inclusion level of 1%. Apart from being described as fungal or bacterial proteases, there were no other details provided about the enzymes. The bacterial proteases appeared to be more effective at breaking down trypsin inhibitors than the fungal protease. Meijer and Spekking (1993) isolated microorganisms

<table>
<thead>
<tr>
<th>Soybean treatment</th>
<th>ADG (kg)</th>
<th>FCR</th>
<th>N retention (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfermented</td>
<td>0.52</td>
<td>2.33</td>
<td>50.6</td>
</tr>
<tr>
<td>Fermented with <em>A. oryzae</em></td>
<td>0.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.7</td>
</tr>
<tr>
<td>Fermented with <em>R. oligosporus</em></td>
<td>0.56</td>
<td>1.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>*P > 0.05.*

*Table 6.5. Summary of results of Zamora and Veum (1979).*
producing enzymes that could utilize purified sources of Kunitz soybean trypsin inhibitor (KSTI) and Bowman-Birk inhibitor (BBI) as sole carbon, nitrogen and sulphur sources, and inactivate them over a time course of several days. Therefore these microorganisms must all possess enzymes capable of hydrolysing the trypsin inhibitors in soybeans. This does not necessarily mean that trypsin inhibitors would be utilized in preference to the other sources of C, N or S in whole soybeans. These enzymes may have potential activity against trypsin inhibitors in other VPMs.

Rooke et al. (1996) aimed to assess whether protease treatment of soybean meal (SBM) could reduce its in vitro antigenicity and improve its nutritional value when fed to newly weaned piglets. Table 6.6 (Ref. 1) describes the protease treatment of SBM in this study. Antigenic soybean proteins were measured by a competitive enzyme-linked immunosorbent assay (ELISA) technique. All diets, including the

Table 6.6. Summary of recent studies utilizing protease pretreatment of SBM.

<table>
<thead>
<tr>
<th>Ref. no.</th>
<th>Vegetable protein</th>
<th>Enzyme</th>
<th>Treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SBM</td>
<td>Acid protease (P2)</td>
<td>0.1% protease added to SBM (800 g kg(^{-1}) moisture) pH 4.5. Incubated for 3 h at 50°C, neutralized, dried at 65°C</td>
<td>Rooke et al. (1996)</td>
</tr>
<tr>
<td>2</td>
<td>SBM</td>
<td>Acid protease (P2)</td>
<td>0.1% protease added to SBM (800 g kg(^{-1}) moisture) pH 4.5. Incubated for 3 h at 50°C, dried at 55°C</td>
<td>Hessing et al. (1996)</td>
</tr>
<tr>
<td>3</td>
<td>Alkaline protease (P1)</td>
<td>0.1% protease added to SBM (800 g kg(^{-1}) moisture) pH 8.5. Incubated for 3 h at 50°C, dried at 55°C</td>
<td>Hessing et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>SBM</td>
<td>Acid protease (P2)</td>
<td>0.1% protease added to SBM (800 g kg(^{-1}) moisture) pH 4.5. Incubated for 2 h at 50°C, fed as a wet mash</td>
<td>Rooke et al. (1998)</td>
</tr>
<tr>
<td>5</td>
<td>Alkaline protease (P1)</td>
<td>0.1% protease added to SBM (800 g kg(^{-1}) moisture) pH 8.5. Incubated for 2 h at 50°C, fed as a wet mash</td>
<td>Hessing et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SBM</td>
<td>Protease (B. subtilis: subtilisin)</td>
<td>0.1% protease added to SBM (1:2 wt:vol water) pH 4.5. Incubated for 16 h at 50°C, freeze dried</td>
<td>Caine et al. (1997)</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>50 ml of enzyme solution (pH 4.5) to give final enzyme concentration of 0.1% sprayed on SBM, air dried at ambient temperature for 24 h</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>FFSBM (autoclaved)</td>
<td>Protease (P4)</td>
<td>0.25% added to soybean meal (1:3 wt:vol water) incubated for 24 h at 20°C, fed as liquid</td>
<td>Beal et al. (1998b)</td>
</tr>
<tr>
<td>9</td>
<td>Raw soybean</td>
<td>Protease (P4)</td>
<td>0.25% added to soybean meal (1:3 wt:vol water) incubated for 24 h at 20°C, fed as liquid</td>
<td>Beal et al. (1999)</td>
</tr>
<tr>
<td>10</td>
<td>Micronized FFSBM</td>
<td>Protease (P3)</td>
<td>0.5% added to soybean meal (1:3 wt:vol water) incubated for 24 h at 20°C, fed as liquid</td>
<td>Beal et al. (1999)</td>
</tr>
<tr>
<td>11</td>
<td>Raw soybean</td>
<td>Protease (P3)</td>
<td>0.5% added to soybean meal (1:3 wt:vol water) incubated for 24 h at 20°C, fed as liquid</td>
<td>Beal et al. (1999)</td>
</tr>
</tbody>
</table>

Vegetable Protein Meals

skim milk diet, contained antigenic soybean proteins, indicating a possible problem with the technique. The SBM treated with protease contained fewer antigenic proteins than the other soybean-containing diets. It is assumed here, as in many studies, that reduction in levels of antigenic proteins as measured by ELISA indicates protein denaturation by exogenous protease treatment. This is not necessarily an accurate assumption as, for example, antiserum from animals immunized with soybean protein may not recognize any protein components of an enzyme-treated soybean product, but the treated product may still contain antigenic epitopes.

Hessing et al. (1996) examined the ability of two microbial proteases (P1 and P2) to degrade ANFs, and to determine whether enzymatically hydrolysed SBM could improve the productive performance of newly weaned piglets or broiler chicks. The SBM was pretreated with the protease before feeding. Table 6.6 (Refs 2–5) describes the protease pretreatments. SDS-PAGE and Western blotting analysis demonstrated that P1 could significantly hydrolyse the storage proteins glycinin and β-conglycinin, and to a certain extent KSTI at inclusion levels of 1000–10,000 U g⁻¹ material, but there were no effects on soybean lectin. It was concluded that the potential of proteases to improve the nutritional value of soybean meal was promising. It was cautioned that in vitro immunochemical analysis of ANFs must be interpreted with care, as the hydrolysis of proteins such as trypsin inhibitors may expose more antigenic epitopes, but the inhibitor itself may not be functional, resulting in misleading results.

Caine et al. (1997) determined the optimum conditions for Bacillus subtilis subtilisin pretreatment of SBM by measuring increases in protein solubility. They found the optimum conditions to be incubation at 50°C and pH 4.5.

Beal et al. (1998a) used an in vitro technique (Boisen and Fernandez, 1997) as an initial evaluation of the potential of three proteases to improve the in vitro nitrogen digestibility of RSB and four processed SBMs. They found significant increases (P < 0.05) of approximately 5–12% over control with the three proteases. These results indicate the possibility of increases of protein digestibility of these meals in vivo. Beal et al. (1998c) also examined the effects of one of these proteases (P4) using SDS-PAGE, and found a reduction in the number and density of protein bands with apparent molecular weights greater than 66 kDa, indicating the hydrolysis of storage proteins.

Rooke et al. (1998) also used SDS-PAGE to examine the effects of proteases P1 and P2 (Table 6.6, Refs 4–5) on SBM. Pretreatment changed the composition of SBM, and soluble α-amino nitrogen concentrations were increased by treatment with P1 and P2. P1 reduced antigenic protein concentration (as determined by SDS-PAGE) to a greater extent than P2.

Table 6.6 summarizes exogenous protease pretreatments of soybean meals in recent studies described above. The in vivo results obtained from these trials are summarized in Table 6.7. The results of these trials are variable. In some studies, enzyme pretreatment produced positive effects on some of the parameters measured whilst in others there were no significant effects. As with all such studies, it is difficult to draw direct comparisons, due to differences in age, weight and genotype of pig and in diet formulation.
Pretreatment of other VPMs with exogenous enzymes

Castanon and Marquardt (1989) investigated the effects of Celluclast (cellulase), SP249 (polygalactouronase), Alcalase (protease) and BAN (α-amylase), in various combinations, on raw, autoclaved, or steeped faba beans for young chickens. The

<table>
<thead>
<tr>
<th>Cross ref.</th>
<th>Animal age/weight and trial duration</th>
<th>Summary of results, enzyme treatment (T) compared with control (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Newly weaned pigs 21 days</td>
<td>Significant (P &gt; 0.01) increase in ADG for 7 days post weaning 155 g day⁻¹(T) vs. 95 g day⁻¹ (C). Significant increase in ADFI for 21 days post weaning 392 g day⁻¹(T) vs. 347 g day⁻¹(C). No significant increase in FCR</td>
</tr>
<tr>
<td>2</td>
<td>7 day old chicks 141 g, 27 days</td>
<td>Significant increase (P &lt; 0.01) in ADG; 41 g day⁻¹(T) vs. 30 g day⁻¹(C), ADFI; 79 g day⁻¹(T) vs. 64 g day⁻¹(C), apparent ileal N digestibility 0.85(T) vs. 0.76(C)</td>
</tr>
<tr>
<td>3</td>
<td>7 day old chicks 141 g, 27 days</td>
<td>No significant increases in ADG, ADFI or apparent ileal N digestibility</td>
</tr>
<tr>
<td>4</td>
<td>Newly weaned pigs 7.5 kg, 14 days</td>
<td>Significant increases (P &lt; 0.01) in ADG over first 7 days postweaning; 120 g day⁻¹(T) vs. 64 g day⁻¹(C) and (P &lt; 0.05) in ADG over 14 days postweaning; 181 g day⁻¹(T) vs.128 g day⁻¹(C). No significant difference in FCR</td>
</tr>
<tr>
<td>5</td>
<td>Newly weaned pigs 7.7 kg, 14 days</td>
<td>Significant decrease (P &lt; 0.01) in ADG over first 7 days postweaning; 78 g day⁻¹(T) vs. 143 g day⁻¹(C) and (P &lt; 0.05) in ADG over 14 days postweaning; 153 g day⁻¹(T) vs. 199 g day⁻¹(C). No significant difference in FCR</td>
</tr>
<tr>
<td>6</td>
<td>Newly weaned pigs 6.4 kg, 2 × 9 day changeover</td>
<td>No significant differences in ADFI, ADG, FCR or apparent ileal amino acid digestibilities</td>
</tr>
<tr>
<td>7</td>
<td>Newly weaned pigs 6.4 kg, 2 × 9 day changeover</td>
<td>No significant differences in ADFI, ADG, FCR or apparent ileal amino acid digestibilities</td>
</tr>
<tr>
<td>8</td>
<td>Growing pigs 33.5 kg, 6 weeks</td>
<td>No significant differences in ADG, ADFI or FCR</td>
</tr>
<tr>
<td>9</td>
<td>Growing pigs 33.5 kg, 6 weeks</td>
<td>Significant (P &lt; 0.05) increases in ADFI; 1.37 kg day⁻¹(T) vs. 1.24 kg day⁻¹(C), ADG 0.606 kg day⁻¹(T) vs. 0.529 kg day⁻¹(C). No significant difference in FCR</td>
</tr>
<tr>
<td>10</td>
<td>Growing pigs 28.7 kg to slaughter (90 kg)</td>
<td>No significant difference in ADG, ADFI, FCR or lean : fat ratio. Significant reduction of 4 ± 1.86 days to reach slaughter weight due to protease addition</td>
</tr>
<tr>
<td>11</td>
<td>Growing pigs 28.7 kg to slaughter (60 kg)</td>
<td>Significant (P &lt; 0.05) improvement in ADG; 0.606 kg day⁻¹(T) vs. 0.515 kg day⁻¹(C) and FCR; 3.042(T) vs. 3.512(C) in finisher period. Significant (P &lt; 0.05) increase of 0.423 ± 0.158 in lean : fat ratio due to protease addition</td>
</tr>
</tbody>
</table>

CP = crude protein; ADG = average daily liveweight gain; ADFI = average daily feed intake; FCR = feed conversion ratio (feed/gain).
authors found that cellulase and protease, and a combination of the two, significantly
increased weight gain and FCR ($P < 0.05$) by approximately 5–10% in both raw and
autoclaved beans, and the combination significantly improved the weight gain
($P < 0.05$) and FCR ($P < 0.01$) of steamed beans. The other enzymes had no effect.

Näsi (1991) investigated the effects of processing, enzyme pretreatment
and multi-enzyme supplementation (cellulase, protease and β-glucanase) of SBM
and rapeseed meal (RSM) diets on the nutrient digestibility, protein utilization and
performance in growing pigs. Extrusion and the addition of enzyme premix
significantly improved the organic matter and protein digestibilities of SBM
($P < 0.05$). No significant differences in growth rate, FCR or carcass quality were
seen between pigs fed diets containing differently treated SBM, relative to an
untreated control. No significant effects of enzyme treatment on any parameters were
observed with RSM.

Schulze et al. (1997) examined the effects of germination or pancreatin
pretreatment of *Phaseolus vulgaris* (white kidney bean) on the ANF activities and
apparent ileal digestibility of nitrogen in young pigs. The authors found germination
significantly decreased ($P < 0.05$) the flow of amino acids at the terminal ileum by
approximately 50% compared with pancreatin or control. Trypsin inhibitor and
lectin activity were reduced by germination, although the significance of these
reductions was not reported.

Direct Addition of Enzymes to Complete Diets Containing
VPMs

A number of pig and poultry trials have been performed using the addition of
exogenous enzyme premixes to dry diets. Pluske and Lindemann (1998) have
extensively reviewed the use of Vegpro, a mixture of protease, cellulase, pentosanase,
α-galactosidase and amylase, in pig and poultry trials using various VPMs. Cowan
et al. (1996) reviewed the use of Biofeed Plus CT (main activity protease) and
Energex MG (main activities β-glucanase and pectinase). Some other work using the
same enzymes is reviewed below.

Enzymes targeted to SBM dietary components

De Koning and van der Wel (1996) supplemented starter broiler maize/SBM diets
with Vegpro at 0.1 and 0.05%. Significant increases ($P < 0.05$) in weight gain after
21 days were observed with both enzyme inclusion levels, but no significant
differences in FCR were observed. Similar results were obtained by Sefton and
Perdok (1996). Swift et al. (1996) examined the effects of Vegpro on expanded
and pelleted maize/SBM diets for broilers. Enzyme treatment significantly
improved ($P < 0.05$) FCR in the expanded diet over the 35-day feeding period.
Vegpro significantly improved ($P < 0.05$) nitrogen and energy digestibility in grower
diets but not starter diets. Schang et al. (1997) evaluated the effects of Vegpro in
maize/SBM and maize/full-fat SBM (FFSBM) diets for broilers, using high and low nutrient density formulations. There were no significant effects of adding Vegpro to high density diets. Significant improvements \( (P<0.05) \) in weight gain were seen with maize/FFSBM at low nutrient digestibilities. Spring et al. (1998) examined the effect of Vegpro on a grain/SBM diet with reduced protein concentration, in weaned piglets. Significant improvements \( (P<0.05) \) in FCR due to Vegpro treatment were observed in diets in which the lysine concentration was reduced to 0.97%.

**Enzymes targeted to other VPM dietary components**

Viveros et al. (1993) examined the effect of addition of a combination of Biofeed Pro, a mixture of protease, amylase, hemicellulase, pentosanase and xylanase, and BAN, a mixture of amylase and \( \beta \)-glucanase to diets containing 10 and 15% faba bean hulls in diets for chickens. Addition of the enzyme mixtures significantly improved \( (P<0.05) \) weight gain in 10 and 15% hull diets, and FCR in 15% hull diets. However, enzyme addition did not improve the feed efficiency of the hull diets to the level of a maize/SBM diet. The same workers investigated the use of tannase in a 10% hull diet. Tannase addition significantly improved \( (P<0.05) \) weight gain, but not FCR.

Pfrirter et al. (1993) investigated the effects of exogenous protease supplementation on broiler feeds containing crude or extracted lupins. No significant improvements over control, crude lupin diets were observed.

Hadron et al. (1993) evaluated the effects of exogenous carbohydrases and proteases on growing-pig diets containing peas. No effects of enzymes were observed on nitrogen or energy digestibility, or on production characteristics.

Bedford and Morgan (1995) examined the effects of the addition of xylanase, xylanase plus alkaline protease, xylanase plus neutral protease, and xylanase plus acid protease on canola meal (CM) diets for broilers. Addition of xylanase alone significantly improved \( (P<0.05) \) weight gain over the CM control diet. Addition of xylanase and alkaline protease significantly improved \( (P<0.05) \) FCR over a SBM-based control diet. The addition of acid protease also significantly \( (P<0.05) \) improved FCR, but the neutral protease had no effect.

Annison et al. (1996) examined the effects of two commercial enzyme preparations on the nutritive value of dehulled lupins for broiler diets. Enzyme A contained xylanase, pentosanase, and hemicellulase; Enzyme B contained \( \beta \)-glucanase, hemicellulase and pectinase. Enzyme A increased the AME of lupins \( (P<0.05) \); Enzyme B had no significant effects.

Stanley et al. (1996) examined the effect of Vegpro addition on various levels of cottonseed meal (CSM) in diets for broiler chicks. The inclusion of Vegpro significantly improved \( (P<0.05) \) FCR in diets containing 7.5, 15 and 30% levels of CSM. Schang and Azcona (1998) evaluated the effects of Vegpro on maize/sunflower diets as a replacement for maize/SBM diets. Sunflower meal significantly \( (P<0.05) \) decreased egg production, and the addition of Vegpro did not compensate for this.
Discussion

Trials conducted to date on the effects of exogenous enzyme supplementation of VPMs show variable results, with some showing significant changes with enzyme inclusion, and others showing trends towards improvement, which are not actually significant. There are a number of possible explanations for these observations. In many cases it is impossible to draw direct comparisons between individual studies due to differences in enzyme type, activity and inclusion level. Where enzyme activities are actually stated, there are often differences in units and the method of determining activity is not always stated.

The majority of trials have been performed under experimental conditions, with high health status animals, which may not necessarily reflect commercial situations. Basal diets can vary widely between different trials depending on the availability of dietary components. It must be remembered that modern diets are formulated to achieve maximum performance from an animal. Any increase in nutrient availability due to enzyme supplementation may not elicit a response from the animal unless the dietary factor in question is reduced to suboptimal levels. This is not necessarily a consideration when substituting proportions of a diet with inferior feedstuffs, provided nutrient specifications do not exceed recommended levels for that species, age and genotype. For example, if the effect of protease inclusion in the diet is being investigated, the protein : energy ratio needs to be reduced to suboptimal levels in order for the animal to respond to any increase in amino acid availability due to enzyme treatment. In the studies outlined in Tables 6.6 and 6.7, Rooke et al. (1996, 1998), Hessing et al. (1996) and Beal et al. (1998b, 1999) indicated that lysine : energy ratios were reduced below recommended levels, whereas Caine et al. (1997) stated that the diets used were formulated to exceed NRC recommendations. This could explain the lack of response to enzyme inclusion in their study. Other studies corroborate this, such as Schang et al. (1997) and Spring et al. (1998), who showed significant differences with diets formulated with nutrient densities below recommendations, but not with diets formulated to meet or exceed nutrient recommendations.

It is difficult to assess the efficacy of enzyme preparations on target substrates when applied to complete diets, as all plant feedstuffs in a diet are likely to contain substrates on which enzymes can act. This does not present a major problem with enzymes designed to target substrates such as NSPs, which can present problems in other major dietary constituents, such as cereals. However, when targeting proteinaceous ANFs in VPMs with exogenous proteases, a number of factors need to be considered. The VPM in a compound feed represents only a fraction of the total feed, typically 20–30%. The target substrate for the enzyme will be even less than this. For example, if full-fat soybean meal with a crude protein content of 40% (see Table 6.1) is included in a diet at a rate of 25%, then the actual soybean protein content of that compound feed will be 10%, and the ANF content even less, at 6–7% of the total protein (Nielsen, 1983). This represents a considerable dilution of the substrate by other feed components; the competition from other protein substrates in a complete diet may result in little or no ANF degradation. Pretreatment of VPMs
prior to inclusion in a diet has two advantages. Firstly, the dilution effect of other dietary components is reduced. Secondly, pretreatment allows the effects of enzyme activity on the specific substrate to be evaluated. Analysis of *in vitro* results of pretreatment have to be interpreted with caution; for example, ELISA techniques may demonstrate changes in protein structure, but not necessarily functionality. However, the development of immunochemical assays which measure KSTI, BBI and soybean lectin (SBL), both by aspects of protein structure and by biological activity, in feed and digesta samples may allow this problem to be resolved (Thorpe *et al*., 1997; Miller *et al*., 1998). Techniques used to measure *in vitro* digestibility cannot necessarily be extrapolated to *in vivo* situations; however, they are useful tools in the initial screening of the suitability of enzyme preparations.

In designing exogenous enzyme studies, the predicted application of the enzyme needs to be considered to allow the transfer of technologies to commercial situations. This is of particular importance with pretreatment strategies. Some of the studies previously described use pretreatments with relatively energy-intensive processes, such as high incubation temperatures followed by drying (Hessing *et al*., 1996; Rooke *et al*., 1996; Caine *et al*., 1997). This may not be economically viable when extrapolated to a commercial situation.

New developments in feeding systems, particularly of those for pigs, have increased scope for effective commercial enzymatic applications. One such development is the increased use of liquid feeding systems, which provide excellent conditions for feed enzymes. On many UK farms, liquid feed systems for pigs are used to enable farmers to take advantage of liquid by-products from food processors; thus diets are often mixed on-farm (Geary, 1997). Enzyme preparations could be added directly into the feeding system, thereby eliminating the risk of enzyme denaturation during processing procedures such as pelleting. These systems provide an ideal opportunity for pretreatment of individual raw materials, such as VPMs, *in situ*, in a commercial environment, before addition of other dietary components. Pretreatment of VPMs prior to incorporation into poultry diets does not appear to have attracted so much attention. Pretreatment may not be so relevant to the poultry industry, as liquid feed systems are generally not used and anatomical features of the fowl digestive system effectively provide a pretreatment chamber in the crop. The activity in the crop may explain the greater response of poultry to exogenous enzyme supplementation in general.

Pretreatment of SBM with exogenous enzymes appears to have the greatest effect in young animals, particularly noted in early weaned pigs (Hessing *et al*., 1996; Rooke *et al*., 1996, 1998; Caine *et al*., 1997). This effect is usually greatest in the 7 days immediately post-weaning, possibly helping to overcome the transient deleterious effect of feeding SBM observed by workers such as Li *et al*., 1991a,b and Dreau *et al*. (1994). At this time the animal is often performing suboptimally, giving a window of opportunity for the use of exogenous enzyme supplementation. Effects of enzyme supplementation do not appear to be as great in grower/finisher pigs. Possible reasons for this include the maturity of the digestive and immune system and the formulation of diets to high nutrient specifications. The major VPM in use in grower/finisher pig diets at present is SBM. This is often highly processed to
maximize digestibility (van der Poel, 1989). Processes used are often expensive and it is possible that the use of enzyme supplementation of less digestible SBMs or other VPMs may allow higher inclusion rates of less expensive ingredients in diets without affecting performance.

Future Research Requirements

To date, the majority of research has been aimed towards the improvement of SBMs. SBM is considered to be a high quality protein source and other, possibly lower quality, VPMs may offer more scope for improvement by exogenous enzyme supplementation. This could allow the use of VPMs from indigenous rather than imported sources.

The pretreatment of individual dietary components with exogenous enzymes has not been fully explored. In particular, the application of exogenous enzyme pretreatments in liquid feed systems in the pig industry provides a potential opportunity for the improvement of the nutritional status of VPMs.

There is little published data on the optimum inclusion levels of exogenous enzymes in animal diets. Inclusion levels are often extrapolated between species and, considering the differences in anatomy and physiology of the digestive tract, these levels may be incorrect. Maximum improvements of VPMs may not be achieved unless optimum enzyme inclusion levels are used. These levels need to be determined.

The implications of the recent European ban of antibiotic growth promoters in animal diets on performance and health status have yet to be fully explored. It is possible that this ban may result in decreased animal performance and exogenous enzymes could be utilitzed to help to alleviate this.

References

Beal, J.D., Brooks, P.H. and Schulze, H. (1998b) The effect of the addition of a protease enzyme to raw or autoclaved soya bean on the growth performance of liquid fed...


Vegetable Protein Meals


Introduction

Feed enzymes have come to be regarded by many nutritionists as being a necessary ‘ingredient’ for formulating poultry rations. This has occurred mainly since the 1990s, but the concept of enhancing animal performance using enzymes is by no means new. For example, in the mid 20th century, various preparations of amylase were used in an attempt to overcome poor performance of chicks fed barley diets by increasing the availability of starch (Hastings, 1946; Fry et al., 1957). The early work focused on the hydrolysis of specific substrates to their simple constituents for absorption (Fry et al., 1957; Moran et al., 1968), but this approach was not successful. Appreciable advances have since been achieved in the use of enzymes in poultry diets with a clear understanding of the target substrates and the development of microbiological technology. The prime example of this is the use of β-glucanases in barley diets and xylanases (pentosanases) in rye or wheat diets.

Enhancing Bird Performance by Enzyme Supplementation

Barley

The macro-nutrient contents of barley and maize are very similar, but their nutritive value for poultry is vastly different. This led scientists to use various treatments, including enzyme supplementation (Hastings, 1946; Fry et al., 1957). These workers used an α-amylase, which significantly increased the liveweight gain and feed conversion efficiency (FCE) of chickens fed barley diets. It is now well established that the starch in barley is totally digestible by the amylase secreted by chickens. Therefore the reported improvements with amylase supplementation were probably due to the impurities in the enzymes used, i.e. the crude enzyme preparation.
contained β-glucanase activity. β-Glucans are glucose polymers containing a mixture of β1,3 and β1,4 linkages that make their physicochemical properties totally different from cellulose that is a straight-chain glucose polymer with only β1,4 linkages. Barley contains a high level of mixed-linked β-glucan (3–4%), which is responsible for its poor nutritive value in chickens (Burnett, 1966). Since this significant finding, there have been numerous studies on the use of enzymes, in particular β-glucanases in barley-based poultry diets (Hesselman and Åman, 1986; Campbell et al., 1989), with outstanding success. The enzymes increase growth performance and feed conversion efficiency. Increases of up to 17% in liveweight gain (Broz and Frigg, 1986) and 19% in feed conversion efficiency (Newman and Newman, 1987) have been reported for broiler chickens fed barley diets supplemented with β-glucanases. Barley also contains an appreciable amount of soluble NSP other than β-glucans and thus the majority of enzymes for barley diets have both β-glucanase and arabinoxylanase activities.

Rye

The poor feeding value of rye was reported more than 60 years ago (Halpin et al., 1936). In the search for an answer to the problem, Fernandez et al. (1973) extracted rye grain with water and freeze-dried the extract. When this extract was added to a maize-based diet, it depressed the growth of the birds and caused sticky droppings, whereas the water-extracted rye was markedly better than normal rye. This water-extractable factor is now known to be the soluble arabinoxylan (Fengler and Marquardt, 1988a,b). Thus, addition of arabinoxylanases to rye-based broiler diets significantly improves the growth performance and feed conversion efficiency (Bedford et al., 1991; Bedford and Classen, 1992). Supplementation with increasing levels (0.11, 0.22, 0.44 and 0.88 g kg⁻¹) of an enzyme preparation having arabinoxylanase and β-glucanase activities to a rye–wheat-based diet improved the weight gain of broilers up to 27% and FCE up to 10% (Pettersson and Åman, 1988, 1989). Although enzymes always substantially improve the performance of birds fed rye diets, they do not seem to have as large an effect on the litter quality.

‘Low-ME’ wheat

Although the large variability (up to 4 MJ kg⁻¹ dry matter) of the nutritive quality of wheat was reported by numerous researchers (Sibbald and Slinger, 1962; Schumaier and McGinnis, 1967), the significance of the problem had not been widely appreciated until recently. Connor et al. (1976) noticed that the AME value obtained for wheats was 7–25% lower than that of sorghum, and Payne (1976) postulated that some wheats may contain a ‘slightly toxic inhibitor’. Subsequently, two major studies found that approximately 25% of the Australian wheats had AME values below 13 MJ kg⁻¹ dry matter (Mollah et al., 1983; Rogel et al., 1987). When these ‘low-ME’ wheats are included at above 50% in the ration, chickens have sticky and
watery droppings accompanied by poor growth and feed efficiency. At very high levels of inclusion (up to 90% of the diet), young broilers can only utilize a very small portion of the energy (2.90 MJ kg$^{-1}$) (Wiseman and McNab, 1998). It is now generally conceded that the occurrence of low-ME wheats is due to an increased level of NSP, in particular the arabinoxylan (Choct and Annison, 1990; Annison, 1991). Choct et al. (1995) demonstrated that supplementation of a low-ME wheat diet with a commercial glycanase preparation increased the AME by 24% and the FCE by 25% in 3–4 week old broiler chickens.

**Mechanisms of Action**

The term non-starch polysaccharide (NSP) represents a diversity of compounds possessing different physicochemical properties; thus their nutritional effects in poultry are also diverse and, in some cases, extreme. It is, however, generally conceded that the detrimental effect of NSP in viscous grains is associated with the viscous nature of these polysaccharides, their physiological and morphological effects on the digestive tract and the interaction with the microflora of the gut. The mechanisms include altered intestinal transit time and modification of the intestinal mucosa, as well as changes in hormonal regulation due to a varied rate of nutrient absorption. To elucidate the action of NSP in poultry diets, Choct and Annison (1990, 1992a) isolated the cell wall polysaccharides from wheat and added them to a semi-purified diet. Two fractions were prepared. Fraction 1 was extracted using water only and hence named water-extractable arabinoxylans (WEP). Fraction 2 was obtained by extraction with NaOH (0.2 mol l$^{-1}$) and called alkali-extractable arabinoxylans (AEP), which were also soluble in water. Addition of these soluble arabinoxylan preparations at an equivalent of 30 g pure arabinoxylans kg$^{-1}$ diet depressed weight gain by 24.6%, feed conversion ratio by 11.2%, DM digestibility by 8.4% and AME of sorghum by 9.9%. The anti-nutritive effects of wheat arabinoxylans were also demonstrated in a practical type of broiler diet (Choct and Annison, 1992a). The arabinoxylans severely depressed bird performance and nutrient digestibilities when added at above 2% to the diet. Part of the data is shown in Tables 7.1 and 7.2.

The wheat arabinoxylans caused a general inhibition of nutrient digestion affecting starch, fat and protein, which indicated that they acted in a similar manner as the anti-nutritive NSP of rye and barley. The arabinoxylans of rye have been reported to depress fat, protein and DM retention (Ward and Marquardt, 1987; Fengler and Marquardt, 1988b). Depressed ileal digestibilities of starch and protein have been observed in chickens fed barley diets which had high levels of extractable viscous β-glucans (Hesselman and Åman, 1986). Variability in the experimental data increased as greater amounts of arabinoxylans were added to the diets. This mimics the low-AME wheat phenomenon. When broilers were fed low-AME wheats, greater between-bird variation was seen than when normal wheats were fed (Rogel et al., 1987). Classen et al. (1988) demonstrated that supplementation of barley diets with
β-glucanase reduced the variability from 11.9 to 3.3% for weight gain and from 5.2 to 2.7% for FCR in broilers.

Viscous nature of soluble NSP

Increased bulk and viscosity of the intestinal contents decrease the rate of diffusion of substrates and digestive enzymes and hinder their effective interaction at the mucosal surface (Edwards et al., 1988; Ikegami et al., 1990). Studies in vitro with guar gum showed that soluble, indigestible polysaccharides interact with the glycocalyx of the

Table 7.1. Effects of soluble arabinoxylans from wheat on AME, nitrogen retention (NR), feed conversion ratio (FCR), weight gain (WG) and feed intake (FI) in broilers (n = 8). (Data from Choct and Annison, 1992a.)

<table>
<thead>
<tr>
<th>Diet1</th>
<th>Arabinoxylan2 (g kg⁻¹ DM)</th>
<th>AME³ (MJ kg⁻¹ DM)</th>
<th>NR (g d⁻¹)</th>
<th>FCR (g : g)</th>
<th>WG (g 6d⁻¹)</th>
<th>FI (g 6d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.9</td>
<td>15.05a</td>
<td>3.05a</td>
<td>1.91a</td>
<td>348a</td>
<td>661a</td>
</tr>
<tr>
<td>WEP(20)</td>
<td>43.8</td>
<td>13.90b</td>
<td>2.79b</td>
<td>2.08ab</td>
<td>312ab</td>
<td>637b</td>
</tr>
<tr>
<td>AEP(5)</td>
<td>30.8</td>
<td>15.00a</td>
<td>2.89a</td>
<td>1.94a</td>
<td>348a</td>
<td>675a</td>
</tr>
<tr>
<td>AEP(10)</td>
<td>34.9</td>
<td>14.70a</td>
<td>2.86a</td>
<td>1.95a</td>
<td>352a</td>
<td>686a</td>
</tr>
<tr>
<td>AEP(25)</td>
<td>48.0</td>
<td>13.34b</td>
<td>2.42b</td>
<td>2.49bc</td>
<td>268bc</td>
<td>640b</td>
</tr>
<tr>
<td>AEP(40)</td>
<td>65.7</td>
<td>12.48b</td>
<td>1.96b</td>
<td>2.70b</td>
<td>216b</td>
<td>552b</td>
</tr>
</tbody>
</table>

SE (pooled) | 0.22 | 0.13 | 0.12 | 15 | 19 |

1Values in parentheses are added arabinoxylans (g kg⁻¹ DM).
2Determined values for total pentosans in diets.
3Columns sharing the same superscripts are not significantly different (P > 0.05). WEP = water extractable arabinoxylans; AEP = alkali extractable arabinoxylans.

Table 7.2. Effects of wheat arabinoxylans on ileal digestibility coefficients of starch, protein and lipid (n = 3). (Data from Choct and Annison, 1992a.)

<table>
<thead>
<tr>
<th>Diet1</th>
<th>Arabinoxylan²</th>
<th>Starch³</th>
<th>Protein</th>
<th>Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.9</td>
<td>0.96a</td>
<td>0.75a</td>
<td>0.93a</td>
</tr>
<tr>
<td>WEP(20)</td>
<td>43.8</td>
<td>0.91b</td>
<td>0.70b</td>
<td>0.87ab</td>
</tr>
<tr>
<td>AEP(5)</td>
<td>30.8</td>
<td>0.96ab</td>
<td>0.75a</td>
<td>0.93a</td>
</tr>
<tr>
<td>AEP(10)</td>
<td>34.9</td>
<td>0.95ab</td>
<td>0.73a</td>
<td>0.92a</td>
</tr>
<tr>
<td>AEP(25)</td>
<td>48.0</td>
<td>0.92ab</td>
<td>0.69ab</td>
<td>0.76ab</td>
</tr>
<tr>
<td>AEP(40)</td>
<td>65.7</td>
<td>0.82c</td>
<td>0.61b</td>
<td>0.69b</td>
</tr>
</tbody>
</table>

SE (pooled) | 0.02 | 0.03 | 0.06 |

1Values in parentheses are added arabinoxylans (g kg⁻¹ DM).
2Determined values for total pentosans in diets.
3Columns sharing the same superscripts are not significantly different (P > 0.05). WEP = water extractable arabinoxylans; AEP = alkali extractable arabinoxylans.
intestinal brush border, producing a thickening of the rate-limiting unstirred water layer, which results in decreased nutrient absorption (Johnson and Gee, 1981). Germination of barleys can lower the viscosity of their aqueous extracts and such barleys are of better nutritive value in poultry compared with normal barleys (Fengler et al., 1990). Barleys with high extract viscosity are more detrimental to chickens than those with low extract viscosity (Campbell et al., 1989). These results lend support to the hypothesis that viscosity is involved in the anti-nutritive effect of NSP in poultry diets. Choct and Annison (1992b) compared the effects of a high molecular weight NSP isolate (Intact-NSP) (854 g arabinoxylans and 42 g glucan kg⁻¹ DM; molecular weight = 758,000) and a partially depolymerized NSP isolate (Depol-NSP; 194,000) (Annison et al., 1992), and pentoses on broiler performance and nutrient digestibility (Table 7.3). When included at a level of 30 g kg⁻¹ diet, the partially depolymerized NSP increased digesta viscosity significantly compared with pentose sugars added at the same level, but it was lower than that in birds fed intact NSP. Bird performance was not significantly affected by the depolymerized NSP, indicating that birds can tolerate small increases in digesta viscosity without a detrimental effect on performance.

The anti-nutritive effects of the arabinoxylans appear largely dependent on the polymeric nature of the polysaccharides and their viscous property. This is supported by studies demonstrating that supplementation with enzymes capable of degrading β-glucans and arabinoxylans increases the nutritive value of barley and rye in poultry diets and this coincides with a significant decrease in the digesta viscosity of the chickens (White et al., 1981; Bedford et al., 1991).

Watery and sticky droppings alone may not be a sufficient indicator of the anti-nutritive effect of the arabinoxylans in poultry, as excreta from birds fed the diet containing the depolymerized arabinoxylans were watery and sticky, and those from birds given the diet containing pentose sugars also appeared more moist compared with controls. In the rat, dietary components that are not completely digested or absorbed in the small intestine give rise to an increased amount of osmotically active materials in the gut content. If these materials are utilized by the hindgut bacteria,

Table 7.3. Effects of high molecular weight NSP, partially depolymerized NSP and pentoses on weight gain (WG), feed conversion ratio (FCR), feed intake (FI) and AME in broilers (n = 8). (From Choct and Annison, 1992b.)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Level added (g kg⁻¹)²</th>
<th>Digesta viscosity (g wk⁻¹)³</th>
<th>WG (g wk⁻¹)³</th>
<th>FCR (g : g)</th>
<th>FI (g wk⁻¹)³</th>
<th>AME (MJ kg⁻¹ DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1.2a</td>
<td>430a</td>
<td>1.589a</td>
<td>681</td>
<td>16.13a</td>
</tr>
<tr>
<td>Intact-NSP</td>
<td>30</td>
<td>3.0c</td>
<td>325b</td>
<td>1.960b</td>
<td>622</td>
<td>14.53c</td>
</tr>
<tr>
<td>Pentoses</td>
<td>30</td>
<td>1.2a</td>
<td>394b</td>
<td>1.635bc</td>
<td>658</td>
<td>16.23bc</td>
</tr>
<tr>
<td>Depol-NSP¹</td>
<td>30</td>
<td>2.2e</td>
<td>404bc</td>
<td>1.649e</td>
<td>661</td>
<td>15.74e</td>
</tr>
</tbody>
</table>

¹Partially depolymerized NSP.
²Levels added at equivalent to pure NSP.
³Columns sharing the same superscripts are not significantly different (P > 0.05).
production of low molecular weight metabolites that are not readily absorbed may result. This can lead to further accumulation of osmotically active substances which attract a large amount of water. This may well relate to the inefficient utilization of five-carbon sugars by chickens (Wagh and Waibel, 1966). The significant improvement in performance of birds fed barley or rye supplemented with β-glucanase or arabinoxylanase is not due to a complete hydrolysis of the polysaccharides and a subsequent absorption of the released sugars (White et al., 1981), but is due to the depolymerization of the polysaccharides into smaller polymers which do not greatly elevate the viscosity of digesta (De Silva et al., 1983).

Modification of secretory response of the gut

Viscous polysaccharides cause physiological and morphological changes to the digestive system of rats, pigs and humans (Brown et al., 1979; Cassidy et al., 1981; Morgan et al., 1985; Ide et al., 1989; Low, 1989). The endogenous secretion of water, proteins, electrolytes and lipids can be increased markedly by NSP supplementation of the diet (Low, 1989). The metabolic cost of such processes can be considerable. Prolonged consumption of diets containing viscous polysaccharides is associated with significant adaptive changes in the digestive system in rats (Ikegami et al., 1990). The changes in the gastrointestinal tract are characterized by enlargement of digestive organs and increased secretion of digestive juices, accompanied by decreases in nutrient digestion. The depressed apparent ileal protein digestibility caused by the wheat arabinoxylans, therefore, may be due to an inhibition of protein breakdown and/or a reduction in amino acid absorption. It may also result from an increase in the secretion of endogenous proteins, which can be derived from gut secretions and losses of intestinal cells. In the following study (Angkanaporn et al., 1994), the effect of soluble wheat arabinoxylans on endogenous protein (sum of amino acids) secretions, in comparison to that of pure cellulose and an inert nutrient diluent (polythene powder), was investigated. Endogenous and exogenous amino acids were distinguished using the homoarginine marker technique (Hagemeister and Erbersdobler, 1985). The apparent protein digestibility was depressed to a similar extent by addition of wheat arabinoxylans at levels of 15 and 30 g kg$^{-1}$, but the true protein digestibility was significantly inhibited only at the higher level of inclusion. This indicates that the anti-nutritive effect of wheat arabinoxylans on the apparent protein digestibility is mainly due to increased endogenous secretions of amino acids at low levels of inclusion, while at high levels a direct inhibition of protein breakdown and/or absorption occurs. Addition of cellulose and polythene did not have an effect although the levels of inclusion were much higher than that of wheat arabinoxylans (Table 7.4).

The biological effects of NSP differ considerably due to their diverse physiochemical properties. Southon et al. (1985) reported that a diet containing 75 g non-cellulosic polysaccharides kg$^{-1}$ and 24 g cellulose kg$^{-1}$ fed to rats induced higher rates of protein synthesis in the jejunum and ileum, and more rapid mucosal cell division than rats given a semi-purified diet containing cellulose as the only source of
The lack of action of cellulose on the endogenous amino acid secretions was further demonstrated in chickens by Parsons (1984), who found that when a nitrogen-free diet containing 400 g raw potato starch kg\(^{-1}\) and 100 g pectin kg\(^{-1}\) was fed to laying hens, the total endogenous amino acid secretion was increased by 40%; whereas the diet containing 500 g cellulose kg\(^{-1}\) elicited little effect. These observations indicate that the action of NSP in modification of the physiological functions of the gastrointestinal tract is not a mere mechanical stimulation of the mucosa. It is possible that the soluble NSPs interact with the gut wall in a way that modifies the endocrine regulation.

Encapsulated nutrients

Although the insoluble NSPs have mainly been regarded as a nutrient diluent in the diet, they can also affect digesta transit time and gut motility. Another facet of the role of insoluble NSP in poultry diets that is worthy of reiteration is the possibility of them acting as a physical barrier to digestive enzymes, such as amylase and proteases, thus reducing their efficient digestion in the upper part of the gut. This is probably particularly important in non-viscous grains, such as sorghum. There is evidence that enzymes with affinity for insoluble NSP can also elicit a positive response in growth performance of broilers (Cowan, 1995; Choct, 1998). This indicates that breakdown of cell wall matrix, especially the insoluble components, may facilitate easier access of digestive enzymes to their substrates within the short feed transit time in birds. Wiseman and McNab (1998) also showed that the rate of \textit{in vitro} starch digestion correlates closely with the AME values in wheat, indicating that the accessibility of amylolytic enzymes to starch granules is vastly different.

NSP and gut microflora

The gut harbours a highly evolved and complex microbial ecosystem containing a vast number of diverse populations. For example, microbes make up approximately...
600 g kg\(^{-1}\) of the wet weight of poultry excreta. The proper feeding of poultry should therefore consider the provision of ‘correct’ substrates for the microflora to keep it stable. The consequences of altered rate of nutrient digestion in the gut may be manifested in the number and type of microorganisms present in the gut (Vahjen et al., 1998). An increased amount of NSP in the digesta has been demonstrated to influence the gut microflora in a negative manner to birds (Choc\(t\) et al., 1996). The overall digestibilities of protein and long-chain fatty acids, especially the saturated ones, were more severely depressed in intact birds than in caecectomized birds when wheat arabinoxylans were added to the diets (Choc\(t\) et al., 1992). Bile salts are essential for emulsification of fats and activation of lipase (Tso, 1985), especially for saturated fatty acids (Johnston, 1977) as opposed to short-chain and unsaturated fatty acids, which are more easily absorbed in the absence of bile acids (Garrett and Young, 1975). Excessive deconjugation of bile salts is a possible deleterious effect of gut microflora (Feighner and Dashkevicz, 1988) and it may be exacerbated by the presence of large amounts of undigested carbohydrates in the hindgut. This is supported in studies by Campbell et al. (1983) where supplementation of the conjugated bile salt, sodium taurocholate, to chickens fed rye diets significantly improved the fat retention. Furthermore, utilization of nutrients through microbial conversion of digestible carbohydrates, such as starch, to VFAs is not efficient compared with a direct absorption of glucose released from enzymatic digestion (Müller et al., 1989; Carré et al., 1995). In a recent study, Choc\(t\) et al. (1996) demonstrated that addition of soluble NSP in broiler chicken diets significantly elevated fermentation in the small intestine. Subsequent in vivo depolymerization of the soluble NSP using glycanases almost totally overcame this problem.

Another factor that may contribute to the negative effect of a high microbial load in the small intestine is increased turnover of intestinal cells. According to LeBond and Walker (1956), a 100 g rat gaining 5 g a day synthesizes 1 g mucosal cells daily, which represents a 20% additional tissue synthesis without showing up as weight gain. Extrapolating this to a 2 kg bird gaining 60 g daily, the bird would synthesize 12 g of mucosal tissue to maintain the integrity of its small intestine. Increased microbial load can exacerbate this loss (Abrams et al., 1963; Lesher et al., 1964), since some of their fermentation products, e.g. putrescine, have been shown to enhance small intestinal and colonic mucosal growth rates significantly (Seidel et al., 1985; Osborne and Seidel, 1989). Furthermore, antibiotics can significantly improve performance of birds fed high-NSP diets (MacAuliffe and McGinnis, 1971; Misir and Marquardt, 1978a,b). Whatever the reason, excess fermentation in the small intestine of the chicken may be detrimental to nutrient digestion and absorption and enzyme supplementation seemed to alleviate this problem. Perhaps reducing the viscosity of the digesta in the small intestine hastens digesta passage and nutrient digestion rate (through removal of the diffusional constraint of viscous gums), thereby giving less substrate and time for the fermentative organisms to proliferate. This may in turn restore the normal and efficient digestion (enzymatically) of starch and protein in the small intestine.
Predicting Enzyme Response

The concept of predicting the effect of enzymes in a particular feed is attractive because the producer could then adjust the amount of enzymes to be added or adjust the nutrient specifications in diet formulations. Although it is extremely difficult to predict the specific effects of enzymes accurately in practice, some approaches have proved to provide useful estimates of enzyme responses in viscous grains such as rye, barley and wheat.

The NSP content of the diet

The negative correlation between NSP content of the diet and its nutritive value has been clearly demonstrated in poultry (Choct and Annison, 1990; Annison, 1991), in pigs (King and Taverner, 1975) and in pet dogs and cats (Earle et al., 1998). This indicates that by measuring the NSP content of a diet, it is possible to predict the amount of enzymes to add to the diet. However, the practicality of such an approach is questionable. Firstly, a least-cost diet contains a number of plant ingredients, which contain different forms of NSP. It is no way to know the amounts and types of different NSP substrates present in a diet by a quantitative measurement of the NSP level. Thus the responses to various types of enzyme activities added to the diet are difficult to predict. Secondly, the NSP assay is tedious and complicated and, hence, is not ideal for use in quality control laboratories. Thirdly, the anti-nutritive effect of soluble NSP is related, to a large extent, to the viscous nature of the polymers, which in turn depends on their molecular sizes and structures. Again a qualitative or a combination of qualitative and quantitative measurements is required.

Gut viscosity

The relationship between gut viscosity and the nutritive value of barley in poultry was first described by Burnett (1966). Only during the past 10 years has the importance of his work been recognized and a wealth of information on viscosity of the gut contents and its effect on nutrient digestion and absorption has since emerged (Bedford et al., 1991; Bedford and Classen, 1992; Choct and Annison, 1992a; Steenfeldt et al., 1998). Whether gut viscosity can be used to predict enzyme response depends on a number of factors, including the section of the intestine where the digesta is sampled, the age of the bird, and antibiotic growth promoters in the diet. Firstly, different sections of the gut will influence the viscosity value, because nutrients are digested and absorbed as digesta moves down the gut, thus leaving the indigestible portion (mainly NSP) to accumulate. On a relative basis, the soluble NSP content per ml of digesta supernatant therefore increases; secondly, digesta viscosity decreases as the bird gets older (Dusel et al., 1998; Steenfeldt et al., 1998). This also relates to the finding that older birds cope better with low-ME wheats (Rogel et al., 1987) or with barley (Salih et al., 1991). In addition, the relationship between gut viscosity
and bird performance is not always apparent (Wiseman and McNab, 1998). For example, Hughes and Zviedrans (1999) reported that two groups of birds fed wheat had ileal viscosity value (mPa s) of 12.5 and 49, but the AME contents (MJ kg⁻¹ DM) for the wheat were 10.6 and 12.0. This does not necessarily mean that the viscosity has no effect on nutrient digestion and absorption; rather, it highlights the complex nature of the interaction between digesta viscosity and the gut microflora. It is hypothesized that as the bird ages, its gut microflora becomes more established and it can adapt to the environment – for example, to cope with the viscous digesta better by producing small amounts of NSP-degrading enzymes (Choct and Kocher, 2000). This highlights the third point: that addition of antibiotic growth promoters in the diet can have a major effect on gut viscosity and hence enzyme response. Fourthly, as discussed earlier, removal of cell wall barriers using enzymes with an affinity for the insoluble NSP can also benefit the bird by releasing encapsulated nutrients. The response of such an action cannot be predicted from gut viscosity measurements.

Generally, gut viscosity values in birds fed viscous grains can be used to predict the response to enzyme supplementation with strong viscosity-reducing property within a grain type under set conditions. However, measuring gut viscosity is neither rapid nor cheap.

**Extract viscosity**

A ground sample of ingredients or compound feed can be extracted in water or in a buffer to measure extract viscosity. The viscosity of the extract comes predominantly from soluble NSP in the feed (Izydorczyk et al., 1991; Saulnier et al., 1995). Since the soluble NSP content of a diet is related to its nutritive value, it is possible that extract viscosity can be used to predict enzyme response. Rotter et al. (1989) demonstrated that the growth responses of chickens fed barley diets were accurately predicted by an extract viscosity method. Choct et al. (1993) developed an extract viscosity assay to distinguish wheats with very low AME from normal samples. More systematic studies with promising outcomes have since been reported on the use of extract viscosity as a predictor of nutritive value for poultry (Carré and Melcion, 1995; Choct and Hughes, 1997; Dusel et al., 1997, 1998; Wiseman and McNab, 1998). The extract viscosity method is simple and rapid and, more importantly, it gives qualitative information on the characteristics of the NSP. However, since the extract viscosity assay is based on the amount of soluble NSP content of the ingredient, it can only be used for predicting the response of viscosity-reducing enzymes. In addition, the reliability of the assay may be influenced by the extraction method and the endogenous enzyme activities in the diet or the individual ingredient.

**Rate of starch digestion**

Wiseman and McNab (1998) used an *in vitro* method to measure the rate of starch digestion by amylase. They demonstrated a close correlation between *in vitro* and
in vivo starch digestion for wheat ($R^2 = 0.65$) to distinguish between wheats with high and low metabolizable energy values in poultry. With validation, the assay may be used to characterize wheat for poultry diets; thus it offers potential as a means of predicting enzyme response.

**Conclusion**

The use of xylanase for wheat and β-glucanase for barley in poultry is a well accepted practice today. Generally, most of the enzymes effectively depolymerize the soluble NSP into smaller polymers, though some products with affinity for both soluble and insoluble NSP are also used. The soluble NSPs elicit anti-nutritive activities in poultry diets, which are closely related to their polymeric nature and ability to increase digesta viscosity. Extract viscosity has great potential to be used as a simple and rapid method to predict enzyme response in poultry diets. Other assays such as the in vitro starch digestibility measurement may prove useful. Further studies on NSPs need to address their effect on nutrient utilization in terms of relative efficiency of enzymatic digestion vs. fermentation, and muscle growth vs. gut cell turnover.

**References**


Enzyme Supplementation of Poultry Diets


Pettersson, D. and Åman, P. (1988) Effects of enzyme supplementation of diets based on wheat, rye or triticale on their productive value for broiler chickens. *Animal Feed Science and Technology* 20, 313–324.


The Role and Efficacy of Carbohydrase Enzymes in Pig Nutrition

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Introduction

The history of use of carbohydrase enzymes into pig diets based on ‘viscous’ grains such as wheat and barley is virtually as long as that of poultry but, until recent years, far less convincing. There are many reasons for this, and certainly not all are related to issues of cost effectiveness, which is always the ultimate arbitrator in the adoption of any new feed technology. The basic structure of the pig and poultry industries in many countries (i.e. ‘highly integrated’ versus ‘semi-extensive’, respectively) has tended to work against the rapid adoption of novel feed technologies into the pig sector. This situation is changing rapidly in swine markets in some parts of the world and, in terms of the potential rapid uptake of new ideas, will undoubtedly be a move for the better.

This chapter examines the role and efficacy of carbohydrase enzymes in grain-based diets. Phytases and enzymes directed against vegetable protein meals are assessed elsewhere in this book. It is recognized that, at the time of writing, the penetration of feed enzymes into the pig sector still lags behind that of poultry but that knowledge has accrued rapidly, particularly in the past 5 years, bringing both more effective enzymes for pig applications and considerably more applications expertise. Due to this rapid change the emphasis in this chapter is placed on the last 10 years of published information.

Enzyme Responses: Pigs vs. Poultry

The physiological reasons why differing responses to exogenous carbohydrates might be expected in pigs and poultry have been covered by a number of authors on a number of occasions, and for this reason will only be summarized here. Readers are referred to a number of excellent papers that have been systematically published over the past 10 years on these and other aspects of enzyme use into pig diets. A number of these compared and contrasted responses in poultry and pigs (e.g. Dierick, 1989;
Chesson, 1993; Dierick and Decuyper, 1994, 1996; Graham and Balnave, 1995; Bedford and Schulze, 1998; Danicke et al., 1999).

The main factors that may influence the potential for enzyme response in pigs compared with poultry include gastrointestinal anatomy, digestive capacity and digesta characteristics.

The presence of the crop in the broiler may, depending on the pH profile of the exogenous enzyme, allow for some enzyme activation at a relatively high pH (approximately 6) prior to passing into the acid environment of the gizzard. Generally in the pig a much lower pH (< 3) will be more rapidly experienced by the enzyme and its substrates in the stomach, though this will be highly dependent upon pig age, the degree of ingredient buffering, and the extent of microbial fermentation in the stomach leading to organic acid production (e.g. lactic acid). The pig is noted (Moran, 1982) for its relatively gentle gastric motility, leading to regional variations across the stomach in terms of pH and endogenous enzyme activity. All of these factors can potentially impinge on the activity of an exogenous enzyme, depending on its pH and temperature characteristics and its inherent rate of reaction ($K_m$).

Mean retention times (MRTs) of solid and liquid digesta in poultry also tend to be reduced relative to that of the pig (Moran, 1982). Solute markers in the upper part of the gastrointestinal tract of broilers showed mean retention times in the crop, gizzard and small intestine of 2.8, 0.3 and 1.0 h, respectively (i.e. about 4 h total to the end of the small intestine: Dierick and Decuyper, 1994). In contrast, Dierick and Decuyper (1994) cite a total MRT of 4–5 h through the stomach and small intestine of the pig and Mahan (1982) quotes studies showing approximately 7.5 h through this same part of the gastrointestinal tract. The absolute figure will clearly vary in both species depending on diet composition, soluble/insoluble fibre level, plane of feeding, age, etc., but the fact remains that the broiler, having less time than the pig for effective digestion and nutrient absorption, will be potentially disrupted more by certain anti-nutritional factors that interfere with this process (e.g. viscous, high molecular weight soluble fibres). This may in turn have implications for enzyme action and the relative magnitude of its effects in the two species, but it should equally be remembered that both species lack the appropriate enzymes to break down these anti-nutrients, irrespective of the length of the digestive tract and residence time. Both species, therefore, will potentially suffer the same consequences of disturbed digestion – not only less nutrients absorbed but also a rise in bacterial numbers in the small intestine, resulting in increased competition for nutrients between the host and its microflora.

The greater bacterial proliferation in the gut of the pig, compared with the broiler, is often quoted as a reason why the magnitude of exogenous enzyme response might be expected to be lower in the pig. Certainly in the pig’s hindgut the long residence time and voluminous cecum and colon offer an ideal opportunity for bacterial fermentation of fibrous residues to yield substantial quantities of volatile fatty acids, which will contribute to the animal’s maintenance requirement. In the small intestine, however, as mentioned above, such proliferation will always be a potentially negative factor. Caution should also be observed in interpretation of some studies reporting substantial breakdown of fibre fractions in the small intestine of
the pig in the absence of exogenous enzymes. Some of these studies used cannulated pigs with T-pieces inserted at the terminal ileum and, occasionally, the duodenum (Graham et al., 1986, 1988). The opportunities for an unnatural microbial colonization in the region of the cannula could influence the results seen.

It is well known that soluble, high molecular weight, non-starch polysaccharides (NSPs) from viscous cereal grains interfere with nutrient digestion and absorption in poultry, with consequences for microbial proliferation in the gut (Apajalahti and Bedford, 1998; Bedford and Schulze, 1998). The naturally higher water content of pig digesta (about 10 percentage units higher than poultry on similar diets) leads to dilution effects that will negate, to some degree, this viscosity problem in the pig’s gut (Fig. 8.1) (Danicke et al., 1999). This is not to say that, in pigs, viscosity effects are totally irrelevant to potential carbohydrase responses in viscous grain diets, but rather that they are likely to be of a lower order of importance to the responses seen in broilers. Reductions in digesta viscosity have been seen in many trials in pigs offered viscous cereal-based diets (Fig. 8.2) but it is clear that starting viscosity levels are always considerably lower than equivalent trials in poultry. It is of interest, however, that soluble fibre levels per se seem to be the key factor involved in negative bacterial changes in the gut of pigs under disease challenge and fed specific types of diet, as reviewed later in this chapter, so viscosity should certainly not be underestimated in the pig as an influence on productive performance. It should be recognized, therefore, that its influence may be more subtle than direct effects on nutrient digestibility and, rather, mediated indirectly through its effects on the gut microflora.

Digesta dry matter flow rates from the stomach of young and grower pigs fed wheat or barley-based diets have been found to be increased by certain exogenous enzymes (Sudendey and Kamphues, 1995) (Fig. 8.3). In these trials, viscosity reduction was apparent immediately in the stomach of the animals offered enzyme supplemented diets. Ellis et al. (1996) noted that an increase in gastric viscosity in

**Fig. 8.1.** Effect of wheat variety on intestinal viscosity (mPa s) in broilers, turkeys and piglets (adapted from Danicke et al., 1999). ■ Broilers (3 weeks) wheat inclusion 73%; □ turkey (4 weeks) wheat inclusion 73%; ○ pig (8 weeks) wheat inclusion 86%. * = small intestine, lower two-thirds for the pig. Wheat variety 1 = ‘Ibis’ 11 g kg\(^{-1}\) DM soluble arabinoxylan; wheat variety 2 = ‘Alidos’ 17 g kg\(^{-1}\) DM soluble arabinoxylan. DM = dry matter.

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**Fig. 8.2.** Effect of wheat variety on intestinal viscosity (mPa s) in broilers, turkeys and piglets (adapted from Danicke et al., 1999). ■ Broilers (3 weeks) wheat inclusion 73%; □ turkey (4 weeks) wheat inclusion 73%; ○ pig (8 weeks) wheat inclusion 86%. * = small intestine, lower two-thirds for the pig. Wheat variety 1 = ‘Ibis’ 11 g kg\(^{-1}\) DM soluble arabinoxylan; wheat variety 2 = ‘Alidos’ 17 g kg\(^{-1}\) DM soluble arabinoxylan. DM = dry matter.
the pig can lead to a failure of the sieving mechanism whereby large digesta particles normally fall to the bottom of the stomach, a feature seemingly unique to the pig. In a viscous environment these large particles stay suspended and are thereby more likely to exit the pylorus before being exposed to gastric secretions or particle size reduction, which may have implications for subsequent digestion. Further down the gastrointestinal tract, viscosity is also known to disturb peristalsis, creating laminar rather than disturbed flow, and also to lead to disrupted pancreatic secretion via its effects on gastric inhibitory polypeptide. Viscous non-starch polysaccharides may also physically coat starch granules, further reducing the rate of digestion (Ellis et al., 1996).

In successful pig trials using enzyme supplementation, a stimulation of voluntary food intake is frequently an important contributor to the observed improvements in daily liveweight gain (Haberer et al., 1998). The fact that gastric emptying rate and distension of the stomach are two of a number of factors involved in satiety signals in the pig (Forbes, 1995) is unlikely to be coincidental and it is proposed that some of the positive responses to exogenous enzymes on voluntary food intake could be due to various influences on digesta flow rate, as well as improvements in nutrient availability and digestibility in the small intestine. Feedback loops involving gut hormones could also be contributors to carbohydrase effects, as outlined by Bedford and Schulze (1998), who proposed that caecal fermentation, enhanced

![Graph showing effects of enzymes on viscosity in the small intestine of piglets.](image1)

**Fig. 8.2.** Effects of enzymes on viscosity in the small intestine of piglets. Ref. 1. Inborr (1994); ref. 2. Sudendey and Kamphues (1995) wheat/piglet and barley/grower; ref. 3. Dusel et al. (1997); ref. 4. Partridge et al. (1998a).

![Graph showing effect of enzyme addition to wheat or barley-based diets on dry matter outflow rate from the stomach.](image2)

**Fig. 8.3.** Effect of enzyme addition to wheat or barley-based diets on dry matter outflow rate from the stomach (Sudendey and Kamphues, 1995). Values are g dry matter leaving the stomach h⁻¹ kg⁻¹ body weight. ■ Control; □ +Enzyme complex (amylase, xylanase, β-glucanase). *P < 0.05, (*)P < 0.10.
by breakdown products from xylanase addition (fermentable oligomers), could
influence enteroglucagon concentrations, which in turn influence stomach motility
by depressing gastrin concentrations. These many possibilities suggest exogenous
enzymes could be potent influencers of gastrointestinal physiology through a variety
of different mechanisms, but these aspects have been little studied to date.

The relatively slow passage rate of digesta through the gut of the pig, when
compared with the broiler, may offer some advantages to exogenous enzymes when it
comes to the opportunity for insoluble cell wall breakdown, or thinning, coupled
with physicochemical changes to fibre (e.g. reduced water-holding capacity) as
found that the water-holding capacity of feedstuffs for young pigs (12–25 kg) was a
major influence on voluntary food intake (Fig. 8.4). As carbohydrase enzymes are
known to reduce the water-holding capacity of feedstuffs in vitro (Fig. 8.5), it follows
that some of the potential benefits of exogenous enzyme addition in the pig may
be related to disruption of water and soluble nutrient entrapment, by both soluble
and insoluble fibre residues in the gut. Whilst the importance of soluble, viscous
polysaccharides to digestive disruption in the broiler seems to be a major mode of
action in wheat and barley-based diets (Bedford and Schulze, 1998), in the pig its
importance, as discussed, is clearly less profound. The conclusion must be that a
broader range of mechanisms is likely to be relevant to the pig, not least the ability of
an exogenous enzyme to influence both soluble and, more particularly, insoluble
fibre in the gut. This has implications for product design, as enzymes will differ in
their affinities and rates of reaction against various fibre structures and it follows that

\[ \text{Scaled feed intake} = \text{g feed kg}^{-1} \text{ liveweight day}^{-1} \]

Fig. 8.4. The effect of water-holding capacity of the feed on voluntary food intake
in pigs (12–25 kg liveweight). Scaled feed intake = g feed kg$^{-1}$ liveweight day$^{-1}$
(Kyriazakis and Emmans, 1995).
enzymes designed for maximum efficacy in the pig may involve both different levels and/or different sources than those ideal for poultry. Xylanases, for example, even derived from the same source organism, can vary widely in their catalytic activities on various xylan substrates (Bedford and Schulze, 1998) and the active concentration of xylanase will also be an important determinant of the extent of cell wall hydrolysis (Tervila-Wilo et al., 1996).

Such issues become particularly important as commercial emphasis switches towards carbohydrase products designed for non-viscous grains such as maize and sorghum, which are the predominant cereals globally. In these grains, factors associated with insoluble fibre such as the packaging of nutrients inside cell wall material, together with starch structure and composition, seem to be more relevant and may require a new range of enzyme solutions.

The rest of this chapter will systematically consider the major grain and grain by-product sources available to pigs globally, examine their feeding value, its variability and reasons for it, and then review recent work on their potential for nutritional upgrading with appropriate exogenous enzymes.

**Barley as a Grain Source for Pigs**

The feeding value of conventional (hulled) barley for pigs was reviewed by van Barneveld (1999). Estimates of DE in 16 cited studies ranged from 11.7 MJ kg\(^{-1}\) dry matter (DM) to 16.0 MJ kg\(^{-1}\) DM. Energy digestibility, rather than gross energy content (a range of about 2 MJ kg\(^{-1}\) DM), had the greatest influence on this value. In the recent studies of Fairbairn et al. (1999) the DE content of 20 barley samples, from three locations and representing five varieties, ranged from 11.2 to 13.1 MJ kg\(^{-1}\) (90% DM), with less variation between varieties than within varieties.
(5.8%, 0.7 MJ kg\(^{-1}\) versus 8.4%, 1.0 MJ kg\(^{-1}\)). This re-emphasizes the importance of growing conditions to proximate analysis, carbohydrate composition and, ultimately, feeding value for both swine and poultry (Aman et al., 1985; Aman and Newman, 1986; Bach Knudsen et al., 1987).

The last decade has seen an increasing interest in the potential of hull-less barley as a feed ingredient for monogastrics. Hull-less barley differs from conventional barley grain in that the hull is less firmly attached to the kernel and consequently becomes detached during threshing. Despite, consequently, having lower insoluble fibre levels and higher levels of protein, its feeding value for pigs and poultry has often failed to live up to its theoretical potential (Baidoo et al., 1998b; Thacker, 1999). The reasons for this are most likely associated with the soluble fibre fractions (particularly β-glucans), which are concentrated in the endosperm of the barley grain. Removing the hull fraction effectively concentrates these soluble components in hull-less barley, with comparative levels of β-glucans in the two grains being 3.5–4.5% (hulled) versus 4.5–7% (hull-less) (Baidoo and Liu, 1998). The negative effects on growth seen after feeding micronized, hull-less barley to growing/finishing pigs are almost certainly due to further solubilization of this fibre during thermal processing of the grain, effectively negating the potentially positive effects of the process on starch gelatinization (Thacker, 1999). The latter study re-emphasized the limitations of measuring total tract digestibility in the pig in isolation from performance studies, whereby ‘apparent’ improvements in digestibility of nutrients can be more than offset by reduced voluntary food intake coupled with implications for microbial proliferation/competition for nutrients in the gut. This aspect will be examined in more detail later in this chapter.

Differences in feeding value for hulled and hull-less barley varieties will also undoubtedly be influenced by other components of the grain apart from fibre, notably the amylose : amylopectin ratio of the starch fraction. Pettersson and Lindberg (1997) investigated ileal and faecal digestibility of hull-less versus hulled barleys, with varying amylose : amylopectin ratios, in growing/finishing pigs. They found that the poorest energy and starch digestibility was in hulled/high amylose grain and the best in hull-less/high amylopectin, at both the ileal and faecal levels. Hull-less barley tended to give higher nutrient digestibilities overall and showed increased hindgut digestion compared with hulled material. This would presumably have implications for rate and pattern of microbial fermentation, but these were not investigated in this study. Equally, productive performance was not measured on the different barley types to see if the digestibility differences seen were actually reflected in growth, feed intake and feed utilization. Miller et al. (1994) also described digestibility experiments looking at the interaction between fibre (ADF – acid detergent fibre) level in barley, β-glucan content and amylose : amylopectin ratio. They concluded that the energy content of barley for both poultry and swine was decreased greatly by ADF content and to a significant but lesser extent by higher total β-glucans. They also found that β-glucans had a larger negative effect on energy digestibility in poultry than in swine and that high amylopectin (‘waxy’) barley starch offset some of the reductions in energy digestibility caused by a high ADF level. This illustrates the complex interrelationships between various fractions in the grain.
which can all have potential implications for feeding value and, importantly to this discussion, subsequent response to exogenous enzymes.

Response of Hulled and Hull-less Barley to Exogenous Enzymes in Pigs

The surface of the aleurone cell walls of barley contains high levels of xylan, surrounding a core of mixed-linked β-glucans (Autio et al., 1996). For this reason, optimal responses in rations based on barley seem to be found when both xylanase and β-glucanase activities are supplied. This is illustrated in the studies of van Lunen and Schulze (1996a) and Ramaswamy et al. (1996) where clear responses, particularly in hulled barley, are only achieved in the presence of significant xylanase activity. Some studies using exogenous enzymes in hulled barley-based rations (Graham et al., 1989; Thacker et al., 1992b) have tended to emphasize the β-glucanase component for reducing levels of soluble β-glucans, which are well known to exacerbate digesta viscosity problems in broilers. In the pig these effects are believed to be of a much lower magnitude, due to pig digesta having a much lower dry matter content than the broiler (Bedford and Schulze, 1998), effectively diluting the effects of these viscous fibres and partially negating their detrimental effects on nutrient digestion and absorption. However, viscous fibre can still be relevant for its impact on the microbial population of the gut and thereby indirectly influence enzyme response, an aspect that will be examined in more detail later in this chapter.

Table 8.1 shows a summary of a number of published trials over the past 10 years into both hulled and hull-less barley-based diets, using a range of enzyme additions. Although an overview is made more difficult by potential influences of a number of factors (e.g. growth versus digestibility trials; faecal versus ileal digestibility measurements; pig age/weight used; choice of enzyme activities; pelleting versus meal/mash, i.e. potential for denaturation with some products; hulled versus hull-less barley), some trends do emerge.

Responses to these various products in young pigs (< 25 kg) are strong and quite consistent, particularly in the hull-less barley-based diets that have tended to dominate recent publications. Baidoo et al. (1998a) describe a declining response in pigs over the weight range 40–60 kg but an increasing numerical response to the enzyme used as animals moved from the 9–20 kg weight range (daily gain +12%; feed : gain −3%) to the 20–40 kg weight range (daily gain +17%; feed : gain −10%). It should be noted that even in this trial the animals in the heaviest weight category (40–60 kg) showed improvements in both growth and feed : gain (+4%; −12%, respectively) after addition of the enzyme but that the large standard errors associated with the data meant they failed to achieve statistical significance. Data from studies with grower/finisher pigs (> 25 kg) range from no response or small responses (Graham et al., 1989; Thacker et al., 1992a,b – but see comment above) to those where clear, significant effects on digestibility and/or growth rate were seen (van Lunen and Schulze, 1996a; McCracken et al., 1996; Ramaswamy et al., 1996; Baidoo et al., 1998a,b). Even in some apparent ‘no-response’ trials (Thacker et al.
1992a) significant, positive effects on faecal digestibility of protein and numerical effects on energy were seen after enzyme addition. This is a reminder, particularly in trials with grower/finisher pigs, that products potentially liberating more nutrients must be tested in conditions that allow this nutrient release to be expressed in terms of improved energy utilization, lean gain, etc. In trials where the products are added on top of an existing formulation, which may already be in excess of an animal’s daily nutrient needs for its lean protein deposition potential, carcass information is a fundamental requirement to aid trial interpretation.

The studies of Baidoo et al. (1998a), McCracken et al. (1996) and Han and Froseth (1993) illustrate that interpretation of productive and economic responses to added feed enzymes in barley-based rations is highly dependent on an appraisal of grain ‘quality’ in terms of energy and amino acid availability. Emphasis thus switches to simple predictors of feeding value in barley and, in particular, their rapid estimation. Fairbairn et al. (1999) described a series of predictive equations for hulled barley for pigs in which ADF content of the barley described DE, with an $R^2$ value of 0.85:

$$\text{DE (kcal kg}^{-1}, 90\% \text{ dry matter}) = 3526 - (92.8 \times \text{ADF}%) .$$

More complex equations, incorporating further factors, increased the $R^2$ value to 0.90; for example:

$$\text{DE (kcal kg}^{-1}, 90\% \text{ dry matter}) = 3964 - (81.3 \times \text{ADF}%) - (273.9 \times \text{acid detergent lignin} (\%)) - (56.4 \times \text{total } \beta\text{-glucans} (%)).$$

Near-infrared spectroscopy (NIRS) appears currently to offer the best potential for rapid estimation of barley DE (Zijlstra et al., 1998) and amino acid acid content (Jaikaran et al., 1998) with sufficient accuracy to allow for its subsequent use as a tool to maximize the cost efficacy of enzyme addition. Emphasis on DE estimation alone as a predictor of potential enzyme response must, however, be tempered with some caution. Experiences with wheat in pigs (Cadogan et al., 1999) have indicated that the factors controlling voluntary food intake of different samples do not appear to be closely related to measured DE, hence any screening method must ultimately take account of this anomaly and understand the key factors responsible.

### Wheat, Triticale and Rye as Grain Sources For Pigs

A review of the literature on wheat feeding value for pigs (van Barneveld, 1999) found a range of 3.7 MJ DE kg$^{-1}$ DM across all cultivars studied. The lowest reported DE was 13.3 MJ kg$^{-1}$ DM and the highest 17.0 MJ kg$^{-1}$ DM. As for other grains, the variability seen is a function of methodology, cultivars, growing regions, sites and years. Most studies saw differences between ‘poor’ and ‘good’ wheats in the range 0.8–1.4 MJ kg$^{-1}$ DM.

The importance of cultivar to feeding value was illustrated in a study in the UK (Schulze et al., 1997) where six varieties of wheat were grown on one site, to negate environmental influences, and then used as the sole grain source in diets for piglets from 9 kg body weight (Table 8.2). Variations in growth rate and feed intake of
Table 8.1. Some published studies on the effects of enzyme preparations on barley-based diets. (Note that where both ileal and faecal digestibility measurements were made, data is presented preferentially at the ileal level.)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Basal diet – main ingredients; age/weight of pig; pelleted or meal/mash (where stated)</th>
<th>Enzyme(s) added, levels and inclusion rates (where stated)</th>
<th>Responses seen (C = control; +E = plus enzyme; * P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graham et al. (1988)</td>
<td>Barley; wheat pollard; meal/mash 19–25 kg ileal cannulated pigs</td>
<td>β-glucanase (16 U g⁻¹)&lt;br&gt;xylanase (1300 U g⁻¹)&lt;br&gt;2 kg t⁻¹</td>
<td>Ileal digestibility of protein (%): C: 64.5 +E: 70.1*</td>
</tr>
<tr>
<td>Graham et al. (1989)</td>
<td>Hull-less barley; soybean meal; meal/mash versus pelleted (93°C) 80 kg ileal cannulated pigs</td>
<td>β-glucanase (20 U g⁻¹)&lt;br&gt;5 kg t⁻¹</td>
<td>Ileal digestibility of protein (%): C: 52.2 +E: 53.3</td>
</tr>
<tr>
<td>Bohme (1990)</td>
<td>Barley; wheat; soybean meal; pelleted 11–25 kg</td>
<td>Cellulase, β-glucanase, α-amylase; glucoamylase 1 kg t⁻¹</td>
<td>Growth performance: C: 381 g day⁻¹ Feed : gain 1.88</td>
</tr>
</tbody>
</table>
| Thacker et al. (1992a) | Barley; soybean meal; pelleted (< 60°C)  
C1 = no growth promoter  
C2 = + salinomycin 22–86 kg | β-glucanase (750 U g⁻¹)<br>pentosanase (650 U g⁻¹)<br>2.5 kg t⁻¹ | Growth performance: C1: 840 g day⁻¹ Feed : gain 2.53  |

Faecal digestibility of protein (%):  
C1: 72.2 +E: 73.7  
C2: 75.3 +E: 81.3*  
Faecal digestibility of energy (%):  
C1: 74.2 +E: 75.6  
C2: 73.5 +E: 78.5
<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Enzyme Activity (U g&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Weight Gain:</th>
<th>Feed: gain:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bedford et al. (1992)</td>
<td>Hull-less barley; soybean meal; mash</td>
<td>β-glucanase (1086 U g&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>C: 5.24 kg</td>
<td>+E: 6.14 kg*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C: 1.75</td>
<td>+E: 1.55 (P = 0.10)</td>
</tr>
<tr>
<td>Thacker et al. (1992a)</td>
<td>Hull-less barley; soybean meal; pelleted (&lt; 60 °C)</td>
<td>β-glucanase (750 U g&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>Growth performance:</td>
<td>Feed: gain:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C1: 830 g day&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>+E: 840 g day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C2: 860 g day&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>+E: 790 g day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Faecal digestibility of protein (%):</td>
<td>C1: 78.6</td>
<td>+E: 75.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C2: 74.3</td>
<td>+E: 79.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Faecal digestibility of energy (%):</td>
<td>C1: 76.3</td>
<td>+E: 79.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C2: 78.7</td>
<td>+E: 78.1</td>
</tr>
<tr>
<td>Thacker et al. (1992b)</td>
<td>Hull-less barley; soybean meal; pelleted (&lt; 60 °C)</td>
<td>β-glucanase (750 U g&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>Growth performance:</td>
<td>Feed: gain:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C1: 254 g day&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>+E: 274 g day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C2: 275 g day&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>+E: 275 g day&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Faecal digestibility of protein (%):</td>
<td>C1: 78.4</td>
<td>+E: 77.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C2: 77.6</td>
<td>+E: 81.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Faecal digestibility of energy (%):</td>
<td>C1: 82.6</td>
<td>+E: 81.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C2: 81.0</td>
<td>+E: 82.3</td>
</tr>
</tbody>
</table>
Table 8.1.  Continued.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Basal diet – main ingredients; age/weight of pig; pelleted or meal/mash (where stated)</th>
<th>Enzyme(s) added, levels and inclusion rates (where stated)</th>
<th>Responses seen (C = control; +E = plus enzyme; *P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Han and Froseth (1993)</td>
<td>Nine barley cultivars of ‘high’ and ‘low’ quality ‘high’: &lt; 7.6% ADF; &lt; 4.7% β-glucans (n = 4) ‘low’: &gt; 9.8% ADF; &gt; 5.4% β-glucans (n = 5) 70 kg duodenal cannulae – mobile nylon bag technique</td>
<td>β-glucanase, cellulase; xylanase; pectinase 1 kg t⁻¹</td>
<td>Faecal DE (kcal kg⁻¹ dry matter): 'high' control: 3455 ‘high’ +E: 3532* (+2.2%) ‘low’ control: 3296 ‘low’ +E: 3414* (+3.6%)</td>
</tr>
<tr>
<td>Inbör (1994)</td>
<td>Hulled or hull-less barley; soybean meal; meal/mash 9.5–14 kg</td>
<td>β-glucanase 2.5 kg t⁻¹ to give 89–95 mU g⁻¹ feed</td>
<td>Growth performance: C (hull-less): 223 g day⁻¹ Feed : gain 1.60 +E (hull-less): 229 g day⁻¹ Feed : gain 1.52 C (hulled): 200 g day⁻¹ Feed : gain 1.71 +E (hulled): 208 g day⁻¹ Feed : gain 1.65 Effect of enzyme on growth (P = 0.07) Effect of enzyme on feeds: gain (P = 0.06)</td>
</tr>
<tr>
<td>van Lunen and Schulze (1996a)</td>
<td>Barley; rapeseed meal; pelleted 35 kg</td>
<td>E1: β-glucanase</td>
<td>Faecal digestibility of protein (%): C: 84.6 +E1: 85.3 +E2: 86.7 Faecal digestibility of energy (%): C: 80.9 +E1: 81.8 +E2: 83.4*</td>
</tr>
</tbody>
</table>
Ramaswamy et al. (1996) Hull-less or hulled barley; soybean meal; canola meal 47 kg

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Faecal digestibility of protein (%)</th>
<th>Measured DE (MJ kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1: xylanase</td>
<td>C (hull-less): 82.0 +E1: 81.0</td>
<td>C (hull-less): 14.40 +E1: 14.73</td>
</tr>
<tr>
<td>E2: xylanase; β-glucanase</td>
<td>+E2: 81.0</td>
<td>+E2: 14.94</td>
</tr>
<tr>
<td>C (hulled): 76.8 +E2: 77.3</td>
<td>+E2: 15.3</td>
<td>+E2: 13.5</td>
</tr>
<tr>
<td>Enzyme P &lt; 0.05</td>
<td>Enzyme P &lt; 0.001</td>
<td>Barley × enzyme P = 0.008</td>
</tr>
</tbody>
</table>

Barley × enzyme P = 0.06

Power et al. (1996) Hulled barley; wheat; wheat middlings; pelleted (~70°C) 6–15 kg

<table>
<thead>
<tr>
<th>β-glucanase</th>
<th>Growth performance and digestibility at optimum inclusion level (2000 p.p.m.):</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0, 500, 1000, 2000 p.p.m.)</td>
<td>C: 201 g day(^{-1}) Feed: gain 2.11 +E: 227 g day(^{-1}) Feed: gain 1.95*</td>
</tr>
</tbody>
</table>

Faecal digestibility of protein (%): C: 77.4 +E: 80.3

Li et al. (1996b) Hull-less barley; soybean meal; meal/mash 6–11 kg

<table>
<thead>
<tr>
<th>β-glucanase (1000 U g(^{-1}))</th>
<th>Faecal digestibility of protein (%)</th>
<th>Faecal digestibility of energy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A dose-response study (note the maximum response was shown at the highest level of inclusion, 2 kg t(^{-1}), so only this data is presented)</td>
<td>C: 81.6 +E: 88.5*</td>
<td>C: 85.2 +E: 89.5*</td>
</tr>
</tbody>
</table>

(significant linear effects of dose rate were seen) (significant linear effects of dose rate were seen)
Table 8.1. Continued.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Basal diet – main ingredients; age/weight of pig; pelleted or meal/mash (where stated)</th>
<th>Enzyme(s) added, levels and inclusion rates (where stated)</th>
<th>Responses seen (C = control; + = plus enzyme; * P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li et al. (1996a)</td>
<td>Hull-less barley; soybean meal; meal/mash 7–11 kg</td>
<td>β-glucanase (1000 U g⁻¹) 2 kg t⁻¹</td>
<td>Ileal digestibility of protein (%): C: 65.2 +: 73.5*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ileal digestibility of energy (%): C: 64.9 +: 71.1*</td>
</tr>
<tr>
<td>Baidoo et al. (1998b)</td>
<td>Hull-less barley (varieties 'Condor' and 'Buck'); canola meal; meal/mash 14 kg ileal-cannulated pigs</td>
<td>Cellulase; β-glucanase; xylanase (11 U g⁻¹; 27 U g⁻¹; 43 U g⁻¹) 100 g t⁻¹</td>
<td>Ileal digestibility of protein (%): C: 57.6 +: 61.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ileal digestibility of energy (%): C: 57.1 +: 63.3*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(effects were seen on both varieties, hence these are overall values)</td>
</tr>
<tr>
<td>Baidoo et al. (1998b)</td>
<td>Hull-less barley; soybean meal; canola meal; meal/mash or pellet (80°C) 9–60 kg</td>
<td>Cellulase; β-glucanase; xylanase (11 U g⁻¹; 27 U g⁻¹; 43 U g⁻¹) 100 g t⁻¹</td>
<td>Growth performance (9–60 kg): C: 721 g day⁻¹ Feed : gain 2.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+: 782 g day⁻¹ Feed : gain 1.84*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive effects to enzyme addition were seen in both mash and pelleted feed, hence overall values are shown</td>
</tr>
<tr>
<td>Baidoo et al. (1998a)</td>
<td>Hull-less barley ('Falcon'); meal/mash 10–14 kg ileal-cannulated pigs</td>
<td>E1: xylanase</td>
<td>Ileal digestibility of protein (%): C: 59.6 +: 80.1*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E2: β-glucanase</td>
<td>Ileal digestibility of energy (%): C: 62.1 +: 64.6*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E3: xylanase; β-glucanase; protease</td>
<td>+: 66.7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+: 69.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+: 71.2*</td>
</tr>
</tbody>
</table>
Baidoo et al. (1998a) Hull-less barley ('Buck', 'Condor' or 'Falcon'); canola meal; meal/mash
14 kg ileal-cannulated pigs
Xylanase; β-glucanase; protease
Ileal digestibility of protein (%): C (buck): 60.3
+ E: 64.5
C (condor): 54.8
+ E: 60.7
C (falcon): 67.6
+ E: 67.2
Ileal digestibility of energy (%): C (buck): 57.7
+ E: 67.0
C (condor): 56.2
+ E: 66.3
C (falcon): 60.6
+ E: 61.3

Yin et al. (2000) Hulled barley (high or normal bushel weight – HBW or NBW); pelleted HBW = 70 kg hl\(^{-1}\)
NBW = 64 kg hl\(^{-1}\)
23 kg ileal-cannulated pigs
Xylanase; β-glucanase (400; 400 U g\(^{-1}\))
1 kg\(^{-1}\)
Ileal digestibility of protein (%): C (HBW): 74.8
+ E: 76.1
C (NBW): 72.6
+ E: 76.8*
Ileal digestibility of energy (%): C (HBW): 67.8
+ E: 69.5
C (NBW): 65.8
+ E: 70.2*

Gill et al. (2000) Hulled barley; soybean meal; pelleted (75°C)
8–18 kg
β-glucanase; xylanase; amylase
Growth performance:
C: 329 g day\(^{-1}\) Feed : gain 1.60
+ E: 350 g day\(^{-1}\) Feed : gain 1.53
DE (MJ kg\(^{-1}\))
C: 14.86
+ E: 15.08
Faecal digestibility of protein (%): C: 76.8
+ E: 75.1

70–80 g day\(^{-1}\) were seen in this trial over a 3-week period. Voluntary feed intake and apparent DE intake (feed intake \(\times\) measured faecal DE) appeared to be influenced by the water-holding capacity (WHC) of the finished feed after pelleting and was described by the equation:

\[
\text{DEI (kcal day}\, \text{\(^{-1}\))} = 3325.1 - 663.64 \times \text{WHC (g g}\, \text{\(^{-1}\))}; \quad (P = 0.015, r^2 = 0.81),
\]

which in turn suggests that the fibre content of the diet (soluble plus insoluble) was a contributory factor. This agrees with the observations of Kyriazakis and Emmans (1995), who found that varying fibre types influenced voluntary food intake in the young pig (12–25 kg) by increasing the WHC of the final feed (Fig. 8.4).

Cadogan et al. (1999) described similar weaner growth studies (7.5–15.6 kg) in which ten Australian cultivars of wheat were compared immediately after harvest, and again after 10 months storage. Growth performance across the ten cultivars varied from 233 to 447 g day\(^{-1}\) (mean 388, SD 62.9) and was heavily influenced by voluntary food intake (mean 438 g day\(^{-1}\), SD 70.0, range 271–514 g day\(^{-1}\)). Apparent DM digestibility of the ten wheats showed no relationship to pig growth performance, illustrating the limitations of faecal digestibility in predicting pig performance, without further knowledge of the factors influencing feed intake. After 10 months of storage the marked differences between the varieties remained, although feed intake and daily gain were, on average, 11.3% and 9.8% higher than in the same samples immediately post-harvest.

Variation in triticale and rye feeding value has been studied relatively little in pigs. Van Barneveld (1999) cited data from Charmley and Greenhalgh (1987), who found triticale feeding value was lower than that of wheat (15.1 versus 16.0 MJ kg\(^{-1}\) DM), but that differences between the three triticale cultivars studied were relatively small. It is likely that the feeding value of both triticale and rye will be at least as variable as that of wheat, and likely more so, allowing for the fact that both raw materials will have higher total levels of non-starch polysaccharides and, particularly, soluble arabinoxylans (Cyran et al., 1995). Rye-based diets offered to poultry are a considerable challenge to the bird, due to their negative effects on digesta viscosity.

### Table 8.2. Effect of variety on the feeding value of wheat (Schulze et al., 1997).

<table>
<thead>
<tr>
<th>Variety</th>
<th>Test wt (lb bu(^{-1}))</th>
<th>Water holding capacity (filter method)</th>
<th>Daily gain (g)</th>
<th>Daily feed (g)</th>
<th>Feed : gain</th>
<th>DE (kcal kg(^{-1}))</th>
<th>DE intake (kcal day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hussar</td>
<td>62.4</td>
<td>1.92</td>
<td>488</td>
<td>636</td>
<td>1.31</td>
<td>3365</td>
<td>2140</td>
</tr>
<tr>
<td>Hunter</td>
<td>61.6</td>
<td>1.85</td>
<td>470</td>
<td>608</td>
<td>1.30</td>
<td>3363</td>
<td>2045</td>
</tr>
<tr>
<td>Brigadier</td>
<td>60.9</td>
<td>2.04</td>
<td>457</td>
<td>609</td>
<td>1.34</td>
<td>3246</td>
<td>1977</td>
</tr>
<tr>
<td>Beaver</td>
<td>60.6</td>
<td>2.22</td>
<td>435</td>
<td>558</td>
<td>1.29</td>
<td>3308</td>
<td>1846</td>
</tr>
<tr>
<td>Dynamo</td>
<td>60.6</td>
<td>1.92</td>
<td>419</td>
<td>514</td>
<td>1.49</td>
<td>3298</td>
<td>2025</td>
</tr>
<tr>
<td>Riband</td>
<td>60.5</td>
<td>2.07</td>
<td>415</td>
<td>554</td>
<td>1.38</td>
<td>3384</td>
<td>1875</td>
</tr>
</tbody>
</table>

*All varieties grown in one location.

*bMeasured digestible energy.
leading to poorer nutrient digestibility and bird growth. In pigs this may be less of a physiological problem than to the bird, but the feeding value of rye is still recognized to be well below that of equivalent wheat-based diets and its fibre content seems to be the main contributory factor (Bayzlo, 1990).

Response of Wheat, Triticale and Rye to Exogenous Enzymes in Pigs

The predominant non-starch polysaccharides present in the endosperm and aleurone layers of wheat, triticale and rye are arabinoxylans, which comprise 50–60% of the total NSPs (Dierick and Decuyper, 1994; Evers et al., 1999). In contrast to barley and oats, β-glucan is a minor component in these grains. The arabinoxylans in the thin cell walls within the endosperm are water-soluble to varying degrees (depending on variety, growing conditions, etc.) whereas those in the thick cell walls of the aleurone layer are predominantly insoluble. Xylanases are therefore the main type of exogenous enzyme used for these grain types, as well as for grain by-products derived from them (e.g. wheat bran, middlings, etc.) Table 8.3 shows a summary of a number of published trials over the past 10 years into wheat, rye and triticale-based diets, using a range of enzyme additions.

As with the barley data in Table 8.1, there is a greater emphasis on trials with young pigs (< 25 kg) than in grower/finishers. This is based on the assumption that potential responses to enzymes will be greater in the younger animal where daily nutrient intake, in terms of both energy and amino acids, is often the limitation to achieving the animal’s lean gain potential. The success rate in showing positive effects of enzymes on pig performance, measured as either growth rate, feed : gain or digestibility, seems to be higher in the younger animal but this trend is influenced by the fact that very few studies have made an attempt to predetermine wheat/triticale/rye feeding value, before adding an appropriate enzyme. In the few recent studies where this has been attempted (Choct et al., 1999; Partridge et al., 1999) the ability of a xylanase to raise significantly the productive performance of a ‘poor’ wheat to the level of that of ‘good’ wheat has been clearly demonstrated, irrespective of the age of the pig (Table 8.4).

The relative importance of soluble pentosans to the response to xylanase in the pig is thrown into question by a number of studies where rye was the predominant grain (Thacker et al., 1991, 1992a; Bedford et al., 1992; Thacker and Baas, 1996). Dusel et al. (1997b) showed that the use of a mixed-grain diet based on a combination of wheat (high extract viscosity), rye and barley gave responses to enzyme addition that went beyond the apparent effects on viscosity reduction (Table 8.5). As in the studies of Choct et al. (1999) and Partridge et al. (1999), addition of xylanase or the xylanase/protease combination released an apparent limitation on voluntary food intake by the animal in these diets such that the growth response seen was a cumulative effect of both increased nutrient availability and intake. Other, more subtle, changes in the microbial flora may also have influenced the response to the enzyme. Pigs in the xylanase-supplemented group appeared to show decreases in bacterial proliferation in the small intestine (Fig. 8.6) when compared with both
Table 8.3. Some published studies on the effects of enzyme preparations on wheat, rye or triticale-based diets. (Note that where both ileal and faecal digestibility measurements were made, data are presented preferentially at the ileal level.)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Basal diet – main ingredients; age/weight of pig; pelleted or meal/mash (where stated)</th>
<th>Enzyme(s) added, levels and inclusion rates (where stated)</th>
<th>Responses seen (C = control; +E = plus enzyme; *P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bohme (1990)</td>
<td>Wheat; barley; maize; soybean meal; pelleted 11–25 kg</td>
<td>Cellulase, β-glucanase, α-amylase; glucoamylase 1 kg t⁻¹</td>
<td>Growth performance:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C: 411 g day⁻¹ Feed: gain 1.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+E: 458 g day⁻¹ Feed: gain 1.56*</td>
</tr>
<tr>
<td>Thacker et al. (1991)</td>
<td>Rye; soybean meal; meal (expt 1) or pellets (55°C, expt 2) 20–98 kg</td>
<td>Pentosanase (650 U g⁻¹) 2.5 kg t⁻¹</td>
<td>Growth performance:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C (meal): 730 g day⁻¹ Feed: gain 3.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+E: 760 g day⁻¹ Feed: gain 3.12*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C (pellet): 760 g day⁻¹ Feed: gain 2.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+E: 780 g day⁻¹ Feed: gain 2.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Faecal digestibility of protein (%):</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C (meal): 79.2 +E: 80.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C (pellet): 77.0 +E: 77.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Faecal digestibility of energy (%):</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C (meal): 80.8 +E: 80.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C (pellet): 81.2 +E: 80.1</td>
</tr>
<tr>
<td>Thacker et al. (1992a)</td>
<td>Rye; soybean meal; pelleted (&lt; 60°C) C1 = no growth promoter C2 = + salinomycin 20–84 kg</td>
<td>β-glucanase (750 U g⁻¹) pentosanase (650 U g⁻¹) 2.5 kg t⁻¹</td>
<td>Growth performance:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C1: 740 g day⁻¹ Feed: gain 2.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+E: 710 g day⁻¹ Feed: gain 2.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C2: 720 g day⁻¹ Feed: gain 2.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+E: 750 g day⁻¹ Feed: gain 2.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Faecal digestibility of protein (%):</td>
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<td></td>
<td></td>
<td></td>
<td>C1: 72.5 +E: 76.5</td>
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<td></td>
<td>C2: 78.9 +E: 78.8</td>
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<td>Faecal digestibility of energy (%):</td>
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<td></td>
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<td></td>
<td>C1: 78.9 +E: 80.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>C2: 82.7 +E: 82.5</td>
</tr>
<tr>
<td>Authors</td>
<td>Feed Ingredients</td>
<td>Enzymes Dose and Formulations</td>
<td>Weight Gain</td>
</tr>
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<td>--------------------------------------------------------</td>
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</tr>
<tr>
<td>Bedford et al. (1992)</td>
<td>Rye; soybean meal; mash/meal</td>
<td>11–15 kg; Xylanase (850 U g(^{-1})) 2 kg t(^{-1})</td>
<td>C: 4.24 kg +E: 4.24 kg</td>
</tr>
<tr>
<td>McLean et al. (1992)</td>
<td>Wheat; soybean meal</td>
<td>Weaner pigs; E1: amylase; xylanase; cellulase; pectinase (700 g t(^{-1})) E2: β-glucanase; pectinase; cellulase; hemicellulase (1 kg t(^{-1}))</td>
<td>Ileal digestibility of protein (%): C: 74.0 +E1: 76.6 +E2: 73.1</td>
</tr>
<tr>
<td>Inborr et al. (1993)</td>
<td>Wheat: barley (50:50); soybean meal; meal/mash</td>
<td>-6–10 kg; E1: β-glucanase #1; xylanase; amylase (35:590; 3300 U g(^{-1})) 1 kg t(^{-1}) E2: β-glucanase #2; xylanase; amylase (41:740; 3300 U g(^{-1})) 950 g t(^{-1})</td>
<td>Growth performance: C: 204 g day(^{-1}) +E1: 223 g day(^{-1}) +E2: 205 g day(^{-1})</td>
</tr>
<tr>
<td>Officer (1995)</td>
<td>Wheat; fishmeal; meat meal; soybean meal; meal/mash</td>
<td>-6.5–14 kg; E1: protease; lipase; β-glucanase; amylase; cellulase (2 kg t(^{-1})) E2: protease; lipase; β-glucanase; amylase; cellulase; hemicellulase; pentosanase (2 kg t(^{-1}))</td>
<td>Growth performance (after weaning): C: 309 g day(^{-1}) +E: 319 g day(^{-1})</td>
</tr>
</tbody>
</table>
Table 8.3. Continued.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Basal diet – main ingredients; age/weight of pig; pelleted or meal/mash (where stated)</th>
<th>Enzyme(s) added, levels and inclusion rates (where stated)</th>
<th>Responses seen (C = control; +E = plus enzyme; * P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campbell et al. (1995)</td>
<td>Wheat; lupin (W/L) or wheat; soybean meal (W/S); pelleted (70°C) 6–13 kg</td>
<td>Xylanases; pentosanases; β-glucanases 400 g t⁻¹</td>
<td>Growth performance:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C (W/L): 355 g day⁻¹ Feed : gain 1.21</td>
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<td></td>
<td></td>
<td></td>
<td>+E: 388 g day⁻¹ Feed : gain 1.15</td>
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<td></td>
<td>C (W/S): 291 g day⁻¹ Feed : gain 1.29</td>
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<td></td>
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<td></td>
<td>+E: 353 g day⁻¹ Feed : gain 1.23</td>
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<td></td>
<td>Overall effects of enzyme on growth rate (P &lt; 0.05) and</td>
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<td>feed : gain (P = 0.08)</td>
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<tr>
<td>Thacker and Baas (1996)</td>
<td>Rye; soybean meal Peletted (&lt; 65°C) 23–63 kg (Trial 1) 28–92 kg (Trial 2)</td>
<td>'Multi-enzyme preparations' E1–E4: E1: 1 kg t⁻¹ E2: 200 g t⁻¹ E3: 500 g t⁻¹ E4: 700 g t⁻¹ Inclusion rates supplied the same pentosanase activity, according to the authors' assay method</td>
<td>Growth performance (Trial 1): C: 750 g day⁻¹ Feed : gain 2.52</td>
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<td></td>
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<td>+E1: 780 g day⁻¹ Feed : gain 2.42</td>
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<td>+E2: 830 g day⁻¹ Feed : gain 2.39</td>
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<td>+E3: 800 g day⁻¹ Feed : gain 2.37</td>
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<td>+E4: 800 g day⁻¹ Feed : gain 2.44</td>
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<td>Growth performance (Trial 2): C: 880 g day⁻¹ Feed : gain 2.42</td>
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<td>+E1: 940 g day⁻¹ Feed : gain 2.33</td>
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<td></td>
<td>+E2: 930 g day⁻¹ Feed : gain 2.37</td>
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<td></td>
<td>+E3: 920 g day⁻¹ Feed : gain 2.37</td>
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<td></td>
<td>+E4: 930 g day⁻¹ Feed : gain 2.37</td>
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</tbody>
</table>
Carbohydrase Enzymes in Pig Nutrition

Schulze et al. (1996b) Wheat; soybean meal; pelleted 8–22 kg

E1: xylanase #1 (5000 U g$^{-1}$) 1 kg t$^{-1}$
E2: xylanase #1/protease (5000/500 U g$^{-1}$) 1 kg t$^{-1}$
E3: xylanase #2 (5000 U g$^{-1}$) 1 kg t$^{-1}$

Growth performance:
C: 304 g day$^{-1}$ Feed: gain 1.69
+E1: 394 g day$^{-1}$ Feed: gain 1.49*
+E2: 385 g day$^{-1}$ Feed: gain 1.49*
+E3: 402 g day$^{-1}$ Feed: gain 1.45*

Gill and Schulze (1996) Wheat; soybean meal; meal/mash (M) or pelleted (P) 33–94 kg
Xylanase (4000 U g$^{-1}$) 1 kg t$^{-1}$

Growth performance:
C (M): 859 g day$^{-1}$ Feed: gain 2.46
+C (P): 836 g day$^{-1}$ Feed: gain 2.43
+E: 1070 g day$^{-1}$ Feed: gain 2.03
Pen fouling ‘greatly reduced’ in pelleted diets + enzyme

van Lunen and Schulze (1996b) Wheat: corn (60 : 0); (40 : 20); (20 : 40); wheat middlings; soybean meal; pelleted 22–80 kg
Xylanase (4000 U g$^{-1}$) 1 kg t$^{-1}$

Overall growth performance:
C: 980 g day$^{-1}$ Feed: gain 2.78
+E: 1070 g day$^{-1}$ Feed: gain 2.63*

Li et al. (1996a) Wheat; soybean meal; meal/mash 7–11 kg
β-glucanase (1000 U g$^{-1}$) 2 kg t$^{-1}$

Ileal digestibility of protein (%): C: 68.8 +E: 75.9
Ileal digestibility of energy (%): C: 66.9 +E: 71.2

Faecal digestibility of protein (%): C (W): 85.1 +E: 89.1
Faecal digestibility of energy (%): C (W): 86.8 +E: 88.4

Li et al. (1996b) Wheat; soybean meal (W) Rye; soybean meal (R) meal/mash 6–11 kg
β-glucanase, 1000 U g$^{-1}$
(2 kg t$^{-1}$ (W) 1 kg t$^{-1}$ (R) numerically max. responses are shown)

Ileal digestibility of protein (%): C (W): 87.0 +E: 89.3
Ileal digestibility of energy (%): C (W): 87.2 +E: 88.1
<table>
<thead>
<tr>
<th>Reference</th>
<th>Basal diet – main ingredients; age/weight of pig; pelleted or meal/mash (where stated)</th>
<th>Enzyme(s) added, levels and inclusion rates (where stated)</th>
<th>Responses seen (C = control; +E = plus enzyme; *P &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haberer et al. (1997)</td>
<td>Rye : barley (50 : 50); wheat bran; soybean meal; meal/mash, wet fed 26–40 kg</td>
<td>Xylanase (800 FXU); (\beta)-glucanase (75FBG) 750 g t(^{-1})</td>
<td>Faecal digestibility of protein (%): Measured DE (MJ kg(^{-1}) dry matter):</td>
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<td></td>
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<td>C: 74.9</td>
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<td></td>
<td></td>
<td></td>
<td>+E: 76.3</td>
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<td>+E: 14.8*</td>
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<tr>
<td>Yin et al. (1997)</td>
<td>Wheat; wheat/wheat middlings; wheat/wheat bran -20 kg ileal-cannulated pigs</td>
<td>Xylanase (4000 U g(^{-1})) 1 kg t(^{-1})</td>
<td>Ileal digestibility of protein (%): Ileal digestibility of energy (%):</td>
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<td></td>
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<td>C: 76.5</td>
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<td>+E: 77.9</td>
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<td>+E: 71.4</td>
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<tr>
<td>Dusel et al. (1997b) and Jeroch et al. (1999)</td>
<td>Wheat (high extract viscosity); rye; barley; soybean meal; pelleted 10–25 kg</td>
<td>E1: xylanase (5000 U g(^{-1})) 1 kg t(^{-1}) or E2: xylanase (5000 U g(^{-1})) Protease (500 U g(^{-1})) 1 kg t(^{-1})</td>
<td>Growth performance:</td>
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<td></td>
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<td></td>
<td>C: 354 g day(^{-1})</td>
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<td></td>
<td>+E1: 482 g day(^{-1})*</td>
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<td>+E2: 480 g day(^{-1})*</td>
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<td></td>
<td>C: 70.4</td>
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<td>+E: 77.9</td>
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<td>+E: 71.4</td>
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<tr>
<td>Rattay et al. (1998)</td>
<td>Barley; wheat; wheat bran; soybean meal meal/mash –Avilamycin (A; 40 mg kg(^{-1})) 23–45 kg</td>
<td>Xylanase (4000 U g(^{-1})) 1 kg t(^{-1})</td>
<td>Faecal digestibility of protein (%): Faecal digestibility of energy (%):</td>
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<td></td>
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<td>C: 77.9</td>
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<td>+A: 83.3</td>
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<td>+E: 83.3</td>
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<td></td>
<td>+A: 83.5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+A+E: 83.5</td>
</tr>
<tr>
<td>Partridge et al. (1998a)</td>
<td>Wheat : barley (50 : 50); soybean meal – C1 Wheat : maize (50 : 50); wheat pollard; soybean meal – C2 Both diets pelleted 8–30 kg</td>
<td>Xylanase; (\beta)-glucanase; amyglase; pectinase (4000; 150; 1000; 25 U g(^{-1})) 1 kg t(^{-1})</td>
<td>Growth performance:</td>
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<td></td>
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<td>C: 575 g day(^{-1})</td>
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<td>+E: 581 g day(^{-1})*</td>
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<td>+E: 15.3*</td>
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<td>+E: 15.9*</td>
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<tr>
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<td>+E: 15.6*</td>
</tr>
</tbody>
</table>

Table 8.3. Continued.
Callesen (1998) Wheat; barley; wheat bran; soybean meal pelleted (81°C min.) 30–97 kg Xylanase (4000 U g⁻¹) 1 kg t⁻¹ Growth performance: C: 824 g day⁻¹ FU kg⁻¹ gain 2.66 +E: 836 g day⁻¹ FU kg⁻¹ gain 2.59 Protein efficiency value (protein consumption/kg lean meat gain): C: 986 g +E: 929 g Gross margin/pen place/year: C: 658 DKK +E: 706 DKK*

Partridge et al. (1999) Wheat – high (H) versus medium (M) versus low (L) performance*: pelleted 28–60 kg Xylanase (4000 U g⁻¹) 1 kg t⁻¹ Growth performance: H: 960 g day⁻¹ M: 918 g day⁻¹ +E: 945 g day⁻¹ L: 876 g day⁻¹ +E: 952 g day⁻¹ Feed : gain: H: 1.84 M: 1.80 +E: 1.81 L: 1.76 +E: 1.81

Dreschel et al. (1999) Triticale; rye; barley; wheat 25–110 kg Xylanase, β-glucanase, 300 and 200 gt⁻¹ in grower and finisher phases, respectively Growth performance: C: 865 g day⁻¹ +E: 893 g day⁻¹ Energy efficiency: C: 44.0 MJ ME kg⁻¹ gain +E: 42.4 MJ ME kg⁻¹ gain

Jeroch et al. (1999) Wheat (86% of diet), low (L) or high (H) extract viscosity; pelleted 10–25 kg Xylanase (5000 U g⁻¹) 1 kg t⁻¹ Growth performance: C (L): 376 g day⁻¹ Feed : gain 1.96 +E (L): 383 g day⁻¹ Feed : gain 1.93 C (H): 398 g day⁻¹ Feed : gain 1.90 +E (H): 400 g day⁻¹ Feed : gain 1.80 DE (measured, MJ kg⁻¹ dry matter): C (L): 16.23 +E: 16.29 C (H): 15.91 +E: 16.07
## Table 8.3.  Continued.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Basal diet – main ingredients; age/weight of pig; pelleted or meal/mash (where stated)</th>
<th>Enzyme(s) added, levels and inclusion rates (where stated)</th>
<th>Responses seen (C = control; +E = plus enzyme; *P &lt; 0.05)</th>
</tr>
</thead>
</table>
| Choc et al. (1999) | High (H), medium (M) and low (L) feed intake wheats – predetermined 7–16 kg | Xylanase; β-glucanase; cellulase 120 g t⁻¹ | Growth performance:  
C (L): 230 g day⁻¹  
+E (L): 466 g day⁻¹*  
C (M): 425 g day⁻¹  
+E (M): 445 g day⁻¹  
C (H): 460 g day⁻¹  
+E (H): 479 g day⁻¹  
Feed : gain 1.38  
Feed : gain 1.23  
Feed : gain 1.27  
Feed : gain 1.20  
Feed : gain 1.14  
Feed : gain 1.20 |
| Gill et al. (2000) | Wheat/soybean meal (W); Wheat/beetpulp/soybean meal (WSBP); pelleted (75 °C) 8–18 kg | Xylanase; amylase; pectinase (W) β-glucanase; amylase; pectinase (WSBP) 1 kg t⁻¹ | Growth performance:  
C (W): 338 g day⁻¹  
+E (W): 360 g day⁻¹  
C (WSBP): 345 g day⁻¹  
+E (WSBP): 363 g day⁻¹  
Feed : gain 1.53  
Feed : gain 1.48  
Feed : gain 1.56  
Feed : gain 1.48  
Faecal digestibility of protein (%):  
C (WSBP) 72.1  
+E (WSBP) 73.9  
DE (MJ kg⁻¹)  
C (WSBP) 14.10  
+E (WSBP) 14.33 |
control rations, suggesting that some degree of nutrient ‘sparing’ may also have contributed to the productive response seen. These studies illustrate the difficulty in interpreting responses, or apparent lack of responses, to exogenous enzymes in the pig unless many parameters are examined synchronously.

Maize and Sorghum as Grain Sources for Pigs

Systematic studies on variability in the feeding value of maize for pigs are relatively difficult to find in the scientific literature. Considering the global importance of maize as the key feed raw material in pig and poultry diets, this apparent dearth of information is surprising but has been remarked upon by others (e.g. Dale, 1994). The oft-held perception that maize, assuming it is mycotoxin-free, is a grain with consistent feeding value is clearly erroneous and this has been demonstrated in a number of poultry trials (Lesseon et al., 1993; Dale, 1994) and alluded to in the pig (Leigh, 1994) (Table 8.6). It is equally clear that this observed variability in animal performance on maize-based diets is a function of many interacting factors, including bushel weight, drying conditions, particle size after grinding, variety (e.g. waxy versus dent) and amino acid and oil content. Research in recent years has concentrated
less on maize variability as an issue and more on the so-called 'designer' versions of the maize crop – e.g. high-lysine, high-oil, low-phytate – all with potentially high agronomic value for the future.

For sorghum, van Barneveld (1999) reviewed a number of digestibility studies with pigs and found that DE estimates ranged from 15.8 to 17.4 MJ kg\(^{-1}\) DM, with energy digestibility coefficients ranging from 0.72 to 0.92. Kopinski (1997) found that the largest variation between cultivars at a single study site was 0.71 MJ kg\(^{-1}\) DM and within a cultivar at different sites it was 0.5 MJ kg\(^{-1}\) DM. Kemm and Brand (1996), summarizing trials with samples of low- and high-tannin sorghum, found that high-tannin varieties had reduced DE content (10–16%) compared with low-tannin varieties. Feed conversion was correspondingly poorer on high-tannin sorghum when compared with low-tannin sorghum and maize meal.

Response of Maize and Sorghum to Exogenous Enzymes in Pigs

The NSP levels in maize and sorghum are similar (9–10%; Dierick and Decuyper, 1994) with insoluble arabinoxylans comprising over 40% of this total. It is known
that standard milling procedures (e.g., roller milling or hammer milling through screens of varying diameter) still result in intact packages of cell wall material, with enclosed nutrients, entering the stomach and small intestine. As the cell wall structure in these particular grains is predominantly insoluble fibre, and unresponsive to endogenous enzymes, varying quantities of packaged material have been observed at the end of the small intestine, entering the hindgut (M.R. Bedford, personal communication, 2000). Exogenous enzyme activities of particular relevance to these fibre components in maize and sorghum are xylanase and, to a lesser extent, cellulase designed to break down this cell wall ‘packaging’ and disrupt its associated negative fibre properties, e.g., water-holding capacity.

Maize starch digestibility per se will be most influenced by factors such as the amylose:amylopectin ratio, which will vary particularly when comparing waxy (high amylopectin) versus dent maize. The recent interest in high amylose maize starch as an ingredient in human foods, to stimulate potentially beneficial butyrate fermentation in the hindgut, is a reminder of the importance of this factor. Topping et al. (1997) fed pigs on diets containing either standard maize starch or a high amylose material and observed large differences in the amounts of starch reaching the caecum (Fig. 8.7). This situation has implications, not only for nutrient digestibility and availability in the small intestine, but also with respect to substrate-related changes in the microflora in the hindgut. The relevance to exogenous enzymes is that various amylase sources, having different properties to endogenous amylase, could have a potential role to play in improving starch digestibility in the small intestine and thereby indirectly influence the microbial population in the gut.

Unfortunately, relatively few studies have been reported on the effects of exogenous enzymes on ‘simple’ maize/soybean or sorghum/soybean diets in pigs (Table 8.7). Lindemann et al. (1997) found positive responses in the grower phase (26–64 kg) after addition of a complex enzyme blend (protease, cellulase, pentosanase, galactosidase and amylase) to a maize/soybean meal-based ration. The same enzyme combination gave no response when 20% wheat mill by-product was

![Fig. 8.7. Concentration of starch (mg g⁻¹ dry matter) in caecal digesta of pigs fed diets containing maize starch or high amylose starch; mean of two pigs/treatment. ■, maize starch; □, high amylose starch. (Topping et al., 1997.)](image-url)
Table 8.7. Some published studies on the effects of enzyme preparations on maize or sorghum-based diets –/+ milling by-products (NB: where both ileal and faecal digestibility measurements were made then data are presented preferentially at the ileal level).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Basal diet – main ingredients; age/weight of pig; pelleted or meal/mash (where stated)</th>
<th>Enzyme(s) added; levels and inclusion rates (where stated)</th>
<th>Responses seen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li et al. (1996b)</td>
<td>Maize; soya bean meal; meal/mash 6–11 kg</td>
<td>β-glucanase, 1000 U g⁻¹ (a dose response study – the numerically maximum responses are shown) 2 kg t⁻¹</td>
<td>Faecal digestibility of protein (%): C: 84.4 +E: 82.7 Faecal digestibility of energy (%): C: 85.8 +E: 85.7</td>
</tr>
<tr>
<td>Schulze et al. (1996a)</td>
<td>Maize; soya bean meal; rice bran; meal/mash 46–93 kg</td>
<td>E1: xylanase #1  E2: xylanase #1 + amylase  E3: xylanase #2</td>
<td>Growth performance: C: 700 g day⁻¹ Feed: gain 3.10 +E1: 741 g day⁻¹ Feed: gain 2.82* +E2: 725 g day⁻¹ Feed: gain 2.93 +E3: 763 g day⁻¹ Feed: gain 2.97</td>
</tr>
<tr>
<td>Lindemann et al. (1997)</td>
<td>CH: maize; soya bean meal (‘high’ energy)  CL: maize; soya bean meal; wheat by-products (‘low’ energy) 28–109 kg</td>
<td>Protease; cellulase; pentosanase; α-galactosidase; amylase 1 kg t⁻¹</td>
<td>Growth performance: CH: 80.1 g day⁻¹ Feed: gain 2.97 +E: 880 g day⁻¹ Feed: gain 2.75 CL: 771 g day⁻¹ Feed: gain 3.48 +E: 748 g day⁻¹ Feed: gain 3.42 Enzyme × energy level P &lt; 0.05</td>
</tr>
<tr>
<td>Schulze and Campbell (1998)</td>
<td>Maize; soya bean meal; wheat middlings; pelleted 42–74 kg</td>
<td>E1: xylanase #1  E2: xylanase #2</td>
<td>Growth performance: C: 706 g day⁻¹ Feed: gain 2.30 +E1: 781 g day⁻¹ Feed: gain 1.83* +E2: 765 g day⁻¹ Feed: gain 2.40</td>
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<tr>
<td>Study</td>
<td>Feed Ingredients</td>
<td>Enzymes Used</td>
<td>Feed Performance</td>
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<tr>
<td>Partridge et al. (1998b)</td>
<td>Maize; wheat pollard; soybean meal, meal/mash 37-78 kg</td>
<td>Xylanase (4000 U g⁻¹)</td>
<td>Feed gain at two sites: C: 699 g day⁻¹; Feed gain: 3.07; +E: 694 g day⁻¹; Feed gain: 2.76*</td>
</tr>
<tr>
<td>Kim et al. (1998)</td>
<td>Sorghum, soybean meal; meal/mash 51–118 kg</td>
<td>Cellulase 500 g t⁻¹</td>
<td>Growth performance: C: 930 g day⁻¹; Feed gain: 3.51; +E: 960 g day⁻¹; Feed gain: 3.52</td>
</tr>
<tr>
<td>Evangelista et al. (1998)</td>
<td>Maize; soybean meal, meal/mash 28–85 kg</td>
<td>Amylase; β-glucanase; xylanase; pectinase (1000; 150; 4000; 25 U g⁻¹) 1 kg t⁻¹</td>
<td>Growth performance: Feed gain: C: 792 g day⁻¹; 2.83; +E: 853 g day⁻¹*; 2.82</td>
</tr>
<tr>
<td>Li et al. (1999)</td>
<td>C: Maize; soybean meal CA: Maize; soybean meal + acidifier; meal/mash 10–21 kg</td>
<td>Amylases; β-glucanase; pectinase; proteases; cellulase 700 g t⁻¹</td>
<td>Growth performance: Feed gain: C: 390 g day⁻¹; 1.78; +E: 412 g day⁻¹; 1.62; CA: 399 g day⁻¹; 1.72; +E: 401 g day⁻¹; 1.67</td>
</tr>
<tr>
<td>Partridge et al. (1999)</td>
<td>Maize; wheat by-product; pelleted 28–60 kg</td>
<td>Xylanase (4000 U g⁻¹)</td>
<td>Growth performance: Feed gain: C: 886 g day⁻¹; 1.88; +E: 938 g day⁻¹; 1.82</td>
</tr>
</tbody>
</table>
added to a lower energy-dense version of the same ration. Li et al. (1999) found no significant effects of adding an enzyme blend (amylases, β-glucanase, pectinase, proteases, cellulase) to maize-based diets for weaner pigs, with or without an acidifier in the ration. Similarly Kim et al. (1998) failed to show benefits of adding cellulase to a sorghum/soybean diet fed to grower/finishers. In contrast, Evangelista et al. (1998) described significant responses in growth rate and feed intake to an enzyme blend (amylase, β-glucanase, xylanase, pectinase) offered to grower/finisher pigs.

Van Lunen and Schulze (1996b) reported significant responses to xylanase independent of the maize : wheat ratio in diets for grower/finishers. However, the diet containing the highest level of maize (400 g kg\(^{-1}\)) still contained 20% wheat and 10% wheat by-products, making interpretation of the response in relation to specific substrates more difficult.

When maize/soybean-based rations are supplemented with various milling by-products (e.g. wheat middlings/pollard/bran; extracted and full-fat rice bran) a number of trials have shown a beneficial effect of exogenous enzyme supplementation. Schulze et al. (1996a), Schulze and Campbell (1998) and Partridge et al. (1998b) all showed significant benefits to xylanase supplementation on growth rate and/or feed conversion ratio in growing/finishing pigs.

Overall, it is clear that the reported responses to enzymes in pure maize/soybean or sorghum/soybean diets are, so far, limited and more work is needed to understand the underlying reasons for variability in these raw materials before consistently cost-effective carbohydrase enzyme solutions can be offered for pigs. At the same time it is clear that when such diets are supplemented with certain fibrous by-products, containing significant quantities of insoluble cell wall material, then the potential for a response to an effective enzyme source (particularly xylanase) is considerably magnified. In commercial practice such by-products are frequently added to maize-based diets for grower/finisher pigs to offer some savings in costs of production, so the economic opportunities for cost-effective enzyme addition become a realistic opportunity in these situations.

Implications for Enzyme Use in Diet-induced Disease Syndromes

Recent studies have highlighted the important interactions between diet composition and microflora changes in the pig gut under both chronic and acute disease challenge. In Australia, Pluske and co-workers have elegantly illustrated the role that diet composition plays in the development of swine dysentery, provoked by the anaerobic spirochaetel bacterium *Serpulina hyodysenteriae*. Both soluble, fermentable fibre and resistant starch from various feed raw materials have been found to be particularly provocative to this disease (Pluske et al., 1996, 1998) (Fig. 8.8). To date, attempts to influence this particular condition by the strategic use of carbohydrase enzymes have given equivocal results (Durmic et al., 1997) but the concept is receiving increased research attention with the continued emphasis on prophylaxis, rather than medication therapy, in modern pig production systems.
Undigested soluble fibre has already been identified as one of the key influences on the non-specific colitis syndrome affecting, particularly, pigs in the 15–40 kg weight range offered pelleted, wheat-based rations (Taylor, 1989). Adding an appropriate xylanase has been shown to have a positive influence on this syndrome (Hazzledine and Partridge, 1996), negating the need for meal feeding or potentially costly diet reformulation on affected units.

**Fig. 8.8.** Incidence of swine dysentery, in relation to soluble non-starch polysaccharide concentration and resistant starch concentration in the diet (Pluske et al., 1996).
Other studies have similarly reported positive interactions between the use of enzymes and diet-induced diarrhoea, particularly in the post-weaned pig (Inborr and Ogle, 1988; Florou-Paneri et al., 1998; Kantas et al., 1998). These, together with reports on the potential synergies between enzymes and therapeutic and subtherapeutic antibiotic addition (Florou-Paneri et al., 1998; Kantas et al., 1998; Gollnisch et al., 1999), offer interesting possibilities for future production methods where strategic rather than routine antibiotic use will become the norm.

Future Trends in Enzyme Research for Pig Applications?

The review of many trials published in the last 10 years, across a range of dietary substrates (Tables 8.1, 8.3 and 8.7), suggests that the question to be asked in the next few years is not so much: 'Do enzymes work in pig diets?' but far more: 'When do proven enzymes for pig application give their most cost-effective responses?' Enzyme technology has moved apace over the last decade to bring, amongst others, a range of carbohydrase activities that have the potential to have a major impact on the economics of pig production. However, some key challenges remain.

1. To identify quickly, cheaply and easily, the key parameters in grains and their by-products that appear to limit both voluntary food intake and nutrient availability in the growing pig (e.g. Schulze et al., 1997; Cadogan et al., 1999; Partridge et al., 1999). Then to be able to relate these to responses to specific enzyme additions, given that different sources of carbohydrases (e.g. xylanases, β-glucanases, amylases) will have differing characteristics and relative efficacies both in vitro and in vivo.

2. To ensure, contrary to a number of published studies, that scientific trials involving enzymes are set up in the most appropriate manner to have the opportunity to show an effect in a marginally nutrient-limited animal. This will almost invariably involve some prior response modelling in animals whose lean deposition rates have been predetermined. At the same time in such trials, to superimpose some predetermined aspects of raw material feeding value, determined in vitro (e.g. Chen et al., 1997; Cadogan et al., 1999; Moughan et al., 1999). More attention to detail in describing diet characteristics, particularly fibre analysis and properties, as well as enzyme sources and levels, will also aid interpretation across a range of studies.

3. To understand better the role that enzymes have to play in gut physiology, particularly aspects such as digesta passage rate and patterns of fermentation in different parts of the digestive tract. The effects of these on gut hormone secretion and the physiological control of voluntary food intake would also merit further study.

4. To understand better the potential role for feed enzymes in manipulating the gut microflora with a view to their future use with synergistic additives in production systems where there is far less reliance on prophylactic and therapeutic medication. Fundamental work on how enzymes can potentially influence fermentation (e.g. volatile fatty acid production) in the fore- and hindgut will be particularly valuable in the pig and offers good opportunities to manipulate the gut microflora in a positive way.
5. To understand more clearly the interactions between different enzymes, particularly carbohydrases, phytases and proteases, in order to maximize their cost effectiveness across a range of dietary substrates and situations.

6. To maximize the use of appropriate enzymes in novel pig feeding applications involving preprocessing of raw materials, with or without liquid feeding technology.

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Carbohydrase Enzymes in Pig Nutrition


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Interaction between Cereal Identity and Fat Quality and Content in Response to Feed Enzymes in Broilers

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Introduction

The use of dietary fat of plant or animal origin as a feed ingredient is a common practice in broiler feeding, especially in intensive production systems where dietary energy concentrations of 13 MJ ME kg\(^{-1}\) and greater are required. Under such conditions, its proportion might reach 5–10% of the diet, which corresponds to approximately 40–85% of total dietary fat. The actual proportion of a supplemented fat in a broiler diet depends on several factors, such as local availability, relative price and the effect on the feed manufacturing process, in addition to physiologically derived constraints for a particular fat. The last point in particular requires consideration as several factors modify or interfere with an effective use of a particular type of dietary fat. For example, fat digestibility in a broiler diet depends not only on fat type but also on the particular cereal with which it is used. Antoniou et al. (1980) and Ward and Marquardt (1983) showed that the combination of beef tallow with rye depressed fat digestibility much more than when wheat was the dominant cereal. In contrast, when both cereals were tested with a vegetable oil only slight differences in fat digestibility were found.

The interaction between cereal identity and dietary fat type became even more interesting with the widespread use of exogenous feed enzymes, especially xylanases and β-glucanases. These endo-enzymes are capable of partial hydrolysis of anti-nutritive pentosans (which are more concentrated in rye than in wheat) and mixed linked β-glucans (most concentrated in barley), respectively, thereby reducing their viscous properties in vivo. This in turn allows the use of higher dietary proportions of such ‘critical’ cereals. The fact that greater enzyme effects were reported for fat digestibility in rye- than in wheat-containing diets (Friesen et al., 1992) further confirms the fat by cereal identity interaction, and moreover suggests that the viscous properties of...
these cereals is the reason for their interaction with fat digestibility. In addition, the observed enzyme effect was greater with increasing proportion of dietary rye.

In the following sections an attempt will be made to contribute to the understanding of the underlying mechanisms and to draw some conclusions for practical purposes.

Principles of Fat Digestion in Broilers

Digestion and absorption of fat in poultry was extensively reviewed by Krogdahl (1985). Briefly, the initiation of fat digestion seems to start with a possible onset of fat emulsification in the upper digestive tract but mainly with the entrance of digesta into the duodenum, where it is mixed with the secretions of the exocrine pancreas and gall bladder, which contain lipase, co-lipase and conjugated bile salts, respectively. Bile salts and phospholipids facilitate progressive fat emulsification. Binding of co-lipase at the surface of these relatively large aggregates is the precondition for the action of the lipase that hydrolyses the triglycerides at the 1 and 3 positions of the glycerol molecule. Released fatty acids, the remaining 2-monoglycerides, phospholipids and bile salts form spontaneously into mixed micelles, which are much smaller in diameter than the emulsified fat droplets. Micelles are able to solubilize non-polar lipid compounds such as fat-soluble vitamins or long-chain saturated fatty acids (LCSFA) within their hydrophobic core. The role of the micelle is to act as a shuttle between the bulk intestinal contents and the aqueous–microvillus interface by overcoming unstirred water-layer resistance (Westergaard and Dietschy, 1976). At this point, micelles release their contents to the luminal membranes, from where they passively diffuse into the cell. Under the conditions of a high fat digestibility these processes take place quantitatively in the proximal regions of the small intestine. Bile salts released from the micelles diffuse back to the lumen, and from this point might be used again for micelle formation in a lower part of the small intestine. Ultimately the majority are reabsorbed by the distal small intestine. They are transported back to the liver, where they are again made available for bile production, thus conserving the metabolic bile salt pool.

A long-chain fatty acid that diffuses through the luminal membrane will only enter the intracellular pool if it is bound to the intracellular fatty acid binding protein (FABP) (Ockner et al., 1972a; Ockner and Manning, 1974; Katongole and March, 1979, 1980; for review, see Hohoff and Spener, 1998). The FABP–fatty acid complex translocates the fatty acid to other intracellular compartments where it is normally re-esterified to a triacylglycerol and further processed. It has been shown for broilers that the greatest concentration of this inducible intestinal FABP is found at the main site of fatty acid absorption, i.e. the proximal small intestine (Katongole and March, 1980).

Factors Affecting Fat Digestibility

From the above section it becomes clear that a variety of events have to take place in a coordinated manner within a relatively short time (see below) if high quantities of
ingested fat are to be absorbed. Hence, it is useful to take note of several factors that influence fat digestibility in broilers before dealing with the effects of intestinal viscosity caused by ingestion of cereal grain soluble non-starch polysaccharides (NSPs) – and consequently the effects of the intestinal viscosity reducing carbohydrases – in modification of fat digestibility. The first and probably the most important factor is the chemical nature of the dietary fat itself. For example, beef tallow is characterized by a lower fat digestibility and a lower ME content than soya oil. The reasons are the differences in fatty acid composition of both dietary fat sources. From their fatty acid profile (Table 9.1) it can be deduced that beef tallow is mainly composed of saturated fatty acids (palmitic and stearic acid) and of mono-unsaturated oleic acid, whereas linoleic acid, linolenic acid and oleic acid are the principal fatty acids in soya oil. Differences in fat digestibility arise from differences in absorbability of saturated and unsaturated fatty acids and can be summarized from the literature as follows.

Degree of unsaturation and polarity of fatty acids

LCSFAs (C16 : 0, C18 : 0) are more non-polar than unsaturated long-chain fatty acids of the C18 family and among these fatty acids the polarity increases as the number of double bonds increases. Among the saturated fatty acids (C8 : 0 . . . C18 : 0), the polarity increases as the chain length decreases. The more non-polar a fatty acid is, the more it relies on an adequate presence of bile salts and phospholipids for adequate emulsification. It has been shown that medium-chain saturated fatty acids (MCSFA) and long-chain unsaturated fatty acids (LCUSFA) can be absorbed in large quantities even in the absence of bile salts (Garret and Young, 1975; Westergaard and Dietschy, 1976). The differences in polarity of fatty acids are reflected by differences in their absorbability. In general figures, absorbability increases in the following order: C18 : 0 < C16 : 0 < C14 : 0 < C18 : 1, C18 : 2, C18 : 3 (Renner and Hill, 1961; Young, 1961; Garret and Young, 1975; Ketels et al., 1987; Blanch et al., 1995; Vila and Esteve-Garcia, 1996a,b; Dänicke et al., 1997b). This order is generally at a higher level for fatty acids derived from soybean oil than for beef tallow, and differences in absorbability between particular fatty acids are more pronounced for the latter. Palmitic and stearic acid from soya oil are found to be better absorbable than those originating from beef tallow. It is thought that this is due to the synergistic effects of LCUSFAs, which support the micellar solubilization of LCSFAs. In this respect, monoglycerides or glycerol seem to be necessary for optimized micellar dissolution (Garret and Young, 1975; Sklan, 1979). The synergistic effects of the ratio of unsaturated to saturated fatty acids in relation to fatty acid absorption and ME values of the fats have been described by several regression approaches (e.g. Wiseman and Lessire, 1987; Ketels and De Groote, 1987, 1989; Wiseman and Salvador, 1991). It should be noted, however, that the order of digestibility of fats in addition to the absolute digestibility value might be modified by other factors, such as dietary fat inclusion level (Ketels et al., 1987; Wiseman and Lessire, 1987), age of the birds (Wiseman and Lessire, 1987) and free fatty
Table 9.1. Fatty acid composition of some dietary fat types (%).

<table>
<thead>
<tr>
<th>Fatty acid (C-atoms : double bonds)</th>
<th>Lard</th>
<th></th>
<th>Soybean oil</th>
<th></th>
<th>Beef tallow</th>
<th></th>
<th>Linseed</th>
<th></th>
<th>Palm</th>
<th></th>
<th>Maize oil</th>
<th></th>
<th>Coconut oil</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
<td>Max</td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>8:0</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>2.6</td>
<td>7.5</td>
</tr>
<tr>
<td>10:0</td>
<td></td>
<td></td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>28.4</td>
<td>49.5</td>
</tr>
<tr>
<td>12:0</td>
<td>1.3</td>
<td>1.7</td>
<td>0.1</td>
<td>0.1</td>
<td>2.0</td>
<td>4.9</td>
<td>0.8</td>
<td>1.0</td>
<td>12.1</td>
<td>19.5</td>
<td>12.1</td>
<td>19.5</td>
<td>12.1</td>
<td>19.5</td>
</tr>
<tr>
<td>14:0</td>
<td></td>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>2.0</td>
<td>4.9</td>
<td>0.8</td>
<td>1.0</td>
<td>12.1</td>
<td>19.5</td>
<td>12.1</td>
<td>19.5</td>
<td>12.1</td>
<td>19.5</td>
</tr>
<tr>
<td>16:0</td>
<td>21.2</td>
<td>26.6</td>
<td>0.7</td>
<td>13.5</td>
<td>23.7</td>
<td>35.4</td>
<td>5.0</td>
<td>10.0</td>
<td>40.7</td>
<td>48.8</td>
<td>9.4</td>
<td>13.6</td>
<td>8.5</td>
<td>12.8</td>
</tr>
<tr>
<td>16:1</td>
<td>2.1</td>
<td>5.3</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>1.7</td>
<td>6.7</td>
</tr>
<tr>
<td>18:0</td>
<td>11.1</td>
<td>17.7</td>
<td>1.8</td>
<td>7.4</td>
<td>13.5</td>
<td>36.5</td>
<td>3.1</td>
<td>7.8</td>
<td>3.9</td>
<td>5.2</td>
<td>1.7</td>
<td>6.7</td>
<td>2.0</td>
<td>8.1</td>
</tr>
<tr>
<td>18:1</td>
<td>40.3</td>
<td>51.8</td>
<td>19.1</td>
<td>27.3</td>
<td>24.5</td>
<td>46.4</td>
<td>18.2</td>
<td>21.4</td>
<td>36.7</td>
<td>41.6</td>
<td>25.8</td>
<td>30.1</td>
<td>6.0</td>
<td>13.7</td>
</tr>
<tr>
<td>18:2</td>
<td>8.0</td>
<td>10.4</td>
<td>44.0</td>
<td>59.4</td>
<td>0.9</td>
<td>5.4</td>
<td>13.9</td>
<td>17.9</td>
<td>7.9</td>
<td>12.7</td>
<td>42.0</td>
<td>58.6</td>
<td>1.5</td>
<td>23.1</td>
</tr>
<tr>
<td>18:3</td>
<td>0.1</td>
<td>2.1</td>
<td>3.3</td>
<td>8.2</td>
<td>0.1</td>
<td>1.1</td>
<td>49.4</td>
<td>66.2</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

No. of references 7 9 11 4 3 3 5

acid content (Young, 1961; Wiseman et al., 1991; Blanch et al., 1995; Vila and Esteve-Garcia, 1996c).

Bile salt availability

Bile salt availability in young broilers plays a central role in determining the extent to which fats differ in their digestibility. It has been suggested frequently that bile availability in the very young chick is insufficient, particularly if high proportions of tallow are provided as dietary fat. It was demonstrated that addition of bile or several bile salts to broiler diets containing beef tallow resulted in an improvement in fat digestibility (Fedde et al., 1960; Gomez and Polin, 1976; Katongole and March, 1980; Polin et al., 1980; Polin and Hussein, 1982). It might be concluded that the addition of bile salts would be beneficial in the presence of large amounts of undigested or non-absorbed fatty acids within the small intestine. The reason for this bile salt deficiency in tallow-fed birds might be due to an increase in bile salt excretion whereby bile salts escape the enterohepatic bile salt cycle. This in turn changes hepatic cholesterol metabolism and the profile of synthesized bile salts and ultimately decreases the intestinal bile acid pool, as shown by Monsma et al. (1996) in an experiment with rats fed different dietary fats. These authors demonstrated a strong linear relationship between dietary intake of stearic acid and muricholic acid-derived bile salt excretion. Furthermore, intestinal microflora might also contribute to bile salt metabolism, as shown by Kussaibati et al. (1982) using conventional and germ-free chickens. They found that the addition of bile salt to the diet markedly improved the digestibility of LCSFAs in conventional birds, whereas only slight effects were observed in germ-free chickens. The benefit of adding bile salts to either bird type was marginal for digestibility of LCUSFAs. The authors concluded that intestinal microbes in conventional birds deconjugate bile salts, which are poorly absorbed and consequently escape the enterohepatic bile salt cycle.

Kritchevsky and Story (1974) demonstrated that non-nutritive fibres from different feedstuffs are able to bind bile salts to a different degree in vitro. Consequently, such binding could contribute to changes in the intestinal bile salt pool.

Triglyceride structure – fatty acid positional effects

Another important point that adversely influences the digestibility of dietary fats is the positional distribution of the fatty acids at the glycerol molecule. Generally, high proportions of the LCSFAs at the 2-position are associated with an increased micellar solubility of these fatty acids in the form of the respective monoglycerides compared with their relative solubility when released as free fatty acids from the 1- and 3-positions by the action of the lipase. It has been shown that a relatively high proportion of the LCUSFAs in beef tallow (mainly oleic acid) are bound to the 2-position, and the majority of palmitic and stearic acid are esterified at the 1- and 3-positions (Sibbald and Kramer, 1977; Ketels et al., 1987; Bracco, 1994). Such
Positional effects are presumed to be detrimental to fat digestion and were demonstrated to influence TME values of fats in broilers (Sibbald and Kramer, 1977), energy absorption in rats (Brink et al., 1995), and fat absorption in rats (Mattson et al., 1979) and human infants (Filer et al., 1969).

**Lipase availability**

Lipase activity was reported to be low in the very young turkey (Krogdahl and Sell, 1989) and chick (Nir et al., 1993) and lipase addition to a tallow-containing diet was shown to improve fat digestibility (Polin et al., 1980). Noy and Sklan (1995) did not detect any increase in ileal fat digestibility in chicks between 4 and 21 days of age (6% unsaturated fat in the diet) despite an age-related increase in lipase secretion into the duodenum. At present, no final conclusion can be drawn under which circumstances lipase secretion might be a limiting factor in fat digestion.

**Mineral soaps**

Finally, it is evident from the literature that calcium or magnesium might reduce fat absorption by forming indigestible soaps. This is of special importance if either fat absorption is low, i.e., high intestinal amounts of fat as in tallow digestion, or higher levels of those minerals are present in the diet (Fedde et al., 1960; Mattson et al., 1979; Antoniou et al., 1980; Brink et al., 1995).

Although the above sections focus mainly on beef tallow and soybean oil, it should be noted that the effects of the other dietary fats listed in Table 9.1 might be deduced from their fatty acid profile and other physical and chemical characteristics in a similar manner.

**Role of Intestinal Viscosity in Interference of Fat Digestion**

**General remarks**

Increases in intestinal viscosity of broilers are mainly caused by feeding higher proportions of soluble NSPs such as the soluble pentosans in rye and wheat and soluble mixed linked β-glucans in barley. Although both rye and barley are comparable in soluble NSP content, the relationship between water extract viscosity profile and soluble NSP concentration is quite different (Fig. 9.1). It has been emphasized that, in addition to chemical analysis of polysaccharides, their physical properties have to be considered in evaluation of their physiological effects (Morris, 1992). Molecular weight and degree of branching of the polysaccharide chain mainly determine the so-called intrinsic viscosity, i.e., the fractional increase in viscosity per unit concentration of polymer (Morris, 1992). Bedford and Classen (1992) reported...
a positive correlation between high molecular weight carbohydrate complexes (molecular weight > 500,000) and viscosity in the intestine of chickens fed diets varying in pentosan content. Hence, the distinct structural properties might account for differences between rye and barley with respect to in vitro and in vivo viscosity. Annison et al. (1995) compared soluble isolates from wheat and rice bran with respect to chemical and physical properties and found that, although both isolates were composed mainly of arabinoxylans, they produced extract viscosities of 64 and 1.6 mPa s, respectively. This was associated with arabinose : xylose ratios of 0.58 and 1.23, respectively. These authors suggested that rice bran xylan backbone carries more arabinose side-chains, whereas the unsubstituted sections of the wheat xylan main chain facilitated the formation of inter-chain hydrogen bonds. Consequently, the degree of branching within wheat varieties could be a contributing factor to differences in extract viscosity.

It has been suggested that a viscosity of a polysaccharide solution of approximately 10 mPa s indicates a critical polysaccharide concentration above which viscosity responds more steeply to increasing polysaccharide concentrations. This is thought to be the result of the onset of formation of an entangled network from individual polysaccharide coils (Morris et al., 1981). In spite of the use of complete cereal matrices for in vitro viscosity measurements as shown in Fig. 9.1, it can be clearly seen that rye solution viscosity started to rise more steeply in the range between 1 : 6 and 1 : 5 dilution, which corresponded to viscosities of approximately 5–10 mPa s. From the same graph it might be concluded that such an entanglement was not initiated in the case of barley. The corresponding ileal viscosities in broilers fed diets containing rye and barley (69% dietary inclusion) were measured as 480.4 and 10.2 mPa s, respectively (Dänicke et al., 1999a).
Feed passage through the digestive tract, gut motility and unстirred water-layer resistance

Carbohydrase supplementation of diets based on barley, rye or wheat has been shown to accelerate the total gastrointestinal tract transit time in chickens (Jeroch et al., 1990; Salih et al., 1991; Almirall and Esteve-Garcia, 1994; Dänicke et al., 1997a) and in turkeys (Großer and Jeroch, 1997), which in turn suggests that an increased intestinal viscosity would result in the opposite effect and would consequently reduce feed intake and performance. Table 9.2 summarizes some results on measurement of feed transit time, which is expressed as the time required for 50% of marker excretion ($T_{50}$). From the results of Van der Klis and Van Voorst (1993) it would appear that dietary soluble NSP concentration is closely related to feed transit time. The effects of

### Table 9.2. Mean total gastrointestinal tract transit time of feed, expressed as time required for 50% of excretion of a marker.

<table>
<thead>
<tr>
<th>Reference</th>
<th>1. Bird type</th>
<th>2. Age</th>
<th>Remarks</th>
<th>Carbohydrase</th>
<th>$T_{50}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salih et al. (1991)</td>
<td>1. Broiler</td>
<td></td>
<td></td>
<td>Without</td>
<td>7.59</td>
</tr>
<tr>
<td></td>
<td>2. 2 weeks</td>
<td></td>
<td></td>
<td>With</td>
<td>6.31</td>
</tr>
<tr>
<td></td>
<td>4 weeks</td>
<td></td>
<td></td>
<td>Without</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>High-viscosity barley based diets</td>
<td>6 weeks</td>
<td></td>
<td>With</td>
<td>6.59</td>
</tr>
<tr>
<td></td>
<td>8 weeks</td>
<td></td>
<td></td>
<td>Without</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>With</td>
<td>7.21</td>
</tr>
<tr>
<td>Van der Klis and Van Voorst (1993)</td>
<td>1. Broiler</td>
<td>5 weeks</td>
<td>Without carboxy methyl cellulose</td>
<td></td>
<td>4.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 g carboxy methyl cellulose</td>
<td></td>
<td>5.35</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>30 g carboxy methyl cellulose</td>
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<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Almirall and Esteve-Garcia (1994)</td>
<td>1. Broiler</td>
<td>3 weeks</td>
<td>High-viscosity barley</td>
<td>With</td>
<td>8.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1. Cocks</td>
<td>High-viscosity barley</td>
<td>Without</td>
<td>5.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. 1 year</td>
<td>High-viscosity barley</td>
<td>With</td>
<td>3.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>With</td>
<td>4.81</td>
</tr>
<tr>
<td>Dänicke et al. (1997a)</td>
<td>1. Broiler</td>
<td>4 weeks</td>
<td>Rye-based diet, 10% soybean oil</td>
<td>With</td>
<td>8.38</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Rye-based diet, 10% soybean oil</td>
<td>With</td>
<td>6.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rye-based diet, 10% beef tallow</td>
<td>With</td>
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<td></td>
<td>Rye-based diet, 10% beef tallow</td>
<td>With</td>
<td>6.88</td>
</tr>
<tr>
<td>Großer and Jeroch (1997)</td>
<td>1. Turkey</td>
<td>3 weeks</td>
<td>Wheat-based diet, 8% soybean oil</td>
<td>With</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wheat-based diet, 8% soybean oil</td>
<td>With</td>
<td>8.36</td>
</tr>
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<td></td>
<td>Wheat-based diet, 8% beef tallow</td>
<td>With</td>
<td>14.52</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Wheat-based diet, 8% beef tallow</td>
<td>With</td>
<td>9.4</td>
</tr>
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<td>Wheat-based diet, 8% coconut oil</td>
<td>With</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wheat-based diet, 8% coconut oil</td>
<td>With</td>
<td>6.07</td>
</tr>
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</table>
different fat types and xylanase supplementation on transit times were investigated by Dänicke et al. (1997a,b) in broiler chickens and by Großer and Jeroch (1997) in young turkeys. Xylanase addition resulted in an accelerated transit time for broilers fed on soya oil and beef tallow. A fat effect was not obvious in broilers. In contrast, different fat and enzyme effects were observed in turkeys. Xylanase addition to diets containing soya oil or coconut oil changed the transit time only slightly, whereas the effect was dramatic (reduced transit by 5 h) in the diet containing beef tallow. Passage rate was fastest with the coconut oil diet followed by the diets containing soya oil and finally beef tallow. A comparison between species makes it clear that the young turkey seems to be more responsive to different fat types than the growing chick.

In a more detailed study, Dänicke et al. (1999b) examined the mean retention time (MRT) of digesta in different segments of the digestive tract of broilers fed rye-based diets with differing levels of added dietary fat type and xylanase (Fig. 9.2). Again, no significant fat effect was observed. The majority of the reduction in MRT on addition of xylanase was observed in the jejunum and ileum. Experiments with humans and pigs revealed that an increased digesta viscosity delayed gastric emptying (Furuya et al., 1978; Blackburn et al., 1984), thereby reducing the overall feed transit time through the entire digestive tract. Sudendey and Kamphues (1995) observed an accelerated rate of gastric emptying when diets based on wheat or barley fed to piglets were supplemented with a carbohydrase. Furthermore, Meyer et al. (1986) concluded from their experiment with dogs that meal viscosity affects gastric sieving, which in turn could influence both gastric and intestinal digestion of nutrients. Such effects of viscosity on gastric emptying rate have not been observed with broilers and

**Fig. 9.2.** Effect of fat type and xylanase supplementation on mean retention time in successive segments of the gastrointestinal tract of male chickens (day 24 of age, average of four birds per treatment ± standard error) (Dänicke et al., 1999b). Crop, ■; proventriculus plus gizzard, ■; duodenum, ■; jejunum, ■; ileum, □; rectum, ■.
the effect of different fat types on the contribution of gastric emptying to overall feed transit changes (Fig. 9.2) has proved inconclusive. In this regard the chicken appears to differ substantially from other monogastrics.

However, a general effect of fat as such on the MRT of digesta in the small intestine cannot be excluded completely since its presence modifies gut motility via stimulation of gastrointestinal neurotransmitters or hormones. Neurotensin has been shown to decrease the frequency and strength of contractions of the gizzard and partially of the duodenum and ileum in broilers (Degolier et al., 1997). The presence of lipids in the duodenum was found to inhibit gastric motility in turkeys (Duke and Evanson, 1972). Degolier et al. (1997) reported that neurotensin was released into the hepatic-portal circulation in response to the presence of oleic acid in the duodenum. It was suggested that, by a parallel decrease in gut motility, the efficiency of digestion could be enhanced by a longer MRT of digesta in the intestine. Moreover, fats differing in their contents of unsaturated and saturated fatty acids are not equivalent in their stimulation of release of neurotensin and other gastrointestinal peptides. According to the results of Sagher et al. (1991) obtained with rats, maize oil and olive oil increased neurotensin and substance P, whereas butterfat did not, when compared with the low fat control group.

It might be concluded from these findings that both intestinal viscosity and intestinal fatty acid composition contribute to differences in the MRT of digesta in intestinal segments and consequently the overall gastrointestinal tract transit time, albeit by different mechanisms. Hence, an interaction between both factors cannot be excluded in interpreting the results shown in Fig. 9.2.

A reduction in gut motility, caused by increased viscosity, decreases the mixing of digesta with pancreatic and biliary secretions, which is of particular importance for emulsification of saturated fatty acids and formation of micelles. It has been suggested by Smulikowska (1998) that gut motility-mediated shuttling of digesta between the duodenum and gizzard increases the time of feed exposed to digestive enzymes and favours enhanced digestion and absorption in the upper parts of the small intestine. At the same time as intestinal viscosity is increased and mixing is decreased, the thickness of the unstirred water layer (UWL), an extracellular barrier covering the intestinal microvilli, is proportionally increased (Johnson and Gee, 1981, 1982; Flourie et al., 1984; Lund et al., 1989). Westergaard and Dietschy (1976) proposed that the principal role of the micelle is to overcome the resistance of that UWL. Consequently, an increased thickness of the UWL would increase this resistance and decrease the transfer rate between intestinal bulk and the absorptive site, which is, again, of particular importance for saturated fatty acids. In addition, Smulikowska (1998) suggested that, especially in the young chicken, an increase in the UWL would decrease the effective intraluminal diameter and as a result the rate of digesta transit and mixing would be further impeded. However, this concept of the UWL is open to debate. Edwards et al. (1988) and Read and Eastwood (1992) pointed out that a reduction in mixing and an increase in the UWL are two ways of describing the same thing. Nevertheless, strong relationships between the thickness of the UWL and the malabsorption of sugars and of cholesterol have been reported (e.g. Lund et al., 1989). Cholesterol absorption by everted intestinal sacs was
significantly reduced even at low concentrations of oat gum and continued to fall as the concentration increased, but proportionately less with each increment (Lund et al., 1989). The authors concluded that this initial decrease could indicate a sieving effect of dispersed β-glucan polymer on the micelles, which have relatively high molecular volume (Phillips, 1986). Interestingly, this decrease coincided with a relatively low in vitro viscosity of approximately 5–20 mPa s, which is the range where polysaccharides in solution start to form an entangled network from individual polysaccharide coils (Morris et al., 1981; see above section), associated with a steep incremental increase in viscosity. Moreover, higher intestinal lipid concentrations are paralleled by a corresponding increase in the size of the formed micelles (Ockner et al., 1972b). This would explain why tallow feeding is more dramatically sensitive to small increases in intestinal viscosity when compared with soybean oil feeding.

Availability of endogenous lipase and bile salts

Experiments dealing with the effects of viscous carbohydrates on lipase activity in the intestinal contents and pancreas in relation to fat absorption are often inconclusive and the underlying mechanisms are not fully understood. Isaksson et al. (1983a) found an increased lipase output in rats fed a pectin-containing diet, whereas lipase concentration per gram wet material was not changed. Total fat excretion in the faeces was increased at the same time. These results show that pectin obviously stimulated pancreatic lipase output but do not suggest that specific lipase activity was responsible for the increased fat excretion. In contrast, feeding of neither low methylated or high methylated pectin nor of wheat bran changed specific lipase activity (units per mg) or total lipase output in rats (Isaksson et al., 1983b), whereas protein concentration and protein content of the pancreas were altered. These results were interpreted as adaptive changes in pancreatic enzyme production. In another experiment, Isaksson et al. (1984) found a decrease in specific lipase activity (units per ml) after consumption of a pectin-containing test meal in pancreatectomized patients treated with a granulated pancreatic enzyme preparation. This decrease was accompanied by a decrease in 14CO2 excretion after 14C triolein ingestion, which would indicate that lipase activity indeed became a limiting factor. However, the effect of pancreatic regulation due to pectin ingestion could not be investigated with such an experimental design.

The decrease of in vitro lipase activity and activity of other pancreatic enzymes in human duodenal juice was shown to be directly related to an increased viscosity (Isaksson et al., 1982). It was also suggested that adsorption of enzymes by specific protein enzyme inhibitors could play a role in activity depression in the case of wheat bran. Moreover, it was shown that inhibition in lipase activity due to increased viscosity occurred in a relatively short period of time and it remained at these depressed levels thereafter. Therefore, it was suggested that these inhibitory effects would be relatively independent of intestinal passage time. Finally, lipase activity decreased as the pH was lowered from 5.7 to 5. Dänicke et al. (1997a) reported a significant decrease in pH in the small intestine of broilers fed tallow-containing
diets. Whether such a decrease could be a contributing factor in lower tallow digestibility remains to be clarified.

Almirall *et al.* (1995) reported the specific lipase activity to be decreased in broiler chickens and cocks after feeding barley, compared with maize. Activity was partially restored after β-glucanase supplementation of the barley-based diets. In addition, chickens had higher specific lipase activities and seemed more susceptible to dietary treatments than cocks.

Dänicke *et al.* (1999c) demonstrated that the provision of a xylanase decreased specific lipase activity (units per gram wet weight) in the jejunal contents and in the pancreas of broilers fed rye-based diets supplemented with beef tallow, whereas no effect was noted in diets supplemented with soybean oil. Total pancreatic lipase activity remained unchanged but was significantly increased on body weight basis in birds fed the unsupplemented tallow-containing diet. In addition, relative pancreatic lipase activity was linearly dependent on relative pancreas weight. It would appear, therefore, that birds fed the unsupplemented tallow-containing diet had attempted to increase lipase activity to compensate for such poor fat digestibility and that this was reflected in low body weights and the highest specific intraluminal lipase activity. A decreased pancreatic lipase activity and an increased intestinal activity in rats fed guar gum was reported by Poksay and Schneeman (1983), which suggests a similar response. It was concluded that the observed reduction in fat digestibility following guar gum addition to the diet could not be the result of an insufficient presence of lipase in the intestine. Furthermore, Silva *et al.* (1997) found intestinal lipase activity (corrected for body weight and weight of small intestine and contents) to be significantly decreased in broilers fed xylanase-added rye-based diets. Interestingly, when intestinal contents where examined *in vitro*, lipase activity was slightly increased after the addition of the xylanase when compared with measured activities before xylanase administration. It was suggested that the xylanase released some lipase activity which had been adsorbed by dietary fibre in the intestines of birds fed the unsupplemented diets. Adsorption of lipase and bile salts to different fibre types was examined by Lairon *et al.* (1985). It was found that binding capacity of cellulose, purified xylan and pectin was negligible, whereas wheat bran was shown to have a moderate capacity. Furthermore, fine wheat bran bound more lipase and bile salts than coarse wheat bran. Pasquier *et al.* (1996) demonstrated, by *in vitro* experiments using reconstituted duodenal medium, that the amount of emulsified lipids was reduced and the size of emulsified droplets was increased as the concentration of viscous fibre was increased. In the range of 0–20 mPa s the correlations to both parameters were $r = −0.79$ and $r = 0.88$, respectively. In addition, triglyceride hydrolysis was reduced by approximately 30% over a similar range of viscosity.

Intestinal viscosity also influences bile salt metabolism in rats. Inclusion of psyllium, a dietary fibre rich in soluble components, was shown to increase the excretion of bile salts and total steroids in rats significantly when compared with inclusion of cellulose at a similar level (Buhman *et al.*, 1998). The authors suggested intestinal viscosity itself to be responsible for a decreased reabsorption of bile-salts rather than bile-salt binding per se, since psyllium does not bind bile salts *in vitro*. Furthermore, the hepatic cholesterol metabolism was up-regulated, as suggested by
elevated activities of cholesterol 7α-hydroxylase and cholesterol 7α-hydroxylase mRNA as a result of psyllium feeding. Ikegami et al. (1990) reported a linear relationship between intestinal viscosity and the secretion of pancreatic enzymes and total bile salts in rats which was accompanied by an increased pancreatic weight. It was suggested that the animal compensates for the inefficiency of nutrient digestion with an enlargement of digestive organs and increased secretion of digestive juices.

**Gut morphology and microbial implications**

Feeding soluble NSP was shown to increase the length and the absolute and relative weight of the small intestine in rats (Johnson et al., 1984; Johnson and Gee, 1986) and broilers (Simon, 1998) (Table 9.3). It was suggested by Johnson et al. (1984) that the presence of large amounts of unabsorbed material in the intestine exerts trophic effects on the intestinal mucosa. Feeding of guar gum and carboxy methyl cellulose was shown to increase the cell division rate in the small intestine, colon and caecum of rats, which was accompanied by reduced activity of some mucosal enzymes (Johnson et al., 1984; Johnson and Gee, 1986). It was hypothesized that increased cellular proliferation gives rise to a reduction in lifespan of mucosal cells as a result of an increased rate of shedding of immature (with respect to enzyme expression) cells at the apex of the villi. Guar gum and carboxy methyl cellulose differed, however, in that the latter induced a greater rate of cell division and in the more distal regions of the small intestine. The authors concluded that factors others than intestinal viscosity per se could be involved, such as microbial activity in the small intestine. Rakowska et al. (1993) found severe damage of intestinal villi and mucous membranes of the duodenum and small intestine in broilers after feeding a rye-based diet. Microbial involvement was concluded from this experiment since the antibiotic Nisine protected the intestinal villi. An impact of intestinal viscosity on gut morphology was also shown by Drakley et al. (1997) in that feeding of a highly viscous wheat resulted in a significantly increased villus height and width when compared with a low viscosity wheat. Crypt cell proliferation rate is also increased with increasing intestinal viscosity in broilers, as demonstrated by a decrease after feeding of xylanase-supplemented rye-based diets (Silva and Smithard, 1997).

Dietary fat type might also contribute to changes in intestinal morphology, as shown by Sagher et al. (1991), who found that feeding of maize oil and olive oil significantly increased villus height compared with a low-fat control diet, whereas feeding of butterfat significantly decreased villus height. The question of whether changes in intestinal viscosity interact with such a response remains unanswered.

Dänicke et al. (1999b) examined the effect of xylanase supplementation of rye-based diets containing either soybean oil or beef tallow offered to tissue-associated bacterial groups in several segments of the digestive tract (Table 9.4). Increased intestinal viscosity is largely influenced by feeding of such diets as shown in related experiments (Dänicke et al., 1997a,c) (Table 9.5; Fig. 9.3) and might have caused the described intestinal morphological changes, which lead to reduced colonization surface, altered adhesion structures and an increased release of epithelial cells.
Table 9.3. Effects of dietary fat type (S, 10% soybean oil; T, 10% beef tallow) and enzyme supplementation (−, without; +, with 1 g Avizyme 1300 kg\(^{-1}\) diet) in a rye-based diet (56% dietary inclusion) on protein metabolism in male broilers (Dänicke et al., 2000a,b).

<table>
<thead>
<tr>
<th>Fat type</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Pancreas</th>
<th>Endogenous digestive N-losses (mg day(^{-1}) per LW (kg)(^{0.67}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g 100 g(^{-1}) LW</td>
<td>(k_3) (% day(^{-1}))*</td>
<td>g 100 g(^{-1}) LW</td>
<td>(k_3) (% day(^{-1}))*</td>
<td>g 100 g(^{-1}) LW</td>
</tr>
<tr>
<td>S−</td>
<td>1.5(^a)</td>
<td>56(^a)</td>
<td>2.1(^a)</td>
<td>51(^a)</td>
<td>1.3(^a)</td>
</tr>
<tr>
<td>S+</td>
<td>1.4(^a)</td>
<td>64(^a)</td>
<td>2.0(^a)</td>
<td>52(^a)</td>
<td>1.2(^a)</td>
</tr>
<tr>
<td>T−</td>
<td>1.9(^b)</td>
<td>84(^b)</td>
<td>2.6(^b)</td>
<td>75(^b)</td>
<td>1.6(^b)</td>
</tr>
<tr>
<td>T+</td>
<td>1.4(^b)</td>
<td>61(^b)</td>
<td>1.9(^b)</td>
<td>58(^b)</td>
<td>1.2(^b)</td>
</tr>
</tbody>
</table>

* Fractional rates of protein synthesis, i.e. daily protein synthesis as percentage of tissue protein.

\(^{ab}\)Values with different superscripts within the columns are significantly different (\(P < 0.05\)).
(and adhering bacteria) into the lumen as a result of stimulation of the proliferation rate of mucosal cells. The observed effects on microbial colonization were most pronounced in duodenal and jejunal samples, which exhibited short digesta retention times and thus low steady-state concentrations of soluble arabinoxylans.

The response of cocci bacteria to enzyme supplementation seemed less drastic, which indicates that this group of bacteria is less affected by morphological changes or nutrient composition. On the other hand, supplementation with tallow resulted in an increased number of cocci in the jejunum and ileum, but seemed to depress growth of total anaerobic bacteria and enterobacteria. Significant higher pH values were measured in most intestinal segments when tallow was used as dietary fat.
Table 9.5. Effect of dietary fat type and carbohydrase supplementation of broiler and turkey diets on body-weight gain (BWG, g), feed-to-gain ratio (FCR, g g\(^{-1}\)), intestinal viscosity (mPa s), apparent fat digestibility (FD\%) and apparent protein digestibility (PD\%).

<table>
<thead>
<tr>
<th>Ref no.</th>
<th>1. Bird type</th>
<th>2. Age (balance exp.)</th>
<th>3. Age (growth exp.)</th>
<th>Dietary cereal inclusion (%)</th>
<th>Dietary fat inclusion (%)</th>
<th>BWG</th>
<th>FCR</th>
<th>Vis(^1)</th>
<th>FD(^2)</th>
<th>PD(^2)</th>
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<tr>
<td></td>
<td>2. Days 7-11</td>
<td></td>
<td></td>
<td>61 10</td>
<td>82.3 90.9</td>
<td></td>
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<tr>
<td></td>
<td>Days 21-25</td>
<td></td>
<td></td>
<td>61 10</td>
<td>87.3 91.3</td>
<td></td>
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<td>Days 32-36</td>
<td></td>
<td></td>
<td>61 10</td>
<td>34.0 88.8</td>
<td></td>
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<tr>
<td></td>
<td>3. Days 1-28</td>
<td></td>
<td></td>
<td>61 10</td>
<td>51.0 89.9</td>
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\(\times\) indicates significant differences within each column for Ref no. 1, 2 and 3: *, **, *** and **** respectively.
## Use of Feed Enzymes in Broilers

### Factors Analyzed
- **Enzyme × age**
- **Fat × enzyme × age**

### Data Table

<table>
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<th>Category</th>
<th>Fat (g/kg)</th>
<th>Enzyme (g/kg)</th>
<th>Age (days)</th>
<th>Data</th>
<th>p-Value</th>
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<td></td>
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<tr>
<td>Enzyme</td>
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### Notes
- NS: Not significant
- **: p < 0.05
- ***: p < 0.001
Table 9.5.  
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NS = Not Significant
*** = P < 0.001
** = P < 0.01
* = P < 0.05
### Use of Feed Enzymes in Broilers

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### Notes

1. Broiler
2. Days 21–24
3. Days 1–21
4. Fat
5. Enzyme
6. Fat * enzyme

### Abbreviations
- R, rye; W, wheat; T, triticale; M, maize; B, barley; S, soya oil; T, beef tallow; L, lard; AB, animal fat blend; C, coconut oil; E, enzyme preparation.
- a–c Values with no common superscript are significantly different within columns ($P < 0.05$).
- Probability: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant.

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1. Intestinal viscosity measured in digesta of jejunum plus ileum (reference [4]) or in digesta of ileum (references [1] to [3]).
2. Digestibility was measured at the faecal level (references [1], [3], [4], [5]) or at the terminal ileum (reference [2]).

Abbreviations: R, rye; W, wheat; T, triticale; M, maize; B, barley; S, soya oil; T, beef tallow; L, lard; AB, animal fat blend; C, coconut oil; E, enzyme preparation.

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2.06 1.76 2.12 1.81 2.33 1.93 2.07 1.83 79.3 80.2 60.5 69.9
instead of soybean oil (Dänicke et al., 1997a). Thus, fat type clearly influences the microbial composition in the intestine independently of viscosity. It is not known if cocci growth was actively stimulated by tallow addition or if it was merely a result of inhibition of other bacteria, possibly due to increased secretion of bile acids by the host. Bile acids have a bacteriostatic effect, but this effect varies with each species of interest. Cocci can be distinguished by their ability to grow in the presence of relatively high concentrations of bile salts; thus the most resistant cocci, such as Enterococci, may become dominant under higher bile acid concentrations. Active de-conjugation of conjugated bile acids, as carried out by some lactobacilli, also occurs in the intestinal tract, but it is not known if the generated bile salt hydrolase activity is sufficient to interfere quantitatively with the bile salt metabolism.

**Fig. 9.3.** Effects of dietary proportion of rye, dietary fat type and xylanase supplementation on jejunal and ileal supernatant viscosity of broilers (Dänicke et al., 1999c). (Without xylanase, —; with xylanase, ---; jejunum, ○; ileum, •.)

interactions between dietary fat type and carbohydrase addition in broiler diets containing different cereals

### General remarks

In the previous section an attempt was made to explain why exogenously supplied carbohydrases capable of reducing intestinal viscosity could result in different effects depending upon, amongst other factors, the chemical composition of the dietary fats employed.

Several experiments have demonstrated that carbohydrase supplementation of broiler and young turkey diets results in far greater responses in nutrient digestibility and performance when the fat source contains more LCSFAs. Additionally, the performance and nutrient digestibility responses are more closely related to changes
in intestinal viscosity in animals fed such fats. Therefore, at a comparable total fat supply, the response to carbohydrase supplementation would increase in the order maize < wheat < wheat/rye, triticale, barley < rye/wheat < rye (Table 9.5) since this is the general order of increasing viscosity. Whilst this order will remain regardless of dietary fat type, the relative differences between grains will be much smaller if fat blends (Table 9.5) or lower dietary fat proportions are used. Under practical conditions, one can expect a fat blend in most cases rather than pure fats in broiler diets, for several reasons. Full-fat seeds (especially soybean) are often used as fat or energy sources for feed-manufacture reasons and to decrease the proportion of ‘free’ supplemented fat. The use of maize or oats will itself increase the dietary plant oil proportion substantially. Thus, the scale of response seen on enzyme supplementation is mediated by total dietary fat concentration, fatty acid profile and triglyceride structure, mineral content and age of the birds (see above sections). The interactions are considered in more detail in the following sections.

Intestinal viscosity

It is now well established that the anti-nutritive effects of soluble NSP in broilers are mediated by an increase in intestinal viscosity. It can be seen from the results presented in Table 9.5 that intestinal viscosity was markedly decreased by carbohydrase supplementation. Significant interactions between dietary fat type and carbohydrase addition were especially observed in rye-fed birds in the experiments by Dänicke et al. (1999c,d) (Fig. 9.3) in that enzyme effects were more pronounced in tallow-fed birds. It was concluded that tallow itself, and other unabsorbed materials, contributed to intestinal viscosity. These results are in contrast to those reported by Smulikowska and Mieczkowska (1996), who found the xylanase-dependent decrease in intestinal viscosity to be more pronounced for birds fed soybean oil than for those fed tallow (Table 9.5). For excreta, however, a substantially higher viscosity was measured in broilers fed unsupplemented diets containing tallow or lard, whereas xylanase supplementation reduced excreta viscosity to comparable levels for all fat types tested that caused the significant interactions between fat type and enzyme addition. It was suggested that more insoluble NSP dissolved in the last part of the digestive tract, due to a decrease in MRT mediated by undigested fat and viscosity that could have contributed to the increased excreta viscosity. It should be borne in mind that the described steep response of viscosity to small incremental changes in polysaccharide concentration above a viscosity of 10–20 mPa s might cause a remarkable variation in viscosity even in the same experimental groups.

Digestibility of fat and fatty acids

Carbohydrase supplementation of broiler diets based on rye, rye/wheat, triticale or barley resulted in larger improvements in fat digestibility if the dietary fat was of
animal origin compared with plant. In simple terms, it could be concluded that there is more scope for improvement in fat digestibility when it is low to begin with. This interpretation is probably the reverse of how such data should be viewed. An increase in intestinal viscosity depresses fat digestibility more in animal fat-based diets, due to the mechanisms explained above. Consequently, a reduction in viscosity due to enzyme addition in such diets should exert a more pronounced effect. Digestibility of different dietary fat types clearly demonstrated what was expected, i.e. a relatively high digestibility of soybean oil, which was less drastically modified over a wide range of dietary soluble NSP concentrations, and consequently intestinal viscosity, than beef tallow. Intermediate effects are observed for lard and animal and vegetable fat blends. That enzyme effect on fat digestibility strongly depends on dietary pentosan concentration was clearly demonstrated in dose–response studies by Smulikowska and Mieczkowska (1996) where significant interactions described by greater enzyme responses with higher rye proportions were observed (Fig. 9.4). Moreover, Dänicke et al. (1999c) showed that this relationship is further modified by dietary fat type.

The use of beef tallow in place of of soybean oil resulted on the one hand in a greater reduction in fat digestibility with increasing proportions of rye in the diet, and consequently on the other hand in greater enzyme effects in such diets (Fig. 9.5) – hence the significant interactions between fat type and rye proportion, and between rye proportion and enzyme supplementation. However, in evaluation of such effects it should be stressed that the effects of enzyme on fat digestibility decrease as birds age (Fig. 9.6). That such age-related effects were not observed in the experiments by Dänicke et al. (1997b) (Table 9.5) might be explained by the restricted feeding

**Fig. 9.4.** Effect of dietary rye proportion and xylanase addition (1.5 g Avizyme Tx per kg of each diet) on apparent fat digestibility and metabolizability of energy in broilers (each diet contained 4.25% soybean oil and 4.25% lard; data from Smulikowska and Mieczkowska, 1996). (Apparent fat digestibility, –––; metabolizability of energy, -----; without xylanase, ●; with xylanase, ■.)
regimen applied in these tests, which might modify the relationship between feed supply, body weight, endogenous fat loss and mean retention time.

The differences in crude fat digestibility can be explained by differences in digestibility of the individual fatty acids (Table 9.6, Figs 9.7 and 9.8). Unsaturated fatty acids were more efficiently absorbed than saturated. The digestibility of saturated fatty acids decreased with increasing chain length. Within the C18 family, digestibility increased with increasing number of double bonds. These general

![Graph](image1)

**Fig. 9.5.** Effect of dietary proportion of rye, dietary fat type and xylanase supplementation on apparent crude fat digestibility at the terminal ileum (from Dänicke et al., 1999c). Without xylanase, ○; with xylanase, □.

![Graph](image2)

**Fig. 9.6.** Effect of age and β-glucanase addition on fat digestibility in broilers fed barley-based diets (60% and 70% barley and 5% and 6% tallow inclusion in starter and grower diets, respectively; data from Salih et al., 1991). Without β-glucanase, ●; with β-glucanase, ■.
### Table 9.6. Effect of dietary fat type and carbohydrase supplementation on faecal digestibility of fatty acids in broilers and turkeys.

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**a-c**Values with no common superscript are significantly different within columns (P < 0.05).

Probability: * P < 0.05; *** P < 0.001; NS, not significant.
relationships were shown to be influenced by dietary rye proportion and dietary fat type, as shown in Figs 9.7 and 9.8. However, the relative decrease in digestibility of saturated fatty acids as rye replaced wheat was greater for the tallow-supplemented rations. Replacing wheat with rye reduced digestibility of palmitic and stearic acid by approximately 24% and 48%, respectively, in birds fed soybean oil, whilst for
tallow-fed birds this increased to 44% and 59%, respectively. In contrast to tallow-fed birds, digestibility of unsaturated fatty acids in those fed soybean oil was less affected by intestinal viscosity. In all cases, increased viscosity reduced saturated fatty acid digestibility more dramatically compared with unsaturated fatty acids, regardless of source. However, the original source of the fatty acids had a marked influence on the absolute response in fatty acid digestibility to increased intestinal viscosity. Increased viscosity reduced digestibility of a given fatty acid to a greater extent when it was derived from tallow than when it was derived from soybean oil.

Significant interactions between fat type and xylanase supplementation are caused by higher enzyme effects in tallow-fed birds and significant interactions between rye level and enzyme supplementation reflect increasing xylanase effects with increasing rye proportions, especially in tallow-fed birds.

The effects of a xylanase addition to a turkey diet containing coconut oil (Table 9.6) have to be evaluated in a different way. Whereas both soybean oil and beef tallow are composed of fatty acids with a chain length of 16–18 C-atoms, more than 50% of coconut oil is saturated fatty acids with fewer than 14 C-atoms. These shorter fatty acids are less dependent on bile salts and micelle formation than their longer-chained counterparts, which is clearly indicated by greater absorption of lauric acid in poults fed coconut oil (Table 9.6). The digestibility of palmitic and stearic acid from coconut oil lies between that of soybean oil and tallow fed to poults. There were virtually no differences in the digestibility of unsaturated fatty acids from turkeys fed tallow or coconut oil. Such results suggest a relative deficiency of bile salts in tallow-fed poults, whereas sufficient bile salts were available in turkeys fed coconut oil to aid in emulsification and absorption of greater amounts of palmitic and stearic acid.

With respect to a species comparison, it should be noted that the young turkey seemed to be more sensitive to dietary treatments than the broiler. Comparable digestibility values were observed between species but the diets differed in that the turkeys received diets containing less pentosans and less supplemented fat.

There were also differences in main sites of absorption observed between saturated and unsaturated fatty acids. The latter disappeared to a greater extent from the proximal segments of the small intestine, whereas the former were digested and absorbed in the more distal segments (Fig. 9.8).

**Digestibility of protein and amino acids**

The effect of intestinal viscosity on protein and amino acid digestibility is generally less dramatic than on digestibility of fat and fatty acids (Table 9.5). Digestibility of crude protein and that of some amino acids at the terminal ileum was decreased as dietary pentosan content was increased and significantly improved by xylanase addition (Fig. 9.9). No fat effect or interactions were detected at this site. In contrast, measurements made over the whole gastrointestinal tract showed significantly lower protein and amino acid digestibility values for tallow-fed birds, and significant greater enzyme effects in tallow-containing diets, especially in diets with higher pentosan concentrations (Fig. 9.9). Langhout *et al.* (1997) reported the
improvement in faecal digestibility of amino acids after xylanase addition to a wheat/rye-containing diet to be greater in birds fed blended animal fat compared with those fed soybean oil. The improvements for the individual amino acids varied between $-0.5\%$ and $1.5\%$ in the soya oil group and $0.9\%$ and $4.3\%$ in the blended animal fat group. Moreover, Rotter et al. (1990) combined a highly viscous barley with either 4% maize oil or 4% tallow and tested these diets without or with an enzyme addition. A significant enzyme-related improvement was found but there was no effect of dietary fat type. Smulikowska and Mieczkowska (1996) found that protein digestibility did not differ significantly between diets containing either 6% tallow or 6% soybean oil in the presence of 30% rye. Xylanase addition improved digestibility only slightly. No interactions were observed in that study. Furthermore, Großer and Jeroch (1997) found a xylanase-related increase in apparent faecal protein digestibility but failed to demonstrate fat effects in turkeys fed either soybean oil or beef tallow containing wheat-based diets.

**Energy metabolism**

Increased intestinal viscosity results in an increase in the absolute and relative weight of the gastrointestinal tract, especially of the small intestine. Increasing the relative proportion of gut and liver in rats results in a simultaneous decrease in utilization of ME for energy retention and an increase in energy maintenance requirement ($\text{ME}_\text{m}$) (Ferrell and Koong, 1986). Furthermore, Pekas and Wray (1991) detected a high correlation between fasting heat production and several gut measurement variables in pigs, such as mass and length but also gastrointestinal fill. In contrast, only weak or no correlations at all were observed between heat production and empty body mass.
or carcass. These results would suggest that changes in the proportions of the gut and gut fill in relation to liveweight due to an increased intestinal viscosity should also affect heat production or energy metabolism. Simon (1998) calculated that a 30% increase in intestines as a proportion of body weight would increase the heat production of a broiler of 1 kg by approximately 5%. Jørgensen et al. (1996) reported an increase in heat production as a proportion of ME in broilers after feeding of pea fibre containing high amounts of soluble NSP, whereas feeding of wheat or oat bran did not increase heat production. Addition of xylanase to rye-based broiler diets was shown to increase the caloric efficiency (Table 9.7), i.e. the proportion between net energy and ME, the partial efficiency of ME for net energy above ME<sub>m</sub> (k<sub>pf</sub>) but also ME<sub>m</sub> itself. Caloric efficiency includes both maintenance heat and heat of energy accretion, thus the proportion between heat production and ME was decreased by xylanase supplementation. An additional effect of dietary fat was mostly obvious for metabolizability of energy, which was probably caused by differences in fat digestibility.

Generally, improved energy utilization might be expected following carbohydrase addition as a result of an improvement in both metabolizability of energy (fat digestion) and an increased metabolic utilization of absorbed nutrients (increased caloric efficiency).

Nitrogen-corrected apparent metabolizable energy (AME<sub>N</sub>) content as a measure of the overall nutrient effect on energy metabolism decreased with increasing dietary pentosan content. This measure was significantly improved by addition of xylanase and was lower in tallow-fed birds (Dänicke et al., 1999f). Again, xylanase effects were found to be greater for tallow-fed birds and at higher pentosan concentrations.

**Protein metabolism**

Expected effects on protein metabolism may also be expected since increases in intestinal viscosity result in an altered pancreatic function and an increased gut

---

Table 9.7. Effects of dietary fat type (S, 10% soybean oil; T, 10% beef tallow) and enzyme supplementation (−, without, +, with 1 g Avizyme 1300 kg<sup>−1</sup> diet) in a rye-based diet (56% dietary inclusion) on energy metabolism in male broilers (Dänicke et al., 1999f).

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<th>Metabolizability of gross energy (kJ kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
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<th>Caloric efficiency (kJ kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Fat retention (% of WG)</th>
<th>Fat (NE&lt;sub&gt;f&lt;/sub&gt;) (kJ kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Protein (NE&lt;sub&gt;p&lt;/sub&gt;) (kJ kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Total (NE&lt;sub&gt;pf&lt;/sub&gt;) (kJ kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>ME&lt;sub&gt;m&lt;/sub&gt; (kJ day&lt;sup&gt;−1&lt;/sup&gt; W&lt;sup&gt;−0.75&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fat type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S –</td>
<td>0.643&lt;sup&gt;a&lt;/sup&gt;</td>
<td>299&lt;sup&gt;a&lt;/sup&gt;</td>
<td>242&lt;sup&gt;a&lt;/sup&gt;</td>
<td>540&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.421&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.665&lt;sup&gt;a&lt;/sup&gt;</td>
<td>468</td>
</tr>
<tr>
<td>S +</td>
<td>0.706&lt;sup&gt;b&lt;/sup&gt;</td>
<td>315&lt;sup&gt;b&lt;/sup&gt;</td>
<td>295&lt;sup&gt;c&lt;/sup&gt;</td>
<td>611&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.449&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.729&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>521</td>
</tr>
<tr>
<td>T –</td>
<td>0.606&lt;sup&gt;a&lt;/sup&gt;</td>
<td>301&lt;sup&gt;a&lt;/sup&gt;</td>
<td>286&lt;sup&gt;b&lt;/sup&gt;</td>
<td>588&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.426&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.662&lt;sup&gt;a&lt;/sup&gt;</td>
<td>482</td>
</tr>
<tr>
<td>T +</td>
<td>0.656&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>354&lt;sup&gt;b&lt;/sup&gt;</td>
<td>340&lt;sup&gt;c&lt;/sup&gt;</td>
<td>694&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.462&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>13.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.886&lt;sup&gt;c&lt;/sup&gt;</td>
<td>720</td>
</tr>
</tbody>
</table>

<sup>a–c</sup>Values with different superscripts within the columns are significantly different (P < 0.05).
weight and consequently an increased contribution of gut protein mass to the overall protein mass of the bird. It was shown by Dänicke et al. (2000a) that tissue fractional protein synthesis, i.e. the daily protein synthesis expressed as percentage of total tissue protein, is significantly greater in small intestinal tissues and in the pancreas of birds fed an unsupplemented tallow-containing rye-based diet (Table 9.3) compared with their soybean oil-fed counterparts. Xylanase supplementation reduced intestinal and pancreatic weights and also the fractional protein synthesis rates of these tissues to a level comparable to birds fed the soybean oil diets. Increased intestinal protein synthesis might be related to increased intestinal endogenous N-losses, since it was demonstrated by Dänicke et al. (2000b) that endogenous N-losses, as measured by an isotope dilution technique, were threefold greater in birds fed the tallow-containing rye-based diet (Table 9.3). Moreover, Langhout et al. (1999) measured an increased number of goblet cells per 100 villus cells when high or low methylated pectin was added to a maize-based broiler diet, which could result in an increased secretion of mucin.

The lack of an enzyme effect on intestinal parameters in soybean oil-fed birds would suggest that soybean oil may have exerted a protective effect on the intestinal villi and cell proliferation rate, since intestinal viscosity is also reduced by enzyme supplementation in these birds.

Protein synthesis in the pancreas is closely associated with synthesis of secretory proteins, i.e. hydrolases such as lipase, protease and amylase. Therefore, it might be concluded that increased pancreatic protein synthesis rate in birds fed the unsupplemented tallow-containing diet might have been related to increased digestive enzyme secretion. Further support for involvement of both intestinal and pancreatic contributions to digestive endogenous N-losses comes from Dänicke et al. (2000b), who reported that the $^{15}$N-enrichment of digesta and faeces of broilers was equally as a result of $^{15}$N-enrichments of pancreas and intestinal tissues of $^{15}$N-labelled animals.

Performance and carcass characteristics

Performance is the most important criterion from a practical point of view. It correlates more or less with fat digestibility but reflects also the described interactions between fat type and enzyme supplementation. In most experiments, liveweight gain and feed-to-gain ratio were improved to a greater extent by carbohydrase addition in broilers fed diets containing animal-originated fats (Table 9.5, Fig. 9.10). Although the interactions were not always significant they should not be neglected, since the tendency is obvious in most cases. As already shown for fat digestibility, carbohydrase effects depend on dietary concentration of soluble NSP (Fig. 9.5). Enzyme effects can be expected to be greater in birds fed animal fat and at higher concentrations of dietary soluble NSP. Total mortality (including losses by culling) depends also on fat type, carbohydrase addition and rye proportion (Dänicke et al., 1999d). Mortality increased significantly with the introduction of rye into the diets and was much greater in groups fed the unsupplemented tallow-containing diets, where mortality
of 23–26% was observed. The viscosity causing stickiness of excreta was further amplified in tallow-fed birds due to large amounts of undigested fat in the litter and around the cloaca of the birds. Their feathers appeared dirty and wet, which undoubtedly increases pathogenic microbial stress.

![Graph showing interactions between rye percentage, fat type, and xylanase supplementation for cumulative feed to gain ratio of male broilers.](image)

**Fig. 9.10.** Interactions between rye percentage (pentosan level), fat type and xylanase supplementation for cumulative feed to gain ratio of male broilers. Day 1–35 of age: without xylanase, □; with xylanase, ○. (From Dänicke et al., 1999d.)

![Graph showing interactions between rye percentage, fat type, and xylanase supplementation for abdominal plus visceral fat of male broilers.](image)

**Fig. 9.11.** Interactions between rye percentage (pentosan level), fat type and xylanase supplementation for abdominal plus visceral fat of male broilers. Day 35 of age: without xylanase, □; with xylanase, ○. (From Dänicke et al., 1999d.)
It should be stressed that interactions between fat type and carbohydrase supplementation were detected both in semi-purified diets and in practical diets.

These quantitative changes in performance as a result of enzyme and fat interactions are paralleled by changes in carcass characteristics and liver lipid composition. Increased fat digestibility was shown to increase broiler fatness (Fig. 9.11), which is closely related to energy availability and energy metabolism and affects skin colour, since the major pigments are fat soluble. It can be seen from the relationships shown in Fig. 9.12 that increasing fatness, as a result of either xylanase supplementation or reduced rye levels, is associated with a more yellow skin colour (increasing b-values), more light (increasing L-values) and less red (decreasing b-values).

The liver concentrations of the fat-soluble vitamins A and E were also enhanced after enzyme addition as a result of improved fat digestibility (Dänicke et al., 1997b, 1999c). Alterations in liver fatty acid composition were also explained by both dietary fat type and xylanase addition (Dänicke et al., 1999c).

Concluding Remarks

There is a strong body of evidence from a number of sources which indicates that dietary fat type is a factor that modifies the overall effect of carbohydrase supplementation in broilers. Generally, greater enzyme effects can be expected if a fat of animal origin is used rather than one of plant origin. The importance of such interactions increases: (i) with increasing dietary proportions and absolute amounts of an animal fat; (ii) with increasing dietary concentrations of soluble NSP; and

![Fig. 9.12. Relationship between abdominal plus visceral fat and parameters of drumstick skin colour. Minolta L, O; a, D; b, ■; S, soybean oil; T, tallow; −, without xylanase supplementation; +, with xylanase supplementation; 0, 33, 66, 100%, respectively, rye of total dietary cereals. (From Dänicke et al., 1999d.)](image-url)
(iii) in early fattening periods. The detection of such interactions even in practical diets leads to the conclusion that animal fat-supplemented diets that are based on rye, triticale, barley or wheat, or on a mixture thereof, should be supplemented with an appropriate carbohydrase preparation to avoid adverse effects on performance.

References


Introduction

Phytase may well be the miracle enzyme of the 1990s, as soybeans were described as the miracle crop for producing high quality plant protein in the 1940s. Dietary addition of microbial phytase or the inclusion of high phytase ingredients in pig, poultry and fish diets is now well documented to release a large portion of the naturally occurring phytate P, and thus greatly reduce the amount of inorganic P that must be added to meet the animal’s P requirement. The net result is a reduction in P excretion that can range from 20 to 50%.

Microbial phytase was initially used as a tool to reduce P excretion because modern commercial production of pigs and poultry had led to large amounts of manure which, when applied to the land in excess, resulted in a build-up of nutrients in and on the soil. This had a potential for environmental pollution that continues to lead to legislation in many countries that require nutrient management plans for manure from these farms.

It is becoming increasingly clear that the use of adequate amounts of phytase in most pig and poultry diets results in improved availability of Ca, Zn, protein/amino acids (AA) and energy. This is because seeds or products from seeds, which are the major ingredients in pig and poultry diets, contain 60–80% of the P in the form of phytic acid or phytate. Phytate is known to complex with other nutrients. Thus, the unavailable phytate P and nutrients complexed with it cannot be utilized and are excreted.

This chapter will provide a brief review of phytate, phytase, and the effectiveness of microbial phytase in pig, poultry and fish diets for enhancing the utilization of P,
Ca, Zn, amino acids/protein and energy. Factors that influence phytase activity will be discussed.

Phytate

Occurrence and structure of phytate

The major ingredients in commercial pig and poultry diets are seeds (cereal grains) or products from seeds (oilseed meal and grain by-products). A large portion (60–80%) of the P in these ingredients occurs in the form of phytates, the salts of phytic acid (Table 10.1). Phytic acid, myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate, is an essential component of all seeds. Seeds rapidly accumulate phytic acid during the ripening period (Asada et al., 1969). The location of phytate in the seed varies (Pallauf and Rimbach, 1995). In small grains, phytate lies mainly in the bran (aleurone layer, testa and pericarp), and in the case of maize it is found mainly in the germ. Generally, with legume seeds, phytate accumulates in the cotyledon. In soybeans, phytic acid is located in protein bodies distributed throughout the seed (Baker, 1991). Rapseed phytates are more concentrated in seed tissues than soybean phytates, which appear to have no specific site location (Kratzer and Vohra, 1986). Detailed information on the phytic acid content of various foods and feedstuffs can be found in the reviews by Oberleas and Harland (1981), Ingelmann et al. (1993), Eeckhout and De Paepe (1994) and Ravindran et al. (1994, 1995b).

Table 10.1. Phytate phosphorus content and phytase activity of some common feed ingredients.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Phytate P&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Phytate P&lt;sup&gt;a&lt;/sup&gt; (% of total P)</th>
<th>Phytase activity&lt;sup&gt;b&lt;/sup&gt; (units kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereals and by-products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>0.24</td>
<td>72</td>
<td>15</td>
</tr>
<tr>
<td>Wheat</td>
<td>0.27</td>
<td>69</td>
<td>1193</td>
</tr>
<tr>
<td>Sorghum</td>
<td>0.24</td>
<td>66</td>
<td>24</td>
</tr>
<tr>
<td>Barley</td>
<td>0.27</td>
<td>64</td>
<td>582</td>
</tr>
<tr>
<td>Oats</td>
<td>0.29</td>
<td>67</td>
<td>40</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>0.92</td>
<td>71</td>
<td>2957</td>
</tr>
<tr>
<td>Oilseed meals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean meal</td>
<td>0.39</td>
<td>60</td>
<td>8</td>
</tr>
<tr>
<td>Canola meal</td>
<td>0.70</td>
<td>59</td>
<td>16</td>
</tr>
<tr>
<td>Sunflower meal</td>
<td>0.89</td>
<td>77</td>
<td>60</td>
</tr>
<tr>
<td>Groundnut meal</td>
<td>0.48</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>0.84</td>
<td>70</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data adapted from Ravindran (1996) and Ravindran et al. (1994, 1995b).

<sup>b</sup>Data from Eeckhout and De Paepe (1994). One unit is defined as that amount of phytase which liberates inorganic phosphorus from a 5.1 mM Na-phytate solution at a rate of 1 µmol min<sup>−1</sup> at pH 5.5 and 37°C (98.6°F).
Bioavailability of phytate P

The bioavailability of P in cereal grains and products from oilseeds is generally very low for pigs and poultry, because they have limited capability to utilize phytate P (Table 10.2). Bioavailability estimates of P in maize and soybean meal for pigs and poultry range from 10 to 30% (Nelson, 1967; Calvert et al., 1978; Jongbloed and Kemme, 1990; Cromwell, 1992). This low availability of phytate P poses two problems for producers: (i) the need to add inorganic P supplements to diets; and (ii) the excretion of large amounts of P in the manure.

Potential for binding nutrients

The phytic acid molecule has a high P content (28.2%) and chelating potential (Fig. 10.1) to form a wide variety of insoluble salts with di- and trivalent cations at neutral pH (Vohra et al., 1965; Oberleas, 1973; Cheryan, 1980). One mole of phytic acid can bind an average of 3–6 mol of Ca to form insoluble phytates at the pH of the small intestine. Formation of insoluble phytate makes both Ca and P unavailable. Zn, Cu, Co, Mn, Fe and Mg can also be complexed, but Zn and Cu have the strongest binding affinity (Maddaiah et al., 1964; Vohra et al., 1965). This binding potentially renders these minerals unavailable for intestinal absorption. Zinc may be the trace element whose bioavailability is most influenced by phytate (Pallauf and Rimbach, 1995). Phytic acid may have a negative influence on dietary protein and amino acids (O’Dell and de Borland, 1976; Knuckles et al., 1985) and inhibits proteolytic enzymes such as pepsin and trypsin under gastrointestinal conditions.

Table 10.2. Bioavailability of phosphorus for pigs and nonphytate phosphorus for poultry.

<table>
<thead>
<tr>
<th>Feedstuff</th>
<th>Bioavailability of P for pigs</th>
<th>Nonphytate-P for poultry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereal grains</td>
<td>(% of total)</td>
<td>(% of total)</td>
</tr>
<tr>
<td>Maize</td>
<td>12%</td>
<td>28%</td>
</tr>
<tr>
<td>Oats</td>
<td>23%</td>
<td>33%</td>
</tr>
<tr>
<td>Barley</td>
<td>31%</td>
<td>36%</td>
</tr>
<tr>
<td>Triticale</td>
<td>46%</td>
<td>33%</td>
</tr>
<tr>
<td>Wheat</td>
<td>50%</td>
<td>31%</td>
</tr>
<tr>
<td>Maize, high moisture</td>
<td>53%</td>
<td>–</td>
</tr>
<tr>
<td>High protein meals – plant origin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groundnut meal</td>
<td>12%</td>
<td>21%</td>
</tr>
<tr>
<td>Canola meal</td>
<td>21%</td>
<td>26%</td>
</tr>
<tr>
<td>Soybean meal, dehulled</td>
<td>25%</td>
<td>35%</td>
</tr>
<tr>
<td>Soybean meal, 44% protein</td>
<td>35%</td>
<td>40%</td>
</tr>
</tbody>
</table>

aAdapted from Cromwell (1992).
bRelative to the availability of phosphorus in monosodium phosphate, which is given a value of 100.
cNRC (1994) – poultry.
Under acidic conditions, the basic phosphate groups of phytic acid may complex with amino groups such as lysyl, histidyl and arginine (De Rham and Jost, 1979; Fretzdorff et al., 1995). Under neutral conditions, the carboxyl groups of some amino acids may bind to phytate through a divalent or trivalent mineral. Phytate–protein or phytate–mineral–protein complexes may reduce the utilization of protein.

Starch is also known to be complexed by phytate. The in vitro hydrolysis of either wheat or bean starch incubated with human saliva was retarded when Na phytate was included in the mixture, but digestion was restored when Ca was added with the Na phytate (Yoon et al., 1983; Thompson, 1986; Thompson et al., 1987). As will be reviewed later, Ravindran (1999) reported studies with broilers that clearly show that apparent metabolizable energy is improved when high-phytate diets are supplemented with microbial phytase.

**Phytases**

Phytases are known to occur widely in microorganisms, plants and certain animal tissues (Nayini and Markakis, 1986; Nys et al., 1996). Phytase of microbial origin (3-phytase, EC 3.1.3.8) hydrolyses the phosphate group at the C₃ position first, whereas phytase of plant origin (6-phytase, EC 3.1.3.26) acts first at the C₆ position.
Phytase produced by *Aspergillus* has two pH optima: one at pH 2.5 and the other at pH 5.5. Wheat phytase has only one pH optimum: at 5.2. *Aspergillus* phytase has been shown to be more effective per unit of activity than wheat phytase, probably due to these differences (Eeckhout and De Paepe, 1996). Phytase activity is generally defined as follows: one unit of phytase activity is the amount of enzyme that liberates 1 µmol of inorganic phosphorus in 1 min from 5.1 mmol solution of sodium phytate at 37°C and pH 5.5. At least three abbreviations are used in the literature for phytase activity: FTU, PU and U. The latter (U kg⁻¹) will be used in this chapter unless otherwise noted.

Four possible sources of phytase could breakdown phytate within the digestive tract of pigs and poultry: (i) intestinal phytase in digestive secretions; (ii) endogenous phytase present in some feed ingredients; (iii) phytase originating from resident bacteria; or (iv) phytase produced by exogenous microorganisms (Nys *et al*., 1996). Contents of the stomach and intestine of pigs (Jongbloed *et al*., 1992; Yi and Kornegay, 1996) and crop, stomach and small intestine of chickens (Liebert *et al*., 1993) have negligible phytase activity. Although endogenous phytase activity of intestinal mucosa has been reported for pigs (Pointillart, 1993) and poultry (Maenz and Classen, 1998), its contribution toward dietary phytate P digestibility is not known. A higher total of small intestinal brush-border phytase activity was reported for 50-week-old hens compared with 4-week-old broilers, but the specific activity per unit of brush-border tissue was similar for broilers and mature hens. Phytase activity has been detected in some animal tissue (McCann and Hart, 1908; Bitar and Reinhold, 1972; Pointillart *et al*., 1985; Yang *et al*., 1991); however, this activity, if present, is negligible for improving the availability of phytate P in non-ruminant animals (Pallauf and Rimbach, 1995). Additionally, the significance of phytase produced by resident bacteria in non-ruminants has not been demonstrated and is probably negligible, considering the lack of significant absorption in the large intestines.

### Plant phytase

It has been known for more than 50 years that plant phytase has the ability to hydrolyse phytate (McCance and Widdowson, 1944; Hill and Tyler, 1954) and its effectiveness for improving P digestibility in pigs and poultry has been clearly shown (Nelson, 1967; Newton *et al*., 1983; Bagheri and Gueguen, 1985). Phytase activity has been reported in a wide range of seeds, such as rice, wheat, barley, maize, rye, soybean and oil seeds (Reddy *et al*., 1982; Gibson and Ullah, 1990; Eeckhout and De Paepe, 1994); however, phytase activity of seeds varies greatly among species of plants (Table 10.1). With the exception of wheat, rye and triticale, most dormant seeds contain very low phytase activity. Phytase activity in maize and soybean meal is so low that it is not of practical importance. Barley may or may not contain nutritionally significant levels of phytase activity (Pointillart, 1993). The majority of the phytase activity in wheat, rye and triticale is in the bran. Diets formulated using ingredients having high phytase activity, such as wheat bran, wheat, triticale, rye bran and wheat middlings, promote greater absorption of phytate P (Pointillart, 1991,
1993). Eeckhout and De Paepe (1991) reported that microbial phytase was 74% more efficient in vivo (fed to pigs) than phytase in wheat middlings when added at an equal in vitro activity level (500 U kg$^{-1}$). They showed that genetically modified microbial phytase was active over a wider pH range than wheat middlings phytase. They suggest that microbial phytase may be more active at pH levels present in the stomach than wheat phytase.

Although some feed ingredients contain native phytase activity, steam-pelleting used in the manufacture of many commercial pig and poultry feeds results in significant losses of this intrinsic phytase activity. Because of variation of phytase activity among and within plant species, damaging effects of pelleting during feed manufacturing and the lack of availability of feed ingredients of high phytase activity, the presence of residual phytase activity often may not be considered in diet formulation when feeds are pelleted.

**Microbial phytase**

Microbial phytases are found in numerous bacteria, yeast and fungi (Harland and Morris, 1995) but they have been detected most frequently in media with the *Aspergillus* genus of ascomycetous fungi (Irving and Cosgrove, 1974; Nair and Duvnjak, 1991). Hydrolysis of dietary phytate by extrinsic microbial phytase was probably investigated first by Nelson et al. (1971) who observed improved P utilization by chicks fed maize–soybean meal diets containing preparations of *Aspergillus*. However, the costs of adding the enzyme were very high and there was little environmental pressure to reduce the P excretion until the late 1980s.

Two major factors led to the development of commercial phytase that could be economically used in pig and poultry diets. The first was the advance in biotechnology that led to techniques for genetically modifying fungi; second was mandate that Dutch livestock farmers had to reduce P excretion. Certainly, the fact that the phytase gene had been identified and isolated made possible the genetic modification of *A. niger*. Also, an improvement in fermentation technology was a contributing factor.

**Sources of phytase**

Currently, there are at least four commercially available microbial phytases, two obtained by fermentation of a genetically modified *Aspergillus* (Natuphos® and Novo phytase), and two obtained by extraction of media with *Aspergillus* (Finase™ and Alltech phytase). Patents influence the availability of these in some countries. Most products are available in a powder or granular form and as a liquid. The genetic information for both Natuphos® and Novo phytase originated from *A. ficuum* and was transferred to either *A. niger* or *A. oryzae*.

Research is also underway expressing the *A. niger* phytase gene in seeds from tobacco and rapeseed (Beudeker and Pen, 1995). Zhang et al. (1998a,b) reported
that Phytaseed® (rapeseed genetically modified with the *A. ficuum* phytase gene) and Natuphos® were equally effective in low P pig and broiler diets for improving P utilization. Similar findings were reported for turkeys by Ledoux *et al.* (1998). Other products may be under development. Van Loon *et al.* (1998) reported from short-term pig trials that a heat-stable phytase enzyme, cloned from *A. fumigatus* and overexpressed in *A. niger* (Pasamontes *et al.*, 1997), was more effective in reducing P excretion than a commercially available genetically modified phytase using the *A. ficuum* phytase gene. The *A. fumigatus* phytase was reported to have retained more than 90% of its enzymatic activity after 20 min heating at 90°C.

**Safety of genetically modified phytase**

Genetically modified microbial and plant phytases were fed to pigs and poultry at levels 5–20-fold higher than recommended levels (500 U kg\(^{-1}\)). In a piglet (5 weeks) and a broiler (4 weeks) trial, the performance, P retention coefficients and bone mineralization values continued to increase (at a decreasing rate) to 2500 U kg\(^{-1}\), the highest level fed in these two trials (Zhang *et al.*, 1998a,b). General necropsy and histological examination of liver, kidney and tibial tissues revealed no adverse effects. In trials for both a pig (29 kg initially for 6 weeks) (Harper *et al.*, 1999) and a turkey (4 weeks after 7 days adjustment) (Kornegay *et al.*, 1999), there were no differences in mortality among phosphorus or microbial phytase treatments. It was evident that the addition of 10,000 U phytase kg\(^{-1}\) did not cause any negative effects based on a general necropsy and histological examination of liver, kidney and tibial bones but, rather, continued to provide small positive improvements in performance, bone mineralization and digestibility of P.

**Site of phytase activity**

Results from several post-slaughter and cannulation experiments with pigs have shown that dietary phytase activity, whether from plants (Gueguen *et al.*, 1968; Schulz and Oslage, 1972; Lantzsch *et al.*, 1992; Kemme *et al.*, 1998) or fungal sources (Jongbloed *et al.*, 1992; Mroz *et al.*, 1994; Yi and Kornegay, 1996), was predominantly active in the stomach. In pigs, phytase activity or breakdown of phytate P was generally very low or was not observed in the lower small intestine. Significant amounts of phytate P (40–50% of the total) would be passed into the large intestine, where most of it would be hydrolysed, but little of the hydrolysed P would be absorbed (Jongbloed *et al.*, 1992; Lantzsch *et al.*, 1992; Kemme *et al.*, 1998). The low phytase activity in the small intestine may be due to a much more basic pH (6.5–7.6) of the jejunum and ileum (van der Meulen and Bakker, 1991) that is less favourable for high phytase activity, or due to proteolytic enzymes in the small intestine that may degrade phytase.

Liebert *et al.* (1993) reported in chickens that 69–86% of added microbial phytase activity was detected in the crop and that 31–38% of added phytase activity
was detected in the proventriculus. No phytase activity was detected in the small intestine. The disappearance of phytate P in the crop and proventriculus supports the observation that the crop and proventriculus are the main sites of phytase activity in poultry.

Effectiveness of Microbial Phytase in Poultry and Swine Diets

Supplemental microbial phytase is well known for its effectiveness in improving the availability of P from plant ingredients containing high levels of phytate P. Some reports have also suggested that the availabilities of Ca, Zn, amino acids and, in some cases, energy are also improved when phytase is supplemented in the diet. However, the use of microbial phytase in poultry and swine diets will depend upon a number of factors, including the cost of P and phytase, the overall effectiveness of phytase to release P and other nutrients, and the environmental need to reduce the excretion of P and other elements which translate into disposal costs. The remainder of this chapter will discuss the use of microbial phytase in pig and poultry diets to enhance the utilization of P, Ca, Zn, amino acids and energy so that excretion of these nutrients can be reduced. Factors to consider that can influence the efficiency of microbial phytase in these diets will be discussed.

Effects of phytase on improving the bioavailability of phosphorus

Phosphorus digestibility response equations were generated using 52 pig experiments representing 32 references in a review reported by Kornegay et al. (1998a). Also, 23 poultry experiments representing 13 references were used to generate P retention equations (Kornegay, 1999). Studies that had very high dietary P levels (NRC or above) for the negative control diets were excluded. Because of limited data, no attempt was made to separate and evaluate data by commercial inorganic phosphorus sources. Several equations were generated for the pig and poultry data sets with the upper levels of phytase varying from 600 to 800, 1000, 1200 or 1500 U kg$^{-1}$ of diet. An evaluation was made of estimates of digestibility (or retention) of P calculated using each of these equations. Within the range of 100–600 U kg$^{-1}$ for pigs and 200–800 U kg$^{-1}$ for poultry, similar values were generally obtained for the various equations. It was decided to use the wider range of data, but equations for the upper level at 600 or 800 U kg$^{-1}$ are also given in the figures. Unless otherwise noted, all digestibility or retention coefficients are apparent.

Phosphorus digestibility

A nonlinear response of supplemental phytase on P digestibility for pigs and P retention for poultry was observed (Fig. 10.2). The nonlinear response of phytase on P digestibility is in agreement with a report by Beers (1992), who found an exponential and logistic relationship between the dosage of microbial phytase and apparent P
digestibility, and other reports where multiple levels of phytase were fed (Denbow et al., 1995; Ravindran et al., 1995a; Kornegay and Qian, 1996; Yi et al., 1996a).

Our response curve was remarkably similar to the nonlinear response curve (corrected for the digestibility of the negative controls) of added phytase on pig and poultry. The graphs illustrate the increase of P digestibility and retention with increasing phytase activity.

**Fig. 10.2.** Increase of P digestibility of pigs (top) and poultry (bottom) fed low P, low phytase activity plant-based diets supplemented with microbial phytase.
digestibility of P reported by Dungelhöef and Rodehutscord (1995) for pigs \[ Y = 28.1(1 - e^{-0.0024X}), \quad r^2 = 0.38 \]. For example, in their data set there was a 19.6% unit increase in digestibility of P when 500 U phytase kg\(^{-1}\) was added, and a value of 19.7% was obtained from our pig data set (Table 10.3).

The magnitude of the response per unit of phytase was much greater at the lower phytase levels. For example, in pigs with 400–500 U phytase kg\(^{-1}\) added, there was a 2.17% unit increase in P digestibility; whereas for 900–1000 U kg\(^{-1}\) there was only a 0.58% unit increase.

In poultry, the increase in P retention was 7.8% units for 500 U phytase kg\(^{-1}\), less than one-half that observed for pigs (19.7%). Similar to pigs, the magnitude of the response per unit of phytase was greater at the lower phytase levels. For example, for 400–500 U phytase kg\(^{-1}\) there was a 1.18% unit increase; whereas for 900–1000 U kg\(^{-1}\) there was only a 0.60% unit increase. This relationship of a decrease in the magnitude of the response to phytase as the amount of phytase added increased was previously described in pigs (Kornegay and Qian, 1996; Yi et al., 1996a), broilers (Denbow et al., 1995; Kornegay et al., 1996a) and turkeys (Ravindran et al., 1995a) for growth and bone mineralization.

**Digested phosphorus**

Digested P resulting from microbial phytase supplementation was calculated by multiplying the total P content of the low P diet (negative control) by the increase in P digestibility or P retention resulting from phytase supplementation. The data are shown in Table 10.3 for pigs and Table 10.4 for poultry.

For pigs at a dietary phytase level of 500 U kg\(^{-1}\), 0.075% units (0.75 g) of P were digested. In the pig data set, the average total dietary P level of the low P diet (negative control) was 0.381 ± 0.008% (mean ± SEM) and the Ca level was 0.588 ± 0.020. The average ratio of total Ca to total P was 1.54.

For poultry, 0.037% units (0.37 g) of P were estimated to be digested when 500 U phytase kg\(^{-1}\) was added to the diet. The average total P level of the low P diet (negative control) was 0.478 ± 0.010% (mean ± SEM) and the Ca level was 0.780 ± 0.036%. The average ratio of total Ca to total P was 1.63. Although total P levels fed are higher and retention coefficients are higher for negative control diets for poultry than for pigs, the increase of retention coefficients due to microbial supplementation was lower for poultry than the increase of digestibility coefficients for pigs. As will be discussed later, the percentage utilization of inorganic phosphate appears to be less for poultry than for pigs.

**Excretion of phosphorus**

Based on calculations using the digestibility equation from the pig data set, P excretion can be reduced by 33.2% when 500 U phytase kg\(^{-1}\) is added to a low P diet, compared with a positive control diet (0.481% P) containing 0.1% units more P (Table 10.3). When the P retention equation from the poultry data set was used to make similar estimates, P excretion was reduced by 31.9% when 500 U phytase kg\(^{-1}\)
is added to a low P diet, compared with a positive control diet (0.57% dietary P) which would be 0.1% units higher in P (Table 10.4). Simply lowering the level of dietary P by 0.1% will decrease P excretion by about 8.3% in pigs and 18.4% in poultry. Unless very high levels of P (well above the minimum requirement of the

Table 10.3. Predicted P digestibility, P digested, and percentage reduction in P excretion based on data generated from the pig data set in this study.

<table>
<thead>
<tr>
<th>Supplemental phytase (U kg(^{-1}))</th>
<th>Total P digestibility(^a) (%)</th>
<th>P digestibility(^b) by phytase (%)</th>
<th>P digested(^c) by phytase (%)</th>
<th>Total P excreted(^d) (%)</th>
<th>Decreased P excretion(^e) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>8.3</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>34.2</td>
<td>6.2</td>
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<td>16.3</td>
<td></td>
</tr>
<tr>
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<td>11.0</td>
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<td>22.3</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>40.9</td>
<td>13.0</td>
<td>0.225</td>
<td>24.8</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>42.6</td>
<td>14.7</td>
<td>0.219</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td>350</td>
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<td>16.2</td>
<td>0.213</td>
<td>28.9</td>
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</tr>
<tr>
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<td>0.208</td>
<td>30.6</td>
<td></td>
</tr>
<tr>
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<td>18.7</td>
<td>0.203</td>
<td>32.1</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
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<td>37.1</td>
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</tr>
<tr>
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<tr>
<td>800</td>
<td>51.6</td>
<td>23.6</td>
<td>0.184</td>
<td>38.4</td>
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</tr>
<tr>
<td>850</td>
<td>52.3</td>
<td>24.4</td>
<td>0.182</td>
<td>39.4</td>
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<td>0.179</td>
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<td>54.3</td>
<td>26.4</td>
<td>0.174</td>
<td>41.9</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Generated from the equation given in Fig. 10.2 [54.86(1 - 0.4908e\(^{-0.00263X}\))].

\(^b\)Calculated by subtracting P digestibility of basal diet without phytase from coefficients at each phytase level.

\(^c\)Calculated by multiplying the P digestibility due to phytase by the average P content (0.381% total P) of the basal diet. The equation of this data is \(0.1026(1 - e^{-0.00263X})\), where \(X = \) phytase level.

\(^d\)Calculated by subtracting total P digestibility coefficients from 100 and multiplying the product by the average P content (0.381% total P) of the basal diet.

\(^e\)Based on an inorganic P digested equation \((Y = -0.167 + 0.755X, r^2 = 0.24, \) where \(X = \) % P from inorganic source); 0.0245% P was excreted for the 0.1% unit of added inorganic P above the basal diet making the total P excreted by the positive control diet (0.481% total P) equal to 0.2995% P (0.275 + 0.0245). Decreased P excretion was calculated by subtracting the total P excreted by the phytase supplemented diets from the P excreted by the positive control diet (0.2995%) and then dividing by the P excreted by positive control diet and multiplying by 100. For example, at 500 U phytase kg\(^{-1}\), 33.2% = \([(0.2995 - 0.200)/(0.2995)]*100.
pig) are fed, the amount of P excreted in pig urine will generally be minimal and was not considered in the calculations in this chapter; this is consistent with conclusions reached by Jongbloed (1987).

Table 10.4. Predicted P digestibility, P digested, and percentage reduction in P excretion based on data generated from the poultry data set in this study.

<table>
<thead>
<tr>
<th>Supplemental phytase (U kg⁻¹)</th>
<th>Total P digestibility (%)</th>
<th>P digestibility by phytase (%)</th>
<th>P digested by phytase (%)</th>
<th>Total P excreted (%)</th>
<th>Decreased P excretion (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.219</td>
<td>21.8</td>
</tr>
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<td>0.018</td>
<td>0.211</td>
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</tr>
<tr>
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<td>0.032</td>
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</tr>
<tr>
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<td>7.3</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>0.045</td>
<td>0.184</td>
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</tr>
<tr>
<td>700</td>
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<tr>
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<td>0.180</td>
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</tr>
<tr>
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<td>0.050</td>
<td>0.178</td>
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<td>850</td>
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<td>11.1</td>
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<td>0.175</td>
<td>37.1</td>
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<tr>
<td>900</td>
<td>63.4</td>
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<td>0.172</td>
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<tr>
<td>950</td>
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<td>0.056</td>
<td>0.170</td>
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<tr>
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<td>0.070</td>
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</tr>
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<td>0.073</td>
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</tr>
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<td>0.075</td>
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<tr>
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<td>0.147</td>
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</tr>
<tr>
<td>1500</td>
<td>67.1</td>
<td>13.8</td>
<td>0.081</td>
<td>0.144</td>
<td>45.6</td>
</tr>
</tbody>
</table>

*a* Generated from the equation given in Fig. 10.1 [68.18(1 - 0.2354e⁻⁰.₀₀₁₃₄₅X)].

*b* Calculated by subtracting P digestibility of basal diet without phytase from coefficients at each phytase level.

*c* Calculated by multiplying the P digestibility due to phytase by the average P content (0.478% total P) of the basal diet. The equation of this data (%) = 0.07672(1 - e⁻⁰.₀₀₁₃₄₅X), where X = phytase level.

*d* Calculated by subtracting total P digestibility coefficients from 100 and multiplying the product by the average P content (0.478% total P) of the basal diet.

*e* Based on an inorganic P digested (retained) equation (Y = -0.000936 + 0.484X, r² = 0.69, where X = % P from inorganic source); 0.0516% was excreted for 0.1% added inorganic P above the basal diet making the total P excreted by the positive control diet (0.578% total P) equal to 0.2804% P (0.2288 + 0.0516). Decreased P excretion was calculated by subtracting the total P excreted by the phytase supplemented diets from the P excreted by the positive control diet (0.2804%) and then dividing by the P excreted by positive control diet and multiplying by 100. For example, at 500 U phytase kg⁻¹, 31.9% =[(0.2804 − 0.191)/(0.2804)]*100.
Digested P vs. P equivalency value of phytase

Phosphorus equivalency value, a term used to describe the replacement or substitution value of phytase, is defined as the amount of inorganic P that can be removed by a given amount of added or intrinsic phytase. For direct comparison of equivalency value of phytase for P and digested P, equivalency values must be adjusted by the estimated digestibility of the inorganic P sources that phytase replaces. Kornegay et al. (1998a) estimated that the digestibility of P in several feed-grade P sources was 76.7% for pigs. The retention of P from several feed-grade P sources was estimated to be 46.2% for broilers and turkeys (Kornegay, 1999). Equivalency values (or equations) are usually obtained from non-linear or linear equations generated from body-weight gain, bone mineralization, and sometimes digested P data obtained by feeding multiple levels of P without phytase addition and multiple levels of added phytase to a low P diet; these equations are set equal and solved. This procedure was described in detailed by Denbow et al. (1995).

Using a small data set of four pig studies from Virginia Tech, Radcliffe et al. (1998a) reported an average P equivalency value of 1.0 g inorganic P for 500 U phytase kg\(^{-1}\) based on average daily gain, bone mineralization and P digestibility. Using growing and finishing pig data reported by Shih and Hsu (1997), Veum (1996), O’Quinn et al. (1997) and Harper and Kornegay (1997), non-linear and linear equations were generated for the response to phytase, and the average P equivalency value of 500 U phytase kg\(^{-1}\) was 1.0 g inorganic P.

For example, if the average equivalency value of 1.0 g P is multiplied by 76.7% (0.767 g P digested per 1 g of inorganic P fed), the product, 0.767 g P \((1 \times 0.767)\) is similar to a digested P value of 0.75 g (0.075%) obtained from the pig data set shown in Table 10.3. Remember that equivalency values must be adjusted by the apparent digestibility of the inorganic P source phytase that is being replaced. Jongbloed et al. (1996a) estimated that 500 U phytase kg\(^{-1}\) was equivalent to 0.8 g digestible P, which was equivalent to 1.0 g P from monocalcium phosphate.

Yi and Kornegay (Virginia Polytechnic Institute and State University, 1998, unpublished data) developed and organized equivalency values from several published broiler studies. For broilers, usually from hatch to 3 weeks of age, the average mean P equivalency of 500 U phytase kg\(^{-1}\) was 0.70 g inorganic P based on body-weight (BW) gain and toe ash percentage. In a 3-week starter turkey trial, the average P equivalency of 500 U phytase kg\(^{-1}\) was 0.82 g inorganic P based on BW gain and toe ash. If the 0.70 g P and 0.82 g P are multiplied by 46.2% (0.462 g P retained per 1 g inorganic P), the products are 0.32 g P \((0.7 \times 0.462)\) and 0.38 g P \((0.082 \times 0.462)\). A value of 0.37 g retained P (0.037%) was obtained in the poultry data set for 500 U phytase kg\(^{-1}\) (Table 10.4).

Higher P equivalency values for BW gain and tibia ash percentage were reported by Kornegay et al. (1998a) for growing–finishing broilers: 1.0 and 0.9 g P, respectively, for 400 U phytase kg\(^{-1}\), the highest level of phytase fed in this study. However, the calculated digested P in this study was only slightly more (0.036 vs. 0.032 g P) than the calculated digested P value obtained for 400 U phytase kg\(^{-1}\) from the poultry data set (Table 10.4).
Although studies have reported that the digestibility of inorganic P sources vary, our research results (Kornegay and Radcliffe, 1996; Kornegay et al., 1996b) with young pigs and turkeys suggest little to no difference in the bioavailability of monocalcium/dicalcium, dicalcium/monocalcium and defluorinated phosphate from 'good quality' commercial sources in the United States. Several commercial feed phosphate sources were used in the studies included in our pig and poultry data sets shown in Tables 10.3 and 10.4, but calculations could not be made for the different P sources, because of inadequate data and variation observed.

Based on the similarity of digested P values calculated from P equivalency estimates, and digested P values derived from equations generated in the pig and poultry data sets, the estimates of P excretion calculated from these data sets should be accurate for a range of situations. However, a larger response than observed in these data sets is possible if careful attention is given to ingredient composition and diet formulation (optimal Ca and P levels), and if quality processing procedures are followed, as will be discussed later.

Response of layers to phytase supplementation

In a series of trials reported by Simons et al. (1992), Peter and Jeroch (1993), Simons and Versteegh (1993), Vahl et al. (1993), Van der Klis et al. (1997) and Gordon and Roland (1997), phytase supplementation of a low P diet for layers was very effective as a replacement for inorganic P. A range of P equivalencies, 0.5–1.2 g P as monocalcium phosphate, have been reported for 200–300 U phytase kg\(^{-1}\). Van der Klis et al. (1997) reported that the effect of phytase supplementation (250 and 500 U kg\(^{-1}\)) on ileal P absorption was 12% units greater when added to a low P basal diet containing 3.0% Ca compared with a low P basal diet containing 4.0% Ca. Leske and Coon (1998) reported that phytate P retention was 36.7, 29.0 and 14.8% units greater with phytase supplementation (300 U kg\(^{-1}\) of diet), respectively, for soybean meal, maize and rice bran, but total P retention was only 16.6, 16.1 and 7.1% units greater. As has been reported for pigs, broilers and turkeys, the response of phytase supplementation was greater at the lower levels of total P. Phytase supplementation is very effective at releasing P in layer diets, which can result in reduced dietary P levels and reduced P excretion. The efficiency appears to be greater for layers than for broilers and turkeys. Phytase supplementation of layer diets is also simplified because most diets are fed in a mash form and thus heat stability issues are averted.

Factors influencing phytase response

The dietary level of P influences the response to phytase (Dungelhoef and Rodehutscord, 1995; Kornegay and Qian, 1996; Kornegay, 1996a,b). Results of studies in pigs (Kornegay and Qian, 1996; Yi et al., 1996a), broilers (Denbow et al., 1995; Kornegay et al., 1996a) and turkeys (Ravindran et al., 1995a) clearly demonstrate that the response to microbial phytase is influenced by dietary levels of
available or non-phytate P, and that digested P per unit of phytase decreases as the amount of phytase increases per unit of diet. Extremely high and low levels of dietary P should be avoided, to optimize the response to phytase.

Vitamin D probably influences phytase activity indirectly by increasing Ca absorption, therefore limiting the formation of insoluble Ca-phytates, which are more resistant for phytase hydrolysis (Pointillart, 1993). High levels of Ca or wide Ca : P ratios will reduce the response for phytase (discussed later).

Effects of phytase on calcium bioavailability

**Broilers and turkeys**

Schoner et al. (1991, 1993, 1994) reported improved Ca retention in broilers fed supplemental phytase. In a broiler study designed to measure the effect of phytase on Ca availability, Schoner et al. (1994) reported that 500 U microbial phytase were equivalent to 0.46 g Ca based on BW gain and phalanx ash. Calcium retention and DM digestibility were improved when phytase was added to broiler diets (Kornegay et al., 1996a, 1998c; Yi et al., 1996b). In our study with turkey pouls (Kornegay et al., 1996c), estimates based on BW gain, gain : feed and digested Ca suggest that 500 U phytase are equivalent to 0.87 g Ca. Both P and Ca retention were sensitive to the addition of phytase at two non-phytate (nP) levels and four Ca : tP ratios in turkeys (Qian et al., 1996b), or two vitamin D₃ levels and four Ca : tP ratios in broilers (Qian et al., 1997). Calcium retention, as well as P retention, linearly increased as the amount of supplemented phytase increased, and decreased as the Ca : tP ratios became wider in both broilers and turkeys.

**Pigs**

Radcliffe et al. (1995) reported results of two pig trials conducted to determine the effectiveness of microbial phytase for improving the bioavailability of Ca. Based on this data, Kornegay et al. (1996c) estimated Ca equivalency values of 1.08 and 0.38 g Ca per 500 U of microbial phytase for pigs in Trials 1 and 2, respectively, with an average of 0.73 g Ca. Calcium equivalency estimates for phytase were based on daily gain during weeks 3 and 4, digestible Ca and tenth-rib ash percentages.

The interaction between dietary Ca and microbial phytase were reported by Jongbloed et al. (1996c) in two trials. In both trials, the apparent total tract digestibility of P linearly decreased with increasing Ca level. No significant interaction could be demonstrated between dietary Ca level and microbial phytase. Microbial phytase enhanced not only the apparent total tract digestibility of P, but also the apparent total tract digestibility of Ca. They estimated that an extra 0.8 g of P and between 0.4 and 0.7 g of Ca were absorbed with 500 U supplemental phytase kg⁻¹.

Using primarily cereal phytase, Pointillart (1993) reported that improved P utilization was generally accompanied by improved Ca retention. Simecek et al. (1995) also reported that P as well as Ca digestibility was improved when two forms of microbial phytase were supplemented in a barley–soybean meal diet for growing pigs. In other studies, the apparent absorption of Ca and N (Kornegay and Qian,
1996; Yi et al., 1996a) in pigs was improved when phytase was added to the diet. Eeckhout and De Paepe (1991) reported a highly positive correlation between Ca and P digestibilities and phytase supplementation to a low P pig diet. They suggested that this relationship could be explained by the fact that phytic acid acts as a Ca-binding agent in the proximal small intestine. Hydrolysis of phytate in the stomach as a result of phytase activity results in increased digestibility, not only of P but, indirectly, of Ca.

**Influence of phytase on zinc bioavailability**

Zinc is probably the most vulnerable mineral to phytate complexation. In early research in chicks, swine, rats and other species it was observed that dietary Zn requirement was affected by dietary phytate (Prasad, 1966). Oberleas and Harland (1996) suggest that it is imperative that much of the endogenously secreted minerals needs to be reabsorbed lest perpetual deficiencies result. Oberleas and Harland (1996) reported results that clearly showed that phytate was a significant factor in the development of zinc deficiency. The presence of Ca aggravated the effect of phytate on zinc utilization, probably through an insoluble Ca–phytate–Zn complex that is formed which prevents the absorption of Zn.

**Broilers and turkeys**

The addition of 800 U phytase kg\(^{-1}\) to a diet containing 27 mg Zn kg\(^{-1}\) increased the retention of Zn and decreased Zn excretion of chicks (Thiel and Weigand, 1992). Thiel et al. (1993) reported that the femoral Zn content of chicks fed a diet containing 30 mg Zn kg\(^{-1}\) plus 700 U phytase kg\(^{-1}\) of diet was equal to that of chicks fed a diet containing 39 mg Zn kg\(^{-1}\) without phytase. Using chicks fed a glucose–soybean concentrate diet (13 mg Zn kg\(^{-1}\) diet) with multiple levels of added Zn, phytase or 1,25-dihydroxycholecalciferol (di-OH-D\(_3\)), Biehl et al. (1995) reported that phytase and di-OH-D\(_3\) supplementation both increased growth rate and tibial Zn to a similar extent. Based on Biehl et al. (1995) estimates using tibia Zn, the Zn equivalency of 600 and 1200 U phytase kg\(^{-1}\) was 3.8 and 5.5 mg, respectively. In contrast, Roberson and Edward (1994) did not consistently observe an improvement in Zn absorption or retention in broilers when 600–750 U phytase kg\(^{-1}\) was added to a maize–soybean diet containing 32 mg Zn kg\(^{-1}\) diet.

In our laboratory, day-old male broilers were fed a maize–soybean isolate basal diet containing 20 mg Zn kg\(^{-1}\) alone and supplemented with multiple levels of Zn and phytase for 21 days (Yi et al., 1996d). Non-linear or linear response equations of the effects of Zn and phytase levels were generated for BW gain, feed intake, Zn retention, Zn concentration of toes, tibia and liver and used to calculate an average Zn equivalency of 5.4 mg kg\(^{-1}\) for 600 U phytase kg\(^{-1}\).

**Pigs**

Based on enhanced growth, increased plasma Zn concentration and alkaline phosphatase activity, the bioavailability of Zn for pigs was improved when phytase
was added to a low P (0.3%) and Zn (30 mg kg\(^{-1}\)) maize–soybean meal diet (Lei et al., 1993). Adding microbial phytase to the diets of young pigs significantly improved apparent absorption of Zn and Mg (Pallauf et al., 1992; Nasi and Helander, 1994). Using a low Zn diet (23 mg kg\(^{-1}\)) containing 0.8% Ca and 0.62% P, Adeola et al. (1995) reported that growth rate and the retention of Zn, Cu, Ca and P were increased when 1500 U phytase kg\(^{-1}\) diet was fed. A Zn equivalency for phytase has not been established for pigs.

**Influence of microbial phytase on amino acids and nitrogen bioavailability**

Phytate can bind with protein/amino acid (AA) at low and neutral pH (De Rham and Jost, 1979; Cosgrove, 1980; Anderson, 1985; Thompson, 1986; Fretzdorff et al., 1995). Phytate–protein/AA complexes may occur in foodstuffs in the native state, and may be formed in the upper gastrointestinal tract. Complexing of phytate with proteolytic enzymes may also occur in the upper gastrointestinal tract. These potential phytate–protein complexes thus may reduce the utilization of proteins and amino acids.

If phytate is hydrolysed, then its inhibitory effects are reduced. Most of the early research with microbial phytase was conducted to measure the effects of phytase on P utilization, and results were inconsistent when total tract N digestibility was measured. Amino acid digestibility was rarely evaluated. Kemme (1998) provides a good review in his dissertation.

The opportunity to show improvements in protein/AA utilization is influenced by the dietary level of protein/AA. If the protein/AA retention is maximum, then the potential to show an improvement is greatly reduced. Furthermore, the use of total tract (faecal) digestibility may not be reliable because of the influence of the microbial population in the large intestine. Ileal digestibility is a more appropriate method of evaluating the influence of phytase on protein/AA utilization.

Binding of free amino acids (particularly lysine) by phytate has been demonstrated *in vitro* (Rutherfurd et al., 1997). Approximately 20% of free lysine-HCl was bound following incubation with rice pollards (rich in phytate). Half of that amount was liberated after the addition of phytase. In broilers, Ravindran and Bryden (1997) and Ravindran et al. (1999a) reported that dietary addition of phytic acid as rice pollards reduced ileal digestibility of N and AA (lysine, threonine, isoleucine, leucine, valine, phenylalanine and histidine). These adverse effects of phytic acid were effectively overcome by supplemental phytase.

**Broilers and turkeys**

The apparent N retention of broilers was improved when phytase was added to a 23% crude protein maize–soybean diet (Kornegay et al., 1996b). Yi et al. (1996c), using Large White turkey female pouls fed a maize–soybean meal diet, reported that apparent and true ileal digestibilities of N and AA were generally improved when 750 U phytase kg\(^{-1}\) was added to both 22.5 and 28.0% CP diets that contained
Improvements were only observed for birds fed 22.5% CP that contained 0.60% nP. Ravindran and Bryden (1997) similarly observed that improvements in AA digestibility were higher in low P wheat–sorghum–soybean meal diets.

Using young broilers, Kornegay (1996b) reported that ileal digestibilities of all amino acids was linearly decreased ($P < 0.001$) as the CP level increased (17, 20 and 23%). Ileal digestibilities of all amino acids except methionine and proline were linearly increased ($P < 0.05$ to 0.001) as phytase (250, 500 and 750 U kg$^{-1}$) was added to the 17 and 20% CP diets. In a broiler finishing study, Kornegay et al. (1998b) reported that when dietary protein/AA levels were reduced from 95 to 85% of the NRC (1994) recommendation (1.5–2.0% units of CP), additions of 300–450 U phytase kg$^{-1}$ diet prevented the decrease in performance (slightly lower), breast meat yield, and ileal CP/AA digestibilities observed.

Supplemental phytase (1200 U kg$^{-1}$) was reported by Ravindran et al. (1999b) to improve ileal digestibilities of protein/AA of three cereals (maize, sorghum and wheat), four oilseed meals (soybean meal, canola meal, cottonseed meal and sunflower meal) and two cereal by-products (wheat middlings and rice polishings). The magnitude of the response varied among feedstuffs and individual AA.

Using a low lysine (85% NRC) wheat–sorghum–soybean meal diet with multiple levels of crystalline lysine or microbial phytase and based on BW gain and feed:gain ratios of broilers, Ravindran and Bryden (1998) reported that 500 U phytase kg$^{-1}$ diet was equivalent to 0.7 g lysine kg$^{-1}$ diet. Apparent metabolizable energy was also linearly increased as phytase was added, but not as lysine was added. Ravindran and Bryden (1998) suggest that BW gain and feed efficiency responses to phytase may have resulted from both lysine and energy effects. In a similar study using broilers fed a single level (600 U kg$^{-1}$) of phytase or multiple levels of crystalline lysine added to a low lysine (0.8%) maize–soybean meal diet, Johnston and Southern (1999) reported an average equivalency, based on BW gain and feed efficiency, of 0.23 g lysine kg$^{-1}$ diet for 600 U phytase kg$^{-1}$ diet.

Pigs

Similar findings have been reported in pigs. Officer and Batterham (1992) and Khan and Cole (1993) reported that microbial phytase improved the apparent ileal and total tract digestibility of crude protein by 2.3–12.8% units. Using cannulated pigs in two studies, Mroz et al. (1994) and Kemme (1998) reported that microbial phytase (800 and 900 U kg$^{-1}$, respectively) enhanced the apparent ileal digestibility of N and most amino acids. Added microbial phytase generally enhanced the total tract digestibility of N and retention of N. Li et al. (1998) reported improved DM digestibility and N retention when phytase was added to a maize–soybean meal diet fed to weaned pigs. In a literature review, Jongbloed et al. (1996d) reported in 17 experiments using microbial phytase that the average apparent total tract digestibility of protein was enhanced by 0.85 ± 1.70% units.

Faecal N digestibility, ileal AA and N digestibilities and N retention were determined in a feeding trial and N-balance study, and a cannulation study using finishing pigs fed diets containing: (i) NRC (1998) CP levels (12% CP); (ii) 7.5%
CP reduction (11% CP); (iii) 15.0% CP reduction (10% CP); (iv) diet 3 (10% CP) plus 250 U phytase kg\(^{-1}\); and (v) diet 3 plus 500 U phytase kg\(^{-1}\) (Kornegay et al., 1998a). Apparent digestion coefficients for N and AA increased as the dietary levels of CP increased and they increased as microbial phytase was added to the 10% CP diet. Coefficients were generally equal to or greater than values for pigs fed the 11% CP diet (a 7.5% reduction in CP). Using a conservative reduction of CP of 1% unit (7.1% reduction), and assuming a N excretion (faeces and urine) value of 40% of N intake, N excretion was estimated to be reduced by 7.1% when phytase was added to 10–11% CP pig diets at a level of 500 U kg\(^{-1}\). Therefore, phytase supplementation has the potential to reduce P and N/AA excretion when P and N/AA levels are reduced appropriately.

**Influence of microbial phytase on energy metabolism**

Thompson and Yoon (1984) indicated that, in the native state, phytate could complex with starch. Rajas and Scott (1969) reported that the apparent metabolizable energy (AME) of cottonseed meal and soybean meal for chicks was improved following treatment of the meals with a crude phytase preparation from *Aspergillus ficuum*. Later, Miles and Nelson (1974), using chicks and a similar product, reported improvements in the AME value of cottonseed meal and wheat bran, but not soybean meal, when treated with the phytase preparation.

More recent studies, primarily in Australia, used a genetically modified phytase (reviewed by Ravindran, 1999). Small but significant improvements in AME were observed for broilers fed sorghum–soybean meal-based diets (Farrell et al., 1993; Selle et al., 1999), and sorghum–soybean meal–canola meal–cottonseed meal–wheat middling diets (Selle et al., 1999) when phytase was supplemented to the diets. The AME values of a sorghum–soybean meal-based diet and in diets with 60% rice bran fed to ducks was improved when phytase was added (Martin and Farrell, 1994).

Findings from three very recent studies (Bryden and Ravindran, 1998; Ravindran et al., 1999a,b), which were designed to determine the influence of microbial phytase on protein/AA and energy utilization in poultry, clearly show that supplemental phytase improves the AME value of wheat- and sorghum-based poultry diets (reviewed by Ravindran, 1999). In agreement, Kornegay and Denbow (Virginia Polytechnic Institute and State University, 1998, unpublished data) observed that phytase supplementation (600 U kg\(^{-1}\)) improved AME coefficients in 4-week-old broilers fed maize–soybean meal, maize–wheat middlings–soybean meal, maize–meat meal–soybean meal or wheat–maize–soybean meal diets. It was suggested, based on literature reported by Ravindran (1999), that phytic acid may exert its effects on starch digestion in one or more ways: (i) by binding α-amylase or by chelating Ca\(^{2+}\), which is needed for the normal activity of amylase; and (ii) by binding starch directly through a protein linkage. Of course, hydrolysis of phytate P would release the enzyme or free the starch.
Influence of dietary calcium and calcium : phosphorus ratio on the effectiveness of phytase

The response to a given level of supplemental phytase will be influenced by dietary Ca level and/or Ca : P ratio, dietary P level and dietary phytate level (Lei et al., 1994; Dungelhoef and Rodehutscord, 1995; Kornegay, 1996b). A high molar ratio of Ca to phytate in the diet can lead to the formation of extremely insoluble Ca–phytate complexes under intestinal conditions, making the phytate molecule inaccessible to phytase. The presence of such strong complexes could explain the apparent inactivity of phytase in calcium-rich diets rather than a direct inhibition of the enzyme by Ca²⁺ (Wise, 1983; Bos, 1988). The importance of maintaining a narrow ratio of total Ca to total P (or for that matter available Ca to available P) has recently been demonstrated in broilers (Qian et al., 1997), turkeys (Qian et al., 1996b) and pigs (Qian et al., 1996a; Liu et al., 1998).

In broilers and turkeys, a total Ca : total P ratio of 1.1 : 1 to 1.4 : 1 appeared to be equally effective. Feeding diets with wider ratios reduces performance, P utilization and bone mineralization. In young broilers, using data reported by Qian et al. (1997), a 14.5, 8.5 and 8.3% decrease was calculated for BW gain, P retention and toe ash percentage, respectively, when the Ca : total P ratio was increased from 1.1 : 1 to 2.0 : 0. In young turkeys, using data reported by Qian et al. (1996b), an 8.7, 10.8 and 6.6% decrease was calculated for BW gain, P retention and toe ash percentage, respectively, when Ca : total P ratio was increased from 1.1 : 1 to 2.0 : 1.

A Ca : nP (weight/weight) ratio of 2 : 1 is suggested for poultry with the exception of laying birds (NRC, 1994). A high level of dietary Ca was shown to affect adversely the availability of phytate P; whereas a high level of vitamin D was shown to have a positive effect (Mohammed et al., 1991; Edwards et al., 1992). Schoner et al. (1993) reported in broilers that feeding high levels of Ca with a constant level of P (0.35%) reduced the increase in BW gain, feed intake and P and Ca retention that was observed when phytase was added. From their lowest (0.6%) to highest (0.9%) levels of Ca, the Ca : total P ratio varied from 1.7 : 1 to 2.57 : 1.

In pig studies, a greater response to phytase (better utilization of P) is obtained when the total Ca : total P ratio is maintained between 1 : 1 to 1.1 : 1 (Qian et al., 1996a). For example, in growing–finishing pigs, Liu et al. (1998) reported a 4.5, 8.2 and 9.7% decrease in ADG, P digestibility and metacarpal breaking strength, respectively, when the Ca : total P ratio was increased from 1.0 : 1 to 1.5 : 1. Lei et al. (1994) reported for pigs fed a maize–soybean meal diet with no added inorganic P (0.31% tP) that the ability of phytase to improve phytate–P availability was greatly reduced at 0.92% dietary Ca (Ca : tP ratio was 3.1) compared with the 0.50% dietary Ca (Ca : tP ratio was 1.6 : 1). Dungelhoef and Rodehutscord (1995) also observed from this review of the literature that high Ca levels reduce the absorption of P and the utilization of phytate. Similar findings have also been reported by Jongbloed et al. (1996c,d).

The decrease in the effectiveness of phytase as the Ca : tP ratio becomes wider could be explained as follows: (i) phytate P utilization in maize–soybean diets fed to broilers is influenced by Ca and P levels in the diet (Edwards and Veltmann, 1983;
Ballam et al., 1984); (ii) the extra Ca can bind with phytate to form an insoluble complex that is less accessible to phytase; or (iii) the extra Ca may directly repress phytase activity by competing for the active sites of the enzyme (Wise, 1983; Pointillart et al., 1985). The negative effect of wide Ca : tP ratios is greater at lower levels of supplemental phytase and at lower levels of aP or nP, because less P would be released as a result of reduced phytase activity.

### Phytase and organic acids

The use of organic acids in combination with phytase to enhance the effectiveness of phytase has produced mixed results. It is known that the inclusion of organic acids in the diet will generally lower dietary pH (Kirchgessner and Roth, 1982; Giesting and Easter, 1985; Risley et al., 1992) with a resultant smaller effect on lowering the stomach pH (Risley et al., 1992; Radcliffe et al., 1998b). Also, it is well documented that there are two optimal pH values for maximizing microbial phytase activity – 2.5 and 5.5 (Shieh et al., 1969; Simons et al., 1990) – with the activity about 50% greater at pH 5.5. Organic acids (fumaric and citric) have been reported to promote some improvement in the total tract apparent digestibility of minerals (Hohler and Pallauf, 1993; Ravindran and Kornegay, 1993). Complexes are known to be formed between organic acids and various cations such as minerals, and these may result in increased intestinal absorption of minerals (Ravindran and Kornegay, 1993).

Jongbloed (1987) pointed out that a lower intestinal pH enhanced the solubility of P and phytate, and thus increased P absorption. Jongbloed et al. (1996e) reported two trials (cannulation and grower feeding trial) in which a combination of phytase and lactic acid was fed in trial 1 and a combination of phytase and lactic acid, formic acid and propionic acid was fed in trial 2. In trial 1, lactic acid enhanced the effectiveness of phytase on P digestibility but not in trial 2. In trial 2, only formic acid enhanced the effectiveness of phytase. In two trials, Radcliffe et al. (1998b) reported that the addition of citric acid to weanling pig diets lowered gastric pH and improved pig performance. However, the lowered gastric pH did not enhance the efficacy of microbial phytase. In fact, when high levels of phytase were supplemented to diets containing citric acid, there was a decrease in the amount of phytase activity recovered from the stomach after slaughter (Yi and Kornegay, 1996). This did not affect phytase efficacy, because no citric acid interaction with phytase was observed on growth performance, feed efficiency, dry matter, Ca and P digestibility or bone measurements.

Valencia and Chavez (1997) reported that 1.0% acetic acid addition to a maize–soybean meal diet enhanced the effectiveness of microbial phytase as measured by performance and apparent mineral utilization of young pigs. In a balance trial with young pigs, Li et al. (1998) reported that microbial phytase supplementation of a maize–soybean meal diet improved digestibilities of P, Ca, N retention and dry matter, and only non-significant trends for further improvements were observed when 1.5% citric acid was added to the diet. Boling et al. (1998)
observed only additive effects of dietary addition of 6% citrate and 1450 U phytase kg\(^{-1}\) using bone ash and weight gain of young chickens.

Further research is needed to understand the relationship between diet acidification and phytase effectiveness.

**Effects of age and physiological status**

The effects of age and physiological status of the pig on the efficacy of phytase in rendering phytate P available for absorption have been inconsistent. Kemme *et al.* (1997a) reported that the efficacy of the phytase for improving digestible P decreased in the order of lactating sows, growing–finishing pigs, sows at the end of pregnancy, piglets and sows at mid-pregnancy. Efficacy of phytase was particularly low in sows on day 60 of pregnancy and high in lactating sows, with the growing–finishing pigs intermediate (Kemme *et al.*, 1997b). They suggested that the differences among the categories probably relate to differences in gastric retention time, but also the requirement for digestible P by the specific category has to be taken into account. Harper and Kornegay (1997) reported slightly higher apparent digestibility coefficients for finisher pigs compared with grower pigs in one trial, but the opposite effect was observed in a second trial. Rodehutsccord *et al.* (1998) recently compared the efficacy of microbial phytase fed in combination with a low P semipurified diet in growing pigs of either 16 or 39 kg body weight (BW). Digestibility of P was very similar for both BW groups. From these studies there is no indication that the efficacy of microbial phytase is dependent on BW of young pigs.

**Effect of soaking on phosphorus availability**

As pointed out by Helander (1995) in her dissertation review, the positive effect of soaking on P availability has been reported in many experiments involving different feedstuffs (Irving, 1980). The P in high-moisture maize or grain sorghum is known to be considerably more available than in dry grain (Trotter and Allee, 1979a,b; Boyd *et al.*, 1983; Ross *et al.*, 1983; Jongbloed *et al.*, 1991). Although phytase is present in mature seeds, it appears to have little effect on phytate in dry or dormant seeds (Maga, 1982). Irving (1980) reported that water was necessary for the hydrolysis of phytate.

Kemme and Jongbloed (1993) reported that the efficacy of microbial phytase was enhanced by soaking the diet in water in one experiment, but in a second experiment, soaking had no effect on P digestibility. A pelleted diet with a different composition was used in the second experiment and the soaking time was much shorter; these factors, together, may explain why soaking had no effect on P utilization. A higher incubation temperature may also have increased phytate hydrolysis (Hill and Tyler 1954; Han, 1988). The pH of the diet is also most important. Tossenberger *et al.* (1993) reported that the degradation of sodium phytate was 81%
and 93%, respectively, for 2.5 and 5.0 h of soaking in a pH 5.0 solution. The respective values were only 38% and 47% when the pH was increased to 6.0.

Beneficial effects of soaking on P utilization with whey were observed for a barley–rapeseed meal diet fed to growing pigs (Nasi et al., 1994), whereas no effect of soaking a pelleted barley–soybean meal diet was observed by Nasi and Helander (1994). Liu et al. (1997) reported improved P utilization when a soybean meal-based diet was soaked. A soaking by phytase quadratic interaction was observed; soaking the 250 U kg\(^{-1}\) diet increased P absorption to a level similar to that obtained with the 500 U kg\(^{-1}\) diet fed dry.

Wet feeding of growing–finishing pigs has increased, especially in Europe and has the potential of enhancing phytase effectiveness and improving P digestibility. Reports from the industry are that phytase is more efficient in these feeding systems.

### Effectiveness of Microbial Phytase in Fish Diets

Feed ingredients of plant origin are being used in greater amounts in intensive fish farming. Like pigs and poultry, several species of fish utilize only one-third of the phytate P in plant feedstuffs. The addition of inorganic P is necessary to meet the P needs of the fish for normal growth. It has also been observed that high dietary phytate levels lead to depressed growth, feed intake and protein utilization, which may be a result of phytate complexing with cations in the gastrointestinal tract so that Zn, protein and energy bioavailability are reduced (Spinelli et al., 1983; Richardson et al., 1985, 1986; McClain and Gatlin, 1988).

A number of studies with striped bass (Hughes and Soares, 1998), carp (Schafer et al., 1995), catfish (Jackson et al., 1996; Eya and Lovell, 1997; Li and Robinson, 1997), rainbow trout (Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995; Lamari et al., 1998; Vielma et al., 1998) and Atlantic salmon (Storebakken et al., 1998) have shown that microbial phytase is very effective in enhancing P digestibility and utilization. Generally, only single and usually high levels of phytase have been fed, but multiple levels have been used in a few studies (Schafer et al., 1995; Jackson et al., 1996; Li and Robinson, 1997). Reported improvements in P absorption and retention range from 15 to 45%, and are influenced by phytase level and sources of ingredients. Reduction in P excretion is reported to range from 30 to 88%.

In summary, microbial phytase is very effective in improving P availability of diets based on plant ingredients and can significantly reduce P excretion and accumulation in the water when dietary P levels are reduced. Because of very limited data where multiple levels of phytase were fed, it is not possible to derive a response curve for any measurement so as to determine equivalency value based on good data sets.

### Summary

When pig and poultry diets are formulated using significant amounts of plant-based ingredients that are low in native phytase, microbial phytase supplementation is very
effective for improving the availability of phytate P. Because phytate is known to complex a number of other minerals, amino acids/protein and even starch, their bioavailability is enhanced when phytate is hydrolysed by phytase. Thus, the excretion of P, Ca, Zn and N can be reduced significantly when diets are properly formulated using phytase. The dose–response curve of phytase for improving P utilization is non-linear for pigs and poultry (broilers and turkeys) and the response has been described over a wide range of phytase levels. The dose–response curves for the effects of phytase on Ca, Zn, amino acid/N and energy utilization are not as clearly understood as they are for P.

The dose response of phytase will, however, depend upon: (i) the level of phytase used; (ii) the level of total P in the diet; (iii) the level of phytate P in the diet; (iv) the level of Ca and the Ca : P ratio; (v) the intrinsic level of phytase in foodstuffs; and (vi) processing and pelleting methods. The effects of physiological age and the influence of organic acids are not clearly understood, if they exist.

Microbial supplementation of diets based on plant ingredients for several species of fish is very effective in improving P availability, which leads to reduced P excretion and accumulation in the water.

References


Role of Phytases in Nutrient Digestion


Introduction

Exogenous enzymes have been used extensively to remove anti-nutritional factors from feeds, to increase the digestibility of existing nutrients, and to supplement the activity of the endogenous enzymes of poultry (Classen et al., 1991; Bedford, 1993). Researchers in the 1960s examined the use of exogenous enzymes in ruminants (Burroughs et al., 1960; Rovics and Ely, 1962; Rust et al., 1965), but responses were variable and no effort was made to determine the mode of action of these products. Furthermore, production of exogenous enzymes was expensive at the time and it was not economically feasible to apply these preparations at the concentrations necessary to elicit a positive animal response. Recent reductions in fermentation costs, together with more active and better defined enzyme preparations, have prompted researchers to re-examine the role of exogenous enzymes in ruminant production (Chen et al., 1995; Beauchemin et al., 1997; McAllister et al., 1999). Several studies have attempted to define possible modes of action of these additives (Judkins and Stobart, 1988; Feng et al., 1996; Hristov et al., 1998a,b; Yang et al., 1999). Exogenous enzymes could exert a number of effects, both on the gastrointestinal microflora and on the ruminant animal itself. It is highly probable, therefore, that physiological responses to exogenous enzymes are multifactorial in origin.

This review will summarize production responses to supplementary exogenous enzymes obtained to date in ruminants. Possible mechanisms by which these products may improve nutrient utilization by ruminants will be discussed and suggestions will be made with regard to strategies that may further enhance the efficacy of these products for ruminants.
Sources of Enzymes

Although enzyme products marketed for livestock number in the hundreds, they are derived primarily from only four bacterial (Bacillus subtilis, Lactobacillus acidophilus, L. plantarum and Streptococcus faecium) and three fungal (Aspergillus oryzae, Trichoderma reesei and Saccharomyces cerevisiae) species (Muirhead, 1996). Other fungal species, including Humicola insolens and Thermomyces lanuginosus, are being marketed to a lesser extent. It is unlikely that this list of source organisms will expand substantially, given that no petition to the Food and Drug Administration to add a new organism has been successful (Pendleton, 1996).

Enzymes are naturally occurring biocatalysts produced by living cells to bring about specific biochemical reactions. In the context of feed additives for ruminants, enzymes are employed to catalyse the degradative reactions by which substrates (i.e. feedstuffs) are digested into their chemical components (e.g. simple sugars, amino acids, fatty acids). These are in turn used for cell growth, either by ruminal microorganisms or by the host animal.

Complete digestion of complex feeds such as hay or grain requires literally hundreds of enzymes. Enzyme preparations for ruminants are marketed primarily on the basis of their capacity to degrade plant cell walls and, as such, are often referred to as cellulases or xylanases. However, none of these commercial products is a preparation of single enzymes; secondary enzyme activities such as amylases, proteases or pectinases are invariably present. Degradation of cellulose and hemicellulose alone requires a number of enzymes, and differences in the relative proportions and activities of these individual enzymes impacts the efficacy of cell wall degradation by the marketed products. Even within a single microbial species, the types and activity of enzymes produced can vary widely, depending on the strain selected and the growth substrate and culture conditions employed (Considine and Coughlan, 1989; Gashe, 1992).

The diversity of enzyme activities present in commercially available enzyme preparations is advantageous, in that a wide variety of substrates can be targeted by a single product, but it presents problems in terms of quality control and extrapolation of research findings among different preparations. For ruminants, enzyme products are usually standardized by blending crude enzyme extracts to obtain specified levels of one or two defined enzyme activities, such as xylanase and/or cellulase. These products are not currently standardized for secondary activities. In fact, these activities, which may well be affecting the overall effectiveness of a given product, are seldom even measured.

Measurement of Enzyme Activity

Enzyme activity is assayed by measuring over time either the disappearance of a defined substrate or the generation of a product from the biochemical reaction catalysed by the enzyme. Activities of enzymes for use in the feed industry are most commonly measured using the latter approach, and are expressed as the amount of
product produced per unit time. These measurements must be conducted under conditions closely defined with respect to temperature, pH, ionic strength, substrate concentration and substrate type, as all of these factors can affect the activity of an enzyme (Headon, 1993). For example, the relative ranking of cellulase activity of three enzyme preparations differs depending on the test substrate (cellulose, carboxymethylcellulose (CMC) or β-glucan) selected for analysis (Table 11.1). Furthermore, release of reducing-sugars from CMC or xylan is not directly proportional to enzyme concentration. Consequently, the ratio of enzyme to substrate will affect enzyme activity estimates (Fig. 11.1) (Hristov and McAllister, unpublished data).

Enzyme activity can also be assessed using synthetic substrates, which usually consist of chromophores linked to molecules chemically similar to the natural substrate. Enzyme activity is measured as the release of the dye or chromophore (Biely et al., 1985). These synthetic substrates offer uniformity among assays, but are subject to criticism in that they do not represent the substrate found in intact feeds such as cereal grains or forages. Furthermore, the assays used to assess enzyme activity are not representative of the conditions in the digestive tract, where ultimately the level and persistence of enzyme activity may be most important. For these reasons, measurement of enzyme activity using traditional assay techniques may have little relevance to the potential efficacy of an enzyme as a feed additive for ruminants.

Researchers have attempted to develop biological assays that may be more indicative of the value of a given enzyme preparation for ruminants. These methods usually involve *in vitro* incubation of enzyme and feed with ruminal contents, and measurement of the disappearance of substrates (e.g. cereal grain, straw, hay) representative of those consumed by the animal (Forwood *et al.*, 1990; Varel *et al.*, 1993; Hristov *et al.*, 1996b, 1998a). Alternatively, the amount of gas produced by the mixed culture can be used as an indication of digestion (Iwaasa *et al.*, 1998), which enables rapid screening of different enzyme products and application rates. These procedures may provide valuable information on the extent to which exogenous enzymes complement the digestive activity of ruminal microorganisms. However, extrapolation of information from these procedures to whole-animal

<table>
<thead>
<tr>
<th>Product</th>
<th>% Protein</th>
<th>Cellulose</th>
<th>CMC</th>
<th>β-Glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.5</td>
<td>102.1</td>
<td>737.9</td>
<td>1016.4</td>
</tr>
<tr>
<td>2</td>
<td>21.3</td>
<td>128.1</td>
<td>727.1</td>
<td>1042.1</td>
</tr>
<tr>
<td>3</td>
<td>15.6</td>
<td>225.6</td>
<td>699.7</td>
<td>1638.3</td>
</tr>
</tbody>
</table>

*For all substrates, activity is expressed as nmol reducing-sugar released mg per product min⁻¹, after a 3 h incubation of the substrate with the enzyme product in 0.2 M sodium phosphate (pH 6.0) buffer at 39°C.*

*Microcrystalline cellulose.*

*Carboxymethylcellulose.*
situations is limited by: (i) variations in microbial composition among inocula from different donor animals; (ii) differences in growth of microbial populations in the *in vitro* system versus in the rumen; and (iii) artefactual accumulation of end products that alter enzyme activity. Additionally, these assays do not consider the possible impact of exogenous enzymes on biological parameters such as feed intake, rate of passage or post-ruminal digestion of nutrients.

Because viscosity of intestinal digesta is closely correlated with growth and feed efficiency in poultry, viscosity measurements have been used as a standard for assessing the biological value of exogenous carbohydrases for poultry (Sabatier and Fish, 1996). For ruminants, however, the value of enzymes can presently be assessed only through expensive and time-consuming production experiments with beef or dairy cattle, which makes screening large numbers of products impractical. This lack of an adequate bioassay for assessing the value of exogenous enzymes is perhaps the greatest impediment to the development of more efficacious enzyme products for ruminants.

**Fig. 11.1.** Release of reducing-sugars (RS) from xylan and carboxymethylcellulose (CMC) during a 5 h incubation with three commercially available enzyme preparations. Note that the relationship between reducing-sugar release and enzyme concentration is not linear, indicating that concentration of enzyme selected can affect estimates of activity. (Hristov and McAllister, unpublished.)
Evidence that exogenous enzymes could improve average daily gain and feed efficiency in beef cattle was first recorded in a series of ten feeding trials reported 40 years ago (Burroughs et al., 1960). When given diets of ground ear maize, oat silage, maize silage or lucerne hay treated with an enzyme cocktail containing amylolytic, proteolytic and cellulolytic activities (Agrozyme®, Merck Sharp and Dohme Research Laboratories), cattle gained 6.8–24.0% more weight and exhibited feed efficiencies improved by 6.0–21.2%, relative to cattle fed untreated control diets. In the same year, four different enzyme preparations (Agrozyme®, Zymo-Pabst®, Rhozyme® and Takamine®; Merck and Company, Rahway, New Jersey), given in combination with diethylstilbesterol, were shown to increase gain by cattle fed a maize–lucerne hay diet by an average of 14.0% (Nelson and Damon, 1960).

Further studies confirmed that enzyme supplements could improve average daily gain (ADG) and feed efficiency in cattle fed high silage diets (Rovics and Ely, 1962), but not all responses by feedlot cattle to enzyme supplementation were positive. Leatherwood et al. (1960) added a fungal enzyme to a grain supplement for calves fed a lucerne hay-based diet and found no improvement in ADG or feed efficiency. Two enzyme preparations containing primarily amylase and protease activities also failed to increase ADG by cattle given a diet comprising 80% concentrate and 20% chopped lucerne hay (Clark et al., 1961). In a separate study, Agrozyme® even reduced the ADG of cattle by 20.4% when it was fed with a maize carrier to beef cattle given a maize silage diet (Perry et al., 1960). Similarly, Kercher (1960) found that ADG was reduced when Zymo-Pabst® was fed with a maize carrier to cattle given a diet of steam-rolled barley, lucerne hay and maize silage. Perry et al. (1966) attributed an 18.2% decline in ADG observed in cattle fed Agrozyme® with maize cob diets to a 6.8% reduction in feed intake, because the enzyme had been shown to enhance fibre digestibility in metabolism experiments.

Although these early studies provided valuable information on the potential benefits of enzymes for beef cattle, they did little to address the impact on animal responses of factors such as the composition of the diet, types and levels of enzyme activities present, or the method of enzyme application. More recent studies have been designed specifically to address these issues. Different feed types (Beauchemin et al., 1995, 1997), application levels (Beauchemin and Rode, 1996; Michal et al., 1996; McAllister et al., 1999), enzyme products (Pritchard et al., 1996) and enzyme application methods (Beauchemin et al., 1998; Hristov et al., 1998b; Yang et al., 1999) have been compared under controlled conditions. Application of different levels (0.25–4.0 l t⁻¹) of a mixture of xylanase and cellulase products and cellulase increased ADG of steers fed lucerne hay or timothy hay cubes by 30 and 36%, respectively, but had no effect on ADG when applied to barley silage (Beauchemin et al., 1995). When this same mixture was applied to a 95% grain diet, feed efficiency of cattle fed barley was improved by 11% but performance of cattle fed maize was unaffected (Beauchemin and Rode, 1996). Application of a different mix of fungal
enzyme preparations at rates up to 5.0 1 t⁻¹, however, increased the final weight and ADG of feedlot cattle given diets based on lucerne silage (Michal et al., 1996; Pritchard et al., 1996) or barley silage (McAllister et al., 1999). Treatment of 82.5% maize diets with ‘multiple stabilized enzymes’ increased ADG and feed conversion by feedlot cattle by 10 and 7.5%, respectively (Weichenthal et al., 1996), and similar improvements in feed efficiency have been reported for cattle fed sorghum-based diets treated with amylase (Krause et al., 1989; Boyles et al., 1992).

Dairy cattle

The effect of exogenous enzymes on milk production in dairy cows was first examined in the mid-1990s (Chen et al., 1995; Lewis et al., 1995; Stokes and Zheng, 1995) and recently there has been a flurry of research activity in this area (Luchini et al., 1997; Nussio et al., 1997; Kung et al., 1998; Yang et al., 1998, 1999; Beauchemin et al., 1999). As in studies using beef cattle, production responses by dairy cattle to exogenous enzymes have also been variable. Application of enzyme mixtures on sorghum did not improve milk production by Holstein cows (Chen et al., 1995). Similarly, milk responses to exogenous enzymes depended on the method of application to barley-based diets (Beauchemin et al., 1999). Applying a cellulase/xylanase mixture to diets containing 45–50% concentrate and lucerne silage, lucerne hay or a mixture of lucerne silage, maize silage and lucerne hay also failed to increase milk yield (Luchini et al., 1997; Nussio et al., 1997). In contrast, spraying two similar enzyme preparations on maize silage in a 50% concentrate diet increased milk production by 2.5 kg day⁻¹ without altering milk composition (Kung, 1996). Although it was demonstrated that these enzyme preparations could increase milk production in cows fed lucerne hay/silage-based total mixed rations (Lewis et al., 1995; Stokes and Zheng, 1995; Sanchez et al., 1996), positive responses in milk production were highly dependent on the level of enzyme applied (Sanchez et al., 1996). For example, research at the Lethbridge Research Centre has shown that increasing the amount of enzyme applied to lucerne cubes from 1 g kg⁻¹ to 2 g kg⁻¹ increased milk production from 23.7 kg day⁻¹ (control) to 24.6 kg day⁻¹ and 25.6 kg day⁻¹, respectively (Table 11.2) (Yang et al., 1999). Responses to this enzyme preparation were even more dramatic in early lactation, when treatment of a diet comprising 24% maize silage, 15% lucerne hay and 61% barley-based concentrate increased milk production by 4 kg day⁻¹. The efficacy of this enzyme was apparently dependent upon the method of its application, as spraying the enzyme on to the total mixed ration did not affect milk production, whereas applying it to the concentrate did (4 kg day⁻¹) (Beauchemin et al., 1998).

Lambs

It was shown in the 1960s that feeding a mixture of amylolytic, cellulolytic and proteolytic enzymes (Agrozyme®; 1.5, 3 and 6 g day⁻¹), as well as a potent
A proteolytic enzyme (Ficin™, Merck and Company; 5, 10 and 20 mg day⁻¹) did not alter feed conversion or the ADG of fattening lambs fed ground maize or lucerne hay (Theurer et al., 1963). Recently, McAllister et al. (2000) have also found that fibrolytic enzymes did not increase feed intake or ADG by lambs fed lucerne hay- or barley-based diets.

Learning from animal experiments

The positive effects of exogenous enzymes on growth production both by beef and by dairy cattle have been demonstrated definitively, but the information required to improve the consistency and increase the magnitude of these responses is unfortunately still lacking. Comparisons between experiments are exceedingly difficult, because many enzyme products are poorly defined. Further, several studies have shown that over-application of enzyme is possible, such that increased application costs are not recovered by corresponding improvements in animal performance (Beauchemin et al., 1996; McAllister et al., 1999). Thus, application of one enzyme preparation at a given concentration provides little information with regard to the potential effect on animal performance of a different application level, let alone a different product. Method of application also influences production responses; they have been shown to differ between dry forage, fresh forage and silage (Beauchemin

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>LH</th>
<th>HH</th>
<th>HT</th>
<th>se²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter intake (DMI)</td>
<td>20.4</td>
<td>20.7</td>
<td>20.7</td>
<td>20.8</td>
<td>0.7</td>
</tr>
<tr>
<td>% of BW</td>
<td>3.29</td>
<td>3.39</td>
<td>3.32</td>
<td>3.42</td>
<td>0.14</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>621</td>
<td>623</td>
<td>626</td>
<td>619</td>
<td>3</td>
</tr>
<tr>
<td>Milk yield (kg day⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actual</td>
<td>23.7</td>
<td>24.6</td>
<td>25.6</td>
<td>25.3</td>
<td>0.6</td>
</tr>
<tr>
<td>4% FCM</td>
<td>22.4</td>
<td>22.9</td>
<td>24.6</td>
<td>24.2</td>
<td>0.7</td>
</tr>
<tr>
<td>SCM</td>
<td>22.2</td>
<td>23.2</td>
<td>24.4</td>
<td>24.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Milk composition (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>3.79</td>
<td>3.70</td>
<td>3.78</td>
<td>3.76</td>
<td>0.11</td>
</tr>
<tr>
<td>Protein</td>
<td>3.36</td>
<td>3.41</td>
<td>3.48</td>
<td>3.49</td>
<td>0</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.56</td>
<td>4.61</td>
<td>4.60</td>
<td>4.62</td>
<td>0</td>
</tr>
<tr>
<td>Milk DMI (kg kg⁻¹)</td>
<td>1.2</td>
<td>1.22</td>
<td>1.29</td>
<td>1.25</td>
<td>0.1</td>
</tr>
</tbody>
</table>

²Within a row, means bearing unlike superscripts differ (P < 0.05).
¹LH = low level of enzyme added to cubed lucerne hay; HH = high level of enzyme added to cubed lucerne hay; HT = enzyme added to both cubed lucerne hay and concentrate.
²se = standard error.
³FCM = fat-corrected milk; SCM = solids-corrected milk.
et al., 1995; Feng et al., 1996), and if the enzyme is infused directly into the rumen, or applied to the complete diet or to the concentrate component only (Lewis et al., 1996; Beauchemin et al., 1998; McAllister et al., 1999). It is obvious that many factors may influence enzyme efficacy in ruminants. Therefore, an understanding of the modes of action by which enzymes improve nutrient utilization in ruminants is key to obtaining consistent positive responses to exogenous enzymes over a broad range of diets and animal types.

**Modes of Action**

Upon initial consideration, exogenous enzymes might be expected to alter feed utilization in ruminants either through their effects on the feed prior to consumption, or through their enhancement of digestion in the rumen and/or in the post-ruminal digestive tract (Fig. 11.2). In actuality, all of these possible modes of action are intertwined and enzyme-mediated alteration of the feed prior to consumption likely has ramifications on ruminal and post-ruminal digestion of nutrients. Preconsumptive effects of exogenous enzymes may be as simple as the release of soluble carbohydrate or as complex as the removal of structural barriers (Fig. 11.2A) that limit the microbial digestion of feed in the rumen. Within the rumen, exogenous enzymes could act directly on the feed or could indirectly stimulate digestive activity through synergistic effects on ruminal microorganisms (Fig. 11.2B). Exogenous enzymes may remain active in the lower digestive tract, contributing to the post-ruminal digestion of fibre, or they could indirectly improve nutrient absorption in the lower tract by reducing viscosity of intestinal digesta (Fig. 11.2C). They may also supplement enzyme activity in the faeces, thereby contributing by accelerating decomposition of waste (Fig. 11.2D). Ultimately, the goal of enzyme supplementation is to improve the efficiency of feed utilization in ruminants and reduce waste production. Undoubtedly, the mode of action of exogenous enzymes in ruminants is exceedingly complex and continues to be a major focus of the research presently being conducted with these additives.

**Preconsumption effects**

There is ample evidence that exogenous enzymes can release reducing-sugars from feedstuffs prior to consumption (Beauchemin and Rode, 1996; Hristov et al., 1996a,b). The degree of sugar release depends on both the type of feed and the type of enzyme. For example, only two of 11 enzyme preparations tested released significant amounts of reducing-sugars from barley silage (Hristov et al., 1996a). Moreover, the preparations most effective at releasing reducing-sugars from lucerne hay were not those that released the most reducing-sugars from barley silage. There is evidence that exogenous enzymes may be more effective when applied to dry forage as opposed to wet forage (Feng et al., 1996; Beauchemin et al., 1998). At first this seems improbable, given that the role of water in the hydrolysis of soluble sugars
from complex polymers is a fundamental biochemical principle (Lehninger, 1982). However, feed offered to ruminants is seldom absolutely dry; even feeds that nutritionists would describe as ‘dry’ (e.g. grain, hay) contain 6–10% moisture. Release of soluble sugars from these ‘dry’ feeds suggests that their water content is sufficient to enable hydrolysis.

Release of sugars from feeds arises at least partially from the solubilization of NDF and ADF (Hristov et al., 1996a; Gwayumba and Christensen, 1997). This is consistent with observed increases in the soluble fraction and rate of in situ digestion (Feng et al., 1996; Hristov et al., 1996a; Dong, 1998; Hristov et al., 1998a; Yang et al., 1999). However, most studies have not found that exogenous enzymes improve the extent of in situ or in vitro DM digestion (Feng et al., 1996; Hristov et al., 1996a). These results suggest that enzyme additives only degrade substrates that would be naturally digested by the endogenous enzymes of the rumen microflora. Using scanning electron microscopy, we have observed that high concentrations of fibrolytic enzymes can cause digestive pits in the cell walls of barley straw (Fig. 11.3). However, no similar degradation was observed when enzymes
were applied at the concentration recommended by the manufacturer (McAllister et al., unpublished data).

Although exogenous enzymes do effect release of soluble carbohydrates, the amount liberated represents only a minute portion of the total carbohydrate present in the diet. It is difficult to attribute observed enzyme-associated production responses solely to the generation of soluble carbohydrates prior to consumption, given that comparable increases in yield were not seen when up to 9% of total dietary DM was supplied as molasses (Wing et al., 1988). Additionally, there is ample evidence that through associative effects soluble carbohydrates can actually depress fibre digestion in ruminants (Huhtanen, 1991). Some exogenous enzyme preparations contain soluble carbohydrates, which can affect in vitro gas production (Varel et al., 1993), but it is unlikely that they contribute significant levels of soluble carbohydrate to the feed at practical application levels. Thus, it is most likely that at least a portion of the positive production responses observed to accompany enzyme supplements is due to alterations in ruminal or post-ruminal digestion.

Ruminal effects

Direct hydrolysis

Until recently it was assumed that, upon introduction into the rumen, exogenous enzymes would be rapidly degraded by the array of proteases produced by ruminal
microorganisms (Kung, 1996). Indeed, fungal cellulases incubated with ruminal fluid were rapidly degraded to the extent that after 6 h of incubation, less than 25% of their original activity remained (Kopency et al., 1987; Vandevoorde and Verstraete, 1987). However, experimentation with other enzyme products showed that CMCase activity and xylanase activity remained constant after 6 h of incubation with ruminal fluid (Hristov et al., 1998b). Furthermore, exogenous enzymes increased xylanase and cellulase activity in the rumen (Hristov et al., 1998a,b, 2000) and there is evidence that declining exogenous enzyme activity in ruminal fluid is associated both with inactivation of the enzymes and with their outflow with the fluid phase of ruminal contents (Fig. 11.4) (Hristov et al., 1996b).

The fact that exogenous enzymes remain active in the rumen raises the possibility that they may improve digestion through the direct hydrolysis of ingested feed. Several researchers have shown that exogenous enzymes can enhance fibre (Fig. 11.4). Decline in endo-cellulase and β-glucanase activities in the rumen during a 15 h period following administration of two enzyme products directly into the rumen. Note that decline of both enzyme activities differs between the two products, and that the declines are related to the passage of fluid from the rumen, as measured by the decline in Cobalt-EDTA. RS, reducing-sugars. (Adapted from Hristov et al., 1996b.)
degradation by ruminal microorganisms in vitro (Forwood et al., 1990; Varel et al., 1993; Feng et al., 1996; Hristov et al., 1996a; Dong et al., 1999) and in situ (Lewis et al., 1996). This effect has been confirmed in some (Beauchemin et al., 1999; Yang et al., 1999) but not in all (Firkins et al., 1990; Varel and Kriekemeier, 1994) studies conducted using cattle with ruminal and duodenal cannulae. Although adding exogenous enzymes may increase the activity of xylanases and cellulases in ruminal fluid, enzyme activity in the fluid usually represents less than 30% of the total enzyme activity in the rumen, the remainder being associated with the feed particles (Minato et al., 1966; Brock et al., 1982). For example, applying fibrolytic enzymes to a grass hay diet for sheep prior to consumption increased endo-glucanase activity and xylanase activity in ruminal fluid, but this activity accounted for only 0.5% of the total endo-glucanase activity in the rumen (Table 11.3) (Dong, 1998). Given that exogenous enzymes represent only a small fraction of the ruminal enzyme activity, and that the ruminal microbiota are inherently capable of readily digesting fibre (McAllister et al., 1994), it is difficult to envision how exogenous enzymes would enhance ruminal fibre digestion through direct hydrolysis.

**Synergism with ruminal microorganisms**

Enhancement of fibre digestion in the rumen would seem more feasible if these products are working synergistically with ruminal microbes. Logically, this concept implies that exogenous enzyme preparations contain enzymatic activities that would normally be limiting to digestion of plant cell walls by ruminal microorganisms.

**Table 11.3.** Effect of exogenous enzymes on endoglucanase and xylanase activities in the rumen of sheep fed grass hay. (Adapted from Dong, 1998.)

<table>
<thead>
<tr>
<th></th>
<th>% Total ruminal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
</tr>
<tr>
<td><strong>Activity in liquid phase (units ml⁻¹ h⁻¹)</strong></td>
<td></td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>0.026ₐ</td>
</tr>
<tr>
<td>Xylanase</td>
<td>0.315ₐ</td>
</tr>
<tr>
<td><strong>Activity in particulate phase (units g DM⁻¹ h⁻¹)</strong></td>
<td></td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>3.14</td>
</tr>
<tr>
<td>Xylanase</td>
<td>2.37</td>
</tr>
<tr>
<td><strong>Total activity in liquid phase (units × 10³)</strong></td>
<td></td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>0.14</td>
</tr>
<tr>
<td>Xylanase</td>
<td>1.76</td>
</tr>
<tr>
<td><strong>Total activity in particulate phase (units × 10³)</strong></td>
<td></td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>3.79</td>
</tr>
<tr>
<td>Xylanase</td>
<td>30.5</td>
</tr>
</tbody>
</table>

ₐ,b Within a row, means bearing unlike superscripts differ \( (P < 0.05) \).

Endoglucanase activity was standardized against a commercial enzyme preparation from *P. funiculosum* (EC 3.2.1.4, Sigma Chemical Co., St Louis, Missouri) and xylanase activity was standardized against a commercial xylanase activity from *A. niger* (EC 3.2.18, Sigma Chemical). Incubations were conducted in sodium phosphate buffer (pH 6.5) at 39°C for 30 min.
Limitations to plant cell wall digestion in the rumen could result from insufficient quantities or types of enzyme production by the ruminal microbes, from an inability of degradative enzyme(s) to interact with the target substrates, or from conditions in the rumen not being optimal for enzyme activity (e.g., low ruminal pH). At least 21 different enzymatic activities have been identified as being involved in the hydrolysis of the structural polysaccharides of the plant cell wall, all of which are produced by a normally functioning ruminal microflora (White et al., 1993). Researchers have shown that extracts from *Aspergillus oryzae* can increase the number of ruminal bacteria (Newbold et al., 1992a, b) and can work synergistically with extracts from ruminal microorganisms to enhance release of soluble sugars from hay (Newbold, 1995). The extent of cross-linking by *p*-coumaryl and feruloyl groups to arabinoxylans has been identified as one factor that limits the digestion of plant cell walls (Hatfield, 1993). *A. oryzae* has been shown to produce an esterase capable of breaking the ester bridges formed between ferulic and *p*-coumaric acids and arabinoxylan (Tenkanen et al., 1991); and these activities have been proposed to function synergistically with ruminal microorganisms (Varel et al., 1993). However, many of the ruminal fungi (e.g., *Neocallimastix* spp.: Borneman et al., 1990) and some species of ruminal bacteria (e.g., *Fibrobacter succinogenes*: McDermid et al., 1990; *Butyrivibrio fibrisolvens*: Dalrymple et al., 1996) produce esterases capable of hydrolysing linkages with phenolic acids. The fact that exogenous enzymes usually only increase the rate and not the extent of digestion (Varel et al., 1993; Feng et al., 1996; Hristov et al., 1996a) suggests that the activities contributed by these additives are not novel to the ruminal environment. Recent work in which oligonucleotide 16S rRNA probes are used to study rumen ecology suggests that there may be several species of fibrolytic ruminal bacteria (e.g., clostridia, ruminococci) that have yet to be cultured in the laboratory (Forster and Whitford, 1998). If this is the case, these microorganisms may also be contributing to the diverse array of enzyme activities required for efficient digestion of plant cell walls.

Hydrolysis of cellulose and hemicellulose is accomplished either by free enzymes or by cellulosomal structures comprising multiple enzymes bound non-covalently to form an organized complex (Teeri, 1997). Aerobic fungi, the principal commercial source of exogenous enzymes, hydrolyse plant cell walls by means of free enzymes, whereas hydrolysis of plant cell walls by *Clostridium* spp. and the anaerobic ruminal fungi involves cellulosomal structures (Beguin et al., 1998; Ljungdahl et al., 1998). There is also evidence that the ruminococci may rely on a cellulosome-like multienzyme complex for fibre degradation (Flint et al., 1998; Ohara et al., 1998). Destruction of these multienzyme complexes during the extraction process may explain why enzymes from mixed ruminal microorganisms failed to release as much soluble sugar from hay and straw as extracts from *A. oryzae* (Newbold, 1995).

Evidence has also been presented that cellulosomes may play a role in adhesion of microbial cells to their substrates (Pell and Schofield, 1993; Beguin et al., 1998). The process of adhesion is absolutely essential for efficient digestion of forages and cereal grains in the rumen (McAllister et al., 1994; McAllister and Cheng, 1996). Cellulose-binding domains may be involved in the attachment of ruminal bacteria to cellulose (Pell and Schofield, 1993). Presently it is not known if exogenous enzymes
block microbial adhesion sites on the surface of feeds, or expose additional ones. Administering an aqueous solution of mixed fibrolytic enzymes (3.3% vol/vol) directly into the rumen of cannulated sheep actually lowered DM digestion (McAllister et al., 1999), and similar results were reported when enzymes were infused into beef steers (Lewis et al., 1996). These studies suggest that enzymes are not as efficacious when ruminally infused as when applied directly to the feed. Alternatively, this response may be enzyme dependent, given that administration of other enzymes directly into the rumen did not affect DM digestion (Hristov et al., 2000). Treating lucerne cubes with exogenous enzymes prior to consumption increased bacterial colonization and in situ DM disappearance of forage between 3 h and 24 h of ruminal incubation (Yang et al., 1999). These responses were supported by a concurrent numerical increase in digestibility of fibre in the rumen, and by significantly increased digestibility of fibre in the total gastrointestinal tract (Yang et al., 1999).

Considering the low fibre content of high concentrate diets, it is surprising that fibrolytic enzymes have improved feed digestion (Krause et al., 1998) and performance of cattle fed high cereal grain diets (Beauchemin et al., 1997; Iwaasa et al., 1997). An explanation of this phenomenon may come from comparing the pH optima of the fibrolytic enzymes produced by ruminal microorganisms with the pH optima of exogenous fibrolytic enzymes produced by aerobic fungi. It is well documented that growth of fibrolytic bacteria is inhibited (Russell and Dombrowski, 1980), and that fibre digestion is severely compromised (Hoover et al., 1984) when pH falls below 6.2. Most of the fibrolytic enzymes produced by ruminal microorganisms function optimally at pH values above 6.2 (Greve et al., 1984; Matte and Forsberg, 1992). In contrast, the pH optima of fibrolytic enzymes produced by aerobic fungi typically range from 4.0 to 6.0 (Gashe, 1992; Muzakhar et al., 1998). This point is illustrated by an experiment in which the extent to which Trichoderma longibrachiatum enzymes enhanced gas production was shown to increase as the pH declined from 6.5 to 5.5 (Table 11.4) (Morgavi et al., unpublished data). Further, although a decline in pH from 6.5 to 5.5 reduced DM disappearance from maize silage in mixed ruminal cultures supplemented with T. longibrachiatum enzymes, the negative effect of low pH on DM disappearance was more pronounced in the absence of added enzyme (Table 11.4). Ruminal pH can be below 6.0 for a significant portion of the day in dairy cattle (Nocek, 1998; Yang et al., 1999) and in feedlot cattle (Krause et al., 1998). Under these conditions, exogenous enzymes could make a significant contribution to ruminal fibre digestion. The higher fibre content of barley, as compared with maize, may explain why exogenous enzymes improved feed conversion in finishing cattle fed barley grain but did not affect feed conversion by finishing cattle fed maize (Beauchemin et al., 1997).

Some evidence also suggests that non-enzymatic factors in crude enzyme extracts may work synergistically with ruminal microorganisms. In early experiments with fungal cellulases, boiling the extract for 20 min failed to reduce its effect on cellulose digestion in vitro (Bowden and Church, 1959). In that study, adding valine or proline to the incubation medium enhanced cellulose digestion to the same extent as adding fungal cellulases. A more recent study showed that autoclaved A. oryzae
extract (8% vol/vol) enhanced cell wall degradation in vitro (Varel et al., 1993). The researchers attributed the effect to the presence of soluble substrate in the extract, but concluded that it would not be relevant at the concentration of extract (i.e. 0.067 mg ml\(^{-1}\)) expected at recommended in vivo dosages. The effects of non-enzymatic factors are likely expressed more in vitro than in vivo, because microbial populations have limited adaptive capabilities in vitro, and they are exposed to higher concentrations of the additive due to the absence of flow or dilution. Enzyme extracts often contain preservatives to prolong their shelf life, as well as emulsifying agents (e.g. surfactants) that maintain the enzymes in suspension and facilitate application of the product to the feed. Work in our laboratory has shown that surfactants can alter microbial activity and feed degradation in the rumen (McAllister et al., 2000). Unfortunately, few enzyme manufacturers list the non-enzymatic components of their products and the consequent difficulty in distinguishing between non-enzymatic and enzymatic effects of enzyme products continues to hamper progress toward defining the mode of action of these products.

**Digesta flow**

Enzymes have been shown to enhance (Feng et al., 1996; Dong, 1998) and to exert no effect on (Beauchemin et al., 1999; Yang et al., 1999) the rate of passage of particulate matter from the rumen. Increases in passage rate can be associated with a faster rate of particle size reduction in the rumen and a corresponding increase in feed intake (Mertens et al., 1984). Although exogenous enzymes have improved both intake and digestion in some experiments (Stokes and Zheng, 1995; Sanchez et al., 1996), in other studies enhanced digestion is not associated with an increase in feed consumption or particulate passage rate (Judkins and Stobart, 1988; Krause et al., 1998; Kung et al., 1998; Beauchemin et al., 1999). These studies suggest that some portion of the effects of exogenous enzymes may be post-ruminal.
Post-ruminal effects

Experiments in our laboratory have shown that exogenous enzymes not only heighten fibrolytic activity in the rumen, but also increase fibrolytic activity in the small intestine (Hristov et al., 1998a,b, 2000). This phenomenon was particularly evident with xylanase activity. Supplementary enzymes increased duodenal xylanase activity by 30% (Hristov et al., 1998a). In the same study, enzyme supplementation increased cellulase activity at the small intestine by only 2–5%, because these enzymes were largely inactivated by the low pH and pepsin in the abomasum. Other researchers have reported that xylanases from both mesophilic and thermophilic microorganisms resist proteolysis (Fontes et al., 1995), possibly due to their high degree of glycosylation (Gorbacheva and Rodionova, 1977), but our work has shown that acidity, not pepsin, is the primary factor responsible for the inactivation of xylanases in the abomasum (Hristov et al., 1998a). At pH 2.0–2.6 (Sturkie, 1970) gastric fluids in the gizzard of poultry are typically less acidic than digesta in the stomach of swine or in the abomasum of ruminants, and they have shorter residence times. This difference in gastrointestinal physiology may explain why positive responses to enzyme supplementation are more consistent in poultry than in swine or ruminants (Campbell and Bedford, 1992; Baas and Thacker, 1996).

Administering large quantities of enzymes (400 g day−1) directly into the rumen can significantly increase cellulase activity in the small intestine (Hristov et al., 2000). Exogenous enzymes supplemented in this manner flow mainly with the fluid phase of ruminal contents (Hristov et al., 2000) and at these elevated feeding levels a portion of the cellulases even escapes inactivation by the low pH and pepsin in the abomasum. We have observed that increased xylanase activity in the small intestine is associated with a decline in intestinal viscosity (Table 11.5) (Hristov et al., 1998b, 2000). Because viscosity of duodenal digesta increases with increasing levels of grain in the diet (Mir et al., 1998), enzyme-mediated reductions in viscosity could improve nutrient absorption in the small intestine of cattle fed grain diets. Reduced intestinal viscosity was associated with 1.2% and 1.5% increases in total tract digestibility of DM when enzymes were applied to the feed or infused into the abomasum, respectively (Hristov et al., 1998a). However, intestinal viscosity in cattle is only between 1 and 2 cP (Mir et al., 1998) whereas intestinal viscosity in poultry may exceed 400 cP (Bedford, 1993). Improved growth performance in poultry supplemented with enzymes is often associated with tenfold reductions in intestinal viscosity (Bedford, 1993; Graham, 1996). Consequently, it is difficult to comprehend how the relatively modest declines in intestinal viscosity observed in ruminants supplemented with high levels of enzymes results in a substantial improvement in nutrient absorption in the small intestine.

In studies with dairy cows fed barley grain diets, improvements in total tract digestion were attributed primarily to an improvement in the digestibility of fibre and starch in the lower tract (Beauchemin et al., 1999). Hydrolysis of complex carbohydrates by exogenous enzymes in the small intestine and subsequent absorption of released sugars would offer energetic and nitrogen balance benefits to the animal that would not be accessible if these substrates remained undigested or were fermented by
microbial populations residing in the large intestine. It is possible that exogenous enzymes work synergistically with the microbes even in the large intestine, given that we have determined that xylanase activity in the faeces increases linearly with increasing levels of enzyme supplementation (Table 11.5) (Hristov et al., 2000). This heightened fibrolytic activity may have important ramifications with regard to the rate at which faecal material decomposes in the environment.

Steps toward Improving Exogenous Enzymes for Ruminants

Matching the enzyme to the feed

Not all exogenous enzymes are equally effective at digesting complex substrates such as lucerne and barley grain (Table 11.6). Feedstuffs are exceedingly complex structurally, and lack of knowledge of the factors that limit the rate and extent of feed

<table>
<thead>
<tr>
<th>Table 11.5.</th>
<th>Enzyme activity¹ in duodenal digesta and faeces of cattle with and without enzyme supplementation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td>Control</td>
</tr>
<tr>
<td>Ruminal contents</td>
<td></td>
</tr>
<tr>
<td>CMCase</td>
<td>42.7</td>
</tr>
<tr>
<td>Xylanase</td>
<td>215.2</td>
</tr>
<tr>
<td>Amylase</td>
<td>190.1a</td>
</tr>
<tr>
<td>Viscosity (mPa s)</td>
<td>3.16AB</td>
</tr>
<tr>
<td>Duodenal contents</td>
<td></td>
</tr>
<tr>
<td>CMCase</td>
<td>ND</td>
</tr>
<tr>
<td>Xylanase</td>
<td>3.6a</td>
</tr>
<tr>
<td>Amylase</td>
<td>0.98</td>
</tr>
<tr>
<td>Viscosity (mPa s)</td>
<td>1.73</td>
</tr>
<tr>
<td>Faeces</td>
<td></td>
</tr>
<tr>
<td>CMCase</td>
<td>NM</td>
</tr>
<tr>
<td>Xylanase</td>
<td>NM</td>
</tr>
<tr>
<td>Amylase</td>
<td>NM</td>
</tr>
<tr>
<td>Total tract digestibility (%)</td>
<td>90.2</td>
</tr>
</tbody>
</table>

¹Expressed as nmol reducing-sugars released ml⁻¹ min⁻¹.
²EF, enzyme applied to feed; EA, enzyme infused into abomasum. Adapted from Hristov et al. (1998).
³Enzyme introduced directly into the rumen at rates of 0 (Control) or 100, 200 and 400 g day⁻¹ (EFL, EFM and EFH, respectively). Adapted from Hristov et al. (2000).
⁴Linear effect of enzyme supplementation on enzyme activity (P < 0.001).
ab Within a row and experiment, means bearing unlike superscripts differ (P < 0.05).
AB Within a row and experiment, means bearing unlike superscripts differ (P < 0.10).
NS, not significant (P < 0.05).
digestion impedes the engineering of enzyme preparations designed to overcome constraints to feed digestion. With some feeds, specific targets can be identified. In maize, for example, the protein matrix surrounding the starch granules, as opposed to the properties of the starch itself, dictates the extent and rate of starch digestion in that grain (McAllister et al., 1993). Thus, exogenous enzymes designed to improve the utilization of maize should contain proteases capable of digesting the protein matrix and exposing starch granules to digestion by endogenous ruminal or host enzymes. An exogenous preparation containing amylase but not protease activity would not be expected to substantially improve utilization of maize by ruminants. In straw, the major barriers to microbial digestion are apparently silica, wax and cutin (Bae et al., 1997), whereas in unprocessed grain the pericarp dictates the extent of grain digestion (Wang et al., 1998). Enzyme preparations that digest the structural components limiting the extent of feed digestion in the rumen would be expected to be more efficacious than preparations that just increase the rate of feed degradation in the rumen. Many enzyme preparations are currently in use, with no attempt being made to define the types or activities of the enzymes they contain. Such random employment of enzymes on feeds, without consideration for specific substrate targets, will only discourage or delay adoption of exogenous enzymes for more standard use in the animal production industry. Ultimately, enzyme cocktails should be designed specifically to overcome the constraints limiting digestion of a particular type of feed. Component enzymes in such cocktails might vary even for a given forage, targeting particular maturity levels and structural barriers. Recent developments in biotechnology make it feasible to engineer such enzyme cocktails containing xylanase and β-glucanase activities, but we presently lack the technology for specific production of many other potentially important enzymes (e.g. cutinase, ferulic acid esterase, acetylxyylan esterase, arabinofuranosidase).

Table 11.6. Release of reducing-sugars\(^1\) from lucerne hay and hull-less barley by a number of commercially available fibrolytic enzyme products.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Lucerne hay</th>
<th>Hull-less barley</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>379.0</td>
<td>4980.32</td>
</tr>
<tr>
<td>B</td>
<td>102.8</td>
<td>1384.4</td>
</tr>
<tr>
<td>C</td>
<td>122.3</td>
<td>1785.5</td>
</tr>
<tr>
<td>D</td>
<td>106.4</td>
<td>1201.8</td>
</tr>
<tr>
<td>E</td>
<td>30.2</td>
<td>387.0</td>
</tr>
<tr>
<td>F</td>
<td>31.0</td>
<td>489.5</td>
</tr>
<tr>
<td>G</td>
<td>134.7</td>
<td>1808.7</td>
</tr>
<tr>
<td>H</td>
<td>156.1</td>
<td>2780.3</td>
</tr>
<tr>
<td>I</td>
<td>170.7</td>
<td>2424.9</td>
</tr>
<tr>
<td>J</td>
<td>62.8</td>
<td>2558.3</td>
</tr>
</tbody>
</table>

\(^{1}\) Activity is expressed as p.p.m. of glucose released by the product (present at 0.250 mg ml\(^{-1}\)) under standardized test conditions (Hristov and McAllister, unpublished observations).
Lowering enzyme cost

Once the mechanisms of action and specific targets for degradative exogenous enzymes have been identified, steps can be taken to optimize the application of these preparations. Application concentrations can be minimized by ensuring that the preparations contain the enzyme activities most likely to elicit an improvement in feed utilization. In some instances, the activity of crude enzyme preparations may be increased by including specific enzymes most likely to overcome the constraints to feed digestion. Recently, application of recombinant DNA technology has enabled manufacturers to increase the volume and efficiency of enzyme production, and to create new products (Ward and Conneely, 1993; Hodgson, 1994). Genes encoding superior enzymes can be transferred from organisms such as anaerobic bacteria and fungi, typically impractical for commercial production, into well-characterized industrial microbial production hosts (e.g. *Aspergillus* and *Bacillus* spp.). Expression of genes encoding novel enzymes in plants such as canola and potato may be a particularly effective means of lowering the cost of enzyme production (Pen *et al.*, 1993; van Rooijen and Moloney, 1994). At the Lethbridge Research Centre, a β-glucanase gene from the ruminal bacterium *F. succinogenes* has been expressed in a number of lines of potato, and a tenfold difference in level of expression has been observed among lines (Table 11.7). The line exhibiting the highest expression levels is now being evaluated in diets for poultry. It is possible that similar technologies (e.g. grasses expressing cutinase, esterase) may have applications in ruminant production.

Conclusion

The use of feed enzymes in the monogastric animal production industry has increased dramatically in recent years and numerous commercial products are presently being marketed. In many instances, the mechanisms and modes of action

<table>
<thead>
<tr>
<th>Line</th>
<th>Leaf tissue</th>
<th>Tuber tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.011</td>
<td>0.004</td>
</tr>
<tr>
<td>2</td>
<td>0.016</td>
<td>0.005</td>
</tr>
<tr>
<td>3</td>
<td>0.020</td>
<td>0.011</td>
</tr>
<tr>
<td>4</td>
<td>0.031</td>
<td>0.023</td>
</tr>
<tr>
<td>5</td>
<td>0.067</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>0.105</td>
<td>0.050</td>
</tr>
<tr>
<td>7</td>
<td>0.047</td>
<td>0.021</td>
</tr>
</tbody>
</table>

β-glucanase from *F. succinogenes* was expressed in potato using the cauliflower mosaic virus (CAMV) 35S promoter (Armstrong *et al.*, unpublished observations).

Table 11.7 Accumulation of glucanase in the leaf and tuber tissue of seven transgenic lines of potato (*Solanum tuberosum*).
of these preparations have been defined. In contrast, few commercial preparations of exogenous enzymes exist for ruminants and many of these just now are entering the marketplace. Although positive responses in animal performance have been observed, results have been inconsistent. Characteristics of the ruminant digestive tract (e.g. complex microbial populations producing numerous endogenous enzymes) complicate the elucidation of the mechanisms of exogenous enzyme action in ruminants. There is evidence that exogenous enzymes initiate digestion of feeds prior to consumption and that they can improve feed digestion in the rumen and lower digestive tract. The challenge for researchers is to determine the modes of action, singly or in combination, that enable exogenous enzymes to improve feed efficiency and increase growth and milk production.

References


Enzymes in Ruminant Diets


Enzymes in Ruminant Diets


Introduction

The intestinal microflora are an integral part of the digestive system of all animals. The fact that they are living organisms means that they have nutritional and spatial requirements. This implies that the ability of the digestive system to digest and absorb nutrients is, in part, dependent upon the species distribution and total population of resident microbes. The species distribution determines the range of metabolic processes that can be found and the total population the degree or extent of such processes. The microfloral population is itself very much dependent upon the diet as the ultimate source of substrates for metabolism (Wagner and Thomas, 1987; Savory, 1992). As a result, changes in dietary composition or nutrient density can have dramatic effects on the intestinal microfloral populations (Gibson et al., 1996; Hillman, 1999; Reid and Hillman, 1999), which in turn influence the ability of the animal to digest and absorb nutrients therein. Exogenous enzymes have frequently been shown to improve nutrient digestibility and some authors have identified concomitant changes in the intestinal flora (Hock et al., 1997; Vahjen et al., 1998). Whether such changes are merely an adaptation to the new environment or whether these changes play a large role in the physiological response seen in the host animal on use of exogenous enzymes is the focus of this chapter. Due to the relative paucity of data in pigs, the majority of this chapter will focus on poultry.

Physicochemical Environment of the Intestinal Tract

There are various estimates of the numbers of different strains of bacteria that inhabit the gut of the broiler, but many settle in the range of 400–500. Most strains are unknown, many unculturable and thus knowledge of this ecosystem is at best rudimentary. The conditions in the intestine range from the relatively aerobic environments of the crop and duodenum to progressively anaerobic conditions of the ileum.
and ultimately the caecum and colon where the redox potential is often less than $-400 \text{ mV}$ (J. Apajalahti, unpublished data), equivalent to that found in the rumen. Differences exist in the pH of these environments, either as a result of the digestive activity of the host (e.g. acid secreted in the stomach/proventriculus or neutralization by secretion of sodium bicarbonate in the duodenum) or the microbes themselves (production of volatile fatty acids, amines, etc.). The substrates available for fermentation range from complex plant and animal polymeric compounds such as cellulose, xylan and proteins to the simplest monomeric sugars, amino acids and fatty acids. The conditions existing in the compartment of the intestine where such substrates find themselves limits and dictates the metabolic processes through which they can be utilized. Anaerobic fermentation dominates in the large intestine whilst facultative anaerobes and aerobes are more frequent in younger animals (Naqi et al., 1970) and in the upper reaches of the intestine. Bacterial fermentation may provide resources to the host in terms of vitamins, essential amino acids and fatty acids, energy in the form of volatile fatty acids (VFAs) and many growth factors, which include VFAs and polyamines (Annison et al., 1968; Just et al., 1983; Dierick et al., 1989, 1990; Choct et al., 1996; Noack et al., 1996). On the other hand they can produce toxins and carcinogens, compete for nutrients directly and create disease conditions directly or indirectly (Muramatsu et al., 1994; Choct et al., 1996; Johnston, 1999; Schutte and Langhout, 1999; Titball et al., 1999; Uchida et al., 1999). Ultimately the microfloral population resident in the gut is dependent upon the resources provided (principally derived from the diet and subsequent metabolites, and from the intestine itself – mucin, endogenous losses, etc.) and the initiating flora (which depends upon environmental exposure and species interactions). Since diet, sex, age and housing, which represent a fraction of all the parameters involved in determining the subsequent microfloral ecosystem, vary with each experiment, it is not surprising to find discrepant data in the literature. The microbial population permutations are potentially limitless. It is therefore currently not possible to describe consistently and adequately a given microfloral population on the basis of the diet offered to the host and it is even more difficult to describe its contribution to the nutritional or health status of the host. Whilst some of the effects and capabilities of individual species may be understood in isolation, in a living gut the roles that each species plays and the potential interactions with others, be it beneficial or detrimental to the host, are not clear at all.

Given these constraints, however, it is still clear that performance responses to exogenous enzyme addition are likely mediated to be in part through changes in the microflora species distribution and total population, and therefore it is important that their role should be determined.

**Microbial Responses to Use of Enzymes**

**Enzyme effects in the ileum**

Much of the early work with exogenous enzymes addressed the problematic grains, rye and barley. The response to enzymes targeting these grains was considered to be
due to their ability to increase nutrient digestibility and hence assimilation through depolymerization of the viscous cell wall carbohydrates in these grains (discussed in more detail in Chapter 7). Viscous digesta was blamed for reduced rates of convection and diffusion, which slowed the rate of nutrient extraction from the intestines (White et al., 1981, 1983; Annison and Choc, 1991; Bedford and Classen, 1992; Classen et al., 1995). Enzymatic depolymerization reduced viscosity and improved nutrient extraction rate and the result was more rapid growth. However, even in such early work it was quickly established that the detrimental effects of feeding rye- and barley-based rations could be partially, if not largely, overcome by use of antibiotics (Moran and McGinnis, 1968; MacAuliffe et al., 1976; Korelski and Rys, 1985). More recent work, in which germ-free and conventional birds were fed highly viscous diets, demonstrated that the negative effects of viscosity were virtually absent in the germ-free bird (Schutte and Langhout, 1999). Such observations suggest that viscosity per se is not relevant unless taken in context with the microbial status of the bird. Subsequent work with wheat and more recently maize and sorghum-based diets has identified additional and alternative mechanisms of direct enzyme action (cell wall mechanisms, starch digestion mechanisms) which, again, may only be relevant when considered along with the microfloral status of the host. The proposition of this chapter is that exogenous enzymes in use today (with the notable exception of phytase) improve animal performance largely through interaction with the intestinal microflora. It is proposed that the exogenous cell wall-degrading enzymes have two types of activity that need consideration: removal and provision of substrates for microbial fermentation. The former is more likely to be an effect seen in the small intestine and the latter in both lower small and large intestine. Each activity and its consequences are discussed in detail below.

Ileal phase – substrate removal

The small intestine of monogastrics, particularly chickens, was for a long time thought to be largely devoid of bacteria, and any counts were assumed to be contaminants rather than established flora. It was later established that in fact there was a very rapid colonization of the small intestine of both turkeys and chickens within the first few days post hatch (Lev et al., 1957; Naqi et al., 1970; Vahjen et al., 1998). Subsequent work has established that there is a specific progression from bacteria staining Gram-positive to those staining Gram-negative in the jejunum of turkeys as they age from 1–21 days old. This evolutionary pattern varies, depending upon the source flock and farm on which the birds were raised (Morishita et al., 1992).

The direct source of substrate for much of this flora is the diet of the host, with poorly digested diets leaving far more substrate for microbial fermentation than highly digestible diets. Dramatically different ileal flora are evident when comparing rye with maize-based diets (Wagner and Thomas, 1987), the rye diet being very poorly digested and supporting much larger numbers of anaerobes, and in particular spore-forming anaerobes, than the maize-based diet. Supplementation of the same maize diet with a small percentage of pectin resulted in a dramatic increase in
numbers of anaerobes and in particular the spore formers. This was likely brought about through two properties of pectin that are relevant to this chapter, namely the rheological properties of pectin, which would slow down the rate of digestibility and reduce oxygen tension through reduced digesta mixing, and its role as a substrate for bacterial fermentation. Reducing oxygen tension and provision of extra substrate will evidently favour increases in the numbers of anaerobes, and the fact that the pectin itself is fermentable will select for those strains capable of using the constituent uronic acids.

Such observations are supported by more recent work, where addition of exogenous enzymes reduces viscosity of wheat- and rye-based diets and as a result reduces populations of small-intestinal flora (Choct et al., 1996, 1999; Hock et al., 1997). Presumably, the addition of the enzyme increases the rate at which starch and protein are removed from the small intestine, which effectively leads to less material being available for microbial fermentation.

In many respects, the numerous studies that have reported significant improvements in ileal digestibility of nutrients on enzyme addition (Pettersson et al., 1991; Bruce and Sundstol, 1994; Jondreville et al., 1995; Dänicke et al., 1997b; Kemme et al., 1999; Ravindran et al., 1999; Wyatt et al., 1999) have underestimated the significance of their results. Whilst extra nutrients captured by the terminal ileum do indeed contribute to a greater extent to the productive energy of the animal than those captured in the large intestine, the effect of such a response on the small-intestinal microflora is ignored by most. For example, if starch digestibility is 80% at the terminal ileum, then there is 20% remaining which has the potential to feed the microflora in the large intestine. If addition of an enzyme accelerates the rate of digestion of the starch such that 85% has been digested by the terminal ileum, then the classic response of a nutritionist would be that the animal has extracted 5% more starch and as such will gain more productive energy from the diet. The microbiologist will recognize that there is now only 75% of the fermentable starch of the control diet entering the large intestine. Moreover, that proportion of the starch that has been removed by enzyme addition was likely the most easily degradable of the residual starch. With less substrate available to support starch fermenters in the lower small intestine, populations fall (Fig. 12.1). Similar conditions will apply for protein digestibility and protein utilizers.

Highly indigestible diets will support greater intestinal microfloral populations, and population reductions will be greatest with the most effective enzyme application. Figure 12.2 demonstrates that in birds fed a reasonable quality wheat-based diet, the application of a xylanase-based enzyme results in a 60% reduction in microbial numbers. The antibiotic evidently reduces populations by direct activity against the target species, and the numbers are reduced more dramatically than that seen with enzyme use. Whilst both reduce microfloral populations, their modes of action are evidently quite different. The performance gain on use of an antibiotic is thought to be due to many factors, such as reduced competition for nutrients in the small intestine, reduced local inflammation due to control of pathogens, and reduced intestinal thickening and length as a result of improved digestibility and pathogen loading (Thomke and Elwinger, 1998a,b). The latter two mechanisms result in a
more efficient digestion and reduced maintenance energy requirement. As a result of the total population changes and species shifts, many of these benefits apply to use of enzymes (Brenes et al., 1992, 1993; Gollnisch et al., 1996; Hock et al., 1997; Simon, 1998; Vahjen et al., 1998). Whilst the issues discussed to date relate to the ability of enzymes to remove fermentable substrate from the small intestine, it is apparent that this is not the only mechanism by which they interact with the microflora.

**Caecal phase – substrate provision**

By their very nature, enzymes that reduce the size of carbohydrates produce smaller, more fermentable products. Xylanases and cellulases ultimately produce xylose and glucose, respectively, and oligomers of various chain length and degrees of
substitution (in the case of xylans). The small intestinal concentration of carbohydrates with an apparent molecular weight of 40,000 Da or less was increased almost twofold on addition of high levels of a xylanase-based enzyme (Bedford and Classen, 1992). More recent work (Apajalahti and Bedford, 1999), shown in Fig. 12.3, breaks such data into additional categories of smaller molecular weight fragments. The effect of the enzyme is to increase dramatically the concentration of all xylo-oligomer fractions, but it is much more pronounced for those that are less than ten xylose sugars long (fivefold increase compared with 2.3-fold for the DP < 500). These oligomers are not, by their very nature, either digested or absorbed by the host and as a result remain in the aqueous phase of the intestinal digesta. Whilst inert to host enzymes, such oligomers are broken down by bacterial enzymes that are present in the caeca (Dänicke et al., 1999a) into their constituent sugars, which can then be utilized by many organisms inhabiting the digesta. Xylo-oligomers fed to diabetic rats were shown to increase caecal acetate levels more than threefold (Imaizumi et al., 1991), indicating their potential in supplying substrate to the large intestine. As a result there is a reduction in the number of starch and protein users (Vahjen et al., 1998) and an increase in xylose users on incorporation of a xylanase enzyme in the diet.

Fig. 12.2. Effect of addition of either an antibiotic (Avoparcin) or a xylanase on the total population of bacteria inhabiting the small intestine of 21 day broilers.

Fig. 12.3. Addition of xylanase to a wheat-based diet increases the concentration of small, soluble xylo-oligomers in the small intestinal tract.
ration (Fig. 12.4., Apajalahti and Bedford, 1999). In this work, broilers were fed a wheat-based diet with or without supplementation with a xylanase enzyme. The entire small intestinal digesta was removed and washed in buffer, and the extracted bacteria were grown in a medium in which xylose provided the only carbohydrate source. Microscopic enumeration of the total bacteria in each treatment prior to incubation revealed that the enzyme-treated birds yielded only 40% of the number of organisms found in the control. Despite this fact, the results demonstrated a considerable degree of adaptation of the microflora towards utilization of xylose in enzyme-treated animals. Clearly the continued generation of this sugar in situ by exogenous enzymes exerts a significant selection pressure on the inhabitants of the intestine. This is seen in alterations in total numbers of bacteria and/or species dominance on feeding xylanase to pigs and poultry (Gollnisch et al., 1996; Hock et al., 1997; Vahjen et al., 1998). As a result of alterations in the intestinal flora profile in both the small and large intestines, it is likely there will be concomitant alterations in intestinal morphology, digestive enzyme secretions, and stability of these secretions and local and systemic immune function.

Some effects are clearly direct. Some species, particularly enterococci and lactobacilli, numbers of which are often reduced on feeding enzymes, have been shown to secrete hydrolases that deconjugate taurine and cysteine from bile acids, rendering them less effective in fat digestion (Campbell et al., 1983; Bovee-Oudenhoven et al., 1999; Schutte and Langhout, 1999; Tanaka et al., 1999). As a result, the digestion of fat, particularly saturated fats, is compromised (Dänicke et al., 1997a, 1999b) and with it the digestibility of many fat-soluble compounds.

**Fig. 12.4.** Influence of previous exposure to xylanase enzyme on ability of total small-intestinal flora isolated from broiler intestines to utilize xylose as the only carbon source. Incubation in media containing only xylose as source of carbon. Total bacterial contents of digesta washed and collected and used as starter culture in each experiment. Since 60% fewer bacteria were harvested and hence used per incubation test for the xylanase-treated birds, the differences between treatments for each parameter is actually much larger on a per bacterial count.
including vitamins and pigments (Roxas et al., 1996). Application of an enzyme results in fewer of these organisms and hence improved digestibility of these nutrients (Hock et al., 1997).

**Indirect Interactions between Host and Microflora Mediated by Enzyme Use**

Some effects of enzyme-mediated changes in the microflora have a less direct effect on host nutrition. For example, use of enzymes has been reported to alter the concentrations of polyamines and volatile fatty acids (Choct et al., 1996, 1999; Jansman et al., 1996; Silva and Smithard, 1996), compounds that are known to influence many processes in intestinal growth and development (Seidel et al., 1985; Osborne and Seidel, 1990; Shinki et al., 1991; Gee et al., 1996; Noack et al., 1996; Raul and Schleiffer, 1996). Furthermore, interaction of the microflora with the intestine will have far-reaching effects on digestion, due to modulations in villus structure and turnover rate, mucin production and growth rates of the animal as a whole (Seidel et al., 1985; Smith, 1990; Bhatt et al., 1991; Furuse et al., 1991; Sharma and Schumacher, 1995; Gee et al., 1996; Noack et al., 1996; Silva and Smithard, 1996; Sharma et al., 1997). The interaction of the microflora with the intestines also has effects on the immune status of the animal which will inevitably interact with and alter the microbial community profile in itself (Ouwehand et al., 1999) and the susceptibility of the host to antigen damage and some nutrient deficiencies (Fukushima et al., 1999; Mengheri et al., 1999). The effects of shifts in fermentation in the caeca can also influence activity elsewhere in the digestive tract. In rats, the provision of fermentable carbohydrates has been shown to have profound effects on secretion of enteroglucagon from the colon, which in turn influences gastric secretions and emptying rates (Gee et al., 1996). As a result the microfloral status of the large intestine can influence the gastric phase of digestion, the extent of which in turn will influence the provision of substrates into the small and large intestines.

In many respects it appears that the nutrition of the animal cannot be divorced from the process of microfloral interaction with the diet. Digestion and absorption of nutrients by the host seem to be accomplished through a two-way negotiated process between host and intestinal microflora that establishes a sustainable partition of the available resources. Use of exogenous enzyme seems to move this equilibrium more in favour of the host, hence the animal benefits.

The absence of a microflora will thus reduce much of the benefit of addition of an enzyme. It is the very fact that the populations of intestinal flora are probably so different between experiments that results vary so much between experiments. The next section focuses on some of the factors that need to be considered when interpreting data from enzyme trials.
Microbial Modulators with Potential to Interact with Response to Enzyme Usage

The species diversity and density within the intestinal tract of an animal will vary, depending upon the environment and diet. Environment evidently influences the composition and density of the resident flora – ranging from the germ-free to the deliberately challenged or seeded environment (Muramatsu et al., 1994; Droleskey et al., 1995; Noack et al., 1996; Hofacre et al., 1998; Schutte and Langhout, 1999). Nipple drinkers are cleaner than bell drinkers (Belyavin, 1995), and floor pens with litter will provide for greater recycling and build-up of bacterial numbers than wire-floor cages. Even litter material can significantly influence intestinal flora and health (Corrier et al., 1993; Bilgili et al., 1999).

Nipple drinkers are cleaner than bell drinkers (Belyavin, 1995), and floor pens with litter will provide for greater recycling and build-up of bacterial numbers than wire-floor cages. Even litter material can significantly influence intestinal flora and health (Corrier et al., 1993; Bilgili et al., 1999).

The diet is of course a major source of the variation in intestinal flora diversity and total population. Provision of specific fermentable sugars can significantly influence composition of the flora, with some (for example, the fructo-oligosaccharides) being put forward as carbohydrates that confer a favourable fermentation pattern (Oyarzabal and Conner, 1995; Grizard and Barthomeuf, 1999), whilst others (such as the raffinose, stachyose and verbascose series of oligosaccharides) have a more questionable value (Brenes et al., 1989; Krause et al., 1994; Iji and Tivey, 1998). In any given study, the dietary concentrations of fermentable compounds derived from ingredients such as soybean meal, canola, peas, etc. are mostly unmeasured.

Antibiotics and coccidiostats with antimicrobial activity markedly influence the population and composition of the resident flora (Ohya and Sato, 1983; Gollnisch et al., 1996; Hock et al., 1997). There are also many dietary nutrients, particularly metals such as zinc and copper, which, when fed at elevated levels, have a pronounced influence on the microfloral populations either directly or through interaction with the immune system (Kidd et al., 1996; Yenigun et al., 1996; Leeson et al., 1997). Many nutrients interact with the immune system, either positively or negatively (Korver and Klasing, 1997; Klasing, 1998), which can significantly alter the populations of the intestines.

When reviewing the literature, it is of value to consider the microbiological challenge of each study as determined by chick handling, caging and rearing, dietary ingredients, antimicrobial compounds present, and the status of the immune system. All such factors, and many more, contribute to the condition and description of the resident flora. Since enzymes alter substrate flow into the small and large intestines, the subsequent response to their use will vary according to the populations present at time of administration and their reaction to such changes. It is not surprising, therefore, that given the huge range in microfloral conditions likely to exist between studies, the responses to enzyme use, rather than being absolute, are a continuum or population of responses from detrimental to highly positive. Determination of the key bacterial species involved in enzyme response will allow for more thorough interpretation of future research where such measurements are made.
Conclusions

Many of the factors that govern the scale of response to enzyme addition are just as relevant to those influencing the response to antibiotics. The response to antibiotics very much depends on the conditions under which the animals are grown, with responses being much more marked in conventional than germ-free animals (Bywater, 1998). Indeed, in conventional birds, the general response to antibiotic administration is very much influenced by the relative performance of the non-supplemented controls. The greatest responses are observed in those trials where the controls performed particularly poorly, and virtually no benefit is reported in those trials where the controls performed very well (Rosen, 1995). The response to exogenous enzymes is subject to the same constraints, the greatest responses being observed with the poorest performing controls (Barrier-Guillot et al., 1995; Classen et al., 1995; Scott et al., 1998). Scale of response to exogenous enzymes seems to depend upon not only initial cereal quality but also the microfloral status of the bird. In studies where both antibiotics and enzymes have been used, in general the response to enzyme in the presence of the antibiotic is reduced in comparison with that observed in its absence (Elwinger and Teglof, 1991; Broz et al., 1994; Vranjes and Wenk, 1996, 1997; Hock et al., 1997). Such observations suggest that the response to enzymes is reduced when the microflora is controlled by the presence of an antibiotic. In those studies where the response to enzyme is just as great in the presence or absence of antibiotic, it is interesting to note that the response to the antibiotic alone is not particularly great (Allen et al., 1996; Esteve-Garcia et al., 1997). Reduced intestinal microbial populations probably limit the scope for improvements in performance due to enzyme supplementation. When digestion is slowed through addition of carboxymethyl cellulose (CMC), which is a viscous non-fermentable carbohydrate, the result is more apparent in conventional compared with germ-free chicks (Smits and Annison, 1996). In essence, addition of an enzyme to a wheat- or barley-based diet has much the same rheological effect as removal of the CMC from the diet in the work of Smits and Annison (1996). The implications of this work and that discussed earlier in this section are that the response to enzyme addition probably depends on the initial microfloral status of the animals, which in turn depends on the digestibility of the ration and microfloral challenge. Nutrition, enzymes, antibiotics, prebiotics and probiotics are in effect all tools for negotiation for nutrient abstraction from the intestines between the host and its residents.

References


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Introduction

Enzymes were discovered in the latter part of the 19th century and have been used in industrial and food processes since the early 1900s. Examples include the use of amylases in textiles for removal of the starch sizing, bovine rennin in cheese making, pancreatic enzymes in production of leather and degumming of raw silk, and proteases for beer stabilization. As a result of the advances in biotechnology over the past 10–20 years, especially in the areas of genetics and protein engineering, there is now widespread use of enzymes in detergent, textile, grain wet milling, food, and pulp and paper applications. Incorporation of enzyme additives in feed rations is more recent. The benefits of enzymes in feed applications include (Annison, 1993; Bedford and Schulze, 1998; Simon, 1998): (i) balancing deficiencies in the endogenous enzyme system of the animal; (ii) elimination of anti-nutritional; and (iii) increased utilization of the feed. As the value of enzymes continues to grow and new markets are developed, technology, which can provide methods for more rapid identification and development of new or improved enzymes, will be a key element for success. This chapter is a review of current and emerging technologies used in the discovery, research and development of new enzyme products with a focus on microbial production hosts.

Background

Enzyme classes and sources

Enzymes are extremely efficient and highly specific catalysts. They also exhibit low toxicity and act over wide pH and temperature ranges. There are several classes of enzymes (International Union of Biochemistry and Molecular Biology, 1992):
1. Oxidoreductases catalyse the transfer of hydrogen from a donor to an acceptor molecule. These enzymes are also referred to as dehydrogenases or reductases. In cases where the hydrogen acceptor is oxygen, the enzymes are referred to as oxidases.

2. Transferases carry out the transfer of a group, such as a glucosyl, from one compound to another.

3. Hydrolases catalyse the cleavage of specific bonds including carbon–oxygen, carbon–nitrogen and carbon–carbon bonds, and oxygen–phosphorus bonds. Essentially these are transfer reactions where water is the acceptor molecule. Many of the enzymes currently used in feed applications, such as amylases, xylanases, cellulases, proteases and phosphatases, are hydrolases.

4. Lyases can cleave similar bonds as hydrolases but do so by a mechanism of elimination resulting in the formation of double bonds or rings. They can also carry out the reverse reaction and add groups to double bonds.

5. Isomerases carry out structural changes within a molecule.

6. Ligases catalyse the coupling of two molecules in conjunction with the hydrolysis of a high-energy phosphate bond such as ATP.

The majority of enzyme products to date have been derived from mesophilic and, to a lesser extent, alkaliphilic microorganisms. While these sources continue to serve as valuable reservoirs of commercial enzymes, other microbial systems, such as extremophiles, are rapidly gaining attention (Kristjansson and Hreggvidsson, 1995; van der Oost et al., 1996; Vielle and Zeikus, 1996; Adams and Kelly, 1998; Davis, 1998; Horikoshi and Grant, 1998). Extremophiles are organisms that reside in extreme environments and they include thermophiles (> 70°C and often in excess of 100°C), psychrophiles (< 20°C), barophiles (> 1 atm) and halophiles (high salt). Plant-derived enzymes as well as genetic engineering of plant cells and transgenic plants are also being explored (Kahl and Weising, 1993, Willmitzer, 1993; Flavell, 1995; Ma et al., 1995; Mazur, 1995; Moffat, 1995; Evangelista et al., 1998; Gale and Devos, 1998; Kusnadi et al., 1998).

**Enzyme properties**

It is not unusual for a microorganism to produce an array of enzyme representatives from each class, subclass and category of enzyme. Many have even evolved multi-enzyme complexes or systems. The cellulolytic enzyme system, which is a mixture of cellulases that act synergistically to degrade cellulose, is one example (Wood and Kellogg, 1988). The cellulolytic system produced by each microorganism is unique and often comprises several types of cellulases, which can differ in their mode of action and specificity (substrate preference). Endoglucanases (endocellulases) attack the internal glucosidic bonds of the cellulose polymer in a random fashion and produce multiple cellulose chains of varying length as well as oligosaccharides. Another type of cellulase, cellobiohydrolases, attacks the cellulose chain ends and produces primarily cellobiose. The more chain ends produced by the endoglucanases, the more substrate there is for the cellobiohydrolases. Build-up of cellobiose, a potent
end-product inhibitor of cellobiohydrolases, is alleviated by β-glucosidase, which converts cellobiose to glucose. Therefore, the complete or whole cellulase system is essential for driving the degradation of cellulose to glucose, an important energy source for many microorganisms. Of further interest is the fact that cellulolytic microorganisms often generate a variety of endoglucanases and/or cellobiohydrolases. These enzymes can differ in their physical and biochemical properties such as molecular weight, isoelectric point (pI), pH and temperature activity profile, stability, and substrate specificity. Production of multiple xylanases, amylases, proteases, phosphatases, esterases, etc., is also not uncommon. This arsenal of enzymes allows the microorganism to access nutrients from a variety of sources and to adapt to a wide range of environmental conditions (pH, temperature, etc.). Through sophisticated control mechanisms, which have evolved over millions of years, the cell can react to these types of changes and turn on genes in order to express the required enzymes and proteins or shut down non-essential protein synthesis and metabolic pathways. In this way, energy is conserved and directed to growth, reproduction and survival of the organism.

Although many of the enzymes that are of value in feed applications can be found in nature, the levels produced by wild-type organisms are relatively low, making the economics unsuitable for most commercial applications. An added issue is the fact that many microorganisms produce a mixture of enzymes. While these mixtures may show a benefit in the application, only one or two of the enzymes present may actually be responsible. Also, while certain enzymes produced by a microorganism may be of benefit, others may exert a negative effect in the application. An example is an enzyme mixture containing both an endo-type xylanase, which is useful in reducing the viscosity of xylan, and a xylosidase, which catalyses the hydrolysis of short xylooligosaccharides to xylose. Given that xylose is known to cause cataracts, diarrhoea and anorexia in some animals, this side activity is not desirable (Schutte, 1990; Schutte et al., 1990, 1991). Therefore, before designing a production organism or host, it is important to know which enzyme(s) provides the benefit in order to develop a host and process that are optimized for its production, thus reducing undesirable side activities while providing improved economics.

### Identification and Development of Novel Enzymes

#### Enzyme identification

The development of enzyme products often relies on screening a large number of organisms for an enzyme activity with a specific set of biochemical and physical characteristics that suit the targeted application. Traditionally, microbial screening processes have involved evaluation of purified enzymes isolated from: (i) pure cultures obtained from in-house or accessible culture collections throughout the world; (ii) microorganisms isolated from environments rich in the substrate of interest; and (iii) microorganisms that have a history of being good enzyme producers, such as *Trichoderma*, *Aspergillus* and *Bacillus*. While these approaches have met with success
over the years, there are limitations in that they provide access to only a very small portion of the immense microbial population or diversity. Indeed it is believed that, to date, only a very small number of microbial species, possibly less than 1%, has been cultured (Pace et al., 1986; Ward et al., 1992; Amann et al., 1996; Pace, 1997). Part of the problem lies in the fact that the majority of microbes are not culturable using standard techniques. While traditional sources and approaches are still valid, several methods for handling unculturables and for speeding up their analysis have recently become available (Olsen et al., 1986; Pace et al., 1986; Saiki et al., 1988; Weller and Ward, 1989; Ward et al., 1992; Amann et al., 1995, 1996). Most of these involve nucleic acid approaches in which the need to culture an organism is eliminated. One such method is sequencing of the total genome of specific organisms in order to screen for DNA sequences that are related to known DNA sequences, which express the desired type of enzyme activity. Other methods include extraction and amplification of DNA from unculturables or mixed culture populations. These approaches and others, including use of RNA, will be discussed in more detail later in this chapter. To complement these techniques much effort is also being invested in development of automated, miniaturized, highly sensitive, accurate and rapid methods for obtaining and analysing nucleic acids and enzymes from various sources.

Despite the diversity of enzymes available in nature, it is sometimes the case that these enzymes do not possess all of the features necessary for a targeted application. Instead of rescreening for an enzyme that possesses one additional property, it is possible to protein-engineer a deficient enzyme by modifying its sequence such that the lacking feature is incorporated into the enzyme without loss of the existing desirable features.

Protein engineering of an enzyme can be carried out by site-directed and/or random mutagenesis. To a limited extent chemical methods, such as acetylation or amidation, can also be used. Although the chemical approach has met with some success, overall it is less predictable. Site-directed mutation involves modification of the enzyme properties by specific changes in the DNA code through the use of recombinant technology (Carter and Wells, 1987; Bott et al., 1988; Estell, 1993; Cleland et al., 1996). These changes can be selective amino acid substitutions, deletions or insertions, or a selective combination of specific structural units of two enzymes to generate hybrid or chimeric enzymes (Schneider et al., 1981; Mas et al., 1986). A protein engineering approach can be carried out by elucidating and studying the three-dimensional structure of the protein by X-ray diffraction, nuclear magnetic resonance (NMR) and other methods. Molecular graphics and modelling studies can be used to predict the effect of single or multiple amino acid substitutions. In the past 40 years or so, over 3000 protein structures have been elucidated (PDB, 1996; Godzik, 1997). Even so, it is still difficult to predict protein structures based on amino acid sequences. Fortunately some alternative approaches exist, such as random mutagenesis and, more recently, directed evolution, which has emerged as a powerful alternative to site-directed protein design. These techniques can be applied to produce designer catalysts without much knowledge of the protein structure.

Random mutagenesis has been used for decades as a tool for increasing genetic variability and improving the properties of microbial strains as well as plant cultivars.
Improved strains or enzymes can be generated by random modification of the nucleic acid sequence followed by screening for the desired properties. Modification or mutation of the DNA sequence, which encodes for the targeted enzyme, can be achieved by physical (heat, freezing, UV, etc.) or chemical methods. With random mutagenesis there is less control over the number and types of mutations made compared with site-directed engineering, which tends to generate modifications in localized regions of the amino acid sequence. This means that a huge number of mutants must be generated and screened. These types of approaches require highly efficient and rapid screening techniques.

Directed evolution involves multiple cycles of mutagenesis with selection or screening. Mutants that show improved properties after each cycle are subjected to further treatment. An accumulation of beneficial mutations is then achieved (Kuchner and Arnold, 1997). A suitable host for expression of the gene, appropriate methods for generating genetic variability, and suitable selection and screening methods are all needed. Again, to ensure a high success rate, a large but manageable number of clones need to be screened. Another approach that can be applied is DNA shuffling. This technique involves random fragmentation of DNA sequences followed by assembly of the fragments into genes by various recombination methods.

By combining enzyme screening with modern techniques such as protein engineering and directed evolution, hybrid molecules with improved performance under specific application conditions can be generated.

**Enzyme development and production**

Once the targeted enzyme has been identified, the gene which encodes the enzyme is then inserted into a suitable production host. As mentioned previously, many natural isolates, from which an enzyme can be derived, are usually not acceptable production hosts. Although it may be possible, to some extent, to alter the type and amount of protein produced by natural isolates or mutants through modification of the fermentation conditions, in most cases this is not sufficient to achieve the desired purity, process reproducibility and economics. Of equal importance are safety concerns and the fact that production hosts are usually limited to those with a history of safe use. Therefore, recombinant methods are often employed. Given that the gene sequence in a recombinant host is well characterized, there should be no safety issues beyond the normal procedures, which regulate all enzymes derived from genetically engineered organisms.

Attractive production systems or hosts include those microorganisms that have the ability to: (i) produce and secrete large amounts of intact protein; (ii) carry out proper folding of the protein and post-translational modifications such as signal sequence cleavage, proteolytic processing, glycosylation, disulphide bridge formation, etc.; (iii) generate stable recombinant cells or transformants; and (iv) comply with regulatory requirements such as ‘generally regarded as safe’ (GRAS) status (van den Hombergh et al., 1997).
Regardless of the nature of the production system developed, it needs to be compatible with industrial-scale fermentation and recovery processes. These processes should be reliable, meet all safety and quality assurance requirements, and provide a reproducible source of high quality product. Ideally, the strain development and fermentation development efforts should be highly interactive. In turn, the fermentation development effort needs to be synchronized with the recovery efforts, since the nature of the fermentation broth exerts a large impact on downstream processes such as cell separation and product recovery. The fermentation and recovery processes must together generate a liquid concentrate that can be formulated as a stable liquid or solid product without significant cost. Successful fermentation development requires: (i) a good understanding of the physiology of the microbial host; (ii) development of a suitable medium; and (iii) optimization of the interactions between the microorganism and its chemical and physical environment in the fermentor (Arbige et al., 1993). The chemical and physical environment, once defined, can be controlled by computers and automated processes. Given all the factors that can exert an effect upon the productivity, statistical–mathematical approaches are often employed to optimize the various parameters.

The type of medium employed as well as by-products produced by the cells can have a large impact on the protein recovery yields and the cost of the downstream processes. Even enzyme products that are sold as crude preparations require downstream recovery after fermentation. Once the enzyme has been isolated from the broth, it can then be concentrated by ultrafiltration, precipitation, extraction or other methods prior to formulation. Where purity is of high concern, further processing involving one or several fractionation steps is needed. The yield and product purity criteria need to be balanced with the economics associated with each step, which is incorporated into the downstream process. Also, the nature of the ingredients used in the fermentation and recovery processes can have a significant effect on the cost of waste disposal.

After the enzyme has been recovered and concentrated, it is usually formulated in order to meet the product stability specifications suited to the application and handling practices employed by the end-user. Food-grade ingredients are required for food and feed applications. For solid products, the enzyme concentrate or formulated product, if suitable, can be granulated or dried in a manner that provides a solid product that meets health and safety requirements, such as low dusting.

Screening

Screening in essence means searching for a particular gene coding for the target enzyme. In the past this involved exclusively the screening of living microorganisms (‘classical microbial screening’), but today, by the application of modern tools of molecular biology, a screening may be performed without the need to culture the organisms involved. As with all methods, there are limitations and a combination of techniques (‘combinatorial screening’) often provides the most success.
The most rewarding starting point for the process of search and discovery is the rich diversity of microorganisms in nature (Bull, 1992). It is seldom appreciated that for 85% of the earth’s history, life was restricted to microbial forms. The metabolic diversity this has created is truly immense. During more than 4 billion years of evolution, enzymes have emerged that are perfectly adapted for maintaining cellular processes. However, many enzymes are required to perform in a milieu that is far removed from the natural physiological conditions and may even involve exposure to chemicals known to degrade or denature enzymes, which leads to loss of activity. Usually, enzyme screening and selection strategies are based on knowledge of the application and the physical and chemical conditions under which the enzyme must operate. Therefore, selection for enzyme performance under the application conditions is an essential early step in the screening process. Access to a large gene pool is a prerequisite for a successful screening programme. The approaches can involve the indiscriminate examination of heterogeneous material (e.g. soil) gathered from the environment, or an ecological approach with a targeted examination of specific habitats where a particular activity is likely to be found. Strategies can be adapted to search specifically in poorly explored environments in order to access a greater variation in genomes from novel microbes. In the search for new genes, exploration of extreme environments such as volcanic hot springs, hypersaline lakes and polar soils has yielded many novel microorganisms with peculiar properties (Horikoshi, 1995; Kristjansson and Hreggvidsson, 1995; Horikoshi and Grant, 1998). Alternatively, a taxonomic-based approach involving a systematic study of known organisms can also be rewarding.

**Natural isolate screening**

The source material can be plant or animal matter, or microbes – both prokaryotes (e.g. bacteria and archaea) and eukaryotes (e.g. yeasts and fungi). Currently, microorganisms are the major source for industrial enzymes in the feed sector. The classical method of screening natural microbial isolates is a well established process having its roots in antibiotic discovery (e.g. penicillins, streptomycin) in the decades after 1945. It involves the examination of thousands of samples of soil, plant material, etc., and the random isolation and screening of the resident microbial flora. Although capable of significant automation, the throughput capacity largely determines the speed of progress. In the past, screening strategies using soil samples as a starting point were highly empirical but much can be done to reduce the need for extensive experimentation. These processes involve subjecting an environmental sample (soil, etc.) to selective pressure using the principles of culture enrichment established early in the 20th century by microbiologists such as Beyerinck and Winogradsky. By manipulating the growth conditions, only those microorganisms expressing a particular enzyme are able to grow and survive. For example, by providing only xylan as the source of carbon and energy, only microbes producing a xylanase either constitutively or by induction will grow and the culture will become enriched in these organisms. The system is capable of considerable refinement; for example,
should an acidic xylanase be required, then the pH of the enrichment culture can be designed to permit growth of microbes producing the enzyme at pH 4–6. The outcome can be improved by a judicious choice of environment for examination – for example, acidic soils, peat bogs and forest litter.

The time between collection of the environmental sample and examination in the laboratory can be critical since changes will inevitably occur. Even in the best-preserved samples chemical and physical changes may occur, leading to the death of some organisms and the reduced viability of others. The longer the time lapse involved, the more serious these changes can be. There are many advantages to treating the sample immediately in the field since this can lead to the isolation of a totally different population of microbes. It can be useful to provide ‘bait’ for specific microbes or types of microbial activity. An example of this in situ enrichment is placing cotton linter in an environment such as lake sediment and retrieving the sample at a later date. The sample can then be taken back to the laboratory and screened for cellulolytic activity.

The first problem in processing of samples is often one of numbers. Composts and soils rich in organic matter contain enormous numbers of microbes. Some form of dilution is required, or targeting of specific groups. Selective techniques can involve, for instance, careful air drying of the (soil) sample, which reduces the numbers of Gram-negative bacteria, or a brief heat treatment at 80°C, which will kill most microorganisms but leave the resistant spores of bacteria such as Bacillus intact. Antibiotics may be incorporated into the growth medium. For example, Gram-positive bacteria are more sensitive towards penicillin than Gram-negative bacteria. Many techniques have been developed empirically, such as the use of water- or chitin-agar for isolation of slow-growing actinomycetes, and such ideas are limitless (Williams et al., 1984).

An ecological approach takes advantage of the natural selective pressure of the environment. For example, an alkaline environment such as a soda lake at pH 9 is inhabited only by alkaliphilic microorganisms. It is likely that these organisms have extracellular enzymes that are stable and perform optimally at a high pH, which is indeed the case (Jones et al., 1998). For screening of feed enzymes such considerations are often less clear-cut. What, for example, is the natural habitat for a phytase-producing microorganism? Phytate, a storage phosphate, is often a major component of some seeds and screening microorganisms in intimate association with the roots of germinating seeds (rhizosphere) would be an appropriate niche to examine.

Whatever the screening strategy adopted, all of these approaches rely on some form of selection criteria for the organism–activity combination being sought. The most widely applied methods involve growing the microbes on an agar plate containing the appropriate enzyme substrate for detecting the desired enzyme activity. Often the methods employed are proprietary and frequently constitute more of an art than an exact science. Some methods, however, are well known. For instance, incorporating casein as sole carbon source into an agar medium providing an opaque surface will detect protease-secreting organisms as a clear halo of hydrolysed casein around the growing colony. Specific protease cleavage reactions
can be detected by dye release from appropriate chemicals carrying a chromophore that is only visible after the specific hydrolysis reaction has taken place.

The selection of microorganisms is a very critical stage in the screening process and can be quite subjective, especially if the numbers of microorganisms involved are very large. Even though the screening has followed some form of selection process, it is often not easy to distinguish the different types of microorganisms growing on an agar medium. This is particularly true of bacteria, which, unless they are clearly coloured, all look much the same. This gives rise to one of the biggest drawbacks of the random approach to isolation and screening of naturally occurring strains: the problem of redundancy. The same microbe will recur time and again from different samples collected from different locations, which may explain why so many of the current products come from a very limited range of microbes. Until relatively recently most screening strategies were fairly conservative. The tendency to use rich media which favour fast-growing, nutritionally undemanding organisms has led to an overemphasis on particular groups of microorganism. This is coupled to the fact that certain fungal genera such as *Aspergillus* and *Trichoderma*, and *Bacillus* and *Pseudomonas* species among the bacteria, have traditionally been a rich and proven source of commercial enzymes.

To ensure greater diversity and as a measure of how extensive a screening has been, some estimate is required of the biodiversity of the population screened. A decade ago this was a daunting task involving a detailed examination of each organism, but nowadays fast molecular methods are available. A range of techniques will rapidly group collections of unknown strains into clusters of related organisms. Now, techniques such as total cell protein patterns, amplified fragment length polymorphism, ribotyping, amplified ribosomal DNA restriction analysis, internally transcribed spacer region–polymerase chain reaction (PCR), single-strand conformational polymorphism combined with sophisticated electrophoresis methods, image analysis for data acquisition and computation can provide information on the diversity of a strain collection. The ease with which small subunit ribosomal RNA (ssu RNA) gene sequencing can be performed today, combined with a large and growing database for comparison, means that specific organisms can often be quickly assigned to recognized taxonomic groups, and that novel microorganisms can be recognized when found. The clustering of organisms into related groups of strains can be combined with screening selection data, thus permitting the identification of specific attributes with recognized groups of organisms. This information allows particular procedures to be devised for the isolation of a specific group and the imposition of more stringent selection criteria. Even so, it is still too easy to overlook potential novel isolates, especially when confronted by many hundreds of culture dishes, each containing several hundred similar-looking microbial colonies. Nowadays, the task can be performed by colony-picking robots and visual imaging systems coupled to computers.

As indicated earlier, one problem often encountered early in the screening process is that natural microbial isolates usually produce commercially important enzymes in exceedingly low concentrations. Environmental manipulation that optimizes the growth and production conditions is limited by the intrinsic maximum
ability of the wild-type organism. Classical genetic manipulation by selecting mutants with improved properties can lead to an increase of potential yield. The classical techniques of mutation and selection for microorganisms with improved attributes have been applied for many decades. Modern methods of molecular biology have in recent years accelerated the possibilities of creating in the laboratory microorganisms with new or improved activities. However, there is increasing evidence suggesting that many of the techniques associated with ‘genetic engineering’ do, in fact, take place in the natural environment between groups of quite diverse microorganisms. It is part of the opportunistic nature of microbes that allows them to cope with changing environmental conditions. This can lead directly to the acquisition of new activities within a microbial community. New activities are often due to selection pressure, especially where recalcitrant chemicals, such as a new herbicide, are introduced into the environment. This can be mimicked in the laboratory by microbial selection in a continuous culture chemostat. This should not be confused with the idea of the creation of a new microbe. Microorganisms react in a manner to preserve their genetic individuality, and in any case, the sudden emergence of an entirely new organism is not an event we are ever likely to be able to witness.

Molecular screening

The last decade has witnessed spectacular progress in the technology enabling the direct sequencing of nucleotides of a DNA strand. Already there are thousands of gene sequences in public databases, although most relate to genes involved in the translation of the genetic code. The complete sequencing of microbial genomes has also accelerated quickly in recent years. At present some 35 prokaryotic (Bacteria and Archaea) and three eukaryotic microbial genomes have been reported in their entirety and the sequencing of some 70 other microbial genomes is currently in progress.

There are several ways this can be done. If a suitable ‘lead’ enzyme (for example, an acid xylanase) has been identified in a particular species, then a search can be made for homologous enzymes in related species. The process requires the application of ‘reverse genetics’. Enzymes are polymers of amino acids covalently bonded in a defined sequence. The order in which the amino acids are arranged in the enzyme can be analysed by a process known as N-terminal sequencing. Since the genetic code is universal it is possible to predict the probable sequence of nucleotide bases in the gene coding for the specific enzyme. Using this information a probe can be constructed consisting of 15–20 nucleotides (primer set) in the correct sequence, which because of the double helix nature of DNA will bind to the complementary strand. By using the primer set in PCR on chromosomal DNA from related organisms, a homologous gene can be amplified and transferred to a host organism such as *Escherichia coli* for the expression of the gene product – a homologous enzyme (Dalborge and Lange, 1998). In practice the procedure can be a little more complex than is described here but it is possible to track down enzymes with identical functionality but with a significantly different sequence of amino acids, where only the crucial elements of the enzyme, such as the active site, have been conserved.
through evolution. In application tests these ‘new’ enzymes may have significantly different properties, such as improved temperature or pH stability, compared with the ‘lead’ molecule. In this way, whole families of related organisms can be screened. Even though this is a very powerful technique it is limited by the ability to grow and extract suitable DNA from the target organisms. It is also confined to known microorganisms that have been isolated, described and deposited in culture collections. It has been estimated that fewer than 1% of all microorganisms that exist in nature have been isolated and even fewer have been adequately characterized and described (Amann et al., 1995). It is thought that fewer than 25% of the microbes in a sample taken from the environment can actually be cultured in the laboratory. There may be many reasons for this ‘unculturable’ portion of the microbial community. Some cells may be in a resting phase, or be damaged or moribund; some may not be true residents and not form part of the active population; others may be opportunists waiting for a shift in environmental conditions in their favour; while others may have unknown requirements for culture that remain unmet in the laboratory.

Fortunately, recent developments have permitted access to at least a portion of these uncultivated microbes (Hugenholtz and Pace, 1996). It is possible to extract DNA directly from the microbial community present in a sample taken from the environment. Although there are many techniques for extracting chromosomal DNA from natural microbial communities, not all cells are equally susceptible to lysis and the DNA recovered is never 100%. Another concern is the detection of organisms that form only a minor proportion of the total community. Their DNA will be under-represented unless efforts are made to normalize the sample. A recent examination of sequences of ssu rRNA genes in environmental DNA clones derived from habitats as diverse as polar seawater from the Southern Ocean and hot springs in Yellowstone National Park revealed a vast biodiversity of ‘yet to be cultivated’ microorganisms unsuspected a few years ago (Hugenholtz and Pace, 1996; DeLong, 1997). It has also revealed that the cultivated microorganisms may not be the dominant flora in these environments, which provides a further challenge to the screening-by-enrichment culture methods. The environmental DNA can be tested for known genes using the PCR techniques described above. This requires good quality DNA since the PCR screening is particularly sensitive to disturbance by contaminating substances in the environment. Or, the DNA can be probed using DNA carrying a radioactive or fluorescent label by a technique called hybridization. Alternatively, the environmental DNA can be cut into smaller fragments using restriction enzymes and the fragments cloned into an expression host such as E. coli. The clones can be screened for new enzymes’ genes by techniques similar to many of those described for the screening of natural microbial isolates.

However, all these molecular methods based on similarity screening have one big drawback. They rely on a comparison with data that has already been recorded. Since more than 70% of the putative genes revealed by total genome sequencing projects (Bacillus subtilis, for example) have no known function, these data indicate that the resources for screening of novel enzymes are still largely untapped. It also remains true that the greatest diversity of life forms on this planet is microscopic. It probably makes little difference whether the exploration and screening of microbial
and gene diversity for novel enzymes for food and feed application use the techniques of the ‘hunters and gatherers’ (conventional microbial screening) or those of the ‘breeders and selectors’ (molecular screening); the resources available are truly immense and the ultimate performance of the enzyme under application conditions still remains the crucial factor in selection.

**Protein Engineering**

The challenges for feed enzymes often require modification of enzymes to function in a new environment or to possess a new property. Through protein engineering techniques, tailored enzymes for cleaning and starch processing have been developed and shown to outperform the best naturally occurring enzymes. The enzymes used in cleaning and starch processing face quite unnatural environments: detergent formulations contain surfactants and buffers to stabilize highly alkaline conditions and starch processing involves liquefaction of the raw starch at temperatures often near 105°C. The challenge has been to improve enzymes taken from quite different environments in nature. Examples include subtilisin (protease) from *Bacillus lentus* (a microorganism growing on plants and in soil) for cleaning, and α-amylase from *Bacillus licheniformis* for starch processing. While neither enzyme would be expected to function under commercial conditions, both perform the best of any found in nature thus far. Although isolated from a mesophilic organism, the α-amylase from *B. licheniformis* is equal or superior to the α-amylase from the thermophile, *B. sterathermophilus*, in starch processing; and *B. lentus* subtilisin, while normally operating in near neutral environments, seems to excel in alkaline detergent environments. Thus natural enzymes can often be found that are already surprisingly robust. These enzymes have evolved over time and as a consequence possess relatively stable structures that can accommodate the accumulation of amino acid changes necessary for the process of evolution. What has been undertaken in the process of ‘engineering’ enzymes is nothing more than a directed evolution of the natural enzyme to be better suited for the commercial task and conditions.

There are a number of complementary approaches to develop an enzyme with improved characteristics. Generally, as previously noted, the first step is to survey naturally occurring enzymes and identify the best starting enzyme. Sampling the available natural diversity and comparing functional properties versus the amino acid sequences may provide clues as to what properties are associated with each desirable molecular trait. The difficulty is that, over the evolutionary process, enzyme amino acid sequences have diverged considerably. With so much variation, the problem of identifying which sequence changes confer the desired trait can be extremely difficult. For example, subtilisin Carlsberg differs from subtilisin BPN’ in 87 of a possible 275 amino acid residues (Siezen et al., 1991). These two enzymes possess quite different kinetic parameters, yet, as will be described below, changes in as few as one to three of the possible 87 amino acids appear to be able to shift the kinetic parameters. The protein tertiary structure plays an important role in identifying the likely structural elements that result in these functional differences.
Subtilisin engineering

Enzymes are synthesized as linear polypeptide chains. The amino acid sequence is dictated by the DNA sequence of the structural gene. After being synthesized, the enzyme folds into a stable three-dimensional structure that brings residues far removed in the linear sequence into close juxtaposition. The juxtaposition of these residues and the creation of a substrate binding surface confer the enzyme molecule with its catalytic properties. As an example, the structure of subtilisin BPN', a serine protease, is shown in Fig. 13.1. In this figure, three residues – aspartic acid (Asp) 32, histidine (His) 64 and serine (Ser) 221 – are brought into position to form the 'catalytic triad' that is common to all serine proteases, not only subtilisin type but enzymes belonging to two other classes as well. There is also a wide depression where a segment of the polypeptide chain, from the substrate, binds to position a peptide bond to be attacked by the activated Ser 221 hydroxyl oxygen.

Knowledge of what residues form the substrate binding surface is invaluable when deciphering which of the multitude of possible changes may contribute to an

Fig. 13.1. Three-dimensional structure of subtilisin BPN’ (Bott et al., 1988). The linear sequence adopts a stable three-dimensional fold to juxtapose the catalytic triad residues: Asp32, His64 and Ser221. The catalytic triad is situated in a depression that forms the substrate binding region.
enzyme having a desired property. Focusing on which residues differ between subtilisin BPN’ from *B. amyloliquefaciens* and subtilisin Carlsberg from *B. licheniformis* allowed the identification of three of 87 possible substitutions that were likely to contribute to the difference in catalytic activity between the enzymes (Wells et al., 1987). Subtilisin Carlsberg has a turnover number, $k_{\text{cat}}$, that is approximately tenfold greater than subtilisin BPN’ and a different specificity (substrate preference) as well. The three-dimensional structures of subtilisin Carlsberg and subtilisin BPN’ share a common folding pattern despite the differences in their sequences. It is possible to superpose the two enzymes and relate residues that, while chemically different, are structurally homologous. This homology in tertiary structure along with the relative restricted fraction of residues forming the catalytic site and substrate binding region allowed the identification of three amino acid residues out of the 87 that were likely to affect the kinetic parameter and specificity of the enzymes. By introducing three substitutions — Ser for glutamic acid (Glu) 156 (E156S), alanine (Ala) for glycine (Gly) 169 (G169A) and leucine (Leu) for tyrosine (Tyr) 217 (Y217L) — a subtilisin BPN’ variant enzyme having the kinetic parameters and specificity corresponding to subtilisin Carlsberg was generated (Wells et al., 1987). Of these changes, a single substitution was found to exert a major effect on the difference seen for the turnover numbers, $k_{\text{cat}}$. Even at this site as shown in Fig. 13.2 for the Y217L variant, only subtle rearrangement is necessary to accommodate the substituted residue (note the superimposed leucine and tryosine residues at amino acid position 217).

![Fig. 13.2. Comparison in three-dimensional structures of native subtilisin BPN’ and a variant having the substitution of Leu for Tyr at position 217.](image)
Amylase engineering

A similar strategy has been successfully employed with amylase. The $\alpha$-amylase from *B. licheniformis* consists of 458 amino acid residues, which in three dimensions consist of three domains as shown in Fig. 13.3. The active site and substrate binding region are situated between domains A and B.

The engineering goal for amylase was to increase the stability of the enzyme. Numerous variants with increased stability were found by exploiting the diversity found in related $\alpha$-amylases from different species. Several clues were initially obtained from variants derived from a screen of randomly created variants. These led to a strategy of exchanging key amino acids with larger structurally homologous residues observed in different $\alpha$-amylases. It was proposed that bulkier residues, known to be accommodated in homologous locations in these other $\alpha$-amylases, would fill surface cavities and thus stabilize the enzyme. An example is presented in Fig. 13.4, where the substitution of Ser for Ala at position 379 has been modelled. This method has been used to create variants having increased pH and thermal stability (Day *et al*., 1998).

The strategy is based on the close homology seen in the structures of different $\alpha$-amylases. A residue found in one position in $\alpha$-amylase from one species can often

![Fig. 13.3. Three-dimensional structure of $\alpha$-amylase from *B. licheniformis*. The molecule consists of three domains A, B and C. The catalytic and substrate binding regions are formed between domains A and B. (Courtesy of Dr Andrew Shaw, Genencor International.)](image-url)
be accommodated in other α-amylases, which are homologous. The strategy thus relies on the diversity of nature to provide clues such as use of crevice-filling residues in the common α-amylase fold. These crevice-filling residues were then recruited into the *B. licheniformis* α-amylase. What is striking about the strategy was that the homologous residues selected for substitution were found in the sequences of other mesophilic α-amylases having lower stability than *B. licheniformis*. These residues were presumed to be very compatible with the α-amylase fold.

The overall process of selecting substitutions resulting in the most stable variant includes rational site-specific substitutions, recruiting changes from variants isolated from random mutagenesis and screening, and from the strategy of homologous recruitment described above. The common thread is that all diversity can be exploited successfully for clues as to how to make an enzyme better suited for a particular function and environment.

**Combinatorial and other methods**

The most recent advance in designing enzymes is the realization that, with advanced recombinant DNA technology, it is possible to entertain the possibility of accelerating evolution to a point where thousands of mutations can be created in successive generations that are subject to either direct selection or to indirect selection by way of
a rapid screening strategy. This approach has been termed ‘directed evolution’. To
date there are numerous examples in the literature when either enzyme functionality
or stability has been improved using this approach. Subtilisin E variants have been
produced to perform in radically different non-aqueous environments using
these techniques (Chen and Arnold, 1993). While these demonstrations may be
academically successful, what is required for an industrial success is the ability to
screen or select for relevant properties and conditions.

These techniques generally produce numerous incrementally beneficial variants.
A leapfrog technique that involves recombining the individual substitutions by shuf-
fling their DNA has also been exploited. All of these techniques share the common
element of exploiting the range of diversity found in homologous functioning
enzymes. We see that a multitude of different but homologous sequences produce
enzymes having a common tertiary structure and function. Different combinations
of substitutions from just a single amino acid substitution to substitutions ranging
over the entire molecule result in enzymes with detectable differences but which are
able to perform a common function.

It may well be an inherent property of natural enzyme structures to have a robust
tertiary fold that is tolerant of considerable sequence variation. Thus evolution is
allowed to sample a relatively broad range of variation, providing sufficient diversity
for enzymes better suited for specific environmental factors and functional
requirements facing a particular organism.

**Engineering Microorganisms for Enzyme Production**

**Classical mutagenesis**

Classical mutagenesis is one of the most powerful techniques used to increase enzyme
yields from microorganisms. Dramatic productivity improvements in the order
of tenfold, or even 100-fold, are common. Classical mutagenesis techniques were
originally developed by the pharmaceutical industry to improve the yields of
antibiotics. The same techniques are used to improve enzyme yields.

Classical mutagenesis techniques are not unlike the process of evolution. Both
require mutations in the DNA of an organism to bring about changes in genetic
traits. While evolution selects for traits that confer greater reproductive fitness upon
an organism, classical mutagenesis can select for traits chosen by the experimenter.
Cells or spores are treated with mutagens to increase the frequency of mutation above
the natural rate. The mutagens can include ultraviolet light, gamma ray irradiation or
chemicals reactive with DNA. After treatment with mutagens, a diverse population
of cells is obtained. A screening process is used to identify cells having mutations
conferring desirable traits. The enzyme productivity of a microorganism is a trait that
can be altered through mutagenesis. While many mutations are detrimental or lethal
to an organism, a few can lead to increased enzyme productivity.

Classical mutagenesis was used to improve the yields of α-amylase enzyme
production by the bacterium *B. subtilis*. The enzyme α-amylase is used for liquefying
maize starch for the production of high fructose maize syrup and for increasing the digestibility of maize starch in animal feed. However, natural isolates of *B. subtilis* produce very low levels of α-amylase. Classical mutagenesis and screening of a wild-type strain resulted in the isolation of a more productive and thus commercially viable strain. Figure 13.5 shows a comparison of the wild-type strain and the improved mutant strain growing on a nutrient agar supplemented with insoluble starch. Degradation and clearing of the starch provides an indication of the presence of α-amylase. Large clear haloes surrounding the cells of the improved mutated strain indicate that the cells are producing and secreting α-amylase. Practically no halo is apparent surrounding the wild-type cells, indicating low levels of α-amylase production.

**Genetic engineering to alter enzyme expression**

Genetic engineering allows the *in vitro* manipulation of DNA with the ability to reintroduce the modified DNA back into host cells. The genetic elements of genes as well as their regulating elements can be altered to achieve changes in enzyme expression. The typical bacteria or fungi used in enzyme production can have between two and 10,000 genes. The bacterium *B. subtilis* encodes its genes in 4.2 million bases of DNA (Kunst *et al*., 1997) on one chromosome (one continuous length of DNA). The fungus *Aspergillus niger* uses approximately 35 million bases of DNA to encode its genes (Debets *et al*., 1990) on eight chromosomes. Each gene specifies the amino acid composition of a protein. One gene confers the sequence for

![Fig. 13.5. Comparison of α-amylase production from Bacillus sp. strains growing on starch agar. The plate on the left shows an industrial overproducing α-amylase strain generated using classical mutagenesis; the plate on the right shows a wild isolate strain. Haloes surrounding the colonies result from α-amylase hydrolysis of the insoluble starch.](image-url)
one protein. The length of a typical bacterial enzyme gene, encoding a protein of 300 amino acids, is in the order of 1000 nucleotides. In addition to segments of DNA that encode genes, there are also segments of DNA called promoters that control the function of genes. Only a fraction of the genes of an organism are ‘on’ at any given time. Coordination of the expression of genes is controlled by promoter elements. These regulatory segments of DNA can be thought of as light switches for genes, able to turn a gene on, off or to a state in between.

A key breakthrough in the 1970s that made genetic engineering possible was the discovery of specialized enzymes called restriction endonucleases. These enzymes are capable of cutting DNA in precise locations, in effect acting as molecular knives. Another group of enzymes, called DNA ligases, can join cut ends of DNA together, in effect acting as molecular splicers. Segments of DNA can thus be excised from chromosomes and recombined.

Genetic engineering requires the ability to transfer recombinant DNA into a host organism, a process referred to as transformation. The bacteria E. coli and B. subtilis can be transformed fairly easily: mixing cells with calcium chloride renders the cells competent (susceptible) to take up DNA. When genetically engineered DNA is added to such competent cells, the DNA adheres to the cell walls and is transported inside the cell. Though transformation of fungi is more complex, a method was found in the early 1980s to transform the fungus Aspergillus nidulans (Balance et al., 1983). Minor variations soon followed to allow transformation (and hence genetic engineering) of the fungi A. niger and Trichoderma reesei, both of which are frequently used for enzyme manufacturing.

To enable high expression levels of enzymes, DNA can be recombined into an overexpression vector. An overexpression vector is a specialized assembly of DNA that when placed into a host cell causes a specific enzyme to be produced (encoded) above normal levels. Several DNA elements are essential to create a suitable vector. An example of an overexpression vector is one that has been used for production of ferulic acid esterase A from A. niger (the enzyme is abbreviated as FAEA and the gene encoding this enzyme is abbreviated as faeA). FAEA (de Vries et al., 1997) can cleave the ester link between ferulic acid and arabinose present in the cell walls of many feed substrates. For this overexpression vector (Fig. 13.6), recombination of four elements was required: a strong promoter glaA, the faeA gene, the glaA terminator and the pyrG selectable marker. The glaA promoter regulates the faeA gene. The glaA promoter is known to result in high-level expression of genes that it regulates, and for this reason was chosen for the vector. When the overexpression vector depicted in Fig. 13.6 was transferred into an A. niger host, production of the FAEA enzyme increased significantly.

The selectable marker gene allows for a way to determine if a host organism has been transformed with the vector DNA. Before transforming the vector into the A. niger host strain, the A. niger cells are initially mutated to make the native pyrG gene non-functional. The pyrG gene produces an enzyme required to synthesize the DNA building block, pyrimidine. If pyrimidine cannot be made, the cell cannot synthesize DNA, which abolishes any possibility of cell growth. After transformation with the vector, the cells are placed on selective nutrient media that lacks any pyrimidine. In
order to grow, cells must take up the overexpression vector DNA containing a pyrG gene. Those cells that take up the vector can synthesize pyrimidine and DNA.

**Genetic engineering to delete enzyme genes**

The use of genetically engineered overexpression vectors does not prevent non-target enzymes from also being produced by a microorganism. Ideally a production microorganism maximizes the expression of the enzyme of interest while minimizing production of inconsequential or deleterious enzymes such as proteases or enzymes with interfering activities. Deletion of genes coding for undesirable enzymes can be performed when necessary to prevent their production.

*Trichoderma reesei* is a filamentous fungus found in warm moist environments growing on wood. The original isolate was put through classical mutagenesis and screening programmes which resulted in higher-producing cellulase strains. However, *T. reesei* produces at least six different cellulase enzymes, each with its own unique enzymatic properties. Additionally, these enzymes are not produced at equal ratios. Typical *T. reesei* cellulase strains produce an enzyme mixture consisting of approximately 40–60% cellobiohydrolase I (CBHI), about 5–20% each of cellobiohydrolase II (CBHII), endoglucanase I (EGI) and endoglucanase II (EGII) and less than a few percent of EGIII (see Fig. 13.7, lane B). While this enzymatic ratio may be useful for
T. reesei to convert the cellulose in plant material to glucose as an energy source, it is problematic for applications where only a single cellulase enzyme is required.

In order to tailor the cellulase enzyme composition to a specific application, a strain of T. reesei was genetically engineered to prevent the production of CBHI, CBHII, EGI and EGII. Deletion of the genes that encode for the non-desired cellulase enzymes resulted in an altered enzyme profile. An example of the gene deletion method, using the \( cbhI \) gene as a model, is illustrated in Fig. 13.8. The \( cbhI \) deletion vector DNA cannot replicate independently upon entering the cell but must integrate itself into an existing chromosome, otherwise the vector DNA will not be replicated. This integration can happen at random. However, since identical DNA has an affinity for itself, the deletion vector was made with the selectable marker, the \( pyr4 \) gene, flanked on either side by \( cbhI \) DNA. The \( pyr4 \) gene is used as a selectable marker in the equivalent manner that the \( pyrG \) gene is used in A. niger. The \( cbhI \) portions of the vector can then recombine with the \( cbhI \) portions of the chromosome of the host strain, resulting in the replacement of the \( cbhI \) gene with the \( pyr4 \) gene. Upon deletion of the \( cbhI \) gene, production of the CBHI enzyme is not possible. Thus, presence of the selectable marker can be used to identify those cells that no longer contain the \( cbhI \) gene.

**Fig. 13.7.** Progression of genetically engineered T. reesei strains. The SDS-PAGE gel shows the fermentation products produced by a series of strains starting from the parent production strain and ending with the EGIII overproducing strain. Lane A, molecular weight markers; B, parent strain; C, double deleted strain (Dcbh1, Dcbh2); D, quad deleted strain (Dcbh1, Dcbh2, Deg11, Deg12); E, eg13 overexpression in quad deleted strain, late fermentation sample.
Deletion vectors were also made for the three other major cellulase genes: cellobiohydrolase II (\textit{cbhII}), endoglucanase I (\textit{eglI}) and endoglucanase II (\textit{eglII}). These deletion vectors were used to delete these three genes sequentially. The effect of gene deletions upon the enzymatic profile of various \textit{T. reesei} strains can be seen in a protein gel (Fig. 13.7). The protein gel (SDS-PAGE) separates proteins on the basis of size (molecular weight). Fermentation broth samples were taken from various genetically engineered strains of \textit{T. reesei}. When the \textit{cbhI} and \textit{cbhII} genes are deleted, the enzyme bands corresponding to these proteins (CBHI, CBHII) disappear (compare lanes B and C in Fig. 13.7). When the \textit{eglI} and \textit{eglII} genes are deleted, the EGI and EGII enzyme bands are no longer present (compare lanes C and D, Fig. 13.7).

The resulting deleted strain was further engineered to achieve higher production of endoglucanase III (\textit{eglIII}) using an \textit{eglIII} overexpression vector. This vector contained the strong \textit{cbhI} promoter to regulate high expression of the \textit{eglIII} gene. The vector was transformed into the strain that had been deleted for \textit{cbhI}, \textit{cbhII}, \textit{eglI} and \textit{eglII}. The resulting strain showed an enhanced production of EGIII as compared
with all the strains that preceded it (compare lane E with lanes B, C and D in Fig. 13.7).

Classical mutagenesis and the genetic engineering techniques of gene deletion and gene overexpression can result in dramatic changes in the enzyme production of a microorganism. By using such techniques, the enzymatic production of a microorganism can be tailored to fit specific applications needs.

**Fermentation Process – Design and Development**

The main goal of fermentation process development is to maximize the productivity and minimize the cost. Also, the process needs to be reproducible and must meet all regulatory, health and safety, and quality assurance requirements. Ideally, fermentation and strain development should take place in an integrated manner. Strain development must take into account the fermentation process design options and limitations, and the fermentation development must address critical strain requirements.

**Fermentor design**

Fermentors are operated in batch, fed-batch, or continuous mode (Fig. 13.9). They are controlled by monitoring and estimating several critical parameters, such as temperature, pH, dissolved oxygen, redox potential, respiration rate, foam level and mass transfer coefficient (Fig. 13.10). During batch fermentation, the production rate increases and then declines. This is often associated with the cell state: exponential growth, stationary or sporulating. In fed-batch fermentation, by careful design of batch and feeding medium, cell growth is regulated and production is maximized. During continuous culture, production is affected by continuous growth, repression, low or non-producing mutants, and dilution. A continuous cell recycle system decouples growth and production and maximizes cell density, which results in high productivity. If extracellular enzyme production under minimal growth is sustainable, then an immobilized viable cell fermentor design would obviate the need for cell separation and result in even higher productivity.

When cells are cultured on a rapidly utilizable carbon source (e.g. glucose and rich nutrients such as amino acids) the production of many enzymes is repressed. This is referred to as catabolite repression. Regardless of how catabolite repression is controlled inside a cell, either production strains must be able to make products in the presence of carbon/nitrogen in the fermentor, or fermentors must be operated under carbon/nitrogen limitation. The maximum rate of product synthesis of many extracellular enzymes occurs during the late exponential or early stationary phase of growth, as the cells become committed to sporulation (Doi, 1989). The use of sporulation-deficient strains is therefore common in the industry (Priest, 1977). For production, the default industrial mode of operation is batch/fed-batch fermentation, with production cycles ranging from 12 to 300 h. Production
Fig. 13.9. Fermentor modes.
maximization with this type of fermentation usually entails optimization of the strain, the medium, the carbon feed and the run conditions (temperature, pH, aeration, agitation, pressure). What exactly determines the limited period of production is not known. The impact of other control systems (e.g. related to nutrient starvation, cell cycle events) is likely to be involved.

Fermentation process design is a highly interdisciplinary effort, requiring integrated use of concepts and methodologies of both chemical engineering and microbial physiology, to accomplish scale-up. Fermentor 'scale' commonly refers to fermentor volume. Scale-up is a procedure for designing and building a large-scale system based on small-scale models. In practice, it involves the study of the effects of scale on physiochemical and biological phenomena in fermentation process design. Scale-up effects are more pronounced for aerobic (carried out in aerated and agitated vessels) than for anaerobic fermentations (Atkinson and Mavituna, 1991). Therefore, as a 'rule of thumb', in an aerobic fermentor, the constant oxygen transfer rate and dissolved oxygen concentration are generally maintained in the scale-up. While thermodynamic and intrinsic kinetic phenomena are independent of scale, momentum, mass and heat transfer are dependent on scale. For example, mixing, aeration and cooling are well controlled and uniform at the 10 l scale. However, not all transport parameters can be maintained in this manner at a greater than 10,000 l scale. Further scale-up complications arise from cell growth, adaptation and decay.
The development of entirely new processes or the improvement of existing processes requires the evaluation of a wide range of strains and cultivation conditions in a short period of time. Shake-flask fermentation studies have a cost advantage but lack the process control options (pH, nutrient addition, aeration). This often leads to use of laboratory scale (about 10 l size) agitated-aerated fermentors, with adequate instrumentation and control. Almost all fermentation processes can be translated to production scale by use of laboratory-scale fermentors. However, pilot-scale fermentors are often necessary for the downstream process scale-up.

**Process development**

A considerable amount of published information is available on laboratory-scale fermentation processes but commercial-scale process information is for the most part proprietary and is generally not published in detail. Mutation, genetic manipulation and strain selection contribute the most to the industrially improved processes.

Once the host of choice has been decided, development of a culture, which can be used to inoculate the seed flask, begins with the process of correctly storing and maintaining the organism. Usual methods include use of culture stocks stored: (i) in 10–20% glycerol or DMSO at liquid nitrogen temperature (−70°C); (ii) as freeze-dried cells; or (iii) on agar nutrient plates/slants kept refrigerated and transferred periodically. In the process of scaling up a fermentation from a flask to the production stage, a proper seed for inoculating the production fermentor needs to be generated. The requirement of the seed is to reach high cell densities in the pre-production fermentor so as to minimize the lag phase in the production vessel, without compromising the cells. A typical seed train for inoculation of a production fermentor is shown in Fig. 13.11.

Seed flask inocula are prepared from frozen, freeze-dried or plated stock cultures. Erlenmeyer flasks, containing nutrient broth designed for optimal growth, are first

**Fig. 13.11.** Production fermentor: typical seed train for inoculation.
inoculated with a stored culture and incubated on an orbital shaker for about 4–48 h. Both the time of transfer and the cycle stage of the cells are critical factors. The purpose of the seed is also to adapt the cells for growth in the production media. Therefore the seed medium must be designed based on the physiological needs of the organism.

All microorganisms have basic requirements, such as water, a source of energy, carbon, nitrogen, salts, trace metals and possibly growth factors. The challenge is to develop a medium and conditions that optimize growth, by meeting the organism’s basic needs, while producing the desired product economically. The media used to isolate and screen production hosts are not necessarily those that can be used in production fermentors. Development of an industrial process requires media components that provide carbon, nitrogen, phosphorus, potassium, magnesium, sulphur and trace nutrients. The carbon source also provides energy for synthesis of cell mass and product. The objective of fermentation-medium development is therefore to maximize productivity, minimize side-products and costs, and ensure steady product quality. Generally, large-scale fermentation media are made up of complex natural materials supplemented with inorganic/organic salts, alkali and acids. Development of a balanced fermentation medium is based on knowledge of physiology as well as empirical work. Medium development combines growth and production data obtained with agar plate, microtitre plate, shake-flask culture and fermentor studies. Statistical methods (Plackett–Burman, Box–Benkhen) are used for media screening and optimization (Monaghan and Koupal, 1990). The media and conditions used may change from shake-flask through the fermentor stages. Although many models have been developed for providing the basic needs of the organism (Pavlou and Fredrickson, 1989; Priest and Sharp, 1989; Arbige et al., 1993), the investigator is usually left to rely on either traditional recipes or media development based on stochiometric requirements (growth, maintenance and production) and statistical design of experiments.

When choosing raw materials for a production-scale process, many inexpensive nutrients are available that provide good sources of the basic requirements, and many contain inducers for product gene expression. Among these are soy flours, cottonseed meal, cornsteep liquor and yeast and protein hydrolysates for nitrogen sources; and glucose, molasses, starch, cellulose and peat for carbohydrate sources. The macro-elements can be added as the salts (e.g. magnesium sulphate, potassium phosphate, sodium phosphate) while the trace elements can be added separately as salts, or may be sufficiently available in the complex raw materials. Among metal ions, only potassium and magnesium are required as bulk salts, since these are involved mostly as osmo- and energy-regulators, while the other trace elements have more specific physiological roles (Hughes and Poole, 1989). Yeast extract usually contains all the metals required for growth, especially copper, iron, magnesium and zinc.

When designing media for product formation, many factors affecting protein expression and secretion need to be considered. In recombinant production hosts, vector selection plays an important part in determining the medium and the process. Also, when choosing a carbon source, the phenomenon of catabolite repression must be considered. Regulation of nitrogen and phosphorus metabolism is an additional
consideration. It has been observed on numerous occasions that some organic and inorganic nitrogen substrates have negative effects on exocellular protease production. While oligopeptides and proteins seem to be the inducers, salts and free amino acids seem to be strong inhibitors (Chu et al., 1992). Repression by amino acids appears to be due not to any particular one, but rather by the supply of a number of amino acids in sufficiently high concentrations. Production can also be affected by high levels of carbon. In amylase production, where the composition of the medium has a profound effect on product yields, the presence of phosphates can have a significant effect on enzyme production (Thirunavukkarasu and Priest, 1980). It has also been shown that production is enhanced by using more slowly metabolized carbon sources, such as starch or lactose, or by slow feeding of glucose to maintain a very low concentration.

When developing media formulations optimized for growth and production, one area often neglected is the elimination of unwanted by-products, either coming from a raw ingredient or produced via metabolism. Common metabolites produced by bacilli are acetate, propionate and the metabolites of the butanediol cycle. These acids not only represent a waste of carbon; they can also lead to inhibition of growth or even cell death at high levels of accumulation.

Preparation of the fermentor is the next step after designing the basic media for growth and production. Some general texts have been written on fermentor preparation (Rehm and Reed, 1993), but because of the advances in genetic, enzyme and pathway engineering, the investigator may also consider special requirements of the production host. Selection and regulation of ingredients can make the difference between a good and bad fermentation run. Another factor is the method of sterilization, which refers to the physical or chemical processes of inactivation or elimination of contaminants present in liquid and gas phases before the fermentation takes place. Besides the initial sterilization procedure, it is also important to prevent contamination during the run. There are several conditions that affect the probability of contamination: temperature, pH, sugar concentration, defined/complex nutrients, oxygen, back pressure, osmotic strength, and antibiotics present in the fermentor (Bader, 1986). Typical fermentation media can support contaminating moulds, yeasts and bacteria but generally fermentations are more susceptible to contamination by spore-forming bacilli and bacteriophages. The sterilization kinetics vary dramatically between vegetative cell and bacterial spore, and between dry and wet heat sterilization. The lower the initial contaminants and particulate matter, the more effective the sterilization procedure. Good housekeeping helps in lowering the bioburden and eliminating difficult-to-sterilize residues. Steam sterilization, as opposed to dry heat, is often preferred because of more efficient heat transfer and higher reactivity, resulting in greater cell kill, most probably by rapid denaturation of cellular protein. A rule of thumb is that all surfaces be exposed to saturated but not superheated steam, for time periods of 10–60 min at 121°C. These conditions are sufficient to inactivate all organisms and heat-resistant bacterial spores. Germination and pasteurization (heating to 63°C for 30 min, or 80°C for 15 s) can also inactivate heat-resistant spores of the Bacillaceae family. On the other hand, phage contaminants are difficult to contain and tend to spread rapidly throughout the
plant. The only effective way to prevent phage attack is to develop resistant cultures. In addition to sterilization, high-solids media are sometimes pretreated by enzymes or acid to avoid mass transfer limitations before seeding the fermentor with cells, since the cells utilize salts and trace nutrients only if available in the right form. As the medium components are utilized by the cells, the broth rheology changes, CO₂ is produced and foaming occurs, making the use of anti- or de-foaming agents necessary. Besides sterilizing all liquids and surfaces in contact with fermenting cells, it is equally important to sterilize the air used for cultivation. Air is first compressed to increase its pressure to as high as 100 psi, resulting in temperatures up to 260°C. Heat of compression reduces contaminants, including heat-resistant spores and phages, but is generally not solely relied upon for sterilization. So, filtration is considered the primary method of sterilization of dry air. The standard air ‘inlet filter’ is membrane based. These membranes are able to eliminate spores as well as cells, and are also able to withstand steam sterilization conditions.

Once the media handling and fermentor sterilization are completed, and the process variables are optimized, the performance of the host production strain in its controlled environment is studied. At this stage, the primary goal is to obtain the maximum biomass or product per unit of substrate in the minimal amount of time. Optimizing growth yields while maintaining cell viability is key to the success of the process development. Avoiding nutrient and O₂ depletion and understanding by-product formation are also important for maximizing the yield and minimizing stress responses.

**Downstream Processing and Formulation of Enzymes**

The overall goal of downstream processing and formulation is to recover the enzyme of interest cost effectively at sufficiently high yield, purity and concentration, and in a form that is stable, safe and easy to use in a target application.

The variety of separations technologies available is large and the possible order and permutation of sequential processing steps is vast. Several general considerations, however, limit the range of practical choices for a process design (Becker, 1995). Intracellular and membrane-bound enzymes are more difficult to recover than those that are secreted, requiring physical or chemical disruption of cells and the challenge of separating the enzyme from viscous or entraining substances such as nucleic acids and cell wall materials. Fortunately, xylanases, proteases, amylases, cellulases and phytases – virtually all feed enzymes of commercial interest today – are extracellular, allowing for a cleaner first-stage separation. Most microbial fermentations are batch or fed-batch, and are typically not harvested until the cell mass, enzyme and other solids are quite concentrated, often requiring some post-harvest dilution to avoid entrainment losses and low fluxes.

Typically, very high purity is not required of feed and food enzymes as it would be of, say, human pharmaceutical proteins for injection. On the other hand, raw materials must be food grade or otherwise shown to be safe and suitable, and new enzyme formulations must be tested for toxicological properties and shown to be safe.
for the intended use. Finally, economic considerations constrain both the range of raw materials and separation processes that can be used. While multiple chromatographic steps are routinely included in the downstream processing of human therapeutic proteins, few feed or food enzymes include even a single chromatographic step. Purification, if any, is accomplished by less expensive techniques such as extraction or crystallization, as described below.

**Harvest and cell separation**

In general, cells should be removed from a fermentation broth within hours after harvest in order to prevent cell lysis. After cell separation, the clarified fermentation broth is usually stable and can be stored refrigerated for days. Upon harvest, the broth may need to be cooled, pH adjusted, and certain stabilizers added in order to minimize enzyme degradation. For the more labile enzymes such as proteases, control of temperature, pH, oxidants, inhibitors and activators will be important throughout the recovery process. For example, it is important to maintain a molar excess of calcium ions to ensure the thermal stability of certain Bacillus proteases and amylases that contain calcium-binding sites (Lloyd et al., 1970; Letton and Yunker, 1982). If minor proteases and other side activities cannot be deleted from the strain itself, finding ways to remove or neutralize them early in recovery becomes imperative.

Cell separation is usually accomplished by means of either depth filtration or centrifugation. Fungal fermentations, such as those of *Trichoderma* or *Aspergillus*, lend themselves particularly well to cell separation by means of filtration through a rotary drum vacuum filter (RDVF) because of the ease with which fungal mats can be thinly shaved off across the drum’s knife, renewing the filter cake surface to maintain high filtration flux. High-capacity filter presses can be a reasonable alternative to RDVFs. Bacterial fermentation broths can usually be processed by either filtration or centrifugation, but the much smaller size of bacteria generally requires the addition of a polymeric flocculant. Cell flocculants are generally cationic, and function by bridging the negative surface charges on neighbouring cells to increase the particle size and facilitate either sedimentation rate or filtration flux and clarity. In addition, the choice of flocculant and optimization of dosage is usually a delicate balance between considerations of separation quality, yield, cost and minimization of residual excess polymer in the product.

The main challenge in centrifugation is to find flocculant and machine-operating conditions such that cell mass or sludge is easily conveyed or discharged from the centrifuge bowl without break-up or carry-over in the centrate. While centrifugation can handle a high concentration of cell solids, filtration may provide more complete removal of the most finely suspended solids, which can interfere with downstream concentration or purification steps. On the other hand, the presence of high solids and sometimes slimy polysaccharide fermentation by-products can limit filtration fluxes without the judicious use of filter aids – usually diatomaceous earth, perlite, or other mined materials. The presence of flocculants and filter aids in cell wastes often gives rise to added disposal costs.
Microfiltration – the use of tangential flow anisotropic membranes to permeate enzyme while retaining suspended solids – would appear to be an attractive cell separation technique because it does not require the use of flocculants or filter aids. However, microfiltration yields are typically low due to progressive fouling of membranes. Equipment and membrane replacement costs are currently higher than capital and operating costs of filters and centrifuges of equal production capacity. Clarified filtrates or centrates are usually too dilute for use in feed applications, so substantial amounts of water must be removed.

Concentration

Industrial concentration methods, such as evaporation and solvent extraction, are unsuitable for dewatering enzymes because of their potential for thermal or chemical denaturation, and evaporation gives rise to high energy costs. Chromatography is generally uneconomical for concentrating feed enzymes, for reasons detailed below. The most common concentration technique in use today for industrial enzymes is ultrafiltration (UF), using hydrophilic tangential flow membranes with molecular weight cutoffs in the range of 10,000–100,000 Da. UF fluxes and yields are often significantly enhanced by removal – or omission – upstream of potential membrane foulants such as certain polysaccharides or anti-foams. Precipitation, crystallization and extraction can also be used for concentration, but are more typically utilized as purification techniques, and therefore will be discussed below.

Purification, decolorization and finishing

The main ‘purity’ consideration for feed enzymes is not the removal of proteinaceous impurities, but rather ensuring that only food-grade raw materials and non-pathogenic, non-toxigenic production organisms are used. In some cases, an undesirable side activity must be removed through some means of purification, particularly when it is not feasible to delete the side activity genetically. Even if minor proteases co-secreted by the host organism do not cause significant degradation of the product enzyme during processing, they are more likely to wreak havoc over several months of storage in the final product formulation. In other cases, side activities must be removed because they interfere in the ultimate application. Although chromatography offers the greatest potential and diversity of mechanisms for fractionation, it is usually not cost effective in feed enzyme manufacture due to the expense and low protein-binding capacity of most chromatography resins, and the complexity and control required for reproducible operation.

Purification can often be accomplished with surprising effectiveness by two of the oldest known protein purification technologies: fractional precipitation and crystallization. Fractional precipitation is most useful when the protein of interest is only one of several enzymes or other proteins present. Chaotropic salts, such as ammonium sulphate, or water-binding polymers, such as polyethylene glycol, are
added in increments to a concentrate containing the enzyme, and those fractional ‘cuts’ are combined that have the most favourable balance of net enzyme purity and recovered yield. When a higher level of purity is desired than can be attained through fractional precipitation, crystallization is an attractive purification technique. To the surprise of those unfamiliar with it, it is also a highly scalable, reproducible and cost-effective technique (Becker and Lawlis, 1991; Visuri, 1992). The purity attainable is comparable to what can be achieved through multiple chromatographic steps. The major challenge is the often time-consuming screening of precipitants, pH and temperature optimization, and mapping of the solubility phase behaviour. Once the development work is completed, however, the process can be linearly scaled to multiple tonnes. It should be noted that highly pure forms of certain proteases and other enzymes are in some cases less stable than the impure forms, which often contain stabilizing peptides and other impurities that inhibit degradation.

In the last one to two decades, aqueous two-phase extraction has become an attractive concentration technique. It provides the selectivity of classical solvent extraction without its denaturing potential. Utilizing incompatible two-polymer and polymer–salt combinations and adjustments in pH and ionic strength, this technique separates proteins based on differences in hydrophobicity, surface charge and molecular weight. One outstanding application of the technique has been the purification of genetically engineered chymosin from multiple side activities produced by the fungal host (Heinsohn et al., 1992).

Decolorization is sometimes preferred in certain applications, mostly as an aesthetic preference. Colour can often be minimized by choice of fermentation medium components and control of the sterilization cycle so as to minimize the browning reaction between nitrogen and carbon sources. Colour can also be reduced by treatment with activated carbon, use of antioxidants, and diafiltration through membranes.

‘Finishing’ refers to one or more filtration steps at the end of downstream processing which have the goal of clarifying and reducing the microbial load of the product. Solids are most conveniently removed using a filter press loaded with cellulosic pads, or precoated with filter aid; disposable submicron-gauge filter cartridges can be used to remove microbes, to the point of sterility if required.

**Liquid formulation**

The majority of feed enzymes are sold as formulated liquid concentrates, which may be further formulated as liquid or solid products suitable for the end-user. The major requirements for a liquid formulation are enzymatic stability and preservation against microbial growth. It is sometimes not appreciated that the dominant factor affecting enzyme stability is the intrinsic stability of the enzyme itself; formulation can do very little to correct for a structurally labile protein. It is advisable, therefore, to make stability an important criterion of the initial screening process.

As most feed enzymes are hydrolases, they are subject to the three principal means of deactivation: denaturing or unfolding, catalytic site inactivation, and proteolysis (Becker et al., 1997). Denaturation is best minimized by controlling
temperature and pH and avoiding the presence of chemical denaturants. Catalytic site inactivation is prevented by supplying sufficient levels of cofactor, typically a metal cation, and preventing oxidation of the active site – for example, by formulating with antioxidants. Alternatively, oxidative resistance can be enzyme engineered into the protein structure. Finally, proteolysis – or autodigestion, in the case of proteases – can be minimized by upstream deletion of proteases, by reducing water activity through the use of water-sequestering compounds, or by addition of inhibitors. Useful water-sequestering compounds include sugars and other polyols, such as glucose, glycerol, sorbitol and propylene glycol. Useful inhibitors include substrate analogues such as peptides and acid salts.

Microbial growth is fairly easy to prevent by the addition of food-grade antimicrobials such as sodium benzoate, methyl paraben and various commercial preparations. The prevention of precipitation and hazes is often a highly empirical challenge and one that is very specific to the enzyme and the specific process raw materials used. The best advice is to use ingredients with high solubilities, and to screen potential formulations by extended storage at high and low temperatures.

**Solid formulation**

Most feed enzymes are supplied as liquid formulations at the end-user level, as these are convenient to use. It is mostly dry enzyme premixes that are being sold to the feed manufacturer. However, solid formulations can provide some significant advantages, such as enhanced stability, delayed or controlled release, and protection against deactivation during harsh applications. One example of the latter is the use of granules to encapsulate enzymes against deactivation in the steam pelleting process. The use of effective stabilizers and coatings can prevent or retard exposure to moisture under high heat, yet allow release of the enzyme in the subsequent feed application.

Dry enzymes, in most industries, are almost universally supplied as encapsulated granules in order to meet strict industry standards for control of airborne dust. The primary technologies for enzyme granulation include prilling, extrusion and spheronization, high-shear granulation, and fluidized-bed spray coating. The latter two techniques are superior in producing low dust granules. The advantage of the fluidized-bed method is that the entire granulation process can be carried out within a single, contained piece of equipment. In addition, the spray-coating process involved in this method allows the sequencing of layers of different thicknesses and compositions with almost infinite flexibility.

**Regulatory and Environmental Aspects**

Along with the transfer of the fermentation, recovery and formulation processes to the manufacturing plant, validated quality control methods and assays, which are suitable for assuring product quality, must also be provided. Various agencies throughout the world are involved in the regulation of enzyme products for feed. In
the USA, all ingredients used in feed, including silage ingredients, need to be listed in the official publication of the Association of American Feed Control Officials (AAFCO Manual), and registered with the states in which they are sold, as well as tonnage fees paid to the states. Any enzyme not listed in the AAFCO Manual requires an assessment of safety and efficacy by the Center of Veterinary Medicine at the Food and Drug Administration (FDA), who will then recommend listing in the Manual. In the EU, all enzymes require submission of a full dossier and specific approval by trade name. Although the regulations can vary from country to country, in general most require (Wohleben et al., 1993; Chapman, 1996): (i) the use of well-characterized non-pathogenic non-toxigenic organisms; (ii) good manufacturing practices (GMPs) involving well-documented, safe and reproducible fermentation and recovery processes in which only approved raw materials are used; (iii) the absence of toxins or pathogens; and (iv) low levels of heavy metals or other contaminants. Not only are genetically engineered organisms well characterized; they have also been engineered for poor survival outside a controlled environment. This, along with the fact that recombinant organisms are grown under contained good industrial large-scale practices (GILSPs), minimizes the safety issues relating to environmental release. Biotechnology has also had a favourable environmental impact compared with traditional techniques. For example, enzymes derived from recombinant organisms usually have higher fermentation yields and therefore reduce the consumption of raw materials used for their production.

Conclusions

Advances in biotechnology over the past few decades have had an immense impact not only in the areas of therapeutics, pharmaceuticals and health care but also in industrial, food and feed applications. Recombinant DNA technology has proved to be an effective means for increasing the production of enzymes, including foreign or minor enzyme components, and for generating tailored enzyme compositions for numerous applications. Recent breakthroughs in screening methods and genomics have allowed greater and faster access to unique enzymes from culturable as well as unculturable organisms. The ability to genetically engineer additional features into these naturally occurring enzymes and to accelerate the evolutionary process in a directed manner has provided routes to the design of unique enzymes that are tailored to a specific application. These technologies, coupled with the development of well-characterized generic hosts and optimized and controlled fermentation, recovery and formulation processes, have assured a reliable flow of safe, stable and cost-effective products.

References


Introduction

Enzymes have found widespread application in those countries where animal diets are based principally upon wheat, barley, oats, triticale or rye. The rapid commercial uptake of this new technology in recent years has resulted from extensive research, yielding a greater understanding of the anti-nutritive properties of these cereals and of the enzyme types and dosages required to reduce their negative effects. This in turn has stimulated the commercial availability of a number of feed enzyme products that can offer consistent and economic benefits to the user.

All animals use enzymes in the digestion of food. These are produced either by the animal itself or by the microbes naturally present in its digestive tract. However, the digestive process is less than 100% efficient. Therefore, the supplementation of animal feeds with enzymes to increase the efficiency of digestion can be viewed as an extension of the animal’s own digestive process.

Recent developments in feed processing technology have resulted in increased processing temperatures (e.g. expanders). This has provided benefits such as improvements in feed hygiene, production efficiency and pellet durability. However, the increases in processing temperature have also focused attention on thermostability of some of the more heat-sensitive feed ingredients, such as vitamins, amino acids and enzymes. This has presented an interesting dilemma for the feed industry in striking a compromise between what these ingredients can endure and what process conditions will allow. The situation is confounded by the fact that there are no set standards worldwide for feed processing. In effect there are almost as many variations in feed conditioning retention times, temperatures and pellet die sizes as there are feed mills (Pickford, 1992).

Enzymes are proteins and can therefore be susceptible to degradation by external factors such as pH, hydrothermal conditioning and frictional forces and by the heavy metals that are added to certain animal feeds. Stability of granular enzymes can be improved through the selection of activities with inherent high thermal stability, selection of specific carriers and through novel manufacturing techniques such as coating. Consequently, some granular feed enzymes have been shown to maintain...
efficacy after exposure to conditioning temperatures of up to 90°C. However, where processing conditions exceed this temperature it is generally advisable to apply enzymes as liquids, post-pelleting, thereby avoiding exposure to high temperatures.

**Application of Liquid Enzymes**

**Use of enzymes in the feed mill**

As various pressures mount on the global animal feed industry, so the demand to produce ‘hygienic food’ for farm animals has increased. This has resulted in the adoption of new processing technologies that have elevated processing temperatures beyond those achieved with conventional steam conditioning. The expander is one such example of a new processing technique. Typically, expanders are capable of processing feed at 105–130°C and at pressures of 10–40 bar (145–580 psi). Hydrothermal processes – expanders or extruders in particular – are often regarded as, at best, neutral or even potentially destructive to certain feed additives (Pickford, 1992).

As feed-processing temperatures increase in areas of the world where viscous cereals are employed, so the intestinal viscosity of the targeted animal (broiler, layer, etc.) fed untreated diets will increase (Table 14.1). The application of enzymes addresses this dilemma, although when feed-processing temperatures exceed 90°C the application of heat-sensitive additives such as enzymes, vitamins, probiotics and certain antibiotics, etc., favours liquid addition at the end of the manufacturing process.

**Addition of liquid enzymes**

A major consideration for successful use of liquids is the method and accuracy of dosing. Whilst other micro-ingredients, such as amino acids, are routinely

<table>
<thead>
<tr>
<th>7–28 days</th>
<th>Pelleted</th>
<th>Expanded and pelleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liveweight gain (g)</td>
<td>1042&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1060&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Feed : gain&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.37&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>1.34&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>Foregut viscosity (mPa s)</td>
<td>6.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.3&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>(CV)</td>
<td>(22)</td>
<td>(13)</td>
</tr>
</tbody>
</table>

<sup>a–c</sup>P < 0.05.

<sup>1</sup>Feed intake corrected to dry matter.

60% wheat, 20% soybean meal, 5% sunflower oil; pelleting = 75°C, expanding = 105°C/Amandus Kahl; batch-type liquid dosing system.
incorporated as liquids, they are dosed directly into the mixer as opposed to post-pelleting. Dosing into the mixer is a relatively simple process typically involving only a dosing pump, flow meter and spray nozzle. Some micro-ingredients, such as flavours/spices, are sprayed on to pelleted feed via low-cost and relatively inaccurate spray systems. However, the relative value of these products and importance of dosing accuracy is typically low compared with feed enzymes. Feed enzymes require a different approach due to the role they play in the feed formulation and the nutritional value they add to the feed. Ineffective application of the enzyme to the feed can result in an energy-deficient diet leaving the feed mill, and subsequent poor performance of the targeted animal.

There are two recognized positions within the feed manufacturing process for the application of enzymes: in process (post-pellet sieving) or at bulk outloading as the finished feed is loaded into the delivery truck.

The advantage of the installation in the loading line is that only one post-pelleting application (PPA) device is required, though it must be correspondingly large. In the other case, several spray systems are necessary, depending on the number of pelleting lines. Application directly before loading additionally offers the option of manufacturing compound feed of one particular composition in large quantities and then adjusting it to individual customer wishes directly prior to loading. Furthermore, unwanted dispersal or cross-contamination with critical components such as liquid medication can be largely ruled out in this way. This simplifies the production process and improves accuracy (Schwarz, 1998).

Application of Enzymes in the Feed Manufacturing Process

In any post-pelleting liquid application, we must first consider the upstream and downstream processing equipment and how it operates. We must also consider the maximum processing capacity, which will not necessarily be the pelleting capacity. These two factors are essential to the successful design and operation of the equipment.

Today, many feed mills have installed counter-flow coolers. These coolers operate in a semi-continuous mode, retaining the product in the cooler for a predetermined time, then discharging the product by opening the discharge gate, allowing product to flow en masse from the cooler. This discharge rate will be higher than the output capacity of the pellet press. Therefore, if a PPA system is installed to match the pelleting capacity, the plant will be bottlenecked at the PPA system, due to the reduction in capacity at this point in the process. In order to overcome this problem, the capacity of the PPA system should be calculated against the maximum transfer capacity (volumetric flow rate × maximum feed bulk density) of the mechanical handling device immediately below the cooler. If the vertical cooler is still employed, the feed rate from the cooler is consistent with the pelleting capacity. It follows, therefore, that if the pelleting capacity changes then this upstream fluctuation will affect the operation of any downstream processing equipment unless adequate precautions are taken.
Another critical aspect in the effective application of liquid enzymes is the location of the PPA system. If the feed is to be screened, for the removal of fines, before being transferred to the finished product bin(s), then it is essential that the application of the enzymes be applied to the feed post-screening. If not, it has been demonstrated that 24–50% of the enzyme activity can be recovered from the fines, which typically amounts to between 5 and 10% of the feed (Gunther et al., 1997; van Laarhoven, 1998). Fines will be particularly high in enzyme activity, due to their large surface area and absorptive capacity. If these fines are removed from the finished product by sieving after the PPA of the enzymes, then the enzyme activity will return to the pelleting system for reprocessing. As the thermal stability of liquid enzymes is far less than their granulated equivalents, then most of this activity risks denaturation during subsequent re-processing of the feed. This will have a major influence on the overall economics of enzyme use.

From Table 14.2 it can be seen that the xylanase activity level of the fines is more than three times higher than the xylanase activity in the pellets. The fines are formed by attrition of the pellets and it can be concluded from these experiments that the absorption of the enzyme into the centre of the pellet is very limited, with the result that the enzyme activity is confined to the outer layers (Engelen, 1998). Consequently the better the pellet quality, in terms of durability and hardness, the higher will be the enzyme recovery during subsequent enzyme analysis.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Xylanase activity in pellets (U kg(^{-1}))</th>
<th>Relative proportion of pellets and fines (%)</th>
<th>Total xylanase activity in a sample (U kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pellets</td>
<td>Fines</td>
<td>Pellets</td>
</tr>
<tr>
<td>A1</td>
<td>6210</td>
<td>19,580</td>
<td>89.6</td>
</tr>
<tr>
<td>A2</td>
<td>6410</td>
<td>19,260</td>
<td>89.8</td>
</tr>
<tr>
<td>A3</td>
<td>6380</td>
<td>19,720</td>
<td>85.5</td>
</tr>
<tr>
<td>A4</td>
<td>5960</td>
<td>19,690</td>
<td>88.3</td>
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<td>A5</td>
<td>5430</td>
<td>19,550</td>
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<td>A6</td>
<td>7480</td>
<td>19,670</td>
<td>92.7</td>
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<td>A7</td>
<td>5480</td>
<td>19,240</td>
<td>94.3</td>
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<td>A8</td>
<td>5995</td>
<td>19,800</td>
<td>91.5</td>
</tr>
<tr>
<td>A9</td>
<td>5490</td>
<td>19,470</td>
<td>91.3</td>
</tr>
<tr>
<td>A10</td>
<td>5720</td>
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<tr>
<td>Average</td>
<td>6056</td>
<td>19,512</td>
<td>91.1</td>
</tr>
<tr>
<td>STD</td>
<td>587.5</td>
<td>216.0</td>
<td>–</td>
</tr>
<tr>
<td>CV</td>
<td>9.7</td>
<td>1.1</td>
<td>–</td>
</tr>
</tbody>
</table>

1. Water activity of feed 0.86 at 25°C.
2. Pellet quality: Holmen 76.4%; Kahl 2.0 ± 0.5.
3. Xylanase is sprayed on the feed in a batch mixer and the resultant pellets and fines are analysed separately for enzyme activity.
Eliminating fines from the feed-manufacturing process may be virtually impossible, but there are devices on the market, in addition to the pellet sieve, to improve pellet quality. Although pellet quality will not directly improve the efficiency of the PPA system, it will reduce the analytical variation of the assay by reducing the percentage of fines in the final sample.

**Post-pelleting Application of Liquid Enzymes**

**Measurement and control of the liquid and dry flow rates**

The accuracy of any liquid application system is based upon a number of essential factors; the most fundamental of these is the measurement and control of the liquid and dry flow rates. To achieve high homogeneity of application (low coefficient of variation) and precision dosing of the enzyme on the feed it is essential to control, and not simply measure, the dry flow rate. This is because fluctuations in the dry flow rate can result from upstream processing equipment, changes in manufacturing capacity, variations in formulation, etc. Without the necessary control logic to manage these immediate changes, long feedback times will render any advantages expected from measuring the dry flow an expensive exercise.

There are principally two methods for controlling the dry and liquid flow rates, namely by volumetric or gravimetric means (Figs 14.1 and 14.2). With the volumetric system, both the liquid and dry elements are dosed independently of each other. No control or adjustment is made for variations in specific gravity or bulk density. Therefore, both elements are dosed on a theoretical capacity. Accuracy of the volumetric system is based upon the variations in the manufacturing process for the pellets (bulk density and product form) and repeatability of the dosing equipment for the liquids, plus any changes in specific gravity of the liquid.

The gravimetric method of measurement is when both the liquid and dry elements are gravimetrically weighed or measured in mass terms (mass/time). The dry flow can be measured in a number of different ways, e.g. impact weigher, belt weigher, batch weight, etc. The liquid flow rate has fewer options for the gravimetric measurement of the flow, being limited to the batch weigher in the case of a batch system or as a loss-in-weigh system for measurement of presumed use. The more common option is the use of a mass flow meter based on the Coriolis measuring principle.

The major benefit of the gravimetric (mass rate of flow) system has to be the accuracy that is achievable. With the gravimetric system, both the dry flow and liquid flow rates are gravimetrically measured, with the flow rate being equated to mass. If the flow rate of either the dry or liquid changes, then the corresponding change is made to the other component to offset any inaccuracies. This type of system is not affected by changes in bulk density or specific gravity, flow rates or flow profile, although the more constant the flow rate, the greater is the degree of accuracy. Economically the level of automation needed for such systems makes it feasible to incorporate a number of 'production aids' to the benefit of the feed mill operator.
and mill manager. It also makes it possible to link the application control system with the mill formulation computer so that the required inclusion rate for a specific formulation can be set by the nutritionist within the formulation computer.

Volumetric systems are lower in specification and therefore lower in cost. For the same reasons the installation of the equipment is much simpler. This type of system comprises a dosing pump without the use of a flow meter and relies solely on the calibrated displacement and repeatability of the dosing pump to deliver a known volume of liquid. The other component of the volumetric system is the volumetric
The major deficiency of the volumetric system is the non-relationship between the two flow rates, dry or liquid, i.e. if the capacity of pellet flow increases, the liquid flow rate does not. It is relatively easy, therefore, to appreciate how the capacity of this type of system can change when manufacturing a different product form or simply a change in bulk density or specific gravity of the liquid.

Fig. 14.2. Typical mill schematic showing the arrangement for a volumetric system.
Application System Types

The three commonest systems for the application of liquids are the semi-continuous, continuous and batch type.

Semi-continuous system

The semi-continuous system is the most commonly adopted system for the post-pelleting addition of liquids. This type of system was introduced for economic reasons, balanced by its flexibility and accuracy. Initially used as a volumetric system for the addition of fat, in recent years the system has been further developed to incorporate gravimetric measurement for the addition of aqueous-based liquids.

The installation of a surge hopper to accumulate the flow of dry product will eliminate any flow fluctuations from upstream processing equipment. The hopper has sufficient capacity to accommodate a volume of product, predetermined to maximize the operation of the dosing system, therefore limiting the frequency with which the complete system has to stop and start. The hopper is fitted with a discharge device that is automatically sequenced to operate by the level of the product in the surge hopper. This is achieved by the inclusion in the hopper of a working-level and low-level probe. The control logic of the system is that when the low level and then the working level are covered by product, the discharge device will operate. When the
working level and then the low level are uncovered, the discharge device will stop. The position of the low-level probe is such that a residue of product will always remain in the feeder and the surge hopper. This mode of operation ensures that when the discharger next operates the volumetric discharge capacity is as the previous operation, eliminating any surging effect.

Advances in technology have seen the introduction of the gravimetric weigh feeder. Although the system operates in the same way as detailed above, the feeder is suspended on a fulcrum immediately under the outlet of the surge hopper. The opposing end of the feeder is suspended on a load cell, which measures the product flow through the feeder.

The volumetric feeder ensures a constant volumetric flow rate from the feeder without compensating for changes in bulk density. This can be a distinct disadvantage if a number of different products are to be manufactured. However, it is possible to compensate for these changes, either manually or automatically, if the changes in the bulk density fall within a range. Manually, the mill operator can adjust a feed gate, precalibrated, at the feeder inlet, and the capacity of the system can be altered by either increasing or decreasing the bed-depth in the feeder. The same can be completed automatically by the use of a variable speed drive fitted to the feeder. This degree of control can provide the flexibility to maintain a constant throughput capacity, given the variations of feed bulk density.

Continuous system

The processing of friable products is best suited to the continuous system, due to the ability of the system to follow the manufacturing rate. As the operating capacity of the application system is matched to the upstream processing equipment, it is possible to optimize the system with only a relatively small surge hopper, so preventing degradation of the product but also optimizing the dry and liquid flow rates (Fig. 14.3).

For the continuous system to operate effectively, it must be capable of adjusting to upstream fluctuations. For this to be possible, it must be capable of determining the upstream capacity and adjust accordingly the operating capacity of the dosing system. This can be achieved by the installation of an ultrasonic level measurement, positioned in the surge hopper before the dosing system. The device is calibrated with an empty hopper and with a full hopper, the scale in between being calibrated against the known manufacturing rates for the given products. The capacity of the dosing system increases as the hopper fills and decreases as the level in the hopper diminishes. The objective is to maintain a consistent capacity through the dosing system by minimizing any large fluctuations in the capacity of the system.

The control of the dosing system must be such that it can respond to the necessary changes in capacity. A continuous liquid dosing system requires extra accuracy in determining the pellet flow and the liquid flow (Engelen, 1998). This means that both the dry and liquid flow rates have to be changed proportionally. For this to be possible, both elements have to be gravimetrically measured to ensure the accuracy of the system.
Batch system

The batch system is a discontinuous system that operates (as the name implies) on a batch-by-batch basis. The system provides the flexibility to apply multiple liquids and combine multiple dry product forms. There are several different arrangements for the batch system and these can be defined in two categories, as either a pre-weighing system or a combined weigher–mixer.

The weigher–mixer combination is when the batch mixer is supported on load cells and performs the dual function of both weighing and mixing. The main disadvantage of this system is that the capacity of the mixer needs to be greater for the same manufacturing capacity as a pre-weighing system, as the batch cycle time is increased. This will increase the cost of the mixer, but this cost may be offset against the cost of the weigh hopper(s) and associated control items. It may also rule out the requirement for additional processing equipment such as conveyors and elevators, as the height required for the installation is reduced.

The pre-weighing system shown in Fig. 14.4 comprises a twin weigh hopper arrangement. This system is configured for the batch weighing of both the dry material and liquid additives. The principle of the batch system is to operate using the actual batch weight and not the theoretical batch weight. For this reason, the system is extremely accurate, proportioning the correct quantity of liquid additives to the corresponding weight of dry material. For this principle to work, the batch weight of the dry material must be known before weighing the liquid additives. Therefore, when the initial batch of dry material is weighed, the corresponding batch weight for the liquid additives is then weighed. For the succeeding batches, the weighing of both the dry material and liquids is completed before the finish of the preceding batch.

Batch-weighing multiple liquids can have its disadvantages. The principle for the addition of micro-liquids to the batch mixers differs from the addition of fat and other high-inclusion liquids. If these liquids are weighed in the same weigher, then a compromise may have to be made regarding the addition method to the mixer, i.e. addition time, nozzle type, nozzle capacity, etc.

The more commonly adopted method for the addition of micro-liquids to the batch mixer is to meter each liquid individually. This provides finite control and flexibility. Unlike other systems, the batch system does not require continuous measurement and control of the liquid flow. The liquid addition is calibrated to dose the theoretical quantity of liquid to the mixer in a predetermined time, the addition time being important to the performance of the system. The dosing capacity of the pump being constant, the enzyme is metered until the set point is achieved.

Measurement and Control of the Liquid Flow Rate

The dosing precision of liquid enzymes is critical, due to the known dose–response relationship that exists between enzyme dose and animal performance (Fig. 14.5). Under-dosing can result in significantly reduced efficacy and bird response,
ultimately reducing feed quality, whilst over-dosing can be costly in terms of wastage of feed enzymes and reduced animal performance under certain circumstances.

Soon, enzymes may not be blanket-fed throughout the animal’s life but targeted with various dose levels at different stages of growth or reproduction (Overfield, 1999a). In addition, some enzyme suppliers are developing modelling tools able to predict the optimum dose rate based on cereal quality. For this to be of practical use within the feed mill, the dosing system needs to be such that the application rate of

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**Fig. 14.4.** Typical mill arrangement for a batch system.
the enzymes can be varied easily, without being recalibrated, and yet still maintain its accuracy.

**Flow meters**

The measurement and control of the liquid flow rate can be achieved with the incorporation of a flow meter in the liquid line. The process signal from the flow meter is used to vary the speed of the drive motor on the dosing pump, thereby altering the liquid flow rate. The process signal from the flow meter is proportional to the required application rate and dry flow capacity.

There are three main types of meters used in the measurement of liquids and a brief explanation of each of these follows.

**Positive displacement or turbine meters**

These are mechanically operated flow meters. The internal arrangement of the meter contains moving parts – either oval gears or a Pelton wheel type arrangement. The rotation of the rotors is sensed by an electronic system, using the interaction of a ferrite material imbedded in the rotor tips. A sensing device is mounted in the top of the meter body to pick up the rotation of the rotors. The sensor will then generate and transmit the resulting signal. The speed of the rotor and the resulting output are directly proportional to the linear flow rate of the liquid through the meter.

The main concern with this type of meter is the low flow rate used in the application of enzymes and the possible build-up of material on the gears of the meter. Either of these parameters can affect the accuracy of the meter or prevent it from operating.
Electromagnetic meters
This type of meter has no moving parts, which eliminates the possibility of entrapment of material in the sensing unit. The measuring principle is based on Faraday’s law of magnetic induction. This is when a voltage is induced into a conductor, which moves in a magnetic field, the flowing liquid being the conductor. The induced voltage is proportionally related to the flow velocity, and through knowledge of the cross-sectional area of the meter sensor, the volumetric flow rate can be calculated. The application of the meter is limited to materials that are electrically conductive above 5 µS cm⁻¹.

Mass flow meters
The measuring principle for these meters is based on the controlled generation of Coriolis forces. These forces are always present when both transitional (straight-line) and rotational movement occur simultaneously. When there is no flow, the measuring tubes both oscillate in phase. When there is flow, the tubes’ oscillation is decelerated at the inlet and accelerated at the outlet. As the flow rate increases, the phase difference also increases. The oscillation of the measuring tubes is determined using electrodynamic sensors at the inlet and outlet. The measuring principle of the mass flow meter is independent of temperature, pressure, viscosity, conductivity or flow profile.

Dosing pumps
The other essential element in controlling the application of liquid enzymes is the correct selection of the dosing pump. There are numerous pumps available and capable of dispensing liquid enzymes. The most commonly adopted type of pump for this application is the diaphragm pump, which can be mechanically or electronically coupled. One of the benefits of the diaphragm pump is its ability to meter volumes of liquid precisely. It is also self-priming and can handle particulate-filled and shear-sensitive liquids.

The pump (Fig. 14.6) operates in two stages, with a positive and negative stroke. For the pump to operate efficiently, the suction valve (1) has to allow liquid into the head on the suction stroke and allow it through the discharge valve (2) on the delivery stroke. This is achieved due to the motion of the diaphragm. As the diaphragm moves backward (negative) on the suction stroke, a partial vacuum is caused in the pump head. This lifts the suction ball valve, allowing liquid to flood into the pump head. The vacuum created also ensures that the delivery ball valve remains tightly seated. When the diaphragm moves forward (positive) on the delivery stroke, the suction ball valve is forced back on to its seat. The pressure in the pump head rises until it is greater than the system pressure. At this point, the ball in the delivery valve lifts and the liquid is displaced. Once the diaphragm has reached its limit of travel, the system pressure on the delivery ball valve forces the ball back on to its seat. The cycle is then repeated.
Flow oscillation is common to all diaphragm pumps. To overcome this and to achieve a consistent and even flow through the flow meter, it is necessary to install a pulsation damper after the pump installation. The gas in the pulsation damper is compressed until the system pressure is overcome, resulting in low-pulsation flow from the discharge of the damper.

**Enzyme Application to the Feed Pellets**

Feed compounders need reassurance that the products they incorporate into their feed are not just present, but present in the desired quantity. It is impossible for enzymes to achieve repeatedly the perfect application criteria of 100% recovery and a coefficient of variation of zero, given the external influencing factors, such as assay procedure, sample collection, sample size, product quality, etc. To achieve a homogeneous distribution, enzyme should be sprayed uniformly on to the feed, the
number of droplets per gram of feed must be sufficiently large, and the pellets sprayed with enzyme must be thoroughly distributed (Gunther et al., 1997).

There are two accepted methods for the application of enzymes on to feed. One involves spraying into a free-falling curtain of material, or a mechanically formed curtain (mist-coater); the other is by spraying into a fluidized area of pellets (batch type mixer, etc.). Fodge et al. (1997) conducted an extensive survey of the different application points and equipment options for the PPA of enzymes. These data will be equally applicable to any heat-labile micro-ingredients, across a range of mill systems, and for this reason are detailed below.

Figure 14.7 shows the application of the enzyme at the mist-coater, followed by subsequent mixing in distribution augers. The left-hand Y-axis indicates the level of enzyme activity in millions of units per ton. The right-hand Y-axis indicates the uncorrected coefficient of variation (CV) (%).

Of the seven systems depicted in Fig. 14.7, system G yielded an uncorrected coefficient of variation (CV) of less than 10% and the smallest spread of average activities. The next best uniformities were observed with systems F, C and D. Systems A, B and E were the least uniform.

Both G and F were continuously fed systems in which an impact-style load cell is positioned in the feed line just prior to the roto-coaters. Feed strikes the impact plate, a proportional voltage signal from the impact plate is forwarded to a variable

![Figure 14.7](image-url)
frequency drive that controls the output of the fat and enzyme pumps in synchrony with feed flowing into the roto-coaters. Feed slides off the impact plate in a curtain-shape, roughly 50 mm thick and 410 mm wide, directly above the roto-coater. One spray nozzle was positioned on either side of this curtain and sprayed the enzyme on the feed rather than applying it inside the roto-coater (Fodge et al., 1997).

A and B roto-coaters were semi-continuous fed systems that were very old and in a feed mill designed for about 50% of the current output. Both systems often had problems and had never performed smoothly. The feed-flow rate varied by 10%, or more, and the mixing of the feed was poor. Systems C and D were batch-fed roto-coaters in a well-maintained old feed mill, also producing feed at greater than the design capacity. Preventive maintenance programmes for C and D were well developed and effective, and the feed mill did not experience unusual problems that slowed their performance. Roto-coater E was constructed by a feed mill maintenance shop. It was fed semi-continuously, and although the accuracy was reasonable, the uniformity of mixing was poor (Fodge et al., 1997).

Applying enzymes to the feed at an existing fat coater is a widely adopted and accurate solution. The above figures help to support this, but they also indicate that, if the correct operating practices are not adopted, then poor results can also occur, as demonstrated by systems A, B and E. In the case of volumetric systems it is vital that changes can be made for variations in product bulk density to compensate for increase or decrease in the system capacity. Roto-coater type systems have been developed primarily for the application of fat; however, testing with enzymes has been conducted and has resulted in excellent uniform coating (Decksheimer, 1998).

Figure 14.8 shows the results of spraying enzyme on to pelleted feed in four different augers. The accuracy of enzyme application was undesirable in three of the four and the CV was slightly less than 20% on two systems and worse than 20% on the third system. In system A, enzyme application was linked to feed flow rate, which was measured, whereas in systems B–D it was not measured. The main problems with using augers are: (i) the need for accurate measurement of feed flow rates in them; and (ii) the difficulty of keeping liquid product from building up on either the walls or the drive shaft in the auger (Fodge et al., 1997).

The results shown for system A are not typical for a measured flow rate, with all samples indicating an over-application of enzyme. It may be the case here that the enzyme and dry flow rate were not correctly calibrated. Either the dry flow rate was low for the given enzyme set point or the enzyme set point was high for the dry flow rate.

The application of enzyme in conveying augers is not generally recommended, due to (i) above. However, in applications such as a fat coater complete with a blending screw, this configuration can provide good opportunities for enzyme application. Additionally, with care and diligence regarding the nozzle selection, positioning and air pressure, it is possible to overcome the associated problems with (ii) above.

The data collected from four feed lines where the enzyme was applied by spraying it on to curtains of feed falling from the end of weigh-belt coaters are shown in Fig. 14.9. Spray nozzles for spraying fat were positioned on either side of this curtain. For adding enzymes, nozzles were positioned on both sides of the curtain of
feed, either above or below the fat nozzles, and occasionally included the enzyme with the fat. System A operates at a constant rate, systems B–D at varying rates (Fodge et al., 1997).

Feed mills may not have roto-coaters, augers or horizontal weigh-belt coaters. To develop an alternative application site the product was applied beneath the cooler just before the feed drops into a chain drag. The transition piece between the cooler exit and the chain drag was replaced with a redesigned transition piece that collected the feed forming a curtain of feed 25–50 mm thick. The new transition piece comprised two compartments, each with two internal baffles that helped to form the feed into a curtain-like shape, and spray nozzles were inserted into the transition behind these curtains. Positioning the nozzles behind the curtain of feed helped to protect the spray patterns from the airflow turbulence that is prevalent at these sites (Fodge et al., 1997).

Figure 14.10 shows the results obtained at four such transition systems installed in poultry feed mills. Accuracy and uniformity were quite good in A and B, acceptable in C but still problematic in D. System D had more airflow turbulence problems than the others. It was concluded that these modified systems were an acceptable means to apply heat-labile products and were cost effective (Fodge et al., 1997).

Spraying at transitions and spouts is a very contentious topic. From the data above it can be seen to work, but also in other circumstances to fail. It is a very
demanding application point, in that it needs constant inspection and continual measurement to ensure the system stays within the predefined limits of acceptance.

Proportion of sprayed pellets

The homogeneous distribution of the additive (liquid) on the feed is critically determined by the proportion of the sprayed pellets. The system should work in such a way that the CV is minimized. A value of less than 20% should be aimed for. The more pellets that are sprayed, the sooner this value is achieved (Schwarz, 1998).

The volume of liquid applied to the feed in a PPA system is relatively small (typically 500–1000 g t\(^{-1}\)). To disperse this quantity of liquid across the feed, the use of air atomizing nozzles is required. Air is used to shear the liquid; this mechanism produces a broad spectrum of droplet sizes, often varying from submicron up to several hundred micrometres in the same spray. A perspective of droplet diameters can be gained by realizing that there are 1000 \(\mu m\) mm\(^{-1}\) and the required range of droplets to be applied to the feed would need to have a mean of 300–400 \(\mu m\). It is critical that the liquid is not over-atomized by the use of too high an air pressure. This will result in a micro-mist, with many of the micro-droplets condensing on the internal surfaces of the application equipment. In addition, the smaller the droplet size, the smaller is
the mass and, as a result, droplets can be affected by the airflow in the application equipment, with the droplets being drawn away from the application point.

To maximize the exposure of enzyme and feed pellets, it is critical to present both elements so that the pellets come into contact with the enzyme spray pattern for as long as possible, and that the pellets are in suspension during this period. The nozzle design, application or orientation should be such that, if used in a continuous or semi-continuous system of varying capacities, the varying flow rate through the nozzle would not in any way affect the exposure of the enzyme and pellets, reducing the efficiency of the system.

**Addition of water with enzymes**

The inclusion rate of some concentrated enzymes can be as little as 50–100 g tt⁻¹. This does not present problems for the metering or measurement of the liquid, but it does make it a considerable task to apply the liquid to the feed accurately. These concentrated enzymes need to be diluted to increase the volume of liquid to feed, improving the exposure of the enzyme to the pellets.

Although the objective is a minimum level of water inclusion, a 1000 g tt⁻¹ total inclusion has proved effective (Decksheimer, 1998). Figure 14.11 demonstrates
that acceptable coefficients of variation were achievable with enzymes sprayed on to samples of feed using different production rates and application points (Decksheimer, 1998).

Predilution of liquid enzyme products to aid application also raises potential issues of product stability. In practice many companies advise against prolonged storage of diluted products, in order to avoid the risk of microbial contamination (e.g. Schwarz, 1998). Any dilution with water should take place immediately prior to the spray nozzle and mixing should be with a static mixer, without moving parts. The mixer, nozzle and connecting parts should also be automatically flushed after each addition (Overfield, 1999b).

Pre- and post-fat addition of enzymes

There are conflicting opinions regarding the application of enzymes at the fat coater. Should enzymes be applied to the feed before the fat and then be subjected to the high fat temperature, or applied post-fat application, with concern then focusing on the enzyme’s ability to penetrate the fat coating on the pellets?

Assay work carried out with samples from commercial feed mills has shown there to be no clear interaction between enzyme recovery and stability and pre- or post-application of enzymes at the fat coater. Research cited by Engelen (1998) (Tables 14.3 and 14.4) also supports this view. The recovery of xylanase LC was shown to be unchanged irrespective of the fat temperature, the fat level and the spraying order.
Liquid Enzyme Stability

Liquid enzymes are inherently less storage stable than their granular counterparts. In liquid form, water activity is sufficiently high for the component enzymes to remain active, whereas in the granular form the low water activity renders the component enzymes relatively inactive prior to ingestion by the animal. Commercially available enzymes are typically less than 100% pure and often contain side enzyme activities in addition to the desired main activity or activities. These side activities can include trace levels of proteases which, even at low activity, can cause some degradation of the main activities (e.g. xylanase). An aqueous environment also introduces a significant risk of microbial contamination and proliferation, which can cause rapid decline in enzyme activity in liquid products. The problem of storage stability is increased further when different liquid enzymes are mixed to produce ‘multi-enzyme’ liquid products and when liquid enzyme products are stored at ambient temperatures in different parts of the world.

Enzyme suppliers generally guarantee the shelf-life of liquid enzyme products for between 3 and 6 months following the date of manufacture, if stored at or below 22°C (e.g. Fig. 14.12). For shipment to tropical countries, where the ambient conditions exceed 22°C, suppliers should ship and store enzymes in refrigerated containers that maintain the product at a constant temperature below 5°C prior to delivery to the feed mill. Once liquid enzymes have been applied to the feed,

### Table 14.3. Effect of spraying fat before or after enzyme addition on measured enzyme recovery (Engelen, 1998).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Control</th>
<th>Enzyme sprayed before fat spray</th>
<th>Enzyme sprayed immediately after fat spraying</th>
<th>Enzyme sprayed 30 min after fat spraying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starter</td>
<td>0</td>
<td>100</td>
<td>90</td>
<td>99</td>
</tr>
<tr>
<td>Grower</td>
<td>0</td>
<td>100</td>
<td>104</td>
<td>99</td>
</tr>
</tbody>
</table>

*50% barley based.

*2 l xylanase LC t⁻¹ of feed.

### Table 14.4. Effect of fat level and fat temperature on the recovery of a liquid enzyme (Engelen, 1998).

<table>
<thead>
<tr>
<th>Fat temperature (°C)</th>
<th>Fat level: 1.5%</th>
<th>Fat level: 3.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>114</td>
<td>118</td>
</tr>
<tr>
<td>80</td>
<td>110</td>
<td>117</td>
</tr>
<tr>
<td>88</td>
<td>105</td>
<td>117</td>
</tr>
</tbody>
</table>

*50% barley based.

*2 l xylanase LC t⁻¹ of feed.
the component enzymes are rendered inactive and stable during the period prior to consumption by the animal.

**Delivery of Enzymes**

Liquid enzymes can be delivered in a range of different sized containers to the feed producer’s requirements. The more commonly adopted sizes are either a 1000 l intermediate bulk container (IBC) or a 210 l barrel. Containers are manufactured from high-density polyethylene with UV stabilizers.

Discharge from the 210 l container is from the 50 mm valve at the top of the container. The discharge from the IBC is via a 50 mm valve located at the bottom of the IBC. The IBC has a 150 mm screw cap, which incorporates a degas/regas vent for balancing internal and external pressure. The 1000 l container is self-emptying, due to the bottom discharge and sloped bottom of the IBC. The IBC is mounted on a pallet for handling by forklift truck.

The IBC offers greater flexibility, ease of handling and self-emptying, and minimizes risk of exposure to operators, etc. The 210 l barrel offers flexibility for installations when access/handling of IBCs is not possible.

**Handling of Liquids**

During the period prior to dosing, liquid enzymes must be stored carefully in the feed mill to maintain stability. Ideally the product should remain in its original container and be stored in cool dry conditions. If transfer to another container or tank is necessary, then this must be sterilized before use and at regular intervals. These containers must also be ‘closed’ to prevent ingress of dust and other sources of contamination.
Microbial contamination of liquid enzymes may not only reduce their stability prior to dosing, but also recontaminate the feed following heat treatment.

Appropriate health and safety precautions must be observed whilst handling liquid enzymes. These precautions will be found in the supplier’s Material Safety Data Sheet.

**Measurement of Dosing Accuracy vs. Bird Performance**

Measurement of the accuracy of dosing enzymes on to feed pellets relies upon a sensitive assay procedure. Currently, feed enzymes stand alone from many other high-value feed additives due to the absence of universally accepted assays for their determination, both as products and when incorporated in-feed. The assay procedures used will be influenced by the properties of the enzyme concerned, e.g. pH, temperature characteristics. The assay is undertaken to measure the effectiveness of the application of the enzymes to the feed, the main criteria for measurement being homogeneity (coefficient of variation) and absolute recovery of enzyme activity from the feed. It can be seen that, provided an accurate dosing system is employed, productive performance is similar whether the product is applied post-pelleting (liquid) or pre-pelleting (granular, Fig. 14.13).

**Summary**

The choices of liquid application system vary both in cost and in accuracy. The use of the simple volumetric dosing system to apply enzymes is an economical choice in

![Fig. 14.13. Effect of temperature on response in broiler performance (0–42 days) to enzyme supplementation in granular (Az 1300) vs. liquid (Az 1310) products (Finnfeeds Int. Technical Report 1300.UK.98.29).](image)
terms of the equipment only. Frequently such systems do not offer the accuracy required to exploit the economic relationship that exists between enzyme dose, target substrate and resultant animal performance.

The installation of the dosing equipment must be at the right location in the process (post-screening) and be correctly sized for the feed mill capacity, which may not necessarily be the same as the pelleting capacity. The measurement and control of the dry flow rate are as vital to accuracy of the system as the measurement and control of the liquid flow rate.

The choice of system must include the present and future requirements of the feed producer, provide the flexibility for the system to dose different numbers of liquids at varied application rates, and handle the different number of product forms manufactured by the feed mill.

References


Introduction

Enzymes are now routinely added to monogastric diets in many parts of the world. It is clear that their use is based on determined benefits in animal performance, but proving their presence in manufactured animal feeds is a daunting task and has been the focus of much research. Enzymes are proteins and as such are susceptible to the rigours of feed manufacture, as are all other feed proteins. Whilst the structural conformation need not be maintained for feed proteins to deliver their benefit in terms of amino acid content, it is critical that feed enzymes are not irreversibly denatured during feed manufacture or they will no longer function. It is clear that detection of their activity in complete feeds is therefore important for confirmation of survival. Whilst it is not within the remit of this chapter to discuss the methods available for their detection (this is covered in Chapters 4 and 15), the idiosyncrasies of each major class of enzyme used in animal feed are of interest and will be covered. Issues addressed will include differences between source enzyme stability to thermal denaturation in vitro, problems with analysis due to interaction with the feed matrix (and methods of limiting this effect), data from in-feed processing trials, and future trends.

The vast majority of mixed feed for poultry and swine undergoes some sort of processing. For many years, feed has been pelleted, a process whereby the feed mixture is conditioned by adding steam, then pressed through a die. Pelleting increases nutrient density, improves the handling characteristics of the feed, and reduces the microbial load. Pelleting commonly uses temperatures between 65 and 95°C (Gibson, 1995), which can be damaging to heat-sensitive nutrients, including enzymes.
In the past few years, concern for feed-borne pathogens and factors of pellet quality have caused feed manufacturers to increase the processing temperature, time and pressure, and feed has been submitted to double pelleting or expansion (Pickford, 1992). The increase in the severity of the processing treatment underlines the importance of enzyme stability. Several approaches have been taken to overcome the difficulties, including avoidance of the treatment altogether by adding the enzymes as a liquid after the pellets have cooled. Despite the possibility of post-pelleting supplementation, feed enzymes are often added to the mash before treatment. The effects of heat may be reduced by protection of the enzymes with hydrophobic coatings or selection of heat-resistant enzymes.

Until recently, there had been limited research published on the survivability of feed enzymes (Chesson, 1993). Yet enzyme stability is of critical importance for the producers of feed enzymes and we must assume that in-house evaluations preceded the marketing of enzyme preparations. Since 1993, several research trials have been reported in the popular press or in symposia proceedings, and a few trials have been described in the peer-reviewed scientific literature. Clearly, measuring enzyme activity in vitro, either in solution or in the feed, is of critical importance. Recent research suggests that measurements of in vitro activity must be tempered by the results of tests on in vivo efficacy.

Phytase

A surprising amount of research has been reported on the temperature stability of phytase, considering that phytase makes up less than 20% of commercial enzyme usage (Bedford and Schulze, 1998). The interest is likely because phytic acid, which renders phosphorus and other nutrients unavailable, is present in many plant sources of feed ingredients (Cheryan, 1980; Eeckhout and dePaepe, 1994; Ravindran et al., 1995) yet endogenous phytase activity in monogastric animals is minimal or absent (Pallauf and Rimbach, 1997). To complicate the situation, many of the same plant sources of phytic acid also contain significant amounts of phytase and the nutritional problem of phosphorus digestion is compounded by the environmental problem of phosphorus deposition on the land. Phosphorus pollution has become a limitation to production in areas with intensive animal production.

Phytase enzymes are diverse in both their sources and their characteristics. Liu et al. (1998) summarized the literature to 1998 and showed that phytases from bacteria, fungi, yeast and plants have optimal temperatures that range from 45 to 77°C, a difference of 32°C. A phytase isolated from Aspergillus niger was described by Dvorakova et al. (1997) as having activity at temperatures between 25 and 65°C with an optimum at 55°C. Incubation of the enzyme for 10 min at 60°C destroyed 5% of its original activity, while incubation at 80°C destroyed 80% of the activity. As part of an effort to find heat-stable enzymes, Wyss et al. (1998) studied heat denaturation of purified phytases isolated from A. fumigatus and A. niger. Both enzymes were denatured at temperatures as low as 55°C. However, even when submitted to a temperature of 90°C, the enzyme from A. fumigatus refolded into an active conformation.
on cooling, while that from *A. niger* did not. Some of the heat-resistant phytases will undoubtedly become available commercially in the near future.

Heat inactivation of enzyme in solution does not translate directly into heat inactivation in the feed, because of an interaction of the enzyme with the feed matrix. In effect, the feed ingredients can protect the enzyme from steam or elevated temperatures for a short period of time (Chesson, 1993). Measuring phytase activity in pelleted feed provides a more accurate evaluation of the commercial importance of inactivation. Simons *et al.* (1990) added phytase to a 'common pig feed', which they heated to either 50 or 65°C before pelleting. Heating to 50°C resulted in a pellet temperature of 78 or 81°C but did not lower the enzyme activity. However, heating to 65°C produced a pellet temperature of 84 or 87°C, resulting in inactivation of 17 and 54%, respectively, of the original enzyme activity. Gibson (1995) added three phytase preparations to a wheat-based feed and pelleted the feed at temperatures of 65–95°C. Two of the preparations suffered inactivation even at 65°C and only a commercially available stabilized preparation retained substantial activity with pelleting at 85°C or above. In addition to studying enzyme stability in solution, Wyss *et al.* (1998) added phytase enzymes to commercial feed before pelleting at 75 or 85°C. Recovery of the activity was similar for the two enzymes after pelleting at 75°C, but pelleting at 85°C resulted in a greater inactivation of the enzyme from *A. niger* than that from *A. fumigatus*, supporting their results on denaturation kinetics. Eckhout *et al.* (1995) added a commercial phytase to feed and reported that pelleting temperatures between 69 and 74°C destroyed 50–65% of the enzyme activity.

Inactivation is not limited to microbial enzymes that may be added to the feed, but also affects the enzymes naturally contained in feed ingredients. Gibson (1995) found that pelleting at temperatures of 85°C or above largely inactivated the endogenous phytase in wheat. Eckhout and dePaepe (1994), in their survey of phytase activity in different feedstuffs, reported that pelleted samples of wheat bran, which is particularly rich in phytase, had only 56% of the phytase activity of unpelleted wheat bran. In three experiments, Jongbloed and Kemme (1990) found that pelleting at temperatures approaching 80°C decreased enzyme activity in pig feeds that were based on feedstuffs that were rich or poor in phytase activity. They carried their experimentation a step further by measuring the effect of pelleting on the apparent absorption of phosphorus. In two experiments, they found that pelleting of phytase-rich diets decreased phosphorus absorption, supporting the data on endogenous phytase inactivation.

Research interest in phytase stability by both the research community and the feed industry is due to the higher processing temperatures currently being used and the increased importance of phosphorus absorption for both nutritional and environmental reasons. Thermal protection of available exogenous enzymes by encapsulation or granulation provides a solution at present. A more fundamental solution may be the isolation of enzymes that are heat-resistant or that can reform their active structure after denaturation. Unfortunately, neither of these solutions prevents high processing temperatures from destroying endogenous enzymes contained in feed sources.
β-Glucanase

The commercial availability of enzyme preparations containing β-glucanase activity has allowed barley to be incorporated into poultry diets without the depressed performance and sticky droppings that are associated with high β-glucan levels (Campbell and Bedford, 1992). The use of β-glucanase is now widespread in areas of the world where barley is commonly grown. There are a limited number of reports on the effect of heat treatment on β-glucanase enzyme that has been added to feed.

Eeckhout et al. (1995) measured the activity of a β-glucanase included in a commercial piglet feed conditioned at temperatures between 50 and 95°C and pelleted at temperatures between 72 and 91°C. Even at the lowest temperatures, 40% of the original activity was lost, and at the highest temperatures, only 7% of the original activity remained after processing. More than two thirds of enzyme inactivation occurred in the conditioner. On the other hand, Esteve-Garcia et al. (1997) did not find major inactivation of enzyme in broiler feed with conditioning and pelleting temperatures approaching 80°C. The enzyme that they used was described as a microgranulate, suggesting that it was incorporated into the feed in a stabilized form.

At least two trials have measured the performance of broiler chicks fed enzyme-supplemented diets that were submitted to heat treatment. McCracken et al. (1993) supplemented a barley-based diet with a stabilized commercial enzyme mixture containing β-glucanase and xylanase activities and heated it to 85°C for 15 min before pelleting. Heat treatment of the feed without exogenous enzyme decreased the apparent digestibility of nutrients, increased the viscosity of the intestinal contents, and reduced faecal dry matter content. The addition of the enzyme improved nutrient digestibility and eliminated the negative effects of heat treatment, demonstrating clearly that the enzymes remained active at this temperature. Vukić-Vranjes et al. (1994) tested a commercial enzyme mixture, including β-glucanase, xylanase, amylase and pectinase activities, in two diets, one of which contained 20% barley. The feed was conditioned at 70–75°C, and pelleted or extruded with the temperature in the extruder barrel reaching 110–120°C. Compared with pelleting, extrusion produced negative effects on chick performance. Extrusion also resulted in increased in vitro viscosity of the feed, suggesting that the higher temperature increased the solubility of non-starch polysaccharides. The enzyme mixture improved chick performance whether the diet was pelleted or extruded, likely indicating that there was enzyme activity even after exposure to the extrusion process. Vukić-Vranjes et al. (1994) also showed that supplementation with the enzyme mixture reduced the viscosity of the feed extract, demonstrating that the enzyme was active in the feed before ingestion by the bird. They did not rule out enzyme action in the feed even before processing.

Inborr and Bedford (1994) measured both enzyme activity in the feed and bird performance after supplementation and heat treatment. These authors found significant inactivation of added β-glucanase (the same product used by McCracken et al., 1993) in a barley-based diet after conditioning at a temperature as low as 75°C followed by pelleting. Conditioning at 95°C for 30 s and 15 min destroyed 84 and 91%, respectively, of the original β-glucanase activity. Inborr and Bedford (1994)
wondered if measuring enzyme activity in vitro or in vivo was the most accurate method of evaluating the effect of processing on enzyme activity. While recovery of enzyme activity was highest at the lowest processing temperatures and lowest at the highest temperatures, this was not accurately reflected in bird performance. The highest value for weight gain and the lowest value for the feed-to-gain ratio were obtained with the intermediate conditioning temperature of 85°C. Clearly, the enzyme remained active at this temperature, and the positive effects of heat treatment outweighed the negative effects of enzyme inactivation.

Pickford (1992) showed that stabilization of β-glucanase protected it from a pelleting temperature of 75 but not 95°C. Cowan and Rasmussen (1993) reported that a commercial β-glucanase lost significant activity with pelleting at a temperature above 65°C, but that coating the same product provided protection to 75°C. Both these reports are brief and there is very little published research relating specifically to increasing the temperature stability of β-glucanase.

Xylanase

Xylanase represents by far the greatest share of worldwide enzyme sales (Bedford and Schultz, 1998) and its use allows high-viscosity wheat to be incorporated into poultry diets without negative effects. Perhaps because of the economic importance, there is considerably more research reported in the scientific literature on the stability of xylanases than there is on the stability of β-glucanases.

Xylanase enzymes are produced by many species of fungi and bacteria, and variation in the optimum conditions for activity of these is not surprising because each organism is best adapted to a specific environment. In a review, Bedford and Schulze (1998) show that the optimum temperature for a number of bacterial and fungal xylanases can range from 30 to 105°C and the optimum pH can range from 2.0 to 10. Pickford (1992) compared the stability of three commercial enzyme preparations without providing information on the type of activities that they contained. At a pelleting temperature of 80°C, 85% of the original activity was retained by one preparation, 55% by a second, and only 33% by the third. No enzyme activity was shown for any preparation with pelleting at 95°C. Expansion, with a higher temperature but shorter exposure, caused greater loss with similar differences between enzymes.

Pettersson and Rasmussen (1997) demonstrated differences in temperature stability of xylanase enzymes isolated from Thermomyces, Humicola and Trichoderma. At a conditioning temperature of 75°C, the Trichoderma enzyme lost significant activity, while at 85°C both the Thermomyces and Humicola enzymes retained more than 80% of their original activity. Even with conditioning at 95°C the Thermomyces xylanase retained 70% of its activity. Gibson (1995) observed variation between nine xylanase preparations, seven of which were commercially available at the time of the trial. Activity remaining after treatment at 90°C was above 90% for one preparation and lower than 10% for several of the others. It is not clear from the report how much of this variation was due to the source of the enzyme and how much was due to stabilization of the enzyme. Perez-Vendrell et al. (1999a) tested the in vitro stability
of eight commercially available preparations, including coated or encapsulated products, after feed processing at 65–70, 75–80 and 85–90°C before pelleting. Measurement in vitro showed that, even at the lowest temperatures, most products lost at least 30% of their activity, while at the highest temperatures, activity loss reached 90%. As they found for β-glucanase, Esteve-Garcia et al. (1997) reported that the microgranulated xylanase preparation that they studied was not inactivated by conditioning and pelleting temperatures that approached 80°C.

In the preceding paragraph reference was made to several methods of thermal protection. The most fundamental protection is that suggested by the study of Pettersson and Rasmussen (1997) in which heat-resistant enzymes are prepared from heat-resistant organisms. At the other extreme, the problem can be avoided altogether by spraying the enzyme on the finished pellets (Perez-Vendrell et al., 1999b), though this requires some additional equipment and an extra step in the manufacturing process.

Dry enzyme preparations can also be stabilized to increase heat resistance and allow their addition to the feed before pelleting without undue inactivation. Methods of stabilization were the subject of a previous chapter. Much of the inactivation of enzymes is due to the steam used for conditioning. Thus, stabilizing a preparation relates largely to protecting the enzyme from the steam by absorbing it on to a carrier or by coating it with a hydrophobic substance (Cowan, 1996). Pickford (1992) provides an example whereby stabilization increased activity remaining after pelleting at 75°C from 48 to 76%, and after pelleting at 95°C from 12 to 34%. Clearly, even protected enzymes have a limit of temperature stability; Steen (1999) suggests that if a feed is to be processed at a temperature above 90°C, even enzymes that have been stabilized should be added after processing.

Tests of in vitro enzyme activity are valuable tools in determining enzyme activity loss, and they suggest that exposure to even relatively low temperatures results in significant destruction. However, in vitro tests are but one tool among many. Clearly, enzyme activity in a buffer solution at the optimum pH provides only a very limited view of activity under the conditions of feed processing and digestion. In fact, most investigators (Vukić-Vranjes et al., 1994; Pettersson and Rasmussen, 1997; Perez-Vendrell et al., 1999a; and others) have recognized that interactions between the enzyme and the feed are important and they have measured activity in complete feeds. Measurement of the viscosity of feed extracts (Spring et al., 1996; Bedford et al., 1997) can provide a measurement of the effects of enzyme action before the feed is ingested by the bird. Preston et al. (1999) demonstrated this action by showing that even with apparently complete enzyme inactivation in the finished feed, supplementation resulted in improved bird performance.

To complete the story of enzyme action in relation to processing, the effects of enzyme addition and heat treatment of the feed must be measured by bird performance. Enzyme inactivation does not always translate directly into performance differences (Bedford et al., 1997; Perez-Vendrell et al., 1999a; Silversides and Bedford, 1999). With processing temperatures between 65 and 105°C, Bedford et al. (1997) found that apparent inactivation of enzyme was linear, whereas performance (body-weight gain, feed conversion ratio) was best when the feed was processed at
81–83°C. Silversides and Bedford (1999) also demonstrated this effect of heat treatment, as is shown in Fig. 15.1. As the processing temperature increases, enzyme activity decreases in a linear fashion ($R^2 = 0.97$) while the change in broiler body weight with increasing processing temperatures was quadratic ($R^2 = 0.84$). Graphing the results for the feed conversion ratio produces very similar results ($R^2 = 0.98$). The highest body weight and lowest feed conversion ratio were at a processing temperature between 80 and 85°C. When such a performance response is achieved but is juxtaposed with apparent enzyme inactivation in an *in vitro* assay, it suggests two possibilities: either the enzyme completes its function almost entirely in the conditioner, making post-pelleting analysis largely irrelevant, or enzyme activity is not effectively measured by the assays used.

Data from enzymes derived from coated *Thermomyces* species do not seem to fall into the same category as those from uncoated *Trichoderma* or *Aspergillus*, probably due to a lack of interaction with the feed matrix of the former. Under such conditions the enzyme is more easily extracted and a correlation between performance and recovered enzyme activity has been demonstrated (Cowan and Rasmussen, 1993; Pettersson and Rasmussen, 1997; Andersen and Dalbøge, 1999). These contrasting observations clearly indicate that there is no single method that can be applied for determining subsequent bird performance from analysis of a feed sample when identity of the enzyme is unknown.

Improved performance with a decline in *in vitro* enzyme activity underlines the danger of relying solely on measuring activity, and it is not as contradictory as it appears. Heat affects many things in addition to enzymes (Pickford, 1992). Moderate heat increases starch gelatinization and cell wall destruction, increasing the availability of nutrients and subsequently improving performance as well. This is, after all, one of the principal reasons for pelleting feed. Higher processing

![Fig. 15.1. Changes in broiler body weight (○) and xylanase activity (▲) with increasing processing temperatures. (From Silversides and Bedford, 1999.)](image-url)
temperatures also cause increased solubilization of non-starch polysaccharides, leading to higher intestinal viscosity and reduced performance, and actually increasing the need for exogenous enzymes. Silversides and Bedford (1999) showed that without exogenous xylanase in a wheat-based diet, the viscosity of the intestinal contents of broilers increased dramatically as processing temperature increased (Fig. 15.2). Addition of xylanase produced low digesta viscosity even with high processing temperatures, and the actual reduction in viscosity was greatest at the highest temperatures. The greater enzyme action with high processing temperatures is likely because more substrate is available for enzyme action. The enzyme may be active before or during processing, reducing the viscosity measured either in vitro or in vivo. High processing temperatures reduce performance not only due to increased digesta viscosity and decreased exogenous enzyme action, but also because vitamins and other enzymes are also inactivated, and starch and protein digestibility is decreased. With heat-resistant or stabilized enzyme preparations available, heat damage to vitamins and nutrients other than exogenous enzymes will likely limit the processing temperatures that will be used commercially.

Conclusions and Trends

The activity of enzymes in solution can be reduced at temperatures of 60°C or lower. Enzymes in mixed feed are somewhat protected by the feed ingredients and most are likely to be reasonably stable at a slightly higher temperature. However, with
processing temperatures as high as 95°C there is a serious loss of enzyme activity. Several approaches have been used or suggested to reduce the negative effects of heat treatment, including protecting the enzymes from steam penetration, using heat-resistant enzymes or simply adding the enzymes as a liquid after processing. Feed enzymes may have some activity before the animal ingests the feed, so that total loss of in vitro activity may not always mean total loss of benefit. This seems to be at least partially dependent on the enzyme and the assay method. In some cases in vitro analysis is misleading, whereas in others there is a clear link between enzyme content and in vivo performance. Common processing temperatures cannot increase indefinitely because of the increased damage to vitamins, proteins and starch, which may be even more susceptible to heat damage than exogenous feed enzymes. The further development of enzymes selected for heat resistance and the utilization of enzyme activity before processing or ingestion by the animal may be promising approaches to obtaining the maximum benefit of added enzymes for the feed industry.

References


Introduction

To date, the use of exogenous enzymes in animal diets has had a great impact on the livestock industry. They are routinely used to improve the nutritive quality of diets containing cereals such as barley, wheat, rye and oats, especially for poultry, and as a result they have improved the quality of the environment by reducing the output of excreta and pollutants, such as phosphate and nitrogen, including ammonia. Since enzymes tend to increase performance of animals fed low apparent metabolizable energy (AME) cereals more so than those fed cereals with higher AME values, a further benefit that results is improved animal uniformity within and between experiments. In commercial situations this results in reduced variance between flocks.

Enzymes have also been shown to decrease the size of the gastrointestinal (GI) tract, which increases partitioning of nutrients into edible tissue. Their use has also been shown to alter microbial fermentation, which again may affect the availability of nutrients and the health status of the animal. Most of the research on enzymes as feed additives has been with poultry but studies with pigs, especially young pigs, have also demonstrated a positive response to enzyme supplements. The recent appearance on the market of recombinant enzymes, especially phytase, should further accelerate the adoption of enzymes by the feed industry. For some recent reviews on the use of enzymes in the feed industry, see Annison and Choct (1991), Bedford (1995) and Jeroch et al. (1995).

Although enzymes have proved to be highly beneficial, their use is still in its infancy. Many problems need to be solved before their full potential is reached. The purpose of this chapter is to highlight areas of further research that should enhance not only the use but also the value of exogenous enzymes in animal nutrition.
Areas Requiring Additional Research

Improved enzyme assays

Of great concern to current users of enzymes is not only how to select an enzyme from the multitude on offer, but also how to assay for the presence of the product in complete animal feed. Currently, there is no single standard procedure for assessing the quality or quantity of all commercial enzyme products, nor has a satisfactory assay been developed for monitoring quantities of enzymes present in complete diets. If xylanases are taken as an example, the current commercial products are derived from at least four different genera of organisms. Even within one genus, some products contain one, two, three or even more xylanases of differing characteristics (pH and temperature optimum, substrate specificity, etc.). As a result it is not valid to assign one assay for determination of the ‘relative activity’ of all products since any one product can be made to appear superior by judicious choice of assay conditions. A further problem is that, once added to a complete feed, some xylanases exhibit a significant binding to the feed matrix, and so estimation of activity is difficult to accomplish (Bedford, 1993).

Some of the commonly used enzyme assays include: measurements of the reducing sugars liberated by the enzymes; the use of coloured substrates; immunological methods, such as the enzyme-linked immunosorbent assay (ELISA); and assays based on the ability of an enzyme to reduce the viscosity of water-soluble non-starch polysaccharides (WSNSPs) (Cowan and Rasmussen, 1993; Hendon and Walsh, 1993). Cowan and Rasmussen (1993) reported that, among the different methods, the release of dye from dyed substrate is one of the easiest and most sensitive methods but it is not sensitive enough to detect enzymes in feeds readily. The ELISA procedure can detect enzymes in feeds, but antisera are not available for all of the different enzymes, and ELISA sometimes shows a weak reaction to inactivated enzymes. Measurement of the reducing-sugars liberated by enzymes in a feed matrix is inaccurate because of the high background level of reduced sugars in feed. A viscosity-based assay has been reported but its use is not common in complete feeds (Vahjen et al., 1997). McCleary discusses the strengths and weaknesses of each of these methods and others in more detail in Chapter 4. The limitations of the current situation do point to a major goal for future research which, if successful, will significantly improve the trust that feed manufacturers place in enzymes. This goal is to produce a meaningful assay, or series of assays, that will determine not only the amount of enzyme present in the complete feed, but also its biological relevance. Clearly, there should be an association between the activity determined in the feed and its activity in vivo.

Government regulatory bodies, enzyme manufacturers, the feed industry and professional societies should therefore benefit from development of standardized assays for feed enzymes. This is important, as standardized assays would: (i) allow the manufacturers and purchasers of enzymes to compare enzymes based on their activity values; (ii) enable livestock producers to determine the amount of active-enzyme product that was added to a diet or the amount that survived the rigours of the...
environment, including heat treatment during processing; (iii) provide a means of assessing how well the enzymes survive in different sections of the GI tract, especially in the section where they are most efficacious (the benefits of this are discussed in the next section); and (iv) bear some relation to the physiological effects that the enzymes are supposed to have (for example, it may be possible to run an assay at pH 8 and 60°C, but the results would be irrelevant to the feed industry).

**Site of action of enzymes**

Fundamental information is lacking on where in the GI tract enzymes produce most of their beneficial effects. It is not known, for example, whether the main site of action of enzymes in chickens is in the crop, proventriculus, gizzard, duodenum, ileum, rectum, or all or part of the GI tract. Data presented in Chapter 15 suggest that enzyme activity may be as much as 60–70% completed during the conditioning process of a wheat-based diet at 85°C. Confirmation of such information may assist in the selection of enzymes appropriate for the target substrate and the conditions at the site where they are most efficacious. The type of enzymes selected for use in poultry, for example, could be very different if the major site of action turned out to be the crop rather than the lower sections of the GI tract. The ability of the enzymes to resist proteolysis, their optimal operating temperature (particularly if most activity is expended during feed manufacture) and pH would be considerations for their selection. Sites of activity may also vary considerably between animal species, since the conditions in the GI tract vary considerably between pigs, poultry and ruminants. As a result, whereas today most enzymes destined for use in pig diets are derived from those used in products designed for poultry, it is likely that future ruminant, swine and poultry products will be designed specifically for the requirements of the target species. Indeed, differences clearly exist between young and older animals of the same species, and within the grouping of poultry there are significant differences between turkeys and chickens, for example. Determination of the sites and modes of action of specific enzymes in each of these species will ultimately lead to better products that are more cost efficient for the industry as a whole.

**Production of new enzymes**

Whilst the current generation of enzymes are highly beneficial, their usefulness will undoubtedly be surpassed when new activities with greater activity are made available. Sources other than those currently used, such as those produced by microorganisms in the rumen of cattle, from thermophilic organisms or from other extremophiles, will probably provide enzymes with the properties desired for maximal activity. Such properties will include: (i) highly specific activities (substrate turnover rates per unit of protein) under the conditions where they act; (ii) high levels of resistance to inactivation by heat treatment, low pH and proteolytic enzymes; (iii) low production costs; (iv) long shelf-life under ambient storage.
conditions; (v) lack of apparent binding to feed matrix to facilitate the quantitative determination of enzyme in finished feed; and (vi) specificity to suit the purpose for which they are designed.

**Substrate and product identification**

The focus so far has been on the developments in enzyme properties that are likely to occur in the next few years. Without a doubt the advances will only be made possible through better identification of the target substrates and the desired products of enzyme activity. Such knowledge will permit more efficient, rapid and focused development of enzymes that will have a biological effect, and will hopefully supplant the current empirical approach that is largely used for product development.

Many researchers have hypothesized that the principal mode of action of most xylanase and β-glucanase enzymes is the destruction of gel-forming polysaccharides leached from cereal cell walls in amounts sufficient to depress performance (Annison and Choct, 1991; Bedford, 1993; Chesson, 1993). Hesselman and Aman (1986) proposed an alternative explanation: namely, that the β-glucans and arabinoxylans that form the endosperm wall of cereals restrict the access of enzymes to nutrients. They postulated that the disruption of intact walls and the release of entrapped nutrients are the major factors in the improvement of the nutritive value of diets with exogenous enzymes. Chesson (1993) postulated that a single enzyme should be effective if the beneficial effects of enzymes are attributable solely to reduced viscosity. The reason is that viscosity is partially a function of chain length and so it is only necessary to break the chain in a few sites to reduce substantially or destroy its gel-forming capacity. However, if the beneficial effects of added enzymes are due to disruption of intact cell walls and release of entrapped nutrients, rather than reduced viscosity, then many more enzymes would be required. Regardless of mechanism, it is fair to say that the actual structure of the anti-nutritive factor in the intestinal lumen is not known. If the structure were known, then in vitro work using such a substrate would enable rapid screening of new enzyme candidates for use in new product design. With modern discovery and mutagenic techniques it is possible to supply many hundreds, if not thousands, of candidates for such screening methods. High-throughput robotic methods allow many thousands of assays to be completed in very short periods of time. As a result, the more faithful the screening method is in reproducing the ‘real life’ conditions and substrate, the more likely it is that the process will lead to provision of successful and cost-effective research.

Of further interest are the saccharides of various degrees of polymerization released in the process of cell wall carbohydrate hydrolysis, i.e. the products of enzymatic activity. Recent work discussed in this book is beginning to show that the response to addition of cell wall hydrolysing enzymes is due in large part to changes in the intestinal microfloral populations that accompany their use. The feeding of a xylanase selects for ileal populations of bacteria that can utilize xylose and
concomitantly alters caecal populations dramatically (e.g. Apajalahti and Bedford, 1999). Such shifts can be seen as a response to the addition of the enzyme and perhaps not a route through which they act on animal performance. Work with germ-free animals, however, tends to suggest that the animal performance responses observed on addition of enzymes may in fact be due to microfloral responses to the alteration in the availability of a number of substrates. Some substrates, such as starch and protein, are more rapidly digested in the presence of an effective enzyme and as a result bacteria that rely on such substrates are less prevalent. Others, such as cell wall oligo- and polysaccharides, increase in concentration and as a result populations that can use such substrates thrive. The areas for future investigation must therefore include a detailed understanding of the microflora of the gut. Desirable and undesirable species need to be identified and their nutritional requirements determined so that enzymes can be designed to supply the correct balance of oligosaccharides and peptides for beneficial organisms, whilst at the same time deny the pathogens their nutrition. Clearly such work is in its infancy but is likely to yield significant benefits.

Raw material pretreatment

Whilst partial hydrolysis of cell wall NSPs reduces intestinal viscosity and may accelerate digestion through permeation of endosperm cell walls, it is also likely that in many cases there is some degree of complete hydrolysis to monosaccharides. Glucose from cellulose and galactose from the α-galactosides can be readily utilized by most animals, whereas the pentoses cannot be readily utilized by all classes of livestock, especially poultry (Schutte et al., 1991, 1992). Most poultry, and even swine to some degree, however, do not have a long enough digesta residence time to enable complete cell wall hydrolysis. The development of raw material pretreatment procedures for the complete hydrolysis of the hemicellulose fraction cereals, and even less digestible feedstuffs such as straw, would be of great economic importance, as it could provide a basis for using products that are present in vast quantities but cannot be utilized to any appreciable extent by monogastric animals. The pretreatment of high-phytate ingredients such as rapeseed meal and cottonseed meal would allow a far greater degree of phytate hydrolysis than is currently achievable in vivo. Pretreatment would yield the added benefit that the content of anti-nutrients can be monitored throughout the process and controlled so that there is consistency from batch to batch. Currently there is variation in efficacy of enzymes added to complete feed, due to variations in dosage rate, survival through processing and intestinal tract conditions. Control of enzyme addition rates, pH, temperature and moisture during processing would likely increase uniformity of product and hence of animal nutrition. Such procedures, in order to be economic, would need to be at minimal cost, which inevitably means low moisture and temperature. Such requirements set the challenge for enzyme discovery projects.
Targeted enzymes

Knowledge of the exact conformation of the substrate to be degraded may confer additional benefits to future enzyme discovery programmes. Often, the target may be a protein, and as a result enzymes are selected on their ability to degrade the anti-nutrient. Blanket administration of a such an enzyme to a complete diet has two problems. The first is that the target protein substrate may represent only a few per cent of the total protein in the diet. In order to be effective, large amounts of protease need to be added in order to provide enough activity to seek out the desired target and render it ineffective. In such a scenario, much of the total protein in the diet is degraded to a lesser or greater extent compared with the target, the extent depending upon its relative susceptibility to the enzyme. Whilst this may be of benefit in speeding up digestion of protein in general, it may also produce bitter peptides in the process, which are thought to decrease diet palatability. As a result, it would be desirable if the added enzyme hydrolysed only the targeted protein and left other proteins intact. Not only would the enzyme be more specific, but in all likelihood far less would be needed. The development of such enzymes, sometimes referred to as catalytic antibodies, is a key area of research. Once the identity of the substrate is well characterized, this technology will lead to the production of antibodies with enzyme-like properties designed to meet the requirements of specific feedstuffs and classes of livestock and poultry (Lerner et al., 1992; Mayforth, 1993). Modifications of these procedures will undoubtedly develop. Alternatively, antibodies could be linked to specific proteases to concentrate the enzyme on its target substrate.

Alternative sources of enzymes

Enzymes are not only produced by fungi that have been improved using traditional methods but also expressed in microorganisms, such as bacteria, and in plants, such as in canola seed. Production in plants is seen as a far less costly method of manufacture. An abundant supply of enzymes in canola seed, for example, would dramatically reduce the cost of enzyme production and hence costs to the livestock producer. Production of phytase in canola or xylanase in rye, if successful, would also result in a higher value feedstuff. Whilst enzyme selection and discovery are still in their infancy, however, it is likely that production techniques need to be able to react rapidly to new discoveries. At present it is quicker to bring a new product to market through fermentation methods than it is through production in plants. Once the rate of improvement in next-generation enzymes slows down, and the base enzyme activities effectively become commodities, then considerations of costs of production will likely drive enzyme production through plant ‘biofactories’. Whilst emphasis here is on reducing costs and economics, it must be born in mind that by reducing costs the scope for enzyme usage increases dramatically. Ten years ago, xylanases were too expensive to consider as viable additives in pig feed. Current prices have allowed rapid expansion into this sector in recent years. Use of enzymes in ruminant feeds is mostly constrained by the cost : efficacy ratio, a ratio that will improve over the next
few years and as a result lead to greater enzyme use in this, as yet, untapped area of animal feed.

An alternative strategy for the hydrolysis of anti-nutritive compounds in animal feeds is to construct transgenic monogastric animals able to digest cellulose, β-glucans, xylans or phytic acid. Forsberg et al. (1993) concluded that it should be feasible to use bacterial DNA constructs to express and secrete glycanases in rat pancreatic acinar cell lines. The major challenge will be to obtain sufficiently high levels of the expression of glucanase genes and other genes in pancreatic cells to effect adequate hydrolysis of the glucans, xylans, etc., in the intestine. Again, such a strategy would require that the enzymes of interest were commodities and not likely to be improved upon by genetic manipulation. Only under these circumstances would the gene inserted into the animal be competitive with the bacterial, fungal, or plant-derived products freely available on the market at the time of introduction of the animal to the marketplace. The animal breeders would have to be certain of such a state of affairs, since the multiplication time required from germ-line breeding stock to food-producing animals can take as long as 5 years, which is more than sufficient time for development of superior products through fermentation methods.

**Enzyme dosage and synergies**

Most enzymes present on the market are simple xylanases, β-glucanases or phytases. It is fair to say that whilst there are some data describing the dose–response characteristics of many of these products, this relationship is for the most part poorly described, particularly when compared with the data available for several essential amino acids, for example. As a result the current usage of such products is far from optimized. Dose-titration studies and linkage of the enzyme dose to the substrate quantity present in any complete diet will certainly help to improve on the economics of current enzyme usage. Zhang et al. (1996) recently showed that they could predict the response of poultry to a particular feed enzyme by using a simple linear model. The model predicts that the response to enzyme supplementation is a function of enzyme concentration. When this is converted to a logarithmic value, a doubling or halving of the response to enzyme treatment can be achieved only by varying the amount of enzyme by a factor of 10, not 2 as may be expected. Clearly, as the costs of feed and enzyme change in proportion to one another, the economically optimum dose of enzyme will change also. The ability to match feed and enzyme costs with substrate concentration and thus predict optimum enzyme dosage via computer models is currently available in wheat- and barley-based rations. The introduction of near-infrared techniques for prediction of substrate concentrations, hence allowing for an ‘on-line’ enzyme dosage optimization at the level of the feed mill, is a clear goal for optimizing the economy of enzyme usage. The advantages of titration of the correct amount of enzyme are realized in terms of not only more cost-efficient enzyme usage, but also greater uniformity of feed and hence animal production.

Nearly all the research done on the response of poultry to enzymes (with the exception of phytase) has used crude fungal extracts with high levels of the desired
activities, such as of β-glucanase or xylanase, but also with considerable amounts of other side activities or contaminating enzymes, including proteases. Research carried out using different combinations of pure enzymes (that is, devoid of contaminating and potentially synergistic enzyme activities) has and will allow determination of whether the principal enzymes have synergistic, antagonistic or additive effects. For example, the ability of enzymes to reduce the viscosity of the water-soluble arabinoxylans in wheat or rye may depend on the amount not only of endo-xylanase but also of arabinofuranosidase, and possibly β-glucanase, acetylxylan esterase and feruloyl esterase, in the preparation (Forsberg et al., 1993). Recent work suggests that there may be synergies between phytases and xylanases (Ravindran et al., 1999). Detailed descriptions of substrates will allow for more focused research into enzyme mixtures based on likely synergistic interactions.

Studies with other animals

Although studies with chickens have clearly established the benefits of enzymes as feed additives, only a limited number of studies have been carried out with other species of poultry, such as turkeys, ducks, geese and ostriches. Also, very few studies have been carried out using fish, eels, alligators, turtles, other exotic animals, pets (such as dogs and cats) and fur-bearing animals that have simple stomachs. Enzymes may be particularly beneficial in the diets of animals that tend to be carnivorous, as they also tend to have GI tracts with smaller large intestines and therefore do not house a large population of anaerobic microorganisms capable of hydrolysing complex carbohydrates.

Conclusions

In the past several years, the use of enzymes as supplements in feeds has expanded dramatically. The research that led to this was mostly carried out at universities and research institutions and funded in large part by the industry, in cooperation with government agencies. Although dramatic progress has been made in the past decade on the use of enzymes in the diets of poultry and livestock, additional research will be needed in many areas to exploit the full potential of this very powerful and beneficial technology. Areas of research and development should include the following:

- Development of more sensitive, accurate and (most importantly) biologically meaningful assays.
- Precise identification of the catalytic properties of enzymes most suited to different classes of livestock and poultry through characterization of the substrates and environments in which they reside.
- A better understanding of the effects that enzymes have on the physiological, microbiological and endocrine responses of animals fed cereal-based diets.
Development of methods to predict substrate concentrations and use of models to predict responses to enzyme treatment and hence optimize economy and efficacy of usage.

It is clear from the above that, in order to be successful, research into this field needs to move from what has been a relatively simple approach to one that is more multidisciplinary. Whilst the roadmap for development of new products from a research viewpoint seems clear, it is by no means clear when viewing the current climate of animal feed production. Bovine spongiform encephalopathy, dioxins in fat sources, sewerage in animal feed and, most importantly, the use of antibiotics in animal production have in recent times increased consumer, regulatory and media interest in food production processes. Genetically modified organisms are currently suffering acute attention and rejection in some parts of the world. Such attention may well increase the time required to introduce new products into some parts of the world, which serves as a reminder that the identification, improvement and production of these products is only one step in the process of their introduction into the marketplace.

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