Enzymes in Farm Animal Nutrition

2nd Edition

Edited by
Michael Bedford and Gary Partridge

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Preface

There have been considerable developments in the feed enzyme industry since the first edition of this book was published in 2001, both in terms of the size and scope of the commercial market and in our scientific understanding of how feed enzymes function. With such rapid changes it became clear that much of the information in the first edition of this book needed to be updated. The reader is referred back to the first edition for a foundation in the fundamentals of the market and science, whereas this edition is more focused on changes in the interim. Most notable is the rapid expansion of both the phytase and non-starch polysaccharide (NSP) enzyme segments. Today, the total feed enzyme market is approximately four times larger in value terms than it was in the early 2000s, but the split in species applications remains broadly similar. Sales are highest in poultry, followed by swine, with the ruminant market in its infancy. Aquatic and pet applications have yet to become commonplace. Penetration of phytase into the poultry and swine sectors is relatively high, while the NSP enzyme market still has some considerable growth potential, particularly in swine. Much of the expansion of the market has been driven by reduced inclusion costs of feed enzymes, which was predicted in the first edition, and significant volatility in feed ingredient prices, which was not. The latter drove many feed manufacturers to seek methods, such as enzymes, to maximize utilization of less costly raw feed materials as the prices of maize, soy, fat and mineral phosphates soared in late 2007.

Investigations into the mode of action of feed enzymes have continued apace. This has particularly been the case in phytase research, where it is clear that the valuable benefit of this enzyme is not simply through the provision of phosphate, but also via the destruction of phytic acid, which has been increasingly reported in the scientific literature as a potent anti-nutrient. Similarly, a better understanding of the complex links between feed enzyme

function and digestive physiology has positively influenced application recommendations for feed enzymes.

The practical challenge remains to identify when feed enzyme use is best justified. The huge number of factors that can contribute to an enzyme response have to be brought together into a composite, and easy to understand, application recommendation. Such descriptive models are beginning to appear, and are making enzyme use more of a science than an art, which was the challenge identified in the first edition of this book. There is still a considerable way to go, however, particularly as the use of more than one enzyme in a feed is now becoming commonplace, and consequently begs the question whether the subsequent response will be sub-additive, additive or potentially synergistic. It is likely in the next decade that enzyme use will be more individually tailored to the needs of specific feed formulations than is currently the case, thereby further maximizing the value of feed enzyme addition.

1 Introduction: Current Market and Expected Developments

A. Barletta

Creating Value through Innovation

Feed enzymes help meet consumer demand for safe, high quality and affordable food. Since the late 1980s, feed enzymes have played a major part in helping radically to improve the efficiency of meat and egg production by changing the nutritional profile of feed ingredients. By targeting specific anti-nutrients in certain feed ingredients, feed enzymes allow pigs and poultry to extract more nutrients from the feed and so improve feed efficiency. They allow the feed producer greater flexibility in the type of raw materials that can confidently be used in feed formulation. In addition, feed enzymes play a key role in reducing the negative impact of animal production on the environment, by reducing the production of animal waste.

Why Use Enzymes in Animal Feed?

All animals use enzymes to digest feed. These are either produced by the animal itself, or by the microbes naturally present in the gut. However, the animal's digestive process is not 100% efficient. Pigs and poultry cannot digest 15–25% of the feed they eat, because the feed ingredients contain indigestible anti-nutritional factors that interfere with the digestive process and/or the animal lacks specific enzymes that break down certain components in the feed.

In many animal production systems feed is the biggest single cost, and on-farm profitability can depend on the relative cost and nutritive value of the feed ingredients available. If feeds are not digested by the animal as efficiently as they could be, there is a cost to both the producer and the environment.

Supplementing the feed with specific enzymes improves the nutritional value of feed ingredients, increasing the efficiency of digestion. Feed enzymes

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help break down anti-nutritional factors (e.g. fibre, phytate) that are present in many feed ingredients. Anti-nutritional factors can interfere with normal digestion, resulting in reduced meat or egg production and lower feed efficiency, and can also trigger digestive upsets. Feed enzymes are used to increase the availability of starch, protein, amino acids and minerals such as phosphorus and calcium from feed ingredients. In addition, they can be used to supplement the enzymes produced by young animals where, because of an immature digestive system, enzyme production may be inadequate. Enzymes are proteins that are ultimately digested or excreted by the animal, leaving no residues in meat or eggs.

The benefits of feed enzymes include:

- improving efficiency and reducing cost – by breakdown of anti-nutrients allowing the animal to digest its feed more efficiently, leading to more meat or eggs per kilogram of feed;
- for a better environment – improving digestion and absorption of nutrients, reducing the volume of manure produced and lowering phosphorus and nitrogen excretion;
- improving consistency – reducing the nutritional variation in feed ingredients, resulting in more consistent feed for more uniform animal growth and egg production; and
- helping to maintain gut health – by improving nutrient digestibility, fewer nutrients are available in the animal's gut for the potential growth of disease-causing bacteria.

What Types of Enzymes are Used in Animal Nutrition?

Enzymes are categorized according to the substrates they act upon. Currently, in animal nutrition the types of enzymes used are those that break down fibre, proteins, starch and phytate.

Carbohydrases

Carbohydrases break down carbohydrates into simpler sugars. In animal nutrition they can be broadly categorized into those that target either non-starch polysaccharides (fibre) or starch.

Fibre-degrading enzymes

All plant-derived feed ingredients contain fibre. Fibre is made up of a number of complex carbohydrates (non-starch polysaccharides) found in the cell walls of plants. There are two main types of fibre: soluble and insoluble. Fibre can act as an anti-nutrient in a number of ways. First, some nutrients such as starch and protein are trapped within the insoluble fibrous cell walls. Pigs and poultry are unable to access these trapped nutrients as they do not produce the

enzymes capable of digesting the fibre within the cell walls. Secondly, soluble fibres dissolve in the bird's or pig's gut, forming viscous gels that trap nutrients and slow down the rates of digestion and passage of feed through the gut. Thirdly, fibre can hold water and trap water-soluble nutrients. Finally, fibre creates bulk in the gut, which slows down the movement of feed, reducing feed intake and subsequent growth.

The two main fibre-degrading enzymes used in animal feed are xylanase and β-glucanase. Xylanases break down arabinoxylans, particularly prevalent in grains and their by-products. β-glucanases break down β-glucans that are particularly prevalent in barley and oats and their by-products. Other fibre-degrading enzymes currently used in animal nutrition, but to a lesser extent, include β-mannanase, pectinase and α-galactosidase.

Starch-degrading enzymes

The degree of starch digestibility in plant-based feed ingredients will vary according to the levels of resistant starch, starch granule size, starch composition and starch encapsulation. Differences in plant genetics, growing conditions, harvesting conditions, handling, drying, storage and feed manufacturing processes are all likely contributors to variability in starch digestibility.

Amylases break down starch in grains, grain by-products and some vegetable proteins. By increasing starch digestibility, amylases potentially allow pigs and poultry to extract more energy from the feed, which can be efficiently converted into meat and egg production. In young pig diets, amylases provide benefits by supplementing an immature digestive system where low feed intake post-weaning is associated with a slow maturation of amylase secretion. In addition, amylase also allows the use of less cooked grain in the diet, with resultant benefits in feed cost reduction, without compromising young pig performance after weaning.

Proteases

Proteases are protein-digesting enzymes that are used in pig and poultry nutrition to break down storage proteins in various plant materials and proteinaceous anti-nutrients in vegetable proteins.

Seeds, particularly of leguminous plants such as soy, contain high concentrations of storage proteins. Storage proteins are proteins generated mainly during seed production and stored in the seed to provide a nitrogen source for the developing embryo during germination. Storage proteins can bind to starch. Proteases can help break down storage proteins, releasing bound energy-rich starch that can then be digested by the animal.

Two major proteinaceous anti-nutrients are trypsin inhibitors and lectins. Trypsin inhibitors are found in raw vegetable proteins, such as soybeans. They can inhibit digestion as they block the enzyme trypsin, which is secreted by the pancreas and helps break down protein in the small intestine. Lectins are sugar-binding proteins that have also been shown to reduce digestibility. While

it is common practice to heat soy products during processing to reduce both the trypsin inhibitors and lectins, excessive heat processing will reduce the availability of amino acids, in particular lysine. Thus optimally processed soybean meal will contain residual levels of trypsin inhibitors and lectins. Proteases can be used to reduce the levels of trypsin inhibitors and lectins, thus improving protein digestibility.

Phytases

Phosphorus is important for bone development and metabolic processes in pigs and poultry. Most of the phosphorus in plant-derived ingredients is in the form of phytate, which is the main storage form of phosphorus in plant seeds. In the plant, phytate forms complexes with minerals (such as phosphorus and calcium), proteins and starch, making them unavailable for absorption. Pigs and poultry do not produce the phytase enzyme that breaks down phytate. Supplementing the feed with phytase releases phytate-bound minerals, proteins and starch, which can then be digested and absorbed by the animal to improve the efficiency of meat and egg production. Phytases also reduce the risk of pollution of watercourses from excess phosphorus excreted by pigs and poultry.

Market Development

In the 1980s, the introduction of feed enzyme technology in Europe revolutionized the poultry industry. Wheat and barley, the main cereal grains used in poultry diets in northern Europe, both contain high levels of soluble fibres that dissolve in the bird's gut, increasing gut viscosity. High levels of gut viscosity reduce bird weight gain and feed efficiency due to a reduced rate of digestion and impaired nutrient absorption. Related to high gut viscosity, wet litter was also a common problem, leading to relatively high incidences of hock burns and breast blisters that reduce carcass quality and the market value of the bird. In addition, wheat and barley can be highly variable in nutritive value, resulting in variable bird growth and feed efficiency. The introduction of fibre-degrading enzymes, specifically xylanases and β-glucanases, provided clearly visible benefits. By breaking down the soluble fibres, litter quality was significantly improved, feed costs were radically reduced due to a marked improvement in feed efficiency and bird uniformity was enhanced. Europe, Canada, Australia and New Zealand are markets where wheat and barley feature prominently in pig and poultry diets. Today, the majority of wheat- and barley-based poultry feeds (particularly broiler) and piglet feeds contain xylanase and β-glucanase feed enzymes. Their use in grower/finisher wheat- and barley-based pig feed, however, is still relatively limited.

The next major breakthrough came in the 1990s with the introduction of phytase feed enzymes. The key driver for phytase adoption was the

environmental benefits of reducing phosphorus excretion from pigs and poultry, particularly in markets such as the Netherlands and Germany and certain states in the USA surrounding Chesapeake Bay, where environmental legislation existed to minimize the negative impact of animal production on the environment. Opportunities for phytase to reduce feed costs as well as provide an environmental benefit have opened up considerably over time. Phytase allows feed producers to reduce the amount of inorganic phosphorus that has to be added to the feed to meet the animal's phosphorus requirements. To reduce feed costs by improving phosphorus digestibility, phytase has to be cost competitive against inorganic phosphorus. As the number of phytase suppliers has increased over time, price erosion from increasing competition, together with improvements in phytase costs of production, have radically reduced the cost of adding phytase to pig and poultry feed. More recently, phytases have been shown to provide additional economic benefits by also improving energy, protein and amino acid digestibility. Consequently the economic benefit of phytase in terms of reducing feed costs has become very attractive and is now the main driver for its use in feed compared with its environmental benefits. Today, it is estimated that more than two-thirds of industrial pig and poultry feeds contain phytase.

In terms of growth potential, there has recently been increasing activity focusing on carbohydrase-based enzyme products for maize- (corn) based diets. The potential is huge. Around 80% of global pig and poultry feed is based on maize. While the majority of wheat- and barley-based poultry feed contains carbohydrase-based enzyme products, it is estimated that only around one third of maize-based poultry feed contains carbohydrase enzymes. Asia and the Americas are the lands of opportunity for this market segment.

Today, enzyme suppliers are actively promoting the additive benefits of combining phytase and carbohydrase products in feed to further drive down costs of producing pigs and poultry. The theory is that each type of enzyme is targeting different anti-nutrients in the diet, and that by adding a combination of the enzyme activities, more energy, amino acids and minerals are released compared with these enzyme activities being used in isolation.

The animal feed enzyme market has grown at an average rate of 13% per year over the period 1998–2008 (Freedonia, 2009). Feed enzymes are now widely used to improve the nutrition of pigs and poultry. Today the market is worth in excess of US$650 million. However, use of feed enzymes in the aquaculture sector is very low and, in ruminants, non-existent. Figure 1.1 summarizes the evolution of feed enzyme products for different applications since the early 1990s.

Drivers for Demand

Feed enzymes improve the efficiency of meat and egg production. It follows, therefore, that the market opportunity for feed enzymes is dependent upon the demand for meat and egg products. The expanding world population and

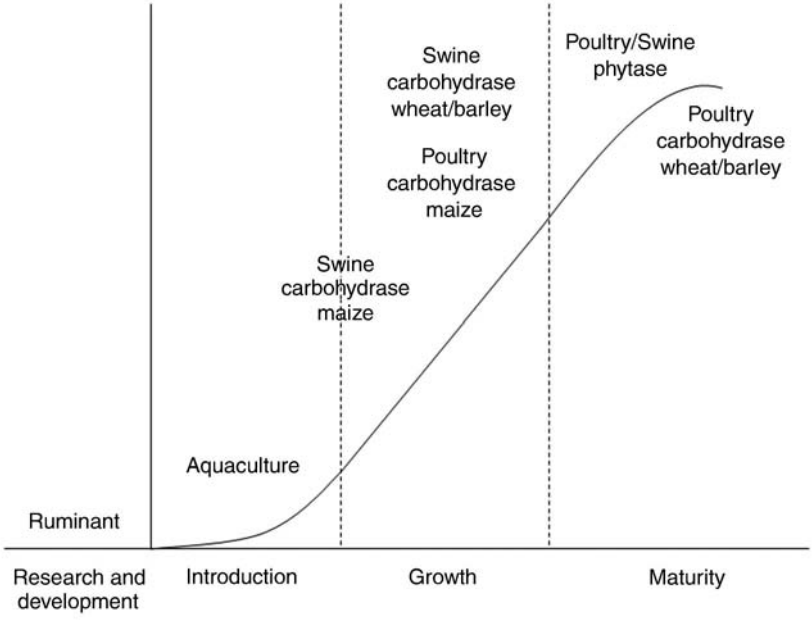

Fig. 1.1. Evolution in the market development of feed enzymes for various applications.

increasing disposable incomes, particularly in developing countries, are the current drivers for the growth in meat and egg consumption. World poultry meat production in 2008 was estimated to be around 94 million t and forecast to grow by around 1% to approximately 95 million t in 2009 (FAO, 2009b), a 42% increase since 2000 (FAO, 2001). World pig meat production in 2008 was estimated to be around 104 million t and forecast to grow by just over 2% to around 106 million t in 2009 (FAO, 2009b), a growth of 16% since 2000 (FAO, 2001 and Table 1.1). The forecast increase in world pig meat production will be driven by sizeable increases in China, which accounts for half of the world pig meat production, as well as increases in Canada, Mexico and Vietnam.

Table 1.1. World meat markets (million t), 2000–2009 (FAO, 2001, 2009b).

	2000	2007	2008 (estimate)	2009 (forecast)	Growth (2008–2009, %)	Growth (2000–2009, %)
Bovine meat	60.0	65.1	64.9	65.1	0.3	8.5
Poultry meat	66.6	90.1	93.7	94.7	1.1	42.2
Pig meat	91.1	99.8	103.9	106.1	2.1	16.5
Ovine meat	11.4	14.0	14.2	14.2	0.5	24.6
Meat production (total)	233.4	274.4	282.1	285.6	1.2	22.4

World feed volumes have risen from just over 610 million t in 2000 to exceed 700 million t in 2008. Global output of feeds for farm animals and fish has grown nearly 15% between 2000 and 2008, with the USA, EU, China, Brazil and Mexico accounting for around two-thirds of the global industrial feed production (Best, 2009).

Feed enzymes also reduce the production of animal waste. Growing concerns over the environmental impact of increasing animal production will also stimulate demand for feed enzymes, particularly phytase, to reduce the risk of phosphorus pollution of watercourses. While a high proportion of animal feed today contains phytase, the opportunity to increase inclusion levels of phytase to further reduce the risk of phosphorus pollution may stimulate further growth in this sector.

Drivers for Value

Reducing feed cost is the principal reason for using feed enzymes. Feed accounts for around 70% of total costs in pig and poultry production. Energy, protein and minerals are the main constituents of pig and poultry feed. Because enzymes improve the digestibility of energy, protein and minerals in the feed, feed manufacturers can reduce costs by reformulating feed to contain lower levels of these nutrients. The value of adding enzymes to feed will be heavily dependent upon the cost of the enzyme versus the cost of energy sources such as maize and fat, protein sources such as soybean meal and inorganic phosphorus sources such as dicalcium phosphate. When maize, wheat, fat and inorganic phosphorus prices increase, the use of enzymes in feed becomes more economically attractive, providing a bigger return on investment. In 2008, the value proposition for feed enzymes was particularly attractive, driven by the exceptionally high cost of edible oils and feed phosphates. The relative cost of oils and fats in 2008 was more than 2.5 times higher than in 2002–2004 (FAO, 2009a). In 2007 and 2008, the price of feed phosphates rocketed due to an imbalance between supply and demand for phosphate fertilizers. Demand for phosphate fertilizers sharply increased due to increased demand for global crop production to feed developing nations and to produce more crops for biofuels.

Ingredient quality is also under pressure. Increasing demand for ingredients such as maize, wheat, barley and soybean meal from the food and biofuels industries means that these ingredients are in shorter supply and become more expensive. Lower-cost, less-digestible ingredients such as cassava and by-products from the food and biofuels industries are being used increasingly in feed. Some of these ingredients tend to be higher in fibre and consequently less digestible. Adding fibre-degrading enzymes improves nutrient availability to the animal, allowing feed manufacturers greater flexibility in the types and levels of high-fibre raw materials that can confidently be used in feed formulation.

The Regulatory Environment: Quality, Efficacy and Safety

Most of the major feed markets have a regulatory approval process in place whereby feed enzyme producers have to provide proof of product quality, efficacy and safety before marketing of the products is permissible. The level of detail and time required to gain approval varies from market to market.

The EU is among the most highly regulated markets. In the EU feed enzymes must be approved under Regulation EC 1831/2003. Its principal aim is to ensure that the feed enzyme approved for use in the EU is safe to the animal for which it is intended, safe for those involved in its handling and also for the consumer. Each enzyme must undergo a series of tests to demonstrate its safety. In addition, data are required to support its efficacy in the target animal(s) for which it is intended. Safety, quality and efficacy data are presented in a dossier which is reviewed by the European Food Safety Authority (EFSA). The conditions of use for approved feed enzymes are published in an authorizing regulation in the Official Journal. A summary list of authorized feed additives is published in the feed additive register, which is published on the European Commission's website. Enzymes are categorized as zootechnical additives as they improve the nutrient status of the animal. The approval process in the EU typically takes up to 2 years.

On the other hand, the requirements and process in, for example, Mexico are currently less severe. While a dossier supporting product quality, safety and efficacy is required, the approval process usually takes around 6–9 months.

Over time the regulatory environment for feed enzymes has become increasingly stringent.

Who's Who in Feed Enzymes?

The feed enzyme market is dominated by four key players at the time of writing. Danisco Animal Nutrition, Novozymes/DSM, BASF and Adisseo account for an estimated 70% of the market. There are many other players in the remainder of the market, including AB Vista, Alltech, Beldem, Chemgen, Kemin and a multitude of Chinese suppliers.

Danisco Animal Nutrition (UK) is a business unit of a leading global food ingredient specialist Danisco A/S (Denmark). Danisco Animal Nutrition (formerly Finnfeeds International Ltd) pioneered the development of feed enzymes in the 1980s. Its enzyme products currently include a phytase Phyzyme XP and a range of carbohydrase-/protease-based products – Avizyme® (poultry), Porzyme® (pigs) and Grindazym® (pigs and poultry).

Novozymes (Denmark) and DSM (the Netherlands) formed a strategic alliance in 2001. DSM is responsible for the sales, marketing and distribution of Novozymes' feed enzymes. Novozymes is responsible for product development and R&D. The alliance covers pig, poultry and pet feed. Their portfolio of feed enzyme products currently includes a protease Ronozyme® ProAct, a phytase

Ronozyme® P and a range of carbohydrase-based products marketed under the brand names Ronozyme® and Roxazyme®. Novozymes reported 744 million DKK sales of feed enzymes in 2008 (Novozymes, 2009a).

As the world's leading chemical company, BASF's feed enzyme products include Natuphos® (phytase) and Natugrain® (carbohydrase). The majority of BASF's feed enzyme sales currently are within the phytase segment.

Adisseo (France) specializes in animal nutrition, providing amino acids, vitamins and enzymes to the animal feed industry. Its feed enzyme portfolio currently includes Rovabio™ Excel (carbohydrase) and Rovabio™ Max (carbohydrase and phytase). The majority of Adisseo's feed enzyme sales are currently within the carbohydrase segment.

How are Enzymes Used in the Feed?

Enzymes can be added to feed in one of two ways. One option is to reformulate the feed to reduce feed costs and at least maintain animal growth, egg production and feed conversion; for example, replace some wheat, barley or maize with lower-cost, higher-fibre by-products and/or reduce the added fat level in the diet. The second option is to add the enzyme to the standard feed formulation and achieve improved animal growth, egg production and feed conversion giving enhanced efficacy of production by improving the efficiency of feed utilization.

In practice, matrix values for least-cost feed formulation are often assigned to the enzyme product. Generated from animal studies, these matrix values will typically be for phosphorus, calcium, protein, amino acids and energy. The matrix values quantify the extent to which nutrients are released by using the enzyme.

In addition, for enzymes to be effective when added to the feed they must be active in the animal, stable during storage and be compatible with minerals, vitamins and other feed ingredients. Equally, they must be stable at the high temperatures reached during feed manufacture, safe and easy to handle and free-flowing to ensure thorough mixing throughout the feed.

Looking to the Future

The animal production industry is in constant flux. Feed ingredients, animal genetics, disease challenges and consumer demand are just some of the factors that are constantly changing and providing new challenges for the feed industry.

The world population is forecast to rise from the current 6.7 billion people (2009) to 9.1 billion people by 2050, with most of the growth coming from developing countries (FAO, 2009d).

With over one-third more mouths to feed, the UN Food and Agriculture Organization (FAO) predicts that 70% more food will need to be produced by

2050. Meat production will have to grow by more than 200 million t to reach a total of 470 million t by 2050, 72% of which will be consumed in developing countries, up from the 58% of today (FAO, 2009c).

In terms of countries offering significant potential for business growth, in the medium term, markets such as Brazil, Russia, China and India are likely to become increasingly attractive. Different factors will contribute to growth in these markets. In Russia, for example, imports from the USA are being replaced by an increase in home-reared pigs and poultry. Overall, meat consumption in developing countries is expected to account for the majority of projected global growth. In China and India, increased economic wealth together with growth in the human population will increase the demand for pig and poultry meat. Today, half of the world's pork is consumed in China. Brazil continues to be able to produce poultry meat at very low cost, making its chicken products commercially attractive in markets such as the EU. Their importance in supplying international meat markets will substantially increase, and are expected to assume one-third of total world meat exports by the end of 2018 (OECD-FAO, 2009).

New market segments such as aquaculture and the dairy sector will open up further opportunities for feed enzymes. The ever-growing population and shift in food habits has resulted in increased demand for fish and related products. The Asia-Pacific region contains the major fisheries and aquaculture markets in terms of production. Prospects for feed enzymes in aquaculture include replacement of expensive fishmeal, a major component of aqua diets, with plant-derived protein sources to radically reduce feed costs and relieve the growing pressure on the world's wild fish and seafood stocks for fishmeal.

For the dairy sector, maximizing milk from forage continues to be a key driver for on-farm profitability. Developing enzyme products that can easily, safely and economically be added to on-farm forage to improve its digestibility is another new opportunity for enzyme producers, e.g. Novozymes has indicated that it plans to launch an enzyme product for ruminants in 2009/10 (Novozymes, 2009b).

The future for technologies such as feed enzymes is very bright. Feed enzymes will play a major role in efficiently supporting the growth in animal-derived food products needed to feed the world in a safe, affordable and sustainable way.

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2 Xylanases and Cellulases as Feed Additives

M. Paloheimo, J. Piironen and J. Vehmaanperä

Introduction

The current global feed enzyme market size is estimated to be about US$550–600 million, phytase having the greatest share of about 50% of this and the non-starch polysaccharide (NSP) enzymes contributing to the remaining 50%, xylanases having a slighter larger share than the β-glucanases (James Laughton, personal communication, Danisco Animal Nutrition, 30 October 2008. The β-glucanases and xylanases have been used as feed additives for over 20 years and their ability to improve the feed conversion ratio and weight gain of monogastric animals (poultry and pigs) has been demonstrated in numerous publications. The use of these enzymes has been restricted primarily to poultry and pigs, although research focusing on supplemental enzymes for ruminants, fish as well as fur and pet animals has been also carried out during recent years (Dawson, 1993; Cowan, 1995; Twomey *et al.*, 2003; Brzozowski and Zakrzewska-Czarnogórska, 2004; Valaja *et al.*, 2004; Farhangi and Carter, 2007).

Starch, proteins and lipids can be easily degraded by the bird's and pig's own digestive systems, whereas the major parts of NSPs (soluble and insoluble) remain intact because of the lack of suitable enzyme activities within the digestive tracts of the animals.

The positive nutritional effects achieved by the addition of enzymes in feed are proposed to be caused by several mechanisms. First, it has been shown that the anti-nutritive effects of 'viscous cereals' (barley, wheat, rye, oats and triticale) are associated with raised intestinal viscosity caused by soluble β-glucans and arabinoxylans ('pentosans') present in those cereals (Bedford and Classen, 1992; Choct and Annison, 1992; Bedford and Morgan, 1996). These hold significant amounts of water and, due to the resulting high viscosity, the absorption of nutrients becomes limited. In practical conditions this can be

seen as reduced feed conversion ratio (FCR) and weight gain, as well as wet droppings in poultry. These problems are overcome by the addition of β-glucanases and xylanases, resulting in improved animal performance. Other benefits of enzyme supplementation in poultry associated with digesta viscosity include reduction in the number of dirty eggs and enhanced egg yolk colour.

Results from several studies indicate that enzymes are able to improve animal performance also with 'non-viscous cereals' such as maize and sorghum (Choct, 2006), or in pigs, where the mode of action differs from that of poultry due to differences in the digestive systems (Dierick and Decuypere, 1994). As a consequence, it is widely assumed that the ability of β-glucanases and xylanases to degrade plant cell walls leads to release of nutrients from grain endosperm and aleurone layer cells. Therefore, this mechanism can also be regarded as important for improving the feed energy value.

A third proposed mechanism having a positive influence on the nutritive value of feed is the prebiotic effect achieved via the release of oligosaccharides (Choct and Cadogan, 2001). Oligosaccharides are reserve carbohydrates, which are mobilized from storage organs such as seeds and tubers during germination. They can be also formed during the degradation of storage and cell wall carbohydrates by supplemental enzymes. Chemically they are defined as glycosides containing between three and ten sugar moieties. In animals the oligosaccharides derived from cell wall digestion resist the attack of digestive enzymes, thus being able to reach the colon, where they work as 'prebiotics' supporting proliferation of beneficial microflora such as *Bifidobacterium* and *Lactobacillus* spp., and at the same time suppressing the growth of pathogenic bacteria such as *Salmonella*, *Clostridium*, *Campylobacter* and *Escherichia coli* (Thammarutwasik *et al.,* 2009).

In addition to the increased energy value obtained through different mechanisms, the use of NSP enzymes also provides other benefits. Diet formulation has become more flexible when differences in feed ingredient quality or animals' digestibility capacity can be controlled by supplemental enzymes. Also, enzyme supplementation allows greater use of raw materials of lower nutritional value. These include by-products, such as bran, which are typically rich in fibre. Furthermore, by increasing digestibility of raw materials NSP enzymes can reduce the amount of faecal mass. However, the best-known environmental benefits have not been obtained by NSP enzymes but by phytase supplementation.

As a simple rule, it can be concluded that β-glucanases are typically used in barley- and oat-based diets, whereas xylanases have been traditionally recommended for wheat-based diets. Due to the complex structure of cereal grains, it has been shown that improved performance can be obtained by appropriate combinations of different enzyme activities (Düsterhöft *et al.*, 1993). In terms of cereals, xylanase/β-glucanase combinations are common.

In this chapter, an overview of β-glucanases (cellulases) and xylanases and their production will be given. The emphasis will be on the current enzyme products on the market. Also, the development of feed enzymes produced over recent years and possible future trends will be discussed.

Substrate

General structure of cellulose and β-glucans

Cellulose is the most abundant biopolymer on Earth, plants producing about 180 billion t per year globally. Plant cell walls typically consist of about 35–50% cellulose, 20–35% hemicellulose and 10–25% lignin by dry mass (Sticklen, 2008). Cellulose is a water-insoluble β-glucan consisting of a linear molecule of up to 15,000 D-anhydroglucopyranose residues linked by a β-(1→4) bond. Anhydrocellobiose is the repeating unit of cellulose in which the adjacent glucose moieties are rotated 180° with respect to their immediate neighbours (Fig. 2.1). The cellulose microfibrils are aligned in a parallel fashion to create crystalline regions with maximal hydrogen bonding. Other regions of the fibril are less organized and form paracrystalline (amorphous) sections. As described below under cellulases, endoglucanases are believed to attack the amorphous regions and produce chain ends which serve as a substrate for the exoglucanases (cellobiohydrolases). These latter enzymes produce the disaccharide cellobiose (Fig. 2.1), which is hydrolysed to two glucose monomers by β-glucosidase (Bhat and Hazlewood, 2001; Zhang and Lynd, 2004; Aro *et al.*, 2005; Sticklen, 2008).

In cellulase studies, several model cellulosic substrates with varying degrees of crystallinity are used. Bacterial micro-crystalline cellulose (BMCC) from

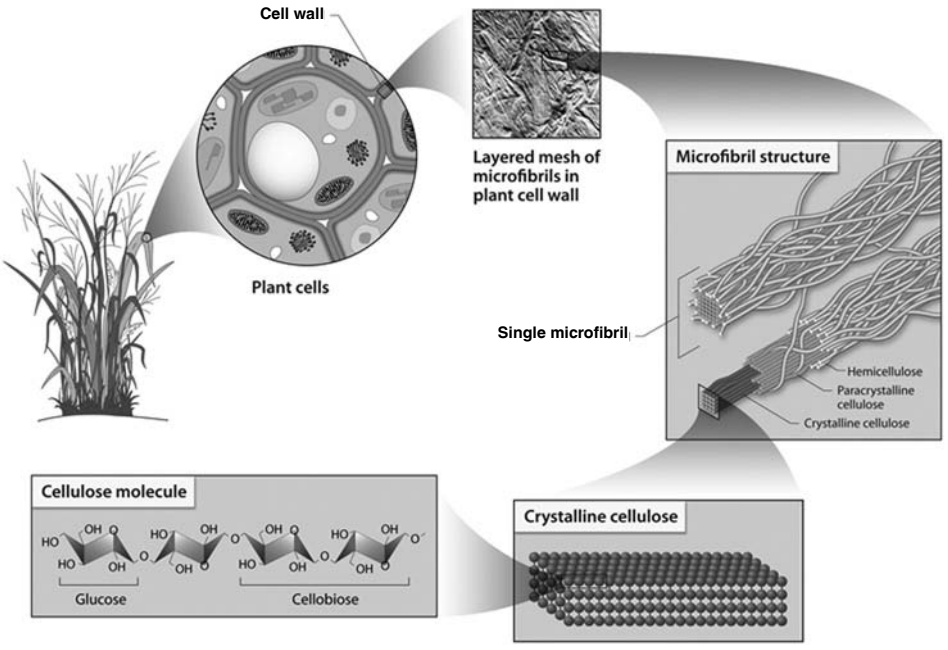

Fig. 2.1. Schematic presentation of cellulose structure (courtesy of the US Department of Energy Genome Program's Genome Management Information System (GMIS), available at http://genomics.energy.gov).

Acetobacter xylinum is a highly crystalline cellulose, whereas model substrates derived from bleached commercial wood pulps, such as Avicel, filter paper (FP) and Solka Floc, are regarded as a mixture of amorphous and crystalline cellulose. Phosphoric acid-swollen cellulose (PASC or Walseth cellulose) is considered amorphous (cited in Zhang and Lynd, 2004). Soluble substituted celluloses like hydroxylethyl cellulose (HEC) and carboxymethyl cellulose (CMC) are mainly used in enzyme activity assays (Ghose, 1987). Chromogenic β-glucosides such as methyl-umbelliferyl-lactoside (MULAC or MUL) or -cellobioside (MUC) are commonly used in research and may help in differentiating between various cellulolytic activities (Tomme *et al.*, 1988a).

The cereal β-glucans are soluble mixed-linkage (1→3),(1→4)-β-D-glucans. The (1→3)-linkages break up the uniform structure of the β-D-glucan molecule and make it soluble and flexible. For example, the β-glucan in barley *(Hordeum vulgare)* consists mainly of β-(1→4)-linked cellotriosyl and cellotetraosyl units linked by β-(1→3) bonds (Buliga *et al.*, 1986; Wood *et al.*, 1994; Planas, 2000).

In fungi and yeasts, cell wall elasticity and strength is provided by a branched β-(1→3)-D-glucan with a degree of polymerization of about 1500 glucose units and having β-1,6 interchain links. Yeasts and fungi have a second shorter and amorphous β-(1→6)-D-glucan, which acts like a flexible glue between the cell wall polymers (Lesage and Bussey, 2006).

In some enzymatic assays, lichenin deriving from an Icelandic moss *Cetraria islandica* and having a similar structure to cereal β-glucans has been used. This polymer consists of predominantly β-(1→3)-linked cellotriosyl units, the linked cellopentaosyl units being the second most prevalent feature (Planas, 2000; Tosh *et al.*, 2004). Laminarin, a β-1,3-glucan polymer derived from the brown alga *Laminaria digitata*, is commonly used in enzymatic characterization of β-glucanases; it has β-1,6-linked D-glucosyl branches substituted at approximately every seven glucose residues, and thus resembles fungal cell walls (Kawai *et al.*, 2005).

General structure of xylan

Hemicellulosic polysaccharides (including xylan) are found in all terrestrial plants, from woods, grasses and cereals (Aspinall, 1959; Wilkie, 1979; Sjöström, 1993). They were originally defined as those plant polysaccharides that could be separated from cellulose by extraction with alkali–water solutions. Hemicelluloses are closely associated in plant tissues with cellulose and lignin, and they are most often structural polysaccharides in these tissues. Hemicellulose consists of a complex and diverse group of polymers that are heterogeneous in their composition, having branched chains and consisting of various sugar units. Hemicelluloses are named according to the main sugar monomer unit in their backbone structure. Thus, xylans are polymers with D-xylose units in the main chain and those with D-mannose, L-arabinose and D-galactose are referred to as mannans, arabinans and galactans, respectively.

Xylan is the major component of hemicellulose and is, after cellulose, the second most abundant polysaccharide in nature. Xylans account for 30–35%

of the cell wall material of annual plants (grasses and cereals), 15–30% of hardwoods and 7–10% of softwoods (Wilkie, 1979; Ladisch *et al.*, 1983; Sjöström, 1993). Due to the significant presence of xylans in plants it serves as a major constituent of animal feed.

The main chain of xylan is composed of 1,4-β-linked D-xylopyranose units (Aspinall, 1959; Wilkie, 1979, Sjöström, 1993). The average degree of polymerization depends on the source, but xylan chains are clearly shorter than cellulose chains, on average about 200 residues in hardwood xylan and more than 120 residues in softwood xylan (Sjöström, 1993). In the majority of xylans there are various substituent groups attached to xylose units. These groups determine the solubility, viscosity and other physico-chemical properties of xylan. The extent and nature of the substituent groups vary depending on, for example, the botanical source, the tissue, the age and the harvest time of the plant (reviewed in Wilkie, 1979). Hardwoods typically contain *O*-acetyl-4-*O*-methylglucurono-β-D-xylan and softwoods arabino-4-*O*-methylglucuronoxylan, whereas the xylan in the cell walls of annual plants, cereals and grasses is typically arabinoxylan. There are two major types of arabinoxylan, those found from endospermic and non-endospermic tissues. However, in both these arabinoxylans, the L-arabinose group is directly linked to the D-xylan backbone, to positions 3 (more usual) or 2, and it is always found in the furanose form (Aspinall, 1959; Wilkie, 1979). The non-endospermic arabinoxylan contains, in addition to α-L-arabinofuranose, some glucuronic acid and/or 4-*O*-methyl-D-glucuronic acid and acetyl and galactose as side-groups. These side-groups are attached to positions 3 and 2 of xylose residues (Aspinall, 1959). The endospermic xylans found in cereals are highly branched and they can be doubly substituted by α-L-arabinofuranose at both positions 3 and 2 (Wilkie, 1979). The uronic acid substitution has been noted only rarely in endospermic arabinoxylan (Wilkie, 1979). Both endospermic and non-endospermic xylans may contain ferulic acid and *p*-coumaric acid that are, when present, attached to the arabinofuranose structures. Xylan chains may be cross-linked with each other by diesterified diferulic acid residues. Figure 2.2 shows a schematic representation of arabinoxylan structural units. Since xylose and arabinose are both pentose sugars, arabinoxylans are often termed pentosans.

β-Glucan and xylan in feed

Cell walls of cereal grains and other seeds consist of hemicelluloses, such as arabinoxylans, and β-glucans, cellulose, pectin substances, lignin, phenolics and proteins (Selvendran *et al.*, 1987). Chemically, polysaccharides of dicotyledonous plants such as legumes or oilseeds are a far more complex group than those of monocotyledons, and their chemical structure is still not well defined. Although many attempts to develop enzyme preparations for dicotyledons can be found, enzyme preparations on the market are still primarily focused on upgrading cereal grains.

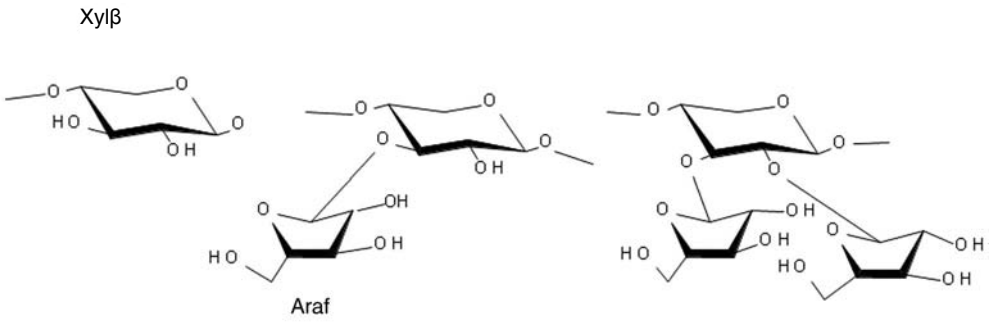

Fig. 2.2. Schematic presentation of arabinoxylan structural units. Only the major substituent group, L-arabinofuranosidase, is marked. Xylβ, D-Xylopyranose; Araf, L-arabinofuranosidase (adapted from Andersson *et al.*, 1992).

Arabinoxylans and mixed-linked β-glucans are predominant cell wall storage polysaccharides in cereal grains, where they are located in the cell walls of the starchy endosperm and aleurone layer, in particular. These are the most valuable fractions of cereal grains and therefore have been subject to extensive research in many applications. The ratio of pentosan (xylan) to β-glucan varies from 1:3 for barley to more than 10:1 for wheat and triticale (Henry, 1985).

Arabinoxylans predominate in wheat and rye, whereas mixed-linked (1→3),(1→4)-β-D-glucans dominate in barley and oats. Most of the β-glucan is located in endospermic cell walls, but the aleurone layer is also rich in β-glucans. β-Glucan isolated from barley consists of linear chains with about 30% (1→3)-linked and 70% (1→4)-linked β-D-glucopyranosyl (reviewed by Hesselman, 1983). The structure of barley β-glucan is illustrated in Fig. 2.3.

The proportion of total cell wall polysaccharides in cereals is affected by genetic factors, climatic factors, stage of maturity, the use of nitrogen fertilizers and postharvest storage time (reviewed by Jeroch and Dänicke, 1995).

The solubility of cell wall polysaccharides varies from grain to grain. This, coupled with the molecular size of the soluble fraction, is an important factor since soluble polysaccharides are known to reduce animal performance, especially in broilers. The amount of β-glucan in the water-soluble fraction

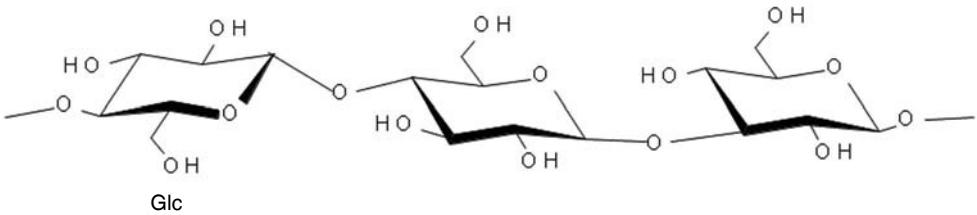

Fig. 2.3. Schematic presentation of barley mixed-linked β-(1→3),(1→4)-D-glucan structure. Glc, glucose (adapted from Bielecki and Galas, 1991).

from barley is more than four times that of pentosan, while in rye, pentosan levels are more than three times those of β-glucan (Henry, 1985). In barley on average 54% of the total β-glucan is soluble and in oats 80% (Åman and Graham, 1987). In wheat and rye one-third or more of the arabinoxylan is soluble in water (Chesson, 1995). Also, heat treatment, such as pelleting of feed, is known to increase the solubility of polysaccharides.

Enzymes

Enzyme classes

The NSP enzymes in feed have traditionally been classified according to the IUB Enzyme Nomenclature (Bairoch, 2000) and belong to the glycosyl hydrolases (EC 3.2.1.x). This classification is based on both the reaction type and substrate specificity, e.g. β-glucanases hydrolysing β-glucan, such as that found in barley, and xylanases acting on xylan. Most of the glycosyl hydrolases are endo-acting enzymes, cutting in the middle of the polymer chain and rapidly reducing viscosity.

Barley β-1,3-1,4-glucan, the major β-glucan in animal feed, consists mainly of cellotriosyl and cellotetraosyl residues linked by a β-1,3-glycosidic bond (Wood *et al.*, 1994). The enzymatic depolymerization of β-glucan is catalysed by at least the following enzyme classes: endo-1,4-β-D-glucan 4-glucanohydrolase (cellulase; EC 3.2.1.4), endo-1,3-β-D-glucan 3-glucanohydrolase (laminarinase; EC 3.2.1.39), endo-1,3(4)-β-glucanase (EC 3.2.1.6) and endo-1,3-1,4-β-D-glucan 4-glucanohydrolase (lichenase; EC 3.2.1.73) (Bairoch, 2000; Planas, 2000).

Endo-1,4-β-glucanase (EC 3.2.1.4) hydrolyses the (1→4)-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans. 1,3(4)-β-Glucanase (EC 3.2.1.6) catalyses endohydrolysis of (1→3)- or (1→4)-linkages in β-D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolysed is itself substituted at C-3. Laminarinase (EC 3.2.1.39) hydrolyses laminarin, paramylon and pachyman and has very limited action on mixed-link (1→3),(1→4)-β-D-glucans. Lichenase (EC 3.2.1.73) acts on lichenin and cereal β-D-glucans, but not on β-D-glucans containing only 1,3- or 1,4-bonds. The main enzyme activity depolymerizing xylan, endo-1,4-β-xylanase catalysing the endohydrolysis of (1→4)-β-D-xylosidic linkages, is designated as EC 3.2.1.8 in the IUB system (Bairoch, 2000). This IUB classification does not reflect the structural features in enzymes and therefore another approach has been taken to classify enzymes (Henrissat, 1991). It is based on fold or sequence similarities between enzymes and has been greatly facilitated by the accumulation of data on gene sequences and three-dimensional structures. The database CAZy (Carbohydrate-Active enZYmes) is maintained at http://www.cazy.org and currently lists 115 glycoside hydrolase families. The β-glucan-hydrolysing enzymes commercially available belong to families GH 5 (EC 3.2.1.4, EC 3.2.1.73), GH 7 (EC 3.2.1.4), GH 12 (EC 3.2.1.4), GH 45 (EC 3.2.1.4) and GH 16 (EC 3.2.1.39, EC 3.2.1.6,

EC 3.2.1.73); the CAZy database indicates β-glucanase entries in ten additional glycoside hydrolase families. According to Collins *et al*. (2005), the xylanases belong to GH families 5, 7, 8, 10, 11 and 43 and, in addition, the GH families 16, 52 and 62 contain bi-functional enzymes which have at least one xylanase domain. Most of the characterized xylanases, however, belong to families 10 and 11 (former families F and G, respectively) (Gilkes *et al.*, 1991; Henrissat and Bairoch, 1993). Moreover, the CAZy database does not classify any sequences with xylanase activity (EC 3.2.1.8) into GH family 7.

The glycoside hydrolase families are grouped in clans based on related three-dimensional structures, and in this classification families 5 and 10 are members of the GH-A clan, families 7 and 16 of the clan GH-B and GH 11 and GH12 belong to GH-C. The clan GH-A has a three-dimensional structure of $(\beta/\alpha)_8$ barrel, whereas clans GH-B and GH-C have a β-jelly roll structure (http://www.cazy.org).

Carbohydrate-binding modules

Most cellulases and many hemicellulases carry an N-terminal or C-terminal carbohydrate-binding module (CBM). CBMs were initially termed cellulose-binding domains (CBDs) as they were first identified from cellulases and were shown to have binding affinity towards cellulose (Tomme *et al.*, 1995). CBMs enhance the association of the enzyme with insoluble substrates but are not essential for hydrolysis of soluble substrates (reviewed in Tomme *et al.*, 1995; Boraston *et al.*, 2004). At the time of writing, CBMs have been grouped into 59 families based on their amino acid sequence similarities (CAZy family of carbohydrate-binding modules: http//www.cazy.org/fam.acc_CBM.html) and into seven 'fold families'; in addition, they are divided into three types according to similarities in their structural folds and structural and functional similarities in respect to their binding to ligands (Boraston *et al.*, 2004). Generally, CBMs range from about 36 to 200 amino acids in size and can be located either at the N- or C-terminus, at both ends and/or in the middle of the enzyme (Meissner *et al.*, 2000). The enzyme can also include more than one CBM and, in the case of multiple CBMs, they can even have similar or different types of binding specificities (Meissner *et al.*, 2000).

All of the *Trichoderma reesei* 'big four' cellulases (CBHI/Cel7A, CBHII/Cel6A, EGI/Cel7B and EGII/Cel5A; see section on cellulases, below) as well as mannanase (Man5A; Stålbrand *et al.*, 1995) and some other minor enzymes involved in cellulose depolymerization carry a family 1-type CBM. Modules of this family are found almost exclusively in fungi. The binding domain is relatively small, 36–38 amino acids long and forms a three-dimensional structure having a flat surface on one side, which is believed to bind to cellulose (Linder *et al.*, 1995; Bourne and Henrissat, 2001). At the time of writing, 369 of CBM family 1 entries are listed in the CAZy database (http://www.cazy.org).

Catalytic modules and CBMs are usually separated from each other with a linker sequence, and the modules are in most cases able to fold and function

independently (reviewed in Gilkes *et al.*, 1991; Gilbert and Hazlewood, 1993). The linkers have two suggested roles: (i) to function as a spacer between the two functional domains; or (ii) as a mediator in the possible interactions of the domains (Teeri *et al.*, 1992). The linker sequences vary in length (6–59 amino acids) and there is no clear sequence identity between the linkers from different organisms. However, the majority of the linkers are rich in proline, glycine, serine and threonine, and several of them contain runs of consecutive repeats of shorter sequences. The linkers in secreted filamentous fungal proteins are often heavily *O*-glycosylated, which has been suggested as protecting the unbound enzyme from proteolysis (Teeri *et al.*, 1992).

The CBM has insignificant catalytic activity but, as already mentioned, it facilitates the hydrolysis of native polymeric substrates; when cellulases with and without a CBM were compared, their activity on soluble substrates like CMC or HEC remained largely unchanged, whereas the lack of a CBM reduced hydrolysis of amorphous or crystalline cellulose to approximately half (Suurnäkki *et al.*, 2000; Voutilainen *et al.*, 2007; Szijártó *et al.*, 2008). As a consequence, since the junction point between the linker and the catalytic core is susceptible to proteolytic cleavage (Tomme *et al.*, 1988b), the loss of the CBM may escape notice if the enzymatic activity of a cellulase preparation is monitored only by assaying a soluble substrate.

In spite of the obvious importance of the CBMs for cellulose hydrolysis, it is interesting to note that some fungi have evolved to harbour main cellulases that in their native form lack CBMs, e.g. *Melanocarpus albomyces* (Cel7A, Cel7B and Cel45A; Haakana *et al.*, 2004) and *Thermoascus aurantiacus* (Cel7A, Cel5A; Hong *et al.*, 2003a,b).

Cellulases

Fungal cellulases and β-glucanases

Only limited hydrolysis or reduction of viscosity, rather than complete hydrolysis to simple sugars, is required from the NSP enzymes used in upgrading animal feed. Several glucanase classes are able to cleave bonds in β-glucan to the extent required. Cellulases are a group of enzymes that hydrolyse cellulose or β-(1,4)-glucan. Enzymes belonging to this class are cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidases or cellobiases (EC 3.2.1.21); the latter are usually included in the cellulase complex even though the enzyme mainly acts on the disaccharide cellobiose. Cellobiohydrolases act on crystalline parts of cellulose, whereas endoglucanases are believed to cleave at the amorphous regions of the polymer.

Commercially available cellobiohydrolases (cellulose 1,4-β-cellobiosidases) and endoglucanases are mainly of fungal origin. The cellobiohydrolases can be divided into CBHI/Cel7 and CBHII/Cel6 classes (the Cel designation refers to cellulases; Henrissat *et al.*, 1998). They have exo-activity, hydrolysing the cellulose chain at the ends with mainly cellobiose as the end product. CBHI/Cel7 acts on the reducing end of the polymer whereas CBHII/Cel6 cuts at the

non-reducing end. Strictly speaking, the IUB code EC 3.2.1.91 is applicable only to CBHII/Cel6, as the number refers to cellulose 1,4-β-cellobiosidases releasing cellobiose from the non-reducing end (Bairoch, 2000).

The difference in mode of action of the Cel7 cellobiohydrolases and endoglucanases is reflected in their three-dimensional structure, the cellobiohydrolases folding to a β-sandwich with the extended loops forming a long, cellulose-binding tunnel, whereas the *T. reesei* and *H. insolens* Cel7/EGIs have an open substrate-binding cleft or groove (Divne *et al.*, 1994; Kleywegt *et al.*, 1997).

Fungal genomes have much a higher number of endoglucanases (EC 3.2.1.4) than cellobiohydrolases. Annotation of the *T. reesei* genome, which is the benchmark organism for cellulases, both as the donor as well as the production platform, revealed eight entries for endoglucanases (Martinez *et al.*, 2008). The currently commercially relevant *Trichoderma* endoglucanases are EGI/Cel7B, EGII/Cel5A and EGIII/Cel12A. The EGV/Cel45A and the GH 61 endoglucanases (Karkehabadi *et al.*, 2008) have not, to the best of our knowledge, been involved in industrial use. *Trichoderma reesei* EGI/Cel7B and EGII/Cel5A are the main endoglucanases secreted into the culture medium of the fungus and constitute about 20% of total cellulases (McFarland *et al.*, 2007). Both enzymes have activity against soluble cellulose (CMC and HEC) and barley β-glucan (Pere *et al.*, 1995; Suurnäkki *et al.*, 2000); interestingly, the specific activity of the catalytic cores is actually slightly higher on these substrates than with the intact enzymes carrying a binding domain (see section on CBDs, above). EGI/Cel7B has broader substrate specificity, and also possesses significant xylanase and some mannanase activity (Bailey *et al.*, 1993), which may be helpful in feed processing. Early expression studies in yeast in the late 1980s indicated that EGI/Cel7B has higher activity on barley β-glucan and on lichenin than EGII/Cel5A cloned subsequently (Penttilä *et al.*, 1987a), although later studies indicate that the latter has higher specific activity on barley β-glucan (Ajithkumar *et al.*, 2006). EGI/Cel7B endoglucanase has found application in feed also as a genetically modified (GM) or recombinant product (Table 2.1).

When assayed with barley β-glucan as the substrate, the optimum pH of yeast-expressed EGI was around 6.0 and the optimal temperature 60°C (Zurbriggen *et al.*, 1991; Karlsson *et al.*, 2002); *Trichoderma*-produced EGI had an optimum pH on β-glucan in the range 5.0–7.0 and optimal activity at 65°C (Jari Piironen, personal communication, 2009). The major β-glucanase activity in commercial *Trichoderma* preparations has an apparent MW of 56 kDa and pI of 4.3 (Vahjen and Simon, 1999), which is in agreement with the values of 55 kDa and pI 4.7 for *T. reesei* EGI reported by Pere *et al.* (1995); further support for this identification comes from the xylanase activity of the 56 kDa enzyme in tested *Trichoderma* preparations (see above; Vahjen and Simon, 1999). A commercial *Trichoderma* β-glucanase and xylanase preparation, Roxazyme® G2, maintained β-glucanase activity reasonably well when challenged with pelleting temperatures of 75°C and 85°C, retaining 58% and 25% of activity, respectively (Wu *et al.*, 2002), indicating some degree of intrinsic thermostability.

Table 2.1. Selected commercial NSP feed enzyme products. The table is based on strain information submitted for EU registration. Not all donor organisms were included as information on these are not available in the public domain. Xylanase, endo-1,4-β-xylanase; β-Glucanase, endo-1,3(4)-β-glucanase; cellulase, endo-1,4-β-glucanase.

Company	Trade name	Declared activity(ies)	Donor organism(s)	Production organism(s)	Reference(s)
Adisseo	Rovabio™ Beta-glucanase GEP	β-Glucanase		*Geosmithia* (*Penicillium*) *emersonii* IMI 133	SCAN (2002)
Adisseo	Rovabio™ Excel LC/AP[d]	Xylanase and β-Glucanase		*Penicillium funiculosum* IMI 101	SCAN (2002); http://www.bioferm.com/downloads/publikace/Rovabio_Info_2.pdf
Agrimex	Belfeed® B1100 MP/ML	Xylanase	*Bacillus subtilis*	*B. subtilis* BCCM LMG s-15136	EFSA (2006)
Alltech Inc.	Allzyme® BG	β-Glucanase		*Trichoderma viride* CBS 517.94	SCAN (2002)
BASF	Natugrain® TS	Xylanase and β-glucanase	*Talaromyces emersonii* FBG1	*Aspergillus niger* CBS 109.713 and DSM 18404	EFSA (2008b)
	Natugrain® Wheat TS	Xylanase	*T. emersonii* FBG1	*A. niger* CBS 109.713	EFSA (2007a)
Danisco-Genencor[a]	Avizyme® 1110 Porzyme® 9110	β-Glucanase		*Trichoderma longibrachiatum* ATCC 2106	SCAN (2002)
	Avizyme® 1310 Porzyme® 9310	Xylanase		*T. longibrachiatum* ATCC 2105	SCAN (2002)
	Avizyme® 1505	Xylanase, α-amylase, alkaline protease	*Trichoderma reesei* RL-P37 Xylanase Y5 (mutated *T. reesei* Xyn2), *Bacillus amyloliquefaciens* BZ53, *B. amyloliquefaciens* ATCC 23844 (apr subtilisin mutant)	*T. reesei* RL-P37, *B. amyloliquefaciens* EBA-1, *B. subtilis* BG125	Fenel *et al.* (2004); EFSA, 2009
	Grindazym™ GV,GP, GPL	Cellulase and xylanase		*A. niger* CBS 600.94	SCAN (2002)
	Xylanase G/L	Xylanase	*T. reesei* (modified, thermotolerant *T. reesei* xylanase)	*T. reesei*	EFSA (2007b)
GNC Bioferm Inc.	Endofeed® DC[d]	Xylanase, β-glucanase		*A. niger* CCFC-DAOM 221137	SCAN (2002); http://www.gncbioferm.ca/about.html

Huvepharma	Hostazym® C	Endo 1,4-β glucanase (EC 3.2.1.4)		*T. longibrachiatum* IMI SD 142	SCAN (2002)
	Hostazym® X	Xylanase		*T. longibrachiatum* IMI SD 135	SCAN (2002)
Kemin[b]	Kemzyme® W Dry	α-Amylase, cellulase, β-glucanase, xylanase, bacillolysin		*B. amyloliquefaciens* DSM 9553, *T. reesei* CBS 592.94 *Aspergillus aculeatus* CBS 589.94, *Trichoderma viride* NIBH FERM BP 4842 and *B. amyloliquefaciens* DSM 9554	SCAN (2002)
LeSaffre	Safizym GP, GL	β-Glucanase		*T. longibrachiatum* CNCM MA 6-10 W	SCAN (2002)
	Safizym X	Xylanase		*T. longibrachiatum* CNCM MA 6-10 W	SCAN (2002)
Lyven	Feedlyve AGL	β-Glucanase		*A. niger* MUCL 39 199	
	Feedlyve AXC	Xylanase		*T. longibrachiatum* MUCL 39 203	SCAN (2002)
Novo-DSM	Bio-Feed Plus	Xylanase and cellulase	*Aspergilllus*	*Humicola insolens* DSM 10442	Cowan *et al.* (1993); SCAN (2002)
	Roxazyme® G	Cellulase, β-glucanase and xylanase		*T. viride* NIBH FERM/BP 447	SCAN (2002)
	Roxazyme® G2 G/Liquid	Cellulase, β-glucanase and xylanase		*T. longibrachiatum* ATCC 74 252	SCAN (2002)
	Ronozyme® WX (Biofeed Wheat)	Xylanase	*Thermomyces lanuginosus* spp.	*Aspergillus oryzae* DSM 10 287	SCAN (2002); Choct *et al.* (2004)
Roal[c]	Econase® Wheat Plus	Xylanase and β-glucanase	*T. reesei*	*T. reesei* CBS 529.94 and CBS 526.94	EFSA (2005)
	Econase® XT	Xylanase		*T. reesei* CBS 114044	EFSA (2008a)
	Econase® BG 300 Econase® Barley P 700	β-Glucanase		*T. reesei* CBS 526.94	SCAN (2002)

[a]Not all marketed varieties are shown.
[b]Only the product with the most widely declared enzyme activities has been included.
[c]Distributed by AB Vista.
[d]Non-GMO product according to the reference cited.

Other filamentous fungi that have been widely been used in the enzyme industry, particularly as sources of starch- and pectin-modifying enzymes, are *Aspergillus niger* and *Aspergillus oryzae*. Preparations with β-glucanase and xylanase activities from *A. niger* have been registered for feed applications (Table 2.1). Analysis of crude commercial samples revealed seven proteins with β-glucanase activity in zymogram analysis with lichenin as a substrate, with two main activities of apparent molecular weight of 38 kDa and 28 kDa. The optimum pH of the crude preparation was 5.0, with single activities having highest relative activities in the range 4.0–6.0 (Vahjen and Simon, 1999). *A. niger* is not known for its potent cellulolytic activity, and few cellulases have been cloned from this microbe in individual studies – these belong to families GH 5 and GH 12 (de Vries and Visser, 2001). However, genome sequence annotation suggests eight cellulase genes in families GH 5, 6 and 7, and four genes for exo-1,3-β-glucanases in family GH 5 (Pel *et al.*, 2007).

Other fungal cellulases used in feed include endoglucanases derived from *Humicola insolens*, *Talaromyces emersonii* and *Penicillium funiculosum* (Table 2.1). *H. insolens* produces a complete set of cellulases, at least seven, which are optimally active at a pH range of 5.0–9.0 (Schülein, 1997). Analysis of crude commercial *H. insolens* samples revealed nine enzyme bands with β-glucanase activity in lichenin zymogram analysis, with two main activities of apparent molecular weight of 102 kDa and 56 kDa. The optimum pH of the crude preparation was 5.5, with single enzymes having optimal activities in the range 4.5–6.5 (Vahjen and Simon, 1999). The major endoglucanase component of Novozymes' *H. insolens* DSM 1800 strain is EGI (GH 7), comprising 50% of the crude enzyme preparation, followed by 10% of EGV (GH 45) (Schülein, 1997; Tolan and Foody, 1999). EGI is the most efficient on soluble substrate and EGV is on amorphous cellulose; EGI in its native form does not harbour a CBM, whereas EGV has a C-terminal-binding domain (Schülein, 1997). A commercial *H. insolens* β-glucanase and xylanase preparation, Ronozyme® W, lost all β-glucanase activity when exposed to a pelleting temperature of 85°C, but retained 39% at the lower temperature of 75°C (Wu *et al.*, 2002).

Talaromyces emersonii (anamorph *Penicillium emersonii,* synonym *Geosmithia emersonii*; Salar and Aneja, 2007) is a moderately thermophilic ascomycete producing an array of glucan-modifying enzymes, many of which are intrinsically thermostable (Murray *et al.*, 2001; McCarthy *et al.*, 2005). Several of the purified enzymes were active on barley β-1,3-1,4-glucan and lichenin. The authors purified a 40.7 kDa endoglucanase having the characteristics of a 1,3-1,4-β-glucanase (EC 3.2.1.73) (Murray *et al.*, 2001) and three endoglucanases of 22.9 kDa (EGV), 26.9 kDa (EGVI) and 33.8 kDa (EGVII), of which EGVI and EGVII were active on laminarin and therefore were determined as belonging to class EC 3.2.1.6 (McCarthy *et al.*, 2003). The 40.7 kDa β-glucanase had optimal activity on β-glucan at pH 5.0 and 80°C. The published databank indicates that endoglucanase sequences from this fungus belong to families GH 5 (accession codes AX254752 and AF440003), GH 7 (AX254754) and GH 45 (AX254756).

Adisseo markets a *P. funiculosum* product, Rovabio™ Excel, containing multiple NSP-activities, including β-glucanase (Table 2.1; Guais *et al.*, 2008). The *P. funiculosum* β-glucanase activity, as assayed with barley β-glucan as the substrate, had a broad pH spectrum having more than 80% of activity between pH 3.0 and 5.0, and the highest activity in the assay was at temperature range 60–65°C, decreasing rapidly at higher temperatures. Enzymatic stability as determined *in vitro* by pre-incubating the preparation at various temperatures for 2 h indicated that the β-glucanase activity was recoverable up to 50°C (Karboune *et al.*, 2009). Proteomics analysis of the enzyme preparation revealed multiple cellulases, listing homologues to, for example, GH 5 endoglucanase and GH 74 xyloglucanase (Guais *et al.*, 2008).

Less information is available from the family GH 12 (*T. reesei* EGIII) and other GH 45 endoglucanases (*Thielavia terrestris* Cel45A and *Melanocarpus albomyces* Cel45A; Haakana *et al.*, 2004). These have found use in the textile industry as so-called neutral cellulases, but have to our knowledge not been applied in feed processing (Haakana *et al.*, 2004). Although *Trichoderma* EGV/Cel45A shares homology with the three other well-characterized GH 45 cellulases (*H. insolens*, *T. terrestris* and *M. albomyces*), its enzymatic characteristics differ greatly from them and it has very little activity, for example, on CMC (Karlsson *et al.*, 2002). A gene (*lam1*) for a β-glucanase (laminarinase) of the family GH 16 (EC 3.2.1.6) has been found in *T. reesei* in expression screening in yeast (Saloheimo, 2004).

Information on the development of thermostable feed β-glucanases is limited. An intrinsically thermostable endo-β-1,4-glucanase belonging to the family GH 5 has been cloned from the thermophilic filamentous fungus *Thermoascus aurantiacus* (Wu *et al.*, 2002; Hong *et al.*, 2003a; the relevant gene accession numbers are AX812161 and AY055121, respectively). This enzyme (Cel5A), expressed in *Saccharomyces cerevisiae,* showed optimal activity in the range pH 4.0–6.0 and was most active on CMC at 70°C. It retained full activity after 1 h incubation at 70°C and over 80% after 2 h (Hong *et al.*, 2003a). Cel5A purified from the original host retained 96% and 91% of barley-β-glucanase activity after pelleting at 75°C and 85°C, respectively. This compared favourably to the benchmark commercial β-glucanase (*Bacillus, Trichoderma* and *Humicola*) preparations used in the experiment, where 0–46% of initial activity remained after pelleting at 85°C (Wu *et al.*, 2002.). The thermostability of this *T. aurantiacus* Cel5A was also evident from its high melting point of 77.5°C at pH 7.0.

Tuohy's group has characterized several intrinsically thermostable glucanases from *Talaromyces emersonii,* of which a 40.7 kDa 1,3-1,4-β-glucanase (see above) showed optimum assay temperature at 80°C at pH 5.0, and a half-life of 136 min and 25 min at 70°C and 80°C, respectively (Murray *et al*., 2001). Pelleting results were not found in the literature.

Bacterial β-glucanases and cellulases

The only commercially available bacterial feed β-glucanases originate, apparently, from the genus *Bacillus* (Table 2.1; Vahjen and Simon, 1999;

Zhang and Lynd, 2004). Most of the characterized *Bacillus* β-glucanases belong to the family GH 16, and have high 1,3-1,4-β-glucanase activity (EC 3.2.1.73) exhibiting a strict substrate specificity for cleavage of β-1,4 glycosidic bonds in 3-*O*-substituted glucopyranose units (Olsen *et al.*, 1991; Planas, 2000). The Ronozyme® enzyme preparation derived from *B. amyloliquefaciens* is reported to have 1,3(4)-β-glucanase (EC 3.2.1.6) activity (Wu *et al.*, 2002). Industrial mutants of *Bacillus* spp. (*B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*) have served as hosts for production of these enzymes (Vahjen and Simon, 1999; Schallmey *et al.*, 2004).

Several family GH 5 endoglucanases have also been found in bacteria and in the genus *Bacillus*, whereas no family GH 7 cellulases have been identified in prokaryotic organisms and only a few for the family GH 45 (Robson and Chambliss, 1989; Cantarel *et al.*, 2009). The different *Bacillus* spp. GH 5 endoglucanases share about 60% identity at the sequence level (Schülein, 2000). Carbohydrate-binding modules of the families CBM 3, CBM 4, CBM 5, CBM 17 and CBM 28 have been assigned to these cellulases, and a tandem arrangement has been described for *Bacillus* sp. 1139 Cel5 endoglucanase (Boraston *et al.*, 2004).

Early protein engineering work has been carried out with *Bacillus* β-glucanase (EC 3.2.1.73) to increase thermostability by constructing hybrid enzymes from the homologous *Bacillus macerans* and *B. amyloliquefaciens* (1→3),(1→4)-β-glucanases. One of the mutants, H(A16-M), has 16 amino acids of the mature N-terminus of the *B. amyloliquefaciens* sequence and the remaining polypeptide is of the *B. macerans* enzyme (Olsen *et al.*, 1991). This protein-engineered hybrid β-glucanase had 44% higher specific activity, a lower optimum pH and retained >80% of optimal activity at 80°C, 5–15°C higher than the parent molecules. In pelleting tests this variant retained about 76% of the activity at 80°C, when only 54% of the control *A. niger* β-glucanase was recovered (Vahjen and Simon, 1999). The three-dimensional structure of this bacterial β-1,3-1,4-glucanase and that of the closely related *B. macerans* have been solved, and they bear some similarity with the fungal Cel7/EGI endoglucanases (Keitel *et al.*, 1993; Hahn *et al.*, 1995; Kleywegt *et al.*, 1997).

Anaerobic bacteria living in the digestive tract of ruminants possess a completely different cellulase system as compared with aerobic fungi and bacteria. They synthesize a multi-component complex of enzymes and binding modules, the cellulosome. The catalytic domains belong mainly to families GH 5, GH 9 and GH 48, whereas families GH 6 and GH 7 have not yet been described. The architecture of a cellulosome consists of scaffoldins carrying cohesins and dockerins which interact with each other in a kind of plug-and-socket arrangement, bring the catalytic cellulase domains and the CBMs into the complex and attach the cellulosome to the cell surface (for a review, see Bayer *et al.*, 2004). Cellulosomes or their subunits have not yet found use in, for example, feed or other industrial applications, probably due to the complexity of the system and the difficulties in producing commercial levels of the components.

Xylanases

General overview

Xylanases (endo-1,4-β-xylanase, EC 3.2.1.8; recently reviewed in Collins *et al.*, 2005; Polizeli *et al.*, 2005) cleave the xylan backbone randomly, resulting in non-substituted or branched xylooligosaccharides. With regard to feed application, only a partial hydrolysis of xylan is needed for viscosity reduction and thus xylanase addition to feed is already highly effective. However, for complete hydrolysis of the complex structure of xylan, a synergistic action of several hemicellulases is needed (Coughlan *et al.*, 1993). The side-chain-cleaving 'accessory' enzymes remove the substituent groups and the 1,4-β-D-xylosidase (EC 3.2.1.37) cleaves xylobiose and xylooligosaccharides into xylose monomers (Coughlan *et al.*, 1993; Sunna and Antranikian, 1997; Shallom and Shoham, 2003). The accessory enzymes for total hydrolysis of arabinoxylan include α-L-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase and feruloylesterase (EC 3.1.1.72 and EC 3.1.1.73, respectively) and α-D-glucuronidase (EC 3.2.1.139). Hydrolysis by xylanases of cereal xylans releases oligosaccharides consisting of xylose or xylose and arabinose residues.

Xylanases are produced by free-living and gut microorganisms and have also been found from algae, protozoa, snails, crustaceans and seeds of terrestrial plants (Woodward, 1984; Sunna and Antranikian, 1997; Dornez *et al.*, 2009). Most of the xylanases are secreted enzymes and they are almost exclusively single subunit proteins. Due to their industrial uses, a large number of xylanases have been isolated from microbes during the last 20–25 years and their enzymology, characteristics and production have been widely reviewed (e.g. Sunna and Antranikian, 1997; Kulkarni *et al.*, 1999; Beg *et al.*, 2001; Bhat and Hazlewood, 2001; Collins *et al.*, 2005; Subramaniyan and Prema, 2002; Polizeli *et al.*, 2005). In general, microorganisms often produce several xylanases with different specificities (reviewed, for example, in Wong *et al.*, 1988; Sunna and Antranikian, 1997; Subramaniyan and Prema, 2002). Also, xylanases exist in several different iso-enzymic forms in culture filtrates due to, for example, differential glycosylation, proteolysis, auto-aggregation or aggregation with other polysaccharides. The existence of multiple distinct xylanases in one organism has been suggested as being essential for efficient hydrolysis of the complex substrates.

Most microbial xylanases act at mesophilic temperatures (40–60°C) and at neutral or slightly acidic pH (4.0–6.0). In general, fungal xylanases are more acidic as compared with bacterial xylanases. Xylanases with more extreme properties have also been isolated that are suitable for applications requiring high thermostability (Collins *et al.*, 2005). As thermostability is essential in several industrial applications, xylanases acting/resisting high temperatures have also been developed by using mutagenetic approaches (see below).

Xylanases may be inhibited by natural xylanase inhibitors, the sensitivity to these inhibitors varying depending on xylanase (Goesaert *et al.*, 2004). Three types of such inhibitors have been described as being present in cereals

(Sørensen and Sibbesen, 2006; Dornez *et al.*, 2009 and references therein). The TAXI-type inhibitors (*Triticum aestivum* xylanase inhibitors) are proteins of around 40 kDa and are divided into TAXI-I and TAXI-II subgroups. Of these the TAXI-type inhibitors are specific for GH family 11 xylanases. The second group consists of about 29 kDa XIP-type inhibitors (xylanase inhibitor proteins or 'chitinase-like' cereal inhibitors). They are able to inhibit both GH family 10 and 11 xylanases due to two independent binding sites (Payan *et al.*, 2004). The third group consists of TL-XI inhibitors (thaumatin-like xylanase inhibitors). This group has not yet been well characterized. Regarding feed and food applications it would be beneficial that the xylanase in the product would not be inhibited by natural xylanase inhibitors. Such xylanases have already been developed (see below).

Xylanase activity from enzyme samples can be quantified by measuring the release of reducing sugars, by dyed (or labelled) xylan fragments from the xylan substrate, by determining the decrease of viscosity and by analysis of products after enzymatic reaction by HPLC. Methods and substrates generally used in xylanase analysis are listed in the review by Bhat and Hazlewood (2001).

GH family 10 and 11 xylanases

Xylanases, as already mentioned above, are classified into enzyme families based on their primary structure and hydrophobic cluster analyses of their catalytic modules (Carbohydrate-Active enZYmes database; http//www.cazy.org/; Coutinho and Henrissat, 1999). The majority of xylanases included in current feed products are members of GH families 10 and 11 (Table 2.1).

Family 11 xylanases generally have lower molecular mass and higher pI compared with family 10 xylanases (Collins *et al.*, 2005). Family 11 xylanases are well-packed molecules that consist mainly of β-sheets ('β-jelly roll' structure), and their overall structure has been described as resembling a 'right hand' (Törrönen *et al.*, 1993). The tertiary fold in family 10 xylanases is an $(\alpha/\beta)_8$ barrel and they have a 'salad bowl'-like shape (Biely *et al.*, 1997). Both family 10 and 11 xylanases are retaining enzymes and they act via a double displacement mechanism in which two catalytic Glu residues act as a proton donor and a nucleophile. However, they differ from one another with respect to their general specificities: family 11 xylanases are exclusively active on substrates containing D-xylose, whereas family 10 xylanases are catalytically more versatile, due to their more flexible structures (Biely *et al.*, 1997). Therefore, GH family 10 xylanases are generally able to hydrolyse substituted xylan to a higher degree and to cleave linkages closer to the substituent groups as compared with GH family 11 xylanases. For more details on the mode of action, catalytic mechanism and products released by different endoxylanases, see, for example, the review by Bhat and Hazlewood (2001).

Multi-domain structure of xylanases

The major xylanases in commercial products are mostly enzymes with a single catalytic domain structure or they have a catalytic core and a terminal CBM

domain (see below). However, a number of different types of basic structures have been identified from xylanases characterized to date. Xylanases can have not only one but two catalytic modules and, in addition, they can contain one or several non-catalytic modules, NCMs (Coutinho and Henrissat, 1999; Henrissat and Davies, 2000). Both catalytic modules can have xylanase activity (e.g. Zhu *et al.*, 1994) or they can show two different types of activity, e.g. that of xylanase and β-(1,3-1,4)-glucanase (e.g. Zhang and Flint, 1992; Flint *et al.*, 1993; Morris *et al.*, 1999). The majority of identified NCMs are CBMs; however, some xylanases also contain dockerin modules which bind the enzyme to the cellulosome, modules homologous with the nodulation proteins in nitrogen-fixing bacteria and uncharacterized modules (reviewed in Kulkarni *et al.*, 1999). The multi-domain structure has been suggested as providing benefits in the hydrolysis of the substrate, via the synergistic effects between the binding module(s) and the catalytic core or between the different catalytic domains (e.g. Fernandes *et al.*, 1999; Bolam *et al.*, 2001). This multi-domain structure is common in xylanases from anaerobic thermophilic bacteria (Meissner *et al.*, 2000).

Most CBMs identified from xylanases bind to cellulose, but CBMs with specificity for xylan have also been identified (Irwin *et al.*, 1994; Black *et al.*, 1995; Dupont *et al.*, 1998; Charnock *et al.*, 2000; Meissner *et al.*, 2000).

Xylanases in commercial feed preparations

Xylanases in commercial preparations are derived from both bacterial and fungal sources (see Table 2.1). According to both public sources and the list of commercial enzymes provided by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP, 2009; http://www.amfep.org/) the commercial xylanases in feed products are produced by both classical and genetically modified strains. The well-known bacterial expression system, *Bacillus* and the filamentous fungi *Aspergillus*, *Humicola*, *Penicillium* and *Trichoderma*, are used for xylanase production (see below).

Bacillus subtilis strain is a donor for bacterial xylanase included in at least one feed product (Table 2.1). Xylanases have been characterized from a large number of *Bacillus* species (for reviews, see e.g. Sunna and Antranikian, 1997; Beg *et al.*, 2001). The pH and temperature optima of these xylanases vary from slightly acidic (5.5) to alkaline (9.0–10.0) and from 50 to 75°C, respectively, depending on the source organism. Some xylanases derived from thermophilic *Bacillus* species are stable at high temperatures. *B. subtilis* 168 produces two xylanases, the 23 kDa family 11 XynA and the 44 kDa family 5 XynC (Wolf *et al.*, 1995; St John *et al.*, 2006). No family 10 glycosyl hydrolase homologues have been found from *B. subtilis* 168 genome data (St John *et al.*, 2006). XynA has been reported to be the major xylan-degrading activity in *B. subtilis* 168 (Wolf *et al.*, 1995). It does not harbour a CBM. The optimal reaction temperature of *B. subtilis* XynA has been elevated from 55 to 65°C by using a directed evolution approach (Miyazaki *et al*., 2006). Also, *B. subtilis* 168 XynA has been developed by modifying the enzyme by the site-directed mutagenesis approach to resist xylanase inhibitors. *B. subtilis* XynA mutants

have been successfully created that are resistant to TAXI or TAXI- and XIPI-type inhibitions (Sørensen and Sibbesen, 2006; Bourgois *et al*., 2007). The specific activity of the best uninhibited TAXI mutant was somewhat decreased (14%) compared with the wild type XynA (Bourgois *et al*., 2007). However, specific activities of TAXI- and XIPI-uninhibited mutants were highly reduced (74–86%) compared with wild-type xylanase. The *B. subtilis* XynA mutant resistant to natural xylanase inhibitor(s), designated BS3, is included in at least two commercial baking products (Grindamyl H640 and POWERBake 900; Olempska-Beer, 2004). This xylanase differs by only two amino acid substitutions compared with wild-type XynA (Olempska-Beer, 2004).

Most of the xylanases in the commercially available feed products are of fungal origin (Table 2.1). The donor and/or production organisms include *Trichoderma*, *Talaromyces*, *Aspergillus*, *Humicola*, *Penicillium* and *Thermomyces* species. Most fungal xylanases are mesophilic enzymes but there are, however, some more thermostable representatives in, for example, *Talaromyces* and *Thermomyces* species, as will be discussed in the sections below.

Trichoderma reesei is one of the best-known organisms producing high amounts of cellulases and hemicellulases. This organism has also been and is used for production of feed xylanases. Four different xylanases have been characterized from *T. reesei* (Tenkanen *et al.*, 1992, 2003; Xu *et al.*, 1998). The 19 kDa Xyn1 (pI 5.5) and 20 kDa Xyn2 (pI 9.0) are endoxylanases belonging to GH family 11. The 32 kDa Xyn3 (pI 9.1) is a family 10 endoxylanase. The Xyn4 (pI 7.0) is a 43 kDa exo-acting enzyme that belongs to family 5. The Xyn4 clearly has a lower specific activity against xylan substrates as compared with other *T. reesei* xylanases, and has been shown to exhibit synergy with Xyn1 and Xyn2. None of the characterized *T. reesei* xylanases contain a CBM domain or domains. In addition to the above four xylanases, the *T. reesei* endoglucanase I (Cel7B/EGI) is active against xylan (Biely *et al.*, 1991). The pH optima of Xyn1 and Xyn2 are 4.0–4.5 and 5.0–5.5, respectively. Xyn3 is the most neutral of the *T. reesei* xylanases, with an optimum pH of 6.0–6.5, and Xyn4 is the most acid, having an optimum pH of 3.5–4.0. Xyn1 and Xyn2 are the major xylanases in wild-type *T. reesei* culture supernatants in standard laboratory cultivations. Of these, Xyn2 has higher specific activity and better stability properties compared with Xyn1 (Tenkanen *et al.*, 1992). Xyn2 is included as having the major xylanase activity in at least some of the first-generation recombinant feed xylanase products.

All *T. reesei* xylanases are mesophilic enzymes, having their temperature optima at around 50°C. They are not stable at high temperatures and thus are not well suited to high pelleting temperatures. *T. reesei* Xyn2 mutant xylanases with increased thermostability have, however, been successfully generated by targeted mutagenesis (Fenel *et al.*, 2004; Xiong *et al.*, 2004). At the time of writing, one of the commercial feed xylanase products is reported to include such a mutant xylanase (Table 2.1). The thermostability of this mutant, named Y5, was increased by about 15°C by engineering a disulfide bridge into the N-terminal region of Xyn2 (Fenel *et al.*, 2004). In total, mutant Y5 xylanase contains three changes in the amino acid sequence

compared with wild-type Xyn2, and one additional amino acid is inserted into the sequence.

As will be discussed below, *A. niger* and *A. oryzae* are widely used as production organisms for industrial enzymes, and also for feed xylanases (Table 2.1). Physical properties have been analysed for a large number of *Aspergillus*-derived xylanases (reviewed in de Vries and Visser, 2001). According to recent genome sequence data (Pel *et al.*, 2007), *A. niger* carries one 36 kDa family 10 xylanase and four family 11 xylanase (or candidate xylanase) genes, with theoretical molecular masses of 22.6 kDa (XynA), 24.1 kDa (XynB), 24.9 kDa (candidate xylanase) and 27.9 kDa (candidate xylanase). The number of xylanase genes seems to depend on the *Aspergillus* species, as more xylanase (or xylanase candidate) genes can be found from the genomes of three other sequenced *Aspergilli*, i.e. six from *Aspergillus nidulans* (three GH 10, two GH 11 and one GH 5), nine from *A. fumigatus* (four GH 10, three GH 11 and two GH 7) and nine from *A. oryzae* genome (four GH 10, four GH 11 and one GH 7) (Pel *et al.*, 2007). Recombinant *A. niger* xylanase A (reAnxA produced in *Pichia pastoris*) and xylanase B (overproduced in *A. niger*) are mesophilic and acid enzymes with temperature optimum of 50°C and pH optima of 5.0 and 5.5, respectively (Levasseur *et al.*, 2005; Liu *et al.*, 2006). From *Aspergillus awamori* (an *A. niger* subspecies), three endo-xylanase proteins (EndoI, II and III) have been isolated and characterized (Kormelink *et al.*, 1993). These are also all acid (pH optima between 4.0 and 5.5) and mesophilic (temperature optima between 45 and 55°C). The molecular masses of these xylanases were 39 kDa for EndoI (pI 5.7–6.7), 23 kDa for EndoII (pI 3.7) and 26 kDa for EndoIII (pI 4.2). All three released xylobiose and xylotriose from xylan substrate but EndoI also released xylose. Xylanases EndoI and EndoII had better specific activity against soluble oat spelt xylan compared with EndoIII. According to their molecular masses, pI and hydrolysis pattern, EndoI represents a GH 10 xylanase and EndoII and EndoIII represent GH 11 xylanases. Two *A. niger* xylanases with similar molecular masses to the above xylanases, 24 kDa endoxylanase A of GH 11 (pI 3.5) and 36 kDa endoxylanase B of GH 10, have also been isolated using affinity chromatography with immobilized endoxylanase inhitors (Gebruers *et al.*, 2005). These authors showed that endoxylanase A (the N-terminal amino acid sequence corresponding to the above-described 22.6 kDa XynA) was sensitive to both TAXI and XIPI wheat inhibitors, whereas endoxylanase B (two peptide sequences corresponding to the above-described 36 kDa GH10 xylanase) was only inhibited by XIP. Endoxylanase A was highly active in bread-making whereas endoxylanase B was not.

Two major xylanases have been characterized from a *H. insolens* commercial enzyme preparation, Ultraflo™, which is used in wort and beer filtration (Düsterhöft *et al.*, 1997), suggesting that at least two different xylanases are also present in the commercial feed enzyme product derived from a *Humicola* CMO (classically modified organism) strain (AMFEP, 2009). The two above purified *H. insolens* xylanases constituted about 85% of xylanase activity in the Ultraflo™ enzyme preparation. These purified enzymes, named Xyl1 and Xyl2, had molecular masses of 6 and 21 kDa and pIs of 9.0

and 7.7, respectively. They both had optimum pH of 6.0–6.5 and temperature of 55–60°C. Xyl1 and Xyl2 xylanases were not highly thermostable and were inactivated at temperatures above 50°C. Xyl2, however, was found to be particularly effective with regard to cereal arabinoxylan. The *Humicola* xylanase included in the commercial GMO feed product Bio-Feed Plus (Table 2.1) is described as containing the major *H. insolens* endo-xylanase (Cowan *et al.*, 1993). The major xylanase in this case, most probably, corresponds to the above Xyl2 even though the *H. insolens* xylanase protein sequence included in public databases is named as Xyl1 (Dalboege and Hansen, 1994). A more thermostable *Humicola*-derived xylanase has been characterized from another *Humicola* species, *Humicola grisea* var. *thermoidea* (Monti *et al.*, 1991). This 23.0–25.5 kDa *H. grisea* family 11 xylanase has a half-life of 20 min at 60°C.

Three xylanases have been characterized from *P. funiculosum* (Furniss *et al.*, 2002, 2005). The 22 kDa XynB (pI 5.0) and 23.6 kDa XynC (pI 3.7) belong to family 11, the 36 kDa XynD (pI 4.6) to family 10. All *P. funiculosum* xylanases are acidic, with their optimum pH in the region of 3.7–5.2. XynB and XynD xylanases contain a family 1 CBM, whereas XynC does not include a CBM. In addition to the above 'true' xylanases, a 48 kDa GH family 7 cellobiohydrolase from *P. funiculosum*, named XynA (pI 3.6), has also been shown to efficiently break down xylan substrates (Furniss *et al.*, 2005). However, the specific activities of XynB and XynD are clearly higher with respect to both soluble and insoluble wheat arabinoxylans compared with XynA. XynA, XynB and XynD were also shown to act on cellulosic substrates (e.g. barley (1→3),(1→4)-β-glucan), the XynD showing the greatest activity on these substrates (Furniss *et al.*, 2005). All *P. funiculosum* xylanases were inhibited by the xylanase inhibitor proteins from wheat, but to different degrees: XynB was inhibited significantly only with TAXI-I, XynC was strongly inhibited by XIP-I, TAXI-I and TAXI-II, and XynD was only inhibited by XIP-I (Furniss *et al.*, 2002, 2005). Results from an analysis of Rovabio™ Excel feed enzyme preparation by using proteomic technology has been published (Guais *et al.*, 2008). This analysis confirms the existence in the commercial product of the above three xylanases and the xylanase/cellobiohydrolase XynA.

Of the thermophilic fungi, *T. emersonii* and *Thermomyces lanuginosus* (formerly known as *Humicola lanuginosa*) have been shown to produce thermostable xylanases, and xylanase(s) originating from these organisms are also included in commercial feed products (Table 2.1). A review by Coughlan *et al.* (1993) reports preliminary characterization of 13 *T. emersonii* xylanases or xylanase isoforms with different molecular masses. All these xylanases or xylanase forms are acidic (pH optima from 3.5 to 4.7) and have relatively high temperature optima (from 67 to 80°C). Purification and characterization of two *T. emersonii* xylanases, XylII and XylIII, are described in more detail by Tuohy *et al.* (1993). These two xylanases are unusual in their properties as they preferentially hydrolyse unsubstituted xylans and are active against aryl β-D-xylosides and xylo-oligosaccharides. They show little or no action against arabinoxylan from wheat straw, probably because they were shown to require long sequences (at least 24 xylose units) of arabinose-free xylan backbone for

their activity. XylII (pI 5.3) is suggested as being a dimer of two subunits each having a molecular mass of ~75 kDa, while XylIII (pI 4.2) is a monomer with a mass of ~54 kDa. Both these xylanases are acidic and thermophilic, the pH and temperature optima determined for XylII being 4.2 and 78°C and those for XylIII 3.5 and 67°C. Two homologous (but not identical with one another) family 10 xylanases from *T. emersonii* strains are described in more detail in the patent application WO01/42433 (Danisco A/C) and US patent 7,514,110 (BASF Aktiengesellschaft). Both above *T. emersonii* xylanases include a family 1 CBM. Their calculated molecular masses are 38.5 (pI 4.5) and 41.6 kDa (pI 3.3). Both xylanases have an acidic pH optimum (3.0 and 4.0–5.0) and are thermostable enzymes, with their temperature optimum being approximately 80°C. The *T. emersonii* TX-1 xylanase in WO01/42433 is also described as being resistant to naturally occurring xylanase inhibitors, a property that is beneficial in feed application.

The *T. lanuginosus* strains produce family 11 xylanases (23–29 kDa, pI 3.7–4.1) that are among the most thermostable xylanases of fungal origin (reviewed in Singh *et al.*, 2003). Several *T. lanuginosus* isolates have been reported as producing single xylanases that have their optimum temperature and pH in the range of 60–75°C and 6.0–7.0, respectively, and are relatively stable at 50–80°C and over a broad pH range (3.0–12.0). These properties make them highly interesting for use in feed and other industrial applications. Of the characterized, published *T. lanuginosus* xylanases, 23.6 kDa xylanase from the isolate SSBP is the most thermostable, having a half-life of 337 min at 70°C (Lin *et al.*, 1999).

Enzyme Production

Cell factories

Introduction

Feed enzymes, like other industrial enzymes, are currently produced on a large scale mostly in submerged or deep-tank bioreactors. The production hosts are microbial, either bacterial such as *Bacillus* spp. (*B. subtilis*, *B. amyloliquefaciens* or *B. licheniformis*) or filamentous fungi, for example *A. niger*, *A. oryzae*, *H. insolens* and *T. reesei.* The history of these hosts originates from their use in the starch processing industry (*Bacillus* and *Aspergillus*), for detergent protease (*Bacillus*) or for cellulase production (*Trichoderma, Humicola).* These hosts naturally secrete a large array of enzymes, are non-pathogenic and easy to cultivate on an industrial scale. Tools exist for genetic engineering of all of these hosts, and representative genomes have been published for most of them (Kunst *et al.*, 1997; Veith *et al.*, 2004; Machida *et al.*, 2005; Pel *et al.*, 2007; Martinez *et al.*, 2008). Intellectual property rights (IPR) protecting DNA transformation, use of certain strong promoters and heterologous or fusion protein production may still block commercial exploitation of gene technology in certain hosts and in certain countries, particularly in the USA,

where the patent term used to be 17 years from the grant rather than from the filing date.

New recombinant fungal hosts have recently been developed, e.g. *Chrysosporium lucknowense* (or C1) by Dyadic International, Inc., with the apparent benefit of a wider range of cultivation temperatures and pH options and favourable morphology (Gusakov *et al.*, 2007). The methylotrophic yeast *Pichia pastoris* is also available for both research and commercial exploitation from Invitrogen Corporation (http://www.invitrogen.com) and Research Corporation Technologies (http://www.rctech.com), for organizations and companies that have no access to other proprietary production platforms (Teng *et al.*, 2007).

Production hosts can be divided into two categories based mainly on regulatory aspects: wild-type or classical (CMO) and genetically modified strains (GMO). Classical strains are usually derived from natural isolates with desired characteristics and have been subject to several rounds of mutagenesis and screening for high enzyme productivity over decades (Bailey and Nevalainen, 1981; Tolan and Foody, 1999; Veith *et al.*, 2004). They typically produce enzyme mixtures with multiple activities, and the profile may be modified by means of strain development and process optimization. Production levels cited in the literature range from 20 to 25 g total secreted protein l^{-1} with the end of cultivation culture broth with *Bacillus* to 40–100 g enzyme protein l^{-1} with fungal production platforms (Durand *et al.*, 1988; Cherry and Fidantsef, 2003; Maurer, 2004). *Bacillus* produces enzyme in the relatively short time of perhaps 48 h, whereas fungal hosts are typically cultivated for several days; thus the economics of both systems are comparable.

There are several limitations in the use of the classical strains as the only method of enzyme manufacture – for example: (i) enzyme diversity is limited to the native enzymes of the host; (ii) expression levels of the desired activities can be limiting; (iii) the strain may secrete side-enzyme activities that are harmful in certain applications; or (iv) the production host may produce harmful secondary compounds such as acids or toxins. With gene technology it is possible to screen biodiversity in nature for enzymes with optimal characteristics for the application in mind and to maximize expression levels of the desired gene by insertion of multiple gene copies and/or by placing the desired gene under the control of a strong promoter. Genes encoding undesirable enzymes or involved in the metabolism of harmful compounds can be inactivated or deleted from the genome. As a result it is possible to produce virtually monocomponent enzyme preparations at low cost, several of which can then be mixed at optimal ratios for customers' needs. The advantages of gene technology are best exploited when combined with the high secretory capacity of proprietary classical host mutants.

The case of Trichoderma reesei

T. reesei provides a good example of production host development. To the best of our knowledge, all industrial *T. reesei* and the vast majority of academically useful strains originate from one single isolate, QM6a, isolated in

the Solomon Islands during the Second World War (Mandels and Reese, 1957; Nevalainen *et al.*, 1994). In the past, industrial *T. reesei* strains have inconsistently been characterized as either *T. viride* or *T. longibrachiatum*, but molecular genetics tools have verified *T. reesei* as being distinct from these two species and to be an anamorph of *Hypocrea jecorina* (Kuhls *et al.*, 1996). Some *T. viride* or *T. longibrachiatum* strains listed in Table 2.1 could possibly benefit from taxonomical molecular approaches.

Figure 2.4 gives an outline of the different *T. reesei* mutant lineages developed for higher cellulase titres in the past, particularly in the late 1970s and early 1980s inspired by the first oil crisis and studies on lignocellulolytic bioethanol. This work was particularly pioneered by groups at Rutgers University in USA, VTT in Finland, Cayla, CNSR and IFP in France and at Kyowa Hakko Kogyo in Japan (Montenecourt *et al.*, 1980; Bailey and Nevalainen, 1981; Kawamori *et al.*, 1986; Durand *et al.*, 1988; Mäntylä *et al.*, 1998; Tolan and Foody, 1999). Industrial genetically modified *T. reesei* strains are based on some of these lineages, and the tools for genetic engineering were developed in the mid-1980s (Penttilä *et al.*, 1987b).

The most prominent secreted protein in *T. reesei* culture medium is CBHI/Cel7A, comprising 60–80% of total cellulase protein (McFarland *et al.*, 2007). Therefore, for maximal expression the gene of interest is placed between the strong *cbh1/cel7A* promoter and the terminator and transformed into the host. Both circular plasmid and isolated fragments can be used, but removal of the sequences required for propagation in the intermediate host

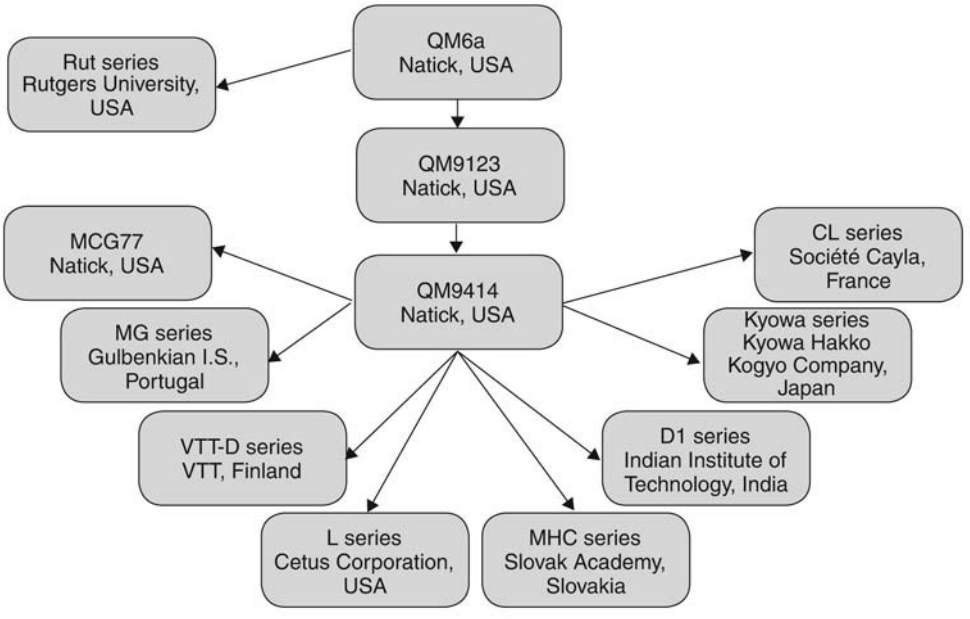

Fig. 2.4. Genealogy of various high-producing *Trichoderma reesei* mutant lineages (adapted from Nevalainen *et al.*, 1994).

E. coli is usually favoured for minimizing the amount of foreign DNA in the production host. The gene integrates randomly into the genome in typically one to three copies, but targeted replacement can also take place, particularly if the construct harbours adequate lengths of both the 5′ and 3′ flanking regions of the gene to be deleted (Mäntylä *et al.*, 1998). Yields of heterologous proteins initially difficult to express in the host can be improved by using native N-terminal carrier proteins or modules and by using low-protease hosts (Penttilä, 1998). The construction of tailored cell lines having genes for major cellulase or xylanase activities deleted in the genome greatly facilitates the detection of the signal of the gene of interest in both enzyme assays and SDS-PAGE analysis. The use of such strain background also accelerates strain development work, as there are often no undesired activities left in the host that could be harmful in the intended applications. Since many of the novel enzymes developed for feed applications are intrinsically thermostable (in order to survive pelleting temperatures), native thermolabile side-activities of the production host now have less importance than previously in the final application.

The development of system biology tools such as transcriptional profiling and proteomics provides exciting possibilities for the analysis and rational development of production platforms. It allows a global view due to the ability to view total gene expression and protein production under different growth conditions and phases. This enables a comprehensive examination of the differences between the representatives of different mutant lines. Ideally, the analysis should reveal the uncharacterized mutations responsible for the beneficial features of the high-producing proprietary mutants.

The publicly available RutC-30 is a three-step mutant derived from the QM6a isolate and presents a different lineage as compared with the many lines deriving from QM9414 (Fig. 2.4). This strain and its sibling RL-P37 (Sheir-Neiss and Montenecourt, 1984) have been the subject of study for many research groups, as they are capable of producing reasonable titres of cellulases. RutC-30 is a glucose de-repressed mutant carrying a truncated version of the repressor *cre1* gene (Ilmén *et al.*, 1996). The sibling strain RL-P37 has been used as a benchmark strain for use in biomass conversion to fermentable sugars by cellulase and related enzymes (Tolan and Foody, 1999; Foreman *et al.*, 2003; Diener *et al.*, 2004). A recent detailed study of the RutC-30 mutant and its immediate ancestor NG14 has revealed a surprisingly high number of mutagenic events (>200), including large deletions, which have accumulated during the three mutagenic steps starting from the wild isolate QM6a, as already suggested by early karyotype studies performed by contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Mäntylä *et al.*, 1992; Le Crom *et al.*, 2009). The high number of random mutations during each step presents a great challenge for the analysis of system biology data and emphasizes the importance of carefully selected screens when developing strains using conventional methods. With costs of sequencing having dropped significantly, it is feasible to sequence the entire genome of newly selected mutants to pinpoint where significant changes have been made.

Recently it has been discovered that *T. reesei* possesses a *MAT1-2* mating type, which allows successful crossing with a *H. jecorina* strain carrying the opposite mating type *MAT1-1* (Seidl *et al.*, 2009). Further developments in the sexual development of *T. reesei/H. jecorina* will hopefully enable industrial microbiologists to combine the desired characteristics of different mutant lines into one superior strain, as well as to eliminate any accumulated harmful mutations.

Production process

Virtually all microbially produced industrial enzymes are secreted, glucose isomerase being an exception to the rule, and consequently enzyme preparations are in essence concentrated, cell-free, spent culture media. Modern feed enzymes are produced in large bioreactors, with the production phase volumes ranging from 50 to 250 m^3; the smaller sizes are most suitable for bacterial cultivation, whereas larger volumes can be used for yeasts and fungi. These are aseptic and aerobic fermentations, where temperature, pH, foaming, aeration and mixing are carefully monitored and controlled (Fig. 2.5). Production strains are typically maintained as pure cultures or working cell banks (WCB) at –80°C or lower, or as freeze-dried preparations, and revived on slants or plates. Seed

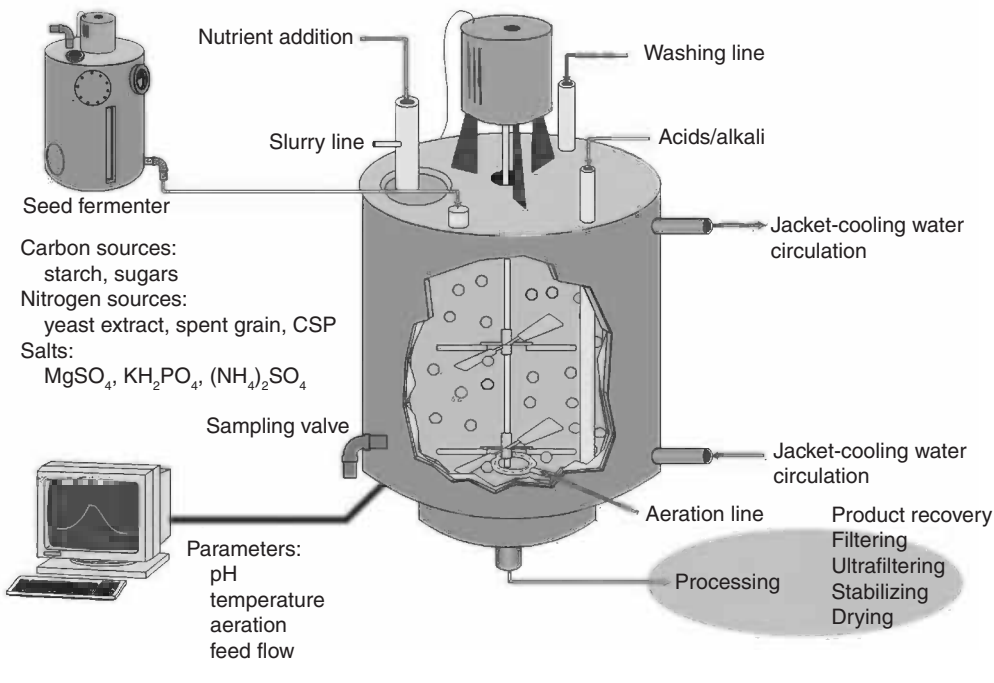

Fig. 2.5. Schematic presentation of the main bioreactor in submerged-type industrial enzyme production.

cultures for the production bioreactor are grown in successively larger volumes, starting from shake flasks and one or two seed tanks before inoculation into the final bioreactor.

The production media should consist of cheap raw materials that are available in large quantities, are not seasonal, are of consistent quality and are non-toxic. Soluble carbon sources such as maltodextrin, glucose syrups, sucrose or lactose are preferred, although insoluble constituents such as cellulose may be used, particularly in small amounts as inducers. The nitrogen source may be a complex industrial by-product, e.g. corn steep powder, spent grain, soy flour, cereal brans, cotton seed or yeast extract. The macro salts typically include potassium, phosphate, sulfate and magnesium, and in some cases calcium for enzyme stabilization. A useful guide for media formulation may be requested from Traders Protein (Memphis, Tennessee, USA). As the osmotic tolerance of the production host allows for only limited initial concentrations of the sugars and salts, the highest volumetric productivity is typically achieved by a fed-batch process, where nutrients are continuously added to the media over time to replenish those consumed by the growing host. Since cultivation conditions usually scale up rather well, strain screening and process optimization can be carried out at laboratory and pilot scale, where the volumes range from a few hundred millilitres to several cubic metres.

In downstream processing the cells and solids are removed by continuous-flow centrifugation, filter presses or rotary drum vacuum filters. Filter aids like diatomaceous earth or kieselguhr and flocculants may be used to facilitate the separation. The spent medium is concentrated by, for example, ultrafiltration with cut-offs around 10,000 Da for enzyme concentration. Chromatographic methods are rarely used in industrial enzyme purification, but selective precipitation or quantitative crystallization of enzymes has been applied on a large scale in special cases, for example with glucose isomerase (Visuri *et al.*, 1990). If the target enzyme is thermostable, the mesophilic host enzymes may be inactivated and precipitated by heat treatment. The need to remove undesired side-activities can largely be avoided by the prior deletion of the genes encoding such activities (such as proteases) from the host.

Stabilizers (NaCl, glycerol, sorbitol, propylene glycol) and preservatives (sodium benzoate, potassium sorbate, methyl paraben) are added as necessary to liquid enzyme preparations, the quantity and extent used depending upon the preparation in question. Boron compounds, in combination with the polyols glycerol and propylene glycol, may be used to inhibit proteases (Stoner *et al.*, 2004).

If a powder product is preferred, the clear and concentrated spent medium must be dried, by for example spray-drying to produce an instantized or granulated product. Fillers like dextrin or salt may be needed as a carrier to start the drying process. The granules formed can be further coated for enhanced dust control or to prevent inactivation in the steam-pelleting process. The dried enzyme preparation is then mixed with fillers such as wheat flour or corn starch to standardize the product on an activity basis.

Enzyme Development and Future Trends

The first commercial feed enzyme products on the market were produced by CMOs with a wide range of side-activities. Several first-generation products were primarily developed for applications other than feed. Such multi-component products are still marketed and used today (Table 2.1) and are preferred by some customers due to the use of a CMO host in their production. However, the use of genetic engineering has improved enzyme production yields of the core enzyme(s), making enzyme products both more economical to use and better defined, and hence suited to the application at hand. The use of genetic techniques also enables more sophisticated product development, i.e. the development of enzymes that meet the specific requirements of the feed application. Enzymes produced by the native host as minor activities, as well as modified enzymes, can be produced in significant quantities using recombinant host strains. Such an achievement is often not possible using classic hosts and techniques. The best-known example of the successful use of genetic engineering to obtain high-value products for the feed industry is the production of phytase, currently widely used all over the world.

One of the major targets for the feed application has been towards more thermotolerant enzymes that can resist high pelleting temperatures. Such tailored xylanase products are already on the market (see above and Table 2.1), and further development can be expected. Information on the development of thermostable β-glucanases for feed is more limited (see above). A new thermostable enzyme can be derived from a natural, thermotolerant isolate, or from mutagenesis of a mesophilic enzyme, or from a combination of a thermotolerant isolate and mutagenesis. A large number of thermophilic enzymes have been isolated from microbial sources (reviewed in Niehaus *et al.*, 1999; Haki and Rakshit, 2003; Collins *et al.*, 2005). Enzymes from archaea are often extremely thermostable and can even withstand boiling for extended periods. However, low production yields from native and recombinant hosts have restricted the commercial exploitation of these extremely thermostable xylanases and thermostable bacterial xylanases in general (e.g. Bergquist *et al.*, 2002). By using rational design and evolution strategies, several successful modifications have been reported which have increased the thermostability of mesophilic xylanases by 15–20°C or even more (e.g. Palackal *et al.*, 2004; Xiong *et al.*, 2004). In spite of that success, however, temperature stabilities of modified mesophilic xylanases often remain lower than those of thermophilic enzymes isolated from native sources, and lower than are ideal for use in the feed industry.

Another recent target of feed enzyme development has been xylanases resistant to natural inhibitors in grains. Such xylanases have already been developed and are commercially available for food use (see above). It is likely that these enzymes will be developed further and that more of these inhibitor-resistant mutants, based on different xylanase backbones, will also enter the market for feed use. One issue with the current inhibitor-resistant xylanases is

that their specific activity is lower compared with that of native enzymes, which can possibly be addressed in future mutants.

Increased specific activity of enzymes employed in feed application would also be beneficial, provided yields during fermentation were not affected. This is because it would allow economically viable dosages to be increased significantly, perhaps enabling the degradation of the cell walls in the aleurone and outer layers of cereal grains. Microscopic images show that endosperm cell walls of wheat can be degraded by xylanase/β-glucanase combinations more easily than those from the aleurone layer, which are much thicker and therefore not easily degraded by supplemental enzymes, as illustrated in Fig. 2.6. In order to make aleurone, or even NSP, from outer layers more digestible, the enzyme dose rate must be substantially increased, which is not usually economically justified. In addition, engineering of substrate selectivity of enzymes might produce further improvements (Moers *et al*., 2005, 2007).

Currently, commercial feed enzyme preparations are focused on upgrading cereal grains. In future, more supplemental enzyme products for diets rich in 'non-viscous cereals' and for legumes and oilseed plants are to be expected. The polysaccharide structures of these substrates are typically very complex, which suggests that a combination of different types of enzyme activities is needed. In addition, even more complex/variable substrates (e.g. by-products from either biofuel production or other volume-wise important sources) might be targeted for feed use and/or more complete hydrolysis of current/future substrates might be found necessary or advantageous. Current feed enzyme products often contain minor side-activities that degrade the side-chains of NSP, e.g. xylan substituent groups such as arabinose. These activities will most

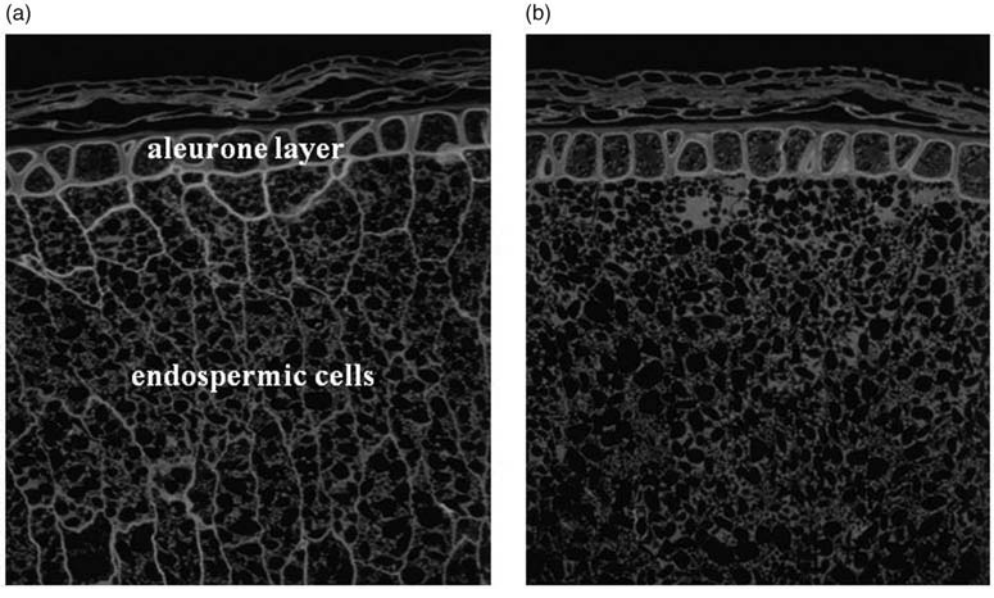

Fig. 2.6. Microscopic image of endospermic and aleurone wheat cell walls before (a) and after (b) treatment with a *Trichoderma* xylanase/β-glucanase preparation.

probably be the subject of further research, as to our knowledge they have not at this point in time been developed and produced for feed use. Products with multiple-tailored major activities can be prepared by mixing separate monocomponent products, but they can also be obtained by overproducing multi-domain native enzymes or engineered fusion proteins that have two or more separate domains with different (and synergistically acting) activities. Such multifunctional enzymes have already been constructed; for example, a fusion protein in which *Thermoanaerobacter ethanolicus* xylosidase-arabinosidase and *T. lanuginosus* xylanase are associated shows enhanced efficiency on arabinoxylan compared with corresponding free enzymes (Xue *et al*., 2009).

One interesting area for further improvements is the formation and utilization of prebiotic oligosaccharides for animal husbandry. Today, the extensively studied oligosaccharides include fructo-oligosacharides and α-galacto-oligosaccharides from plant origin and yeast-derived manno-oligosaccharides. The latter are known to compete with the gut cell wall for the binding site of bacteria, e.g. *E. coli* and *Salmonella* spp. contain mannose-specific lectins on their surface (Rehman *et al.*, 2009). The mechanisms related to the fermentation of oligosaccharides, however, require further research to be fully understood and usable. Regarding xylanases, it has been shown that the xylo-oligosaccharides formed during degradation of xylans can be hydrolysed by *Bifidobacterium* and *Lactobacillus* spp., resulting in an increase in the population of beneficial bacteria and a decrease in the number of harmful examples (Thammarutwasik *et al.*, 2009). The arabinoxylans and their oligosaccharides are fermented to a different degree and by different species. For example, an arabinoxylan polymer can be fermented by *Bifidobacterium longum* and *Bacteroides ovatus*, whereas *Bacteroides vulgatus* and *Bifidibacterium adolescentis* were able to ferment branched oligosaccharides completely but showed no activity towards the arabinoxylan polymer (van Laere *et al.*, 1977). Thus, in this respect, the specificities of xylanases might play a role in determining gut flora populations as a result of the identity of the dominant oligomers produced.

Most feed enzymes have been developed for use in swine and poultry diets. Products for a variety of target species will most probably follow in time. Such species include ruminants, fish, pets and fur animals. The feedstocks used for these animals and conditions of the intestinal tract differ significantly from swine and poultry and, as a result, the enzyme products envisaged may well be different from those employed today. In the more distant future genetically modified plants more suited to feed use or animals with better capability to utilize feed ingredients might be developed. Reports on successful expression and production of a xylanase (a catalytic domain of a fungal xylanase) and a cellulase (a hybrid 1,4-β-glucanase) into barley endosperm are already available (Patel *et al*., 2000; Xue *et al*., 2003).

Due to strict regulations and extensive testing requirements, development and registration of feed enzymes typically takes several years. This has delayed or constituted a barrier towards development and/or introduction of new feed enzyme products. It would be beneficial if the time frame from the development of an enzyme product to market were shorter. A reduction in the number of

animal trials and less time for the registration procedure could be achieved parallel to the development of *in vitro* model systems, and implementation of systems such as GRAS (Generally Regarded As Safe) in the USA and QPS (Qualified Presumption of Safety) in the EU. This subject is further discussed in another chapter of this book.

Conclusions

Xylanases and β-glucanases will remain as the major NSP enzymes in the feed industry. Several hurdles present themselves to any new candidate. In addition to performance in the animal, the enzyme needs to be produced at commercially competitive levels and successfully achieve regulatory authorization, which typically requires an extensive and time-consuming process. Consequently, at this moment in time commercial NSP enzymes are derived from only a limited number of potential donor organisms. Currently, research is focusing on the development of enzymes that are resistant to high temperatures, to natural inhibitors and are, at the same time, most suited for the conditions in the digestive tract of the target animal. It can be expected that, concurrent with increasing knowledge on digestive physiology and enzyme mechanisms, more tailored xylanases, β-glucanases and other enzymes, either singly or combined, will be appearing on the market.

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3 Mannanase, Alpha-Galactosidase and Pectinase

M.E. JACKSON

Mannanase

Introduction

Mannans occur in the forms of glucomannan, galactomannan, gluco-galactomannan and glucurono-mannans in non-starch polysaccharides (NSPs) contained in plants. Mannan and heteromannans are a part of the hemicellulose fraction of plant cell walls in all leguminous plants (Reid, 1985). Hemicelluloses are defined as those plant cell wall polysaccharides that are not solubilized by water or chelating agents but are solubilized by aqueous alkali (Selvendran and O'Neill, 1985). According to this definition, hemicelluloses include mannan, xylan, galactan and arabinan. β-mannan, also referred to as β-galactomannan, is a polysaccharide with repeating units of mannose with galactose and/or glucose attached to the β-mannan backbone (Carpita and McCann, 2000).

Since the 1990s, β-mannanases have emerged as key enzymes in the biotechnology industry. Natural occurrence and industrial use of β-mannan-containing substances has spurred the use of β-mannanases in both industrial and animal food applications owing to their multifaceted properties.

Industrial applications of mannanases

Mannanases have been used in the pulp and paper industry to extract lignin from wood as an initial step in the bleaching process. This is a favourable alternative to pretreating pulp with alkaline, which poses environmental concerns (Cuevas *et al*., 1996).

Mannanases have also been used as processing agents in the manufacture of instant coffee (Nunes *et al*., 2006). Coffee polysaccharides comprise half of the coffee extract dry weight and mannans are abundant, making the extract highly viscous. Addition of mannanase facilitates processing of coffee extracts.

Whereas several classes of enzymes, including amylases and cellulases, have been used in the detergent industry for many years, mannanases active in alkaline conditions are only now starting to be used. Detergents must remove stains of all types and, since many household products (e.g. shampoos, hair-styling gels) and food products (e.g. ice cream and barbecue sauce) contain mannan-based gums used as stabilizers, mannanases have been shown to aid in the cleaning process (Wong and Saddler, 1992), since they break down β-1,4 linkages of mannan resulting in smaller, more soluble polysaccharide fragments that can be extracted with water.

Mannanases have been used in oil-drilling operations for several years. In secondary oil recovery, fissures in the bedrock containing oil are pumped with a mixture of guar gum, a concentrated source of mannan, and sand in order to extract the oil. Mannanases are added at a later point in the operation in order to reduce the viscosity of the solution for pumping purposes (Christoffersen, 2004).

Since the early 1990s, the usage of β-mannanase in diets for monogastric animals as a nutritional aid has become widespread, due to the ubiquitous use of soybean meal or other leguminous plants as protein sources. The mechanism of action and experimental results with various species will be discussed.

Mannanases in farm animals

β-Mannans are most prevalent in a wide variety of animal feed ingredients, including soybean meal, palm kernel meal, copra meal and sesame meal (Table 3.1). Since soybean meal is a major protein source in feeds produced around the world, β-mannan is present in most feeds. Other common ingredients, such as corn distillers' dried grains and canola meal, also contribute to the β-mannan content of many diets for monogastric animals. The β-mannan content of a large number of soybean meal samples from various parts of the world has been reported and shown to be reasonably consistent (Hsiao *et al*, 2006).

A large number of studies have been reported examining the effects of β-mannanase on animal performance under various circumstances. Unless indicated otherwise, all reports tested a commercial source (Hemicell)[1]. Although this product is predominantly a β-mannanase source, it also contains low levels of amylase, β-glucanase, α-galactosidase, xylanase and others. Research with the purified enzyme suggests, however, that β-mannanase is the active ingredient and that other enzymes contained within the product have little or no influence on its efficacy with maize–soybean meal-type diets (Hsiao *et al*., 2004; Jackson *et al*., 2004a).

Table 3.1. β-Mannan content of various feed ingredients (adapted from Dierick, 1989).

Ingredient	β-Mannan content (%)
Palm kernel meal	30–35
Copra meal	25–30
Soybean hulls	8.0
Guar meal[a]	3–9
Sesame meal	3.2
Soybean meal (non-dehulled)[a]	1.61
Soybean meal (dehulled)[a]	1.26
Sunflower meal (33%)[a]	1.20
Rye	0.69
Peanut meal	0.51
Canola meal	0.49
Barley	0.49
Lupinseed meal	0.42
Cottonseed meal	0.36
Rice bran	0.32
Oats	0.30
Corn DDGS	0.27
Wheat middlings	0.15
Wheat	0.10
Bakery meal	0.10
Maize	0.09
Sorghum	0.09
Wheat bran	0.07

DDGS, distillers' dried grains with solubles.
[a]Hsiao *et al.* (2006).

Mode of action

The mode of action of β-mannanase in monogastric animals is complex and is linked to the removal of β-mannans from the animals' diet. It is well accepted that β-galactomannan inhibits insulin secretion in swine (Leeds *et al.*, 1980; Sambrook and Rainbird, 1985), suggesting a deleterious effect on energy metabolism. This is supported by studies showing a reduced glucose and water absorption in swine fed maize–soybean meal-based diets supplemented with guar (Rainbird *et al.*, 1984). Given the effects of guar, it is likely that the beneficial effects of β-mannanase on energy metabolism may be associated with an increased stimulation of insulin secretion and a blocking of the adverse effect of β-galactomannan on glucose absorption (Jackson *et al.*, 1999a). The mechanism may also be associated with the enzyme's effect on viscosity in the gut. β-Galactomannan is a viscous polysaccharide, which may contribute to

hyperplasia of digestive organs resulting in an increased secretion of pancreatic fluid (Ikegami *et al*., 1990), thus increasing the energy demand of the intestine.

Experiments have also clearly demonstrated that β-galactomannans are potent stimulators of the innate immune system. β-Galactomannans have been shown to increase the proliferation of monocytes and macrophages, resulting in secretion of cytokines (Peng *et al*., 1991; Ross *et al*., 2002). Aloe vera leaf has been used as a natural remedy for accelerating the healing process for minor injuries in humans. Acemannan, a gel extracted from the aloe vera leaf, has similar properties to β-galactomannan in soybean meal. This has been demonstrated to stimulate the innate immune system, resulting in macrophage proliferation and cytokine production as well as increased nitric oxide release in mice (Zhang and Tizzard, 1996). The monitoring of specific acute-phase proteins can provide a measure of the stimulation of the innate immune system. Acute-phase proteins are an aspect of the innate immune system, and are known to accumulate in blood at high levels in response to various forms of stress. One acute-phase protein, known as α-1-acid glycoprotein (AGP), was monitored in a series of cage and pen trials with poultry (Anderson *et al*., 2006). These experiments revealed that, by the exclusion of an antibiotic from the diet, the AGP level was significantly elevated in broilers. This effect was also observed with normal diets after infection with three *Eimeria* species, thus establishing a relationship between AGP level and disease-related stress. The addition of β-mannanase to the diets significantly reduced the blood AGP in all trials, demonstrating that a reduction in the β-galactomannan level in the diet can directly reduce the extent of immune stimulation. A reduction in the stimulation of the innate immune system with β-mannanase may result in a reduced expenditure of energy for non-productive purposes.

In summary, the mechanism of action of β-mannanase in monogastric animals is associated with the removal and deactivation of the β-mannan components from the animals' normal diet. Supplementation with β-mannanase has been shown to increase insulin secretion and improve energy metabolism, reduce viscosity of substrates in the digestive tract and reduce stimulation of the innate immune system. It is also possible that the production of prebiotics as a result of β-mannan breakdown may exert a beneficial effect, although this has not been documented. The specific effects from these different modes of action are discussed below in the context of animal feeding study results.

Broiler studies

Graded levels of β-mannanase were added to maize–soybean meal-type diets in a 42-day broiler pen trial, with the results shown in Table 3.2. Data showed a curvilinear improvement in growth and feed conversion, levelling off at the 80–110 MU t^{-1} inclusion level. In addition, the data suggest a possible benefit with regard to mortality at the highest inclusion level. The enzyme, at its highest level of inclusion, improved growth and feed conversion rate (FCR) by approximately 4.4 and 3.7%, respectively ($P < 0.05$). This can be compared

Table 3.2. Effect of varying levels of β-mannanase on 0–42-day broiler performance (from Jackson *et al*., 2004b).

	β-Mannanase addition rate (MU t^{-1})[a]			
Parameter	0	50	80	110
Weight gain (g)	2547[d]	2529[d]	2651[c]	2660[c]
FCR[b] (g g^{-1})	1.970[d]	1.965[d]	1.924[c]	1.899[c]
Mortality (%)	5.00[c,d]	6.33[c]	4.50[c,d]	2.83[d]

[a]MU = 10^6 enzyme activity units.
[b]Feed conversion rate values corrected for mortality.
[c, d]Means without a common superscript differ significantly (P <0.05).

with a larger response to the enzyme (7% in growth and 6% in FCR) reported in a 42-day broiler trial using low-energy maize–soybean meal-based diets containing 4–12% wheat bran (Torki and Chegeni, 2007). The larger effect in the latter study may be a result of very low energy levels in the basal diets.

Guar gum is a rich source of β-galactomannan (Vohra and Kratzer, 1964; Couch *et al*., 1967) and is known to depress growth in chicks (Ray *et al*., 1982). The structure of β-mannan in guar is virtually identical to that of β-mannan in soybean meal (Whistler and Saarnio, 1957), suggesting that it can be a useful tool in assessing β-mannanase enzymes in diets varying in β-mannan content. Two experiments were conducted examining the effect of β-mannanase on broiler chick performance to 14 days of age using guar gum to vary the β-mannan content of diets (Daskiran *et al*., 2004).

In the absence of guar gum, β-mannanase improved feed conversion by 2.9% (P <0.05), with no effect on weight gain (Table 3.3). With the addition of 2% guar gum, performance clearly was depressed compared with the control, and addition of β-mannanase in this case improved weight gain and feed conversion by 5.5 and 6.0%, respectively (P <0.05). Results suggest that β-mannanase will improve early chick performance with maize–soybean-type diets but that its effect increases dramatically as the β-mannan content of the diet is increased. This is supported by results of a study conducted with diets devoid of soybean meal but containing canola meal and sunflower meal as protein sources (Magpool *et al*., 2010). β-Mannanase significantly improved weight gain and feed efficiency (P <0.05) when guar meal was included in the diets at 5–7%.

Using graded levels of β-mannanase, Daskiran *et al*. (2004) observed a curvilinear response to the enzyme that is typical of some other feed enzymes (Rosen, 2002; Table 3.4). However, there was no body weight response to the enzyme. This is in agreement with Jackson *et al*. (2004b) for the feed conversion response but differs in the absence of a weight gain response, possibly as a result of the younger age of bird tested.

Disease challenge studies

Two experiments were conducted to determine the effects of β-mannanase on broiler chick performance under disease challenge (Jackson *et al*., 2003a). In

Table 3.3. The effect of β-mannanase on broiler chick performance to 21 days in diets varying in guar gum content (from Daskiran *et al.*, 2004).

Guar gum (%)	Enzyme[a]	BW (g)	FCR (g g^{-1})
0	–	394.8[b]	1.182[d]
0	+	390.2[b]	1.149[e]
2	–	335.7[d]	1.417[b]
2	+	354.0[c]	1.337[c]

BW, body weight; FCR, feed conversion rate.
[a]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.
[b–e]Means in columns without a common superscript differ significantly (P <0.05).

Table 3.4. The effect of β-mannanase at graded levels on broiler chick performance to 21 days in diets containing 1% guar gum (from Daskiran *et al.*, 2004).

β-Mannanase (MU t^{-1})[a]	BW (g)	FCR (g g^{-1})
0	346.5	1.336[b]
100	346.9	1.304[c]
200	348.1	1.291[d]
300	345.5	1.286[d]

BW, body weight; FCR, feed conversion rate.
[a]MU = 10^6 enzyme activity units.
[b–d]Means without a common superscript differ significantly (P <0.05).

the first experiment, performance was poor as expected with infection, but application of β-mannanase increased gain by 14% and improved FCR by 11%, both being significant (P <0.05) for disease-challenged birds (Table 3.5). A significant reduction in upper lesion score was also observed. Medication, comprising an antibiotic and coccidiostat, also significantly improved performance, and to a larger extent than did β-mannanase.

In the second experiment, an antibiotic and coccidiostat were examined, separately and in conjunction with and without β-mannanase (Table 3.6). In the absence of medication, β-mannanase significantly increased weight gain and reduced both upper and lower coccidial lesion scores (P <0.05) in infected birds. A significant reduction in lesion score in the lower intestine was also observed with the enzyme (P <0.05). No further improvement in performance was observed when both the antibiotic and coccidiostat were present. These results demonstrate that β-mannanase is highly effective in birds exposed to disease stress, possibly through reducing the luminal concentration of β-galactomannans, which are potent stimulators of the innate immune system. In effect, the enzyme is reducing the inflammatory response, which occurs as a result of overstimulation and proliferation of monocytes and macrophages by the intact mannans.

Table 3.5. Effect of infection[a], β-mannanase enzyme[b] and medication[c] on broiler chick performance from 8 to 21 days of age (from Jackson *et al*., 2003a).

						Lesion score (day 14)[d]	
Infection	Enzyme	Medication	Gain (g)	FCR (g g^{-1})	Mortality (%)	Upper	Lower
–	–	+	540[e]	1.446[g]	0.00[f]	0.00[g]	0.00[g]
–	+	+	548[e]	1.424[g]	1.78[f]	0.00[g]	0.00[g]
+	–	–	429[h]	1.704[e]	9.78[e]	1.38[e]	1.56[e]
+	+	–	490[g]	1.536[f]	3.75[e,f]	1.16[f]	1.44[e]
+	–	+	522[f]	1.447[g]	0.89[f]	1.03[f]	0.88[f]

FCR, feed conversion rate.

[a]Orally inoculated with a mixed solution with approximately 70,000 oocysts of *Eimeria acervulina* and 1250 oocysts of *Eimeria maxima* per bird on day 7. On days 11, 12 and 13, birds were given broth cultures of *Clostridium perfringens* containing approximately 1.5×10^8 cfu per bird.

[b]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.

[c]Salinomycin (SAL; 60 g t^{-1}) plus bacitracin methylene disalicylate (BMD; 50 g t^{-1}).

[d]Upper intestine, *E. acervulina*; lower intestine, *E. maxima*.

[e–h]Means within columns not sharing common superscripts are significantly different ($P < 0.05$).

Effects on intestinal morphology and function

The effect of β-mannanase on intestinal function and morphology has been examined in several experiments. In a broiler trial using maize–soybean meal-based diets containing approximately 8% wheat, Saki *et al*. (2005) reported significantly increased protein and dry matter digestibility and reduced uric acid content in the litter with the addition of β-mannanase (Table 3.7). In a histological study, Adibmoradi and Mehri (2007) examined several components of gut morphology in 42-day-old broilers provided with four levels of β-mannanase (0, 100, 140 and 180 MU t^{-1}) in maize–soybean meal-based diets.

Increasing β-mannanase dosage showed a linear improvement in several criteria, with significant increases in duodenal villus height and crypt depth, and decreased epithelial thickness and goblet cell numbers with enzyme supplementation at 140 MU t^{-1} ($P < 0.01$). Crypt depth increased and goblet cell numbers in the ileal villi were reduced at this level of inclusion ($P < 0.01$). A linear decrease in ileal viscosity was also observed with increasing levels of enzyme addition. The authors commented that reduced goblet cell numbers may be expected to lower mucin production, and endogenous nitrogen losses and decreased epithelial thickness may benefit the absorption of nutrients. In an experiment using three levels of mannanase (0, 0.49 and 1.225 MU kg^{-1}, Quest international Company, Ireland; note: these units are not comparable to those used in other studies), Ouihida *et al*. (2002) reported no improvement in weight gain or feed conversion in broilers provided with the enzyme from 6 to 42 days of age. However, a decrease in the concentration of purine bases in the ileum was observed at 21 days ($P < 0.04$) and 42 days ($P < 0.06$). The

Table 3.6. Effect of infection[a], β-mannanase enzyme[b] and medication[c] on broiler chick performance at 8 to 21 days of age (from Jackson *et al.*, 2003a).

Treatment	Enzyme	Gain (g)	FCR (g g^{-1})	Mortality (%)	Lesion score (day 14)[d]	
					Upper	Lower
Non-infected						
1 Non-medicated	–	427^{e,f}	1.695^{g}	1.25	0.00^{i}	0.00^{i}
2 Medicated	–	437^{e}	1.656^{g}	2.50	0.00^{i}	0.00^{i}
Infected, non-medicated						
3	–	296^{i}	1.909^{e}	1.25	2.44^{e}	2.31^{e}
4	+	338^{h}	1.849^{e,f}	3.75	1.94^{f}	1.34^{g,h}
Infected, BMD						
5	–	352^{h}	1.770^{f,g}	5.00	2.25^{e,f}	1.94^{e,f}
6	+	348^{h}	1.772 ^{f,g}	5.00	2.09^{e,f}	1.34^{g,h}
Infected, SAL						
7	–	368^{g,h}	1.720^{g}	3.75	1.00^{g}	1.09^{h}
8	+	397^{f,g}	1.688^{g}	3.75	0.97^{g}	1.09^{h}
Infected, BMD + SAL						
9	–	397^{f,g}	1.671^{g}	5.00	1.59^{h}	1.63^{f,g}
10	+	390^{g}	1.666^{g}	5.00	0.78^{g,h}	1.16^{gh}

FCR, feed conversion rate.
[a]Orally inoculated with a mixed solution comprising approximately 70,000 oocysts of *Eimeria acervulina* and 5000 oocysts of *Eimeria maxima* per bird on day 7. On days 11, 12 and 13, birds were given broth cultures of *Clostridium perfringens* containing approximately 1.5 × 10^{8} cfu per bird.
[b]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.
[c]Salinomycin (SAL; 60 g t^{-1}) and bacitracin methylene disalicylate (BMD; 50 g t^{-1}).
[d]Upper intestine, *E. acervulina*; lower intestine, *E. maxima*.
[e–i]Means within columns not sharing common superscripts are significantly different (P <0.05).

authors speculated that this decrease may be a result of reduced microflora in the ileum and caeca caused by reduced undigested polysaccharides escaping foregut digestion. The lack of live performance response is noteworthy of comment. This may be as a result of an inappropriate trial design, the use of highly digestible ingredients or exceptionally clean conditions. For example, a relatively small number of birds were tested in this experiment (six birds per cage by six replications).

Effects on variability in animal weights

In any population of broilers, a degree of variability in live weights will exist. This variability is caused by a number of factors including genetic variability,

Table 3.7. Effect of β-mannanase enzyme[a] on protein digestibility and uric acid and litter moisture in 42-day-old broilers (from Saki *et al.*, 2005).

	Protein digestibility (%)		Dry matter digestibility (%)	Litter (%)	
Enzyme[a]	*In vitro*	Ileal	*In vitro*	Uric acid	Moisture
–	68.30[c]	61.80[b]	62.49[c]	79.94[b]	4.68[b]
+	71.31[b]	62.48[b]	64.96[b]	66.61[c]	4.64[b]

[a]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.
[b,c]Means within columns not sharing common superscripts are significantly different (P <0.05).

management conditions and inherent stresses caused by local disease challenges and climatic conditions. The efficiency of modern-day processing operations may be improved by a reduction in the variability of live weights. This can result in an increased throughput, as automated equipment is typically adjusted for the average body weight of one or more flocks entering the plant. In addition, the consistency of carcasses or parts can result in a higher value in the marketplace.

Live weight uniformity can be determined in pen trials by weighing all birds at various ages. Although this is a labour-intensive process, it can provide useful information as to the benefits of a feed additive. The percentage coefficient of variation (CV) can be determined for each individual pen, and data may be statistically analysed. A number of studies have been conducted with β-mannanase in maize–soybean meal-based diets to evaluate its effect on broiler live-weight uniformity. These studies determined individual live weights at various ages using pen populations ranging from 18 to 70 birds. Testing 54-day-old mixed-sex broilers, Piao *et al*. (2003) reported that β-mannanase significantly decreased live weight CV from 11.58 to 9.17% (P <0.05), representing a 26% reduction. In agreement with these results, a 19% (P <0.05) decrease in CV with 42-day-old male broilers was reported (Jackson *et al*., 2005). In a pen trial where individual live weights were determined at multiple ages, Jackson *et al*. (2004c) reported a decrease of 20 and 21% in CV at 21 and 49 days of age, respectively (both significant at P <0.05). In each of these reports, the improved uniformity was caused by a smaller percentage of underweight birds provided with β-mannanase. These uniformity improvements provide additional evidence that β-mannanase is most effective in improving performance of birds exposed to high levels of stress, as discussed earlier.

Turkey studies

As a result of longer growing periods, turkeys may be exposed to greater levels of stress compared with broilers. Testing β-mannanase with diets containing hulled and dehulled soybean meal, Odetallah *et al.* (2002) reported on one turkey hen and two turkey tom studies. The results of the hen study are shown in Table 3.8. There were no significant weight differences at 98 days of age,

but at 70 days of age a significant enzyme × soybean meal source interaction was observed. β-Mannanase significantly increased live weight by approximately 4.5% ($P < 0.01$) with the 44% soybean meal. Consistent with the weight gain results, at 98 days of age the enzyme improved FCR with 44% soybean meal (SBM) only ($P < 0.01$). The lack of a significant performance response with 48% SBM may also be related to above-average rearing conditions in the experimental facility. Like all feed additives, including antibiotic growth promoters, a positive response cannot be expected 100% of the time.

The pooled results of the two tom studies are summarized in Table 3.9. Although the enzyme × soybean meal source interaction was not significant, it tended to show a trend for body weight ($P < 0.087$). β-Mannanase increased live weight by 0.8 and 2.6% with 48 and 44% protein soybean meal, respectively. This tends to support the hen trial, where a larger effect was observed with the 44% protein soybean meal source. The lower performance response using 48% SBM may be a result of the lower β-mannan levels in dehulled compared with non-dehulled SBM (Table 3.1). Across both soybean meal sources, β-mannanase improved feed conversion by 3.2% ($P < 0.001$). Overall, the studies demonstrated positive effects of supplementing turkey feed

Table 3.8. Effect of β-mannanase on turkey hen performance[a] (from Odetallah *et al.*, 2002).

SBM (% protein)	Enzyme[b]	Body weight (kg, day 70)	FCR (g g^{-1}, 0–98 days)
48	–	4.661	2.155
48	+	4.577	2.205
44	–	4.278	2.234
44	+	4.471	2.212
P value for SBM × enzyme		0.001	0.034

SBM, soybean meal; FCR, feed conversion rate.

[a]30 birds per pen, four pens per treatment.

[b]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.

Table 3.9. Effect of β-mannanase on turkey tom performance[a] (from Odetallah *et al.*, 2002).

SBM (% protein)	Enzyme[b]	Body weight (kg, day 126)	FCR (g g^{-1}, 0–126 days)
48	–	14.91	2.704
48	+	15.03	2.633
44	–	14.40	2.794
44	+	14.77	2.695
Average	–	14.66	2.749
Average	+	14.90	2.664
P value for enzyme		0.001	0.001
P value for SBM × enzyme		0.087	0.241

SBM, soybean meal; FCR, feed conversion rate.

[a]Two experiments pooled, each experiment with 17 birds per pen, seven pens per treatment.

[b]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.

with β-mannanase and suggest that these effects may be larger when lower-protein soybean meal containing higher levels of β-mannan is used.

Several additional trials testing β-mannanase with toms and hens have been conducted with corn and dehulled soybean meal-based diets under various circumstances. Examining toms to 18 weeks of age, Jackson *et al*. (2002a) reported a final body weight improvement of 4.9% ($P < 0.002$), while improvements in FCR were 2.3% ($P < 0.02$), 2.0% (NS) and 3.6% (NS) from 0–6, 6–12 and 12–18 weeks of age, respectively. This is comparable to a 20-week tom trial where improvements in final body weight of 5.8% ($P < 0.05$) and feed conversion of 20.7 points ($P < 0.05$) were reported (Jackson *et al*., 2008a).

Examining two protein regimes with toms grown to 155 days of age, improvements of 8% ($P < 0.05$) and 4.2% (NS) were observed for high- and low-protein feeding programmes, respectively (Jackson *et al*., 2002b). Corresponding improvements in FCR were not significant, but ranged from 7.8 to 4.1 points in the high- and low-protein feeding programmes, respectively, suggesting that the level of soybean meal may play a role in anticipated responses to the enzyme. In partial contrast to this study, Jackson *et al*. (2003b) reported improvements of 4.1 and 1.9% in weights of 14-week-old hens (both $P < 0.05$) in moderate- and high-density feeding programmes, respectively. A 2.9% improvement in FCR ($P < 0.05$) was observed in the moderate-density programme only. It should be pointed out, however, that in this study the differences in density involved protein as well as energy, so the lower-density programme was probably also limited in energy. Adjusting only energy (in increments of 60 kcal kg^{-1}) in a hen trial, Jackson and Mathis (2006) reported improvements in 14-week weights of 4.1 and 3.5% in very low- and low-energy regimes, respectively, with numerical improvements in FCR. Results suggest that a higher response may be anticipated with feeding programmes limited in energy.

Corn distillers' dried grains with solubles (DDGS) are becoming readily available, and are used partially to spare soybean meal and reduce diet costs in increasing frequency by turkey producers. Comparing the influence of β-mannanase on diets containing 0 and 15% DDGS, but with similar nutrient profiles, Jackson *et al*. (2008b) reported improvements of approximately 2.3% in weight gain and 14 points of feed conversion (both $P < 0.05$) regardless of the DDGS inclusion level, with no interactions between the enzyme and DDGS inclusion. These results suggest that a reduction in β-mannan level associated with lower soybean meal inclusion is insufficient to offset the benefit of β-mannanase in turkey diets, and that the little mannan present in corn DDGS may be proportionately more responsive to this enzyme than that in SBM.

Live weight uniformity has been evaluated in turkeys in much the same way that it has been evaluated in broiler pen trials. Several studies have determined individual live weights at various ages using pen populations ranging from 12 to 40 birds. Testing toms at various ages, Jackson *et al*. (2002a) reported that β-mannanase significantly decreased live weight CV from 11.49 to 7.34% ($P < 0.001$), from 9.56 to 5.94% ($P < 0.020$) and from 10.57 to 7.40% ($P < 0.001$) at 6, 12 and 18 weeks of age, respectively.

A series of four experiments testing the effects of β-mannanase on live weight uniformity was summarized by Jackson *et al.* (2002b). In the first experiment using hens with six commercial starter feeds to 21 days of age and 40 birds per pen, significant reductions in percentage CV were observed in five of the six comparisons ($P < 0.05$). Averaged across feeds, the percentage CV decreased from 22.7 to 16.8. Hens grown to 98 days were examined in the second study with 18 birds per pen, showing a reduction in percentage CV from 7.63 to 4.95 ($P < 0.05$). The third study tested toms to 42 days of age with 36 birds per pen. In this experiment, the percenage CV decreased from 11.90 to 8.64 ($P < 0.05$). The fourth and final study tested pens of 20 toms at two protein levels grown to 155 days of age. Very similar improvements in uniformity were observed across protein levels, with percentage CV decreasing from approximately 14.5 to 11.3 ($P < 0.05$). In several of these studies reported, graphic inspection revealed that the uniformity improvement was a result of fewer underweight birds in the populations provided with β-mannanase. These uniformity improvements are consistent with those observed in broiler populations but are generally of a larger magnitude, suggesting that turkeys may be exposed to greater levels of stress compared with those of broilers.

Laying hen studies

Laying hens are exposed to different forms of stress when compared with broilers or turkeys. Since they are commonly reared in cages, direct exposure to litter-related microbes and pathogens is lower. However, since they are reared for much longer periods, age-related stresses are more of a concern. A 48-week layer trial with maize–soybean meal-based diets was conducted with 6144 laying hens placed in cages starting at 18 weeks of age, and results are given in Table 3.10. The trial tested β-mannanase in two diets varying by 100 kcal kg^{-1}. Increasing the metabolizable energy (ME) by 100 kcal kg^{-1} resulted in significant improvements in egg production during latter three periods of the experiment only. There were no significant ME × enzyme interactions observed. Little or no effects of β-mannanase were observed for feed intake or body weight, but a small improvement in egg weight was reported during the first period only. The addition of β-mannanase resulted in significant egg production improvements with advancing periods, and the magnitude of these improvements increased with age. It is interesting to note that the level of soybean meal decreased with age from approximately 27 to 23%, indicating that the degree of response to β-mannanase appears to be unrelated to the β-mannan content of the diets. Furthermore, the absence of an energy × enzyme interaction suggests that, rather than furnishing an energy source, the beneficial effects of the enzyme are more likely to be associated with physiological improvements related to age-related stress.

A second experiment was conducted in the same facility, but examined varying levels of amino acid density as opposed to energy (Jackson *et al.*, 1999b). A significant enzyme × amino acid density interaction revealed that the enzyme had the greatest effect on egg production (1.06% increase,

Table 3.10. Effects of β-mannanase at two levels of metabolizable energy (ME) on percentage hen-day production (from Jackson *et al.*, 1999a).

	Age (weeks)			
Parameter	18–30	31–42	43–54	55–66
ME level[a]				
Low	70.11	86.11	79.31	74.75
High	71.49	87.02	80.23	74.75
Difference (%)	1.38	0.91	0.92	0.00
Enzyme[b]				
–	70.75	86.21	79.23	74.00
+	70.86	86.91	80.30	75.50
Difference (%)	0.11	0.70	1.07	1.50
P *statistics*				
ME level	0.091	0.001	0.001	0.988
Enzyme	0.885	0.007	0.001	0.001
ME × enzyme	0.429	0.331	0.540	0.577

[a]Low ME level is 100 kcal kg^{-1} lower than high ME level.
[b]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.

$P < 0.05$) with the lowest amino acid density tested (0.70% lysine). This suggests that β-mannanase may be instrumental in improving amino acid utilization under the conditions of the experiment.

Using 98-week-old hens which were moulted at 66 weeks of age, a 12-week trial with maize–soybean meal-based diets was conducted. The trial tested β-mannanase with two levels of energy varying by 120 kcal kg^{-1}, and results are shown in Table 3.11. Although egg production and egg mass were not significantly affected by treatment over the 12-week period, significant effects of β-mannanase and energy were reported during the period from 5 to 8 weeks into the study ($P < 0.05$). The enzyme treatment exceeded its control in all periods tested, and resulted in a numerical 2.2% increase in percentage production and a 2.5 % increase in egg mass. Most striking was a significant 4.4% improvement in FCR ($P < 0.001$) due to the enzyme over the course of the study. The lack of overall significant differences in egg production may be related to the reduced numbers of birds used in this study compared with the above-mentioned trial. The large improvement in FCR due to β-mannanase addition demonstrates the enzyme's ability to improve the efficiency of egg production in older laying hens.

Swine studies

A series of experiments was conducted with maize–soybean meal-type diets to determine the effects of β-mannanase at various stages of growth in swine, with results presented in Tables 3.12, 3.13 and 3.14. In the first experiment

Table 3.11. Effects of β-mannanase on laying hen performance in second-cycle hens (from Wu *et al.*, 2005).

ME[a]	Enzyme[b]	Egg production (%)	Feed intake (g hen^{-1} day^{-1})	Egg mass (g hen^{-1} day^{-1})	FCR (g egg mass g feed consumed^{-1})
High	–	72.14	96.94	48.26	2.01[c]
Low	–	69.44	98.78	46.11	2.15[d]
Low	+	71.65	97.63	47.30	2.06[c]
P value		0.173	0.503	0.131	0.001

ME, metabolizable energy; FCR, feed conversion rate.
[a]Low ME level is 120 kcal kg^{-1} lower than high ME level.
[b]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.
[c,d]Means within columns not sharing common superscripts are significantly different (P <0.05).

(Table 3.12), pigs at approximately 6.27 kg BW were allotted to four treatments with two levels of diet complexity, with and without β-mannanase. The test involved three, two-week phases where the simple and complex diets were iso-nutritional but the complex diets contained lower levels of soybean meal in the early phases, replaced by varying levels of spray-dried blood meal, blood plasma and fishmeal. There were no significant interactions between β-mannanase inclusion and diet complexity. Across diet types, β-mannanase significantly improved feed efficiency by approximately 4%.

In the second experiment (Table 3.13), pigs at approximately 13.6 kg BW were provided with one of three treatments that comprised a control, a diet containing 2% soybean meal oil plus an additional 100 kcal ME kg^{-1} and a control diet plus β-mannanase for a 21-day period. As anticipated, the soybean oil treatment resulted in improved feed efficiency. Likewise, β-mannanase improved feed efficiency (P <0.05) by approximately 4.8%, the improvement being close in magnitude to that resulting from an increase in ME of 100 kcal kg^{-1}.

In the third experiment (Table 3.14), pigs at approximately 109 kg BW were provided with three treatments that comprised a control, a diet containing 2% soybean meal oil and a control diet plus β-mannanase. Similar to experiment 2, the soybean oil treatment resulted in improved feed efficiency (P <0.10) and

Table 3.12. Effects of β-mannanase and diet complexity on growth performance of weanling pigs (from Petty *et al.*, 2002).

	Complex diet[a]		Simple diet[a]	
Parameter	–	+	–	+
ADG (g)	383	387	377	391
ADFI (g)	620	602	621	622
Gain:feed[b]	0.618	0.646	0.607	0.628

ADG, average daily gain; ADFI, average daily feed intake.
[a]β-Mannanase was either added (+) or not added (–) to the feed at a rate of 100 million units t^{-1}.
[b]Significant effects of β-mannanase and diet type (P <0.01).

Table 3.13. Effects of β-mannanase and soybean oil on growth performance of weanling pigs (from Petty *et al.*, 2002).

Parameter	Control	Soybean oil	Enzyme[a]
ADG (g)	543	553	558
ADFI (g)	955	941	938
Gain:feed	0.568[b]	0.588[c]	0.595[c]

ADG, average daily gain; ADFI, average daily feed intake.
[a]β-Mannanase was either added or not added to the feed at a rate of 100 million units t^{-1}.
[b,c]Means within rows not sharing common superscripts are significantly different ($P < 0.05$).

β-mannanase improved feed efficiency ($P < 0.10$) by approximately 4.2%. Again the effect of β-mannanase was similar to that of the 100 kcal ME kg^{-1} increase. In this experiment, the addition of β-mannanase increased the rate of fat-free gain by 5.6% ($P < 0.05$). The authors concluded from these three experiments that the improvement in feed efficiency in all three stages of production tested as a result of β-mannanase inclusion is comparable to 100 kcal kg^{-1}.

Consistent feed efficiency improvements with the inclusion of β-mannanase in maize–soybean meal-based diets reported by Petty *et al.* (2002) have been supported by additional studies. In two finishing pig experiments, Hahn *et al.* (1995) reported a trend in gain:feed improvement ranging from 2.4% ($P = 0.16$) to 3.0% ($P < 0.06$), as well as a large (8%) improvement in lean gain ($P < 0.13$). In a swine trial testing diets varying widely in net energy, Kim *et al.* (2003) reported improvements in feed efficiency ranging from 4.6 to 5.5% in low and high net energy diets, respectively ($P < 0.05$). In a large commercial trial with 5350 pigs grown for 20 weeks, O'Quinn *et al.* (2002) observed a

Table 3.14. Effects of β-mannanase and soybean oil on growth performance of growing-finishing pigs (from Petty *et al.*, 2002).

Parameter	Control	Soybean oil	Enzyme[a]
Growth performance			
ADG (kg)	0.842[b]	0.829[b]	0.872[c]
ADFI (kg)	2.50[b]	2.32[c]	2.48[b]
Gain:feed	0.337[e]	0.358[f]	0.351[f]
Carcass traits			
10th rib fat depth (cm)	2.24[c]	2.06[d]	2.13[c,d]
Longissimus muscle area (cm^2)	40.8	40.6	43.2
Fat-free lean (%)	49.46	50.36	50.40
Fat-free lean gain (g day^{-1})	322[b]	327[b]	340[c]

ADG, average daily gain; ADFI, average daily feed intake.
[a]β-Mannanase was either added or not added to the feed at a rate of 100 million units t^{-1}.
[b,c,d]Means within rows not sharing common superscripts are significantly different ($P < 0.05$).
[e,f]Means within rows not sharing common superscripts are significantly different ($P < 0.10$).

large improvement in health status using β-mannanase. Along with a significant improvement in ADG ($P < 0.04$), they reported a 24% reduction in mortality ($P < 0.04$), a 60% reduction in culling of lightweight pigs ($P < 0.001$) and a trend for increased dressing percentage ($P < 0.08$). Improvements in health are consistent with observations in disease-challenge broiler studies discussed earlier. Improvements in energy utilization support observations by Radcliffe *et al.* (1999), where it was demonstrated that β-mannanase increased apparent ileal digestibility of dry matter and apparent total tract energy digestibility ($P < 0.05$) in swine.

Summary

A large number of studies have been reported examining the effects of β-mannanase in maize–soybean-based diets with broilers, turkeys, laying hens and swine. Although commercial β-mannanase contains low levels of other enzymes including amylase, β-glucanase, α-galactosidase and xylanase, research with the purified enzyme suggests that β-mannanase is the active ingredient and that other enzymes have little or no influence on its efficacy. It is well established that β-mannan is highly anti-nutritional and that β-mannanase is effective in breaking down this undesirable component in animal feeds. The mode of action of β-mannanase is complex, but is probably related to: (i) its effect on insulin secretion, glucose absorption and energy metabolism; (ii) its effect on viscosity in the gut; and (iii) reduced stimulation of the innate immune system, resulting in a reduced expenditure of energy for non-productive purposes.

The preponderance of data demonstrating the potential of β-mannanase to improve live performance and determine various facets of its mechanism is derived from broiler experiments. However, a large database for turkeys, laying hens and swine studies also exists, demonstrating significant improvements in live performance of these species. Broiler disease-challenge trials show particularly large benefits in animals exposed to stress. This is supported by trials demonstrating highly significant improvements in uniformity within broiler and turkey populations. It is also supported by laying hen studies, which show increased benefits in older birds.

Since the β-mannan content of the diet is partially dependent upon the level of soybean meal in the diet, one might expect an increasing benefit from β-mannanase with diets containing higher levels of soybean meal. There is limited evidence for this phenomenon, indicating that the enzyme is effective within a practical range of soybean meal use.

α-Galactosidase

Introduction

α-Galactosidase is a glycoside hydrolase enzyme that hydrolyses the terminal α-D-galactose moiety from galactoside oligosaccharides, glycoproteins,

glycolipids and other galactose-containing molecules. This enzyme is best known in human medicine by its association with a rare genetic disorder, Fabry's disease, which is caused by a mutation of the GLA (α-galactosidase) gene resulting in decreased production of α-galactosidase A. Patients with Fabry's disease experience abnormal lipid metabolism resulting in fatty depositions in several organs of the body such as the eyes, kidneys, autonomic immune system and cardiovascular system. Symptoms vary widely but include burning sensations in the hands, skin blemishes and increased risk of strokes and heart attacks, at a relatively young age.

Commercially, α-galactosidase is used in the food processing industry for the production of sugar from sugarbeets, as an over-the-counter human digestive aid and as an animal feed supplement (USFDA, 2009). It is also being tested as a therapeutic treatment for Fabry's disease (National Institute of Neurological Disorders and Strokes, 2009).

While it is well accepted that α-galactosidase is not produced endogenously by monogastric animals (Gitzelmann and Auricchio, 1965), low levels of this enzyme can be produced by microflora in the large intestine and this can be influenced by oligosaccharides and other substrates contained within the diet (Zdunczyk *et al.*, 2007; Juskiewicza *et al.*, 2008).

α-Galactosides in soybean meal

SBM, used as a high-quality protein source in most poultry- and swine-producing countries, contains significant quantities of galactose-containing carbohydrates (Table 3.15). Whereas the protein fraction of soybean meal is known to be highly digestible for monogastric animals (NRC, 1994, 1998), its carbohydrate energy contribution is limited. Early studies to determine the ME of common poultry feed ingredients indicate that dehulled SBM contains gross energy of about 4695 kcal kg^{-1} (Sibbald, 1986), while its ME is approximately 2440 and 3380 kcal kg^{-1} in poultry and swine, respectively (NRC, 1994, 1998). This represents a utilization rate of only 52 and 72% for poultry and swine, respectively. The reason for this low ME contribution is not fully understood, but is probably due to reduced availability of the carbohydrate fraction.

The carbohydrate fraction of SBM is made up of almost equal amounts of various polysaccharides and oligosaccharides (Table 3.15). Whereas the polysaccharides comprise approximately 15–18% of SBM, the starch content is negligible at 0.5%. The greater part of polysaccharides are acidic polysaccharides, arabinogalactans and cellulosic material, all of which are essentially non-digestible. Oligosaccharides account for 11–15% of the carbohydrates in SBM, with sucrose accounting for the majority, at around 7%. Sucrose is highly digestible for monogastric animals, but the other oligosaccharides are considered poorly digestible.

One of the primary drawbacks to SBM polysaccharides is the loss of potential energy. This would be more severe in poultry than swine because the intestinal tract of swine is more extensive, with greater microfloral activity in the hindgut accompanied by a greater potential for carbohydrate digestion.

Table 3.15. Carbohydrate content of dehulled soybean meal (adapted from Honig and Rakis, 1979, p. 1265).

Carbohydrate	Percentage (by weight)
Polysaccharide content (total)	15–18
Acidic polysaccharides	8–10
Arabinogalactans	5
Cellulosic material	1–2
Starch	0.5
Oligosaccharide content (total)	15
Sucrose	6–8
Stachyose	4–5
Raffinose	1–2

Another potential drawback to SBM is that the polysaccharide fraction may also result in fluid retention and an increased flow rate in the gastrointestinal tract, which could negatively impact absorption of energy and other nutrients (Wiggins, 1984). Conversely, it is possible that polysaccharides such as α-galactosides could have positive impacts on the intestinal tract (Karr-Lilienthal *et al.*, 2005). For example, in a study comparing soybean meal with soy protein concentrates and isolates, Zdunczyk *et al.* (2009) concluded that 1% α-galactosides in a growing turkey diet can be beneficial, whereas their total removal can reduce performance. It was hypothesized that their total removal may result in an undesirable hypotrophy of tissue in the small intestine and a decreased activity of mucosal disaccharides, which could result in reduced growth rate.

True metabolizable energy studies

The magnitude of the negative impact of oligosaccharides derived from SBM has been investigated, with varying results. Large increases in true metabolizable energy (TME) of more than 20% were observed when α-galactosides were removed from SBM via ethanol extraction (Coon *et al.*, 1990; Leske *et al.*, 1993); however, this may have been partly associated with the simultaneous removal of other deleterious compounds. Parsons *et al.* (2000) evaluated several genetic lines of soybeans selected for low levels of raffinose and stachyose compared with those found in conventional soybeans. The two soybean lines with the lowest total raffinose, stachyose and galactinol levels had average TME values that were 9.8% higher than their respective genetic controls. In contrast to these results, the removal of oligosaccharides using endogenous soybean α-galactosidase failed to produce any beneficial effects on the apparent nutritional value of soy flakes. This was measured by growth rate, feed conversion, apparent metabolizable energy (AME) studies with young broilers and TME studies with adult roosters (Angel *et al.*, 1988). Irish *et al.* (1995) used ethanol extraction and incubation of SBM with α-galactosidase to

decrease concentrations of α-galactosides in soybean meal, from a starting value of 6.50% to 0.81% and 1.43%, respectively. There were no improvements in TME when these SBMs were precision fed to adult cockerels. It was concluded that removal of up to approximately 90% of the α-galactosides of sucrose had no beneficial effect on the nutritional value of SBM for chickens. Similar results have been observed with removal of oligosaccharides from canola meal using ethanol extraction (Slominski *et al.*, 1994).

In a study where an α-galactosidase enzyme treatment of SBM degraded raffinose and stachyose by 55–70%, the TME of SBM increased by 12% but no improvement in chick growth performance was observed (Graham *et al.*, 2002). This is in partial contrast to a study involving a series of experiments testing an α-galactosidase in broiler chicks (Ghazi *et al.*, 2003). The α-galactosidase used in this study appeared to significantly improve both TME and weight gain. Similarly, Knap *et al.* (1996) reported a linear improvement in TME, weight gain and feed conversion with increasing levels of α-galactosidase supplementation. It was unclear in the report whether or not the product used contained additional enzymes. In conclusion, removal of galactosyl oligosaccharides by various methods has shown mixed results with respect to TME values, and an improvement in TME does not necessarily translate to an improvement in animal performance.

Broiler studies

A series of broiler experiments was conducted at the University of Arkansas, USA, aimed at determining the potential benefit of a commercial α-galactosidase enzyme product under several conditions. One experiment examined five levels of α-galactosidase inclusion at up to eight times the manufacturer's recommendation to provide 0, 45, 90, 135 and 180 α-galactosidase units kg^{-1} soybean meal (Waldroup *et al.*, 2006). Negative control diets assumed a 10% increase in ME of soybean meal. Broilers tested to 42 days of age showed no benefit in live performance to enzyme addition. In a series of three experiments, Waldroup *et al.* (2005) tested four energy values for soybean meal assuming that the enzymes increased the ME of soybean meal by 0, 10, 20 and 30%. α-Galactosidase was tested in combination with and without xylanase. Similar to the first study, no benefit in live performance was observed in broilers to 42 days of age.

Another series of experiments examining α-galactosidase using a different commercial enzyme source was conducted at Mississippi State University, USA (Kidd *et al.*, 2001a,b). The product tested was a liquid blend containing primarily α-galactosidase, but also having α-amylase, β-glucanase, protease, xylanase and cellulase activities. A large pen trial with 36 replications and 50 birds per pen conducted in hot temperatures (Kidd *et al.*, 2001a) showed no performance benefit to 28 days but a highly significant improvement in feed conversion and liveability ($P < 0.01$), although no improvement in weight gain was observed to 49 days of age. Interestingly, there was no response in mortality-adjusted feed conversion in this study as a consequence of mortality

differences between treatments. Mortality was high in this experiment, ranging from 7 to 13%. A 21-day chick battery trial in the same report showed no responses. Two subsequent pen trials, one in warm and the other in thermoneutral environments (Kidd *et al.*, 2001b), with 18 replications again showed no improvements in weight gain, but demonstrated a trend for improved feed conversion (P <0.60–0.78). It appears that the large number of replications was needed to demonstrate the feed conversion response under the conditions of this facility.

Two experiments examined the potential of α-galactosidase to improve broiler performance in the presence of citric acid, with the rationale being that the optimum pH for the fungal enzyme product used is approximately 4.5 (Ao *et al.*, 2009). The experiments demonstrated that α-galactosidase had no beneficial effects on performance, except where performance was depressed due to citric acid supplementation.

Swine studies

The effect of α-galactosidase on apparent and true ileal digestibility in swine was examined with various substrates (Smiricky *et al.*, 2002). Soybean solubles were used in order to increase significantly raffinose and stachyose levels. Inclusion of soybean solubles effectively depressed the true and apparent digestibilities of most amino acids. Addition of α-galactosidase failed to increase the digestibility of most amino acids or that of stachyose, but significantly increased the digestibility of raffinose. Results suggest that the α-galactosidase used in this study may have acted on the raffinose fraction only, with little or no influence on amino acid availability.

In two experiments with swine, live performance was not improved with two levels of α-galactosidase supplementation in experiment 1 (Pan *et al.*, 2002). However, in the second experiment, 1% stachyose was added to the diets and the α-galactosidase enzyme improved the ileal digestibility of stachyose, raffinose, energy and protein. Improvement in the ileal digestibility of unspecified α-galactosides has also been reported by Veldman *et al.* (1993).

Summary

Application of α-galactosidase in animal feed for monogastrics where soybean meal is used as a primary protein source has theoretical potential. The benefits of supplementing poultry diets may be expected to be greater than those of swine diets as a consequence of the higher metabolizability of energy of soybean meal in swine. Approximately one-third of dehulled soybean meal is comprised of carbohydrate, and the digestibility of most of this fraction is considered very poor. Stachyose and raffinose, the most prevalent oligosaccharides in soybean meal, make up approximately 6% of soybean meal and are both non-digestible and potentially detrimental to the gastro-intestinal system.

There is wide variability in the response to α-galactosidase supplementation in poultry and swine diets. Possible reasons include: (i) differences in the source of enzyme products tested; (ii) the specificity of the enzyme on the key substrates, raffinose and stachyose (activity units vary and are often determined using other test substrates); (iii) pH optima; (iv) additional or side-enzyme activities of the products; and (v) other factors. Environmental conditions, soybean meal source and other nutritional factors may also affect the efficacy of the enzyme products tested. For example, a series of *in vitro* and *in vivo* studies with several enzyme products concluded that minerals such as calcium carbonate and calcium phosphate, both common in monogastric diets, can inhibit the activity of α-galactosidase (Slominski, 1994). It would be beneficial in future studies to develop an assay method that defines a standard unit of activity for all α-galactosidase products under investigation. The enzyme activity should ideally be measured under conditions similar to the physiological conditions of monogastrics, using the substrates raffinose and stachyose that are most prevalent in soybean meal.

Pectinase

Introduction

Pectinase is a term used for a class of enzymes that break down pectin, a polysaccharide contained within cell walls of plants and which functions in the ripening process of fruits. Pectins are large molecules comprised mainly of galacturonic acid residues. They form a jelly-like matrix, which binds plant cell walls together. Pectinase enzymes are used to release cell wall components such as cellulase. The most common pectinase used in industrial processes is endo-polygalcturonase. Fungi such as *Aspergillus niger* naturally produce pectinases in order to break down plant tissues to extract nutrients and insert fungal hyphae.

Commercial pectinases have been used in industrial processes for decades. Common uses of pectinases are in the food industry, where they are used to extract fruit juices from fruit and to improve flavours and reduce the cloudiness of wine. Other uses include aiding in the bleaching process in cotton production and, more recently, in combinations with other enzymes in animal feeds as a nutritional enhancer.

Pectinase in farm animals

Pectinase has been studied in a number of *in vitro* and *in vivo* experiments. While most reports examine pectinase in combination with other non-starch polysaccharide-degrading enzymes, a number of studies have tested pectinase enzymes either in purified forms or in enzyme products containing pectinase as the predominant enzyme activity. The efficacy of enzyme combinations containing pectinase is affected by a large number of factors, including the

animal species and stage of production examined, feed substrates tested and activity levels of pectinase and other NSP-degrading enzymes tested. In addition, the pectin content of feed ingredients varies widely, as shown in Table 3.16.

A common definition of pectinase activity is that one unit of pectinase liberates one micromole of D-galacturonic acid from polygalacturonic acid per minute at 37°C and pH 5.0. However, a viscometric definition method is also a widely used method to measure the activity of pectinolytic enzymes (Maiorano *et al.*, 1995).

In vitro digestibilty studies

Using a two-stage *in vitro* digestion assay, changes in viscosity and sugar release were reported using mixtures of enzymes including xylanase, cellulase and pectinase (Malathi and Devegowda, 2001). When compared with mixtures containing only xylanase and cellulase, a mixture containing pectinase in addition to the other two enzymes resulted in significantly greater ($P < 0.05$) reductions in viscosity and a higher total sugar release using soybean meal as a substrate. However, when tested using different substrates of sunflower meal, de-fatted rice bran or a maize–soybean broiler starter diet containing 10% sunflower meal, addition of pectinase to the enzyme mixture did not result in further reductions in viscosity nor in increases in sugar release. It was concluded that pectinase, when in combination with xylanase and cellulase, may assist in the digestion of soybean meal.

By measuring free galacturonic acid as an index of pectin breakdown (Tahir *et al.*, 2008), researchers examined the effects of purified cellulase, hemicellulase and pectinase and combinations of these enzymes applied *in vitro* to a maize–soybean meal broiler diet. No single enzyme increased crude protein and dry matter digestibility and only hemicellulase increased galacturonic acid release. When tested in combination, the highest release of galacturonic acid and crude protein and dry matter digestibility were observed with all three enzymes tested. This experiment indicates that pectinase in maize–soybean

Table 3.16. Pectin content of various feed ingredients (adapted from Malathi and Devegowda, 2001).

Ingredient	Pectin (%)
Maize	1.00
Sorghum	1.66
Soybean meal	6.16
Rapeseed meal	8.86
De-oiled rice bran	7.25
Peanut meal	11.60
Sunflower meal	4.92

meal-based broiler diets may only be effective when in combination with cellulase and hemicellulase.

Examining three substrates (canola meal, soybean meal and peas), another group of researchers (Meng *et al.*, 2005) observed significant NSP degradation ranging from 8.8 to 10.2% using pectinase. A combination of four enzymes including pectinase was shown to result in an increased NSP degradation with canola meal and soybean meal when compared with pectinase only.

When full-fat flaxseed was used as a substrate, Slominski *et al.* (2006) determined the degradation of components of NSP using pectinase, cellulase and some enzyme combinations. Both cellulase and pectinase, when used alone, resulted in a significant degradation of NSP. Pectinase alone or in combination in enzyme mixtures significantly degraded rhammose. The most pronounced degradation was achieved when a combination of enzymes containing pectinase was used.

Using a wheat-based diet as a substrate and testing phytase with and without pectinase, Zyla *et al.* (2000a) reported increased release of pentoses, reducing sugars and dialysable protein when pectinase was added to the enzyme mixture.

In vivo digestibility assays

Most reports examining digestibility tested combinations of enzymes, many of which contained pectinase. In an experiment comparing balanced diets comprising maize, soybean meal and peas as predominant sources of NSP, Meng and Slominski (2005) tested the efficacy of an enzyme combination in broilers containing xylanase, glucanase, pectinase, mannanase, cellulase and galactanase. Increases in NSP digestibility and AME were observed with the corn and soybean meal diets ($P < 0.01$). In a more recent trial with broilers, Meng *et al.* (2006) observed significant increases in TME and NSP digestibilities with three blends of enzymes containing pectinase for full-fat canola seed ($P < 0.05$). Using a wheat and soybean meal diet that also contained canola meal and peas, Meng *et al.* (2005) reported increases in AME and NSP, starch and protein digestibility ($P < 0.05$) in broilers with a combination of cellulase and pectinase.

Examining broilers provided with a semi-purified diet based on corn and soybean meal and using a commercial enzyme product that contained mainly pectinase, hemicellulase and β-glucanase, Kocher *et al.* (2002) observed an increase in AME, ileal protein digestibility and reduced excreta moisture levels when using five times the recommended dosage of the enzyme product ($P < 0.05$), but saw no change in digesta viscosity. Following this, at the recommended dosage of the enzyme product, the researchers saw no benefits.

Using full-fat flaxseed, Slominski *et al.* (2006) reported that various combinations of enzymes equally increased fat and NSP digestibility in adult roosters ($P < 0.05$), as well as TME. All combinations contained pectinase and cellulase.

A combination of pectinase, β-glucanase and hemicellulase was shown to increase digesta viscosity in broilers ($P < 0.05$) when added to a diet containing 30% lupins (Annison *et al.*, 1996), but had no effect on AME or ileal digestibility.

Two blends of enzymes containing different levels of pectinase were examined in broilers fed diets rich in canola meal and sunflower meal (Kocher *et al.*, 2000a). Whereas neither blend of enzymes affected digesta viscosity or AME with either substrate, the enzyme blend containing the highest pectinase activity resulted in a decreased AME ($P < 0.05$) with canola meal. Interestingly, the enzyme blend containing the lower pectinase activity resulted in a reduction in soluble NSP concentration in the jejunum.

Testing the effect of a combination of pectinase and β-glucanase in broilers using lupin seeds, Kocher *et al.* (2000b) observed no change in AME but reported a significant ($P < 0.05$) increase in digesta viscosity and an increased concentration of soluble NSPs in several sections of the gastrointestinal tract.

An experiment looking at liver fat and blood parameters was conducted in broiler chicks provided with diets containing 0 and 4% citrus pectin (Patel *et al.*, 1981). Pectin addition resulted in reduction in live weight, liver fat and serum cholesterol ($P < 0.05$), suggesting a reduction in energy utilization. Pectinase enzyme partially or fully reversed these effects of pectin, demonstrating the potential of enzyme preparations containing pectinase to impact pectin in the diet.

Animal performance studies

In an 8-week experiment with 252 laying hens provided with 65% peas, Igbasin and Guenter (1997) were unable to detect any benefit from including a crude enzyme preparation containing pectinase at 50 or 100 units per kg in the diet. The lack of response may have been a result of either pectinase activity levels or the relatively small number of hens tested.

Examining pectinase as well as phytase in broilers fed wheat-based diets varying in calcium levels, Zyla *et al.* (2000a) reported that the addition of pectinase in addition to phytase, when averaged across calcium levels, resulted in increased weight gains, feed intake and toe ash percentages, as well as decreased intestinal viscosity in 21-day broilers. It was concluded that pectinase enhanced performance and phosphorus utilization of wheat-based diets containing low levels of phosphorus and phytase in broilers.

Other animal performance studies tested combinations of enzymes containing pectinase. Testing enzyme combinations with maize–soybean meal-type diets, improvements in weight gain and feed conversion have been reported in some studies (Saleh *et al.*, 2005; Tahir *et al.*, 2008) but not in others (Zyla *et al.*, 1996; Meng and Slominski, 2005). Using wheat-based diets, Zyla *et al.* (2000b) observed a significant increase in feed intake only, while improvement in feed conversion only has been reported with canola meal (Meng *et al.*, 2006) and flaxseed (Slominski *et al.*, 2006). No performance improvement with lupin kernels (Annison *et al.,* 1996) or peas (Igbasan and Guenter, 1997) was observed.

Summary

Almost all experiments examining pectinase used this enzyme in combination with various other NSP-degrading enzymes as it is hypothesized that, whereas pectinase may be effective in breaking down a matrix that binds plant cell walls together, other enzyme activities are needed to break down cell wall components.

In vitro studies suggest that pectinase may increase the effectiveness of mixtures of cellulase, hemicellulase and other enzymes in maize, soybean meal or wheat and may also be effective when substrates include canola meal, peas or flaxseed. However, digestibility assays have yielded mixed results, ranging from a possible increase in the AME when pectinase is used in combination with xylanase, glucanase, cellulase, mannanase and galactanase enzymes, to no benefit at all when pectinase was used with glucanase and hemicellulase, to a decrease in AME when the enzyme mixture was used on canola meal as the substrate.

As with AME assays, pectinase included in various enzyme combinations has yielded mixed results in its effect on the viscosity of digesta. It is well established that increased concentrations of soluble NSP are associated with increased digesta viscosity and poorer nutrient digestibility (Annison, 1991). In circumstances where pectinase and other enzymes have significantly increased digesta viscosity, it is likely that the breakdown of NSP has been incomplete and substrate has been converted from an insoluble to a soluble form.

A true potential benefit of enzyme mixtures containing pectinase requires animal performance studies, which may or may not directly translate from *in vitro* and digestibility information. Very limited animal data have been published on the use of pectinase alone in animal diets. Therefore, it is difficult to draw a conclusion as to the efficacy of this enzyme on improvement in animal performance. More often, pectinase has been tested in enzyme combinations, with mixed results. The potential benefit of pectinase included in an enzyme combination is dependent on several factors, including the choice of and activity of enzymes used, ingredients included in the diets and stage of development of the animal species, as well as other factors.

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Note

[1]Hemicell is a registered trademark of ChemGen Corp., Gaithersburg, Maryland, USA.

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4 Starch- and Protein-degrading Enzymes: Biochemistry, Enzymology and Characteristics Relevant to Animal Feed Use

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Introduction

Poultry and swine are omnivorous and, given the opportunity, would satisfy their nutrient requirements by consuming a range of seeds, roots, inorganic materials and insects. However, in order to satisfy consumer preference for 'vegetarian' animal production and to minimize feed costs associated with the commercial production of farm animals, the feed that is presented is rarely optimized for the animal's digestive system, especially in the neonate. For example, the non-starch polysaccharide (NSP) fraction of some cereals such as wheat and barley increases viscosity in the gut, which compromises the diffusion of nutrients. This anti-nutritional effect can be reduced by addition of exogenous xylanase and/or β-glucanase that fragment the hemicellulose polymers, xylan and β-glucan, respectively (see Chapter 2). Another example is degradation of phytic acid, the plant's phosphate store, which is not readily hydrolysed by enzymes produced by the animal. Addition of phytase to the feed ensures release of phosphate from phytic acid, and can thereby partly or totally cover the animal's need for phosphorus (see Chapter 7).

So, in some instances, exogenous enzymes can bridge a gap between the composition of the feed and the animals' own digestive enzyme complement. However, although both poultry and swine are capable of significant amylase and protease secretion, there may still be an opportunity to augment these systems through the use of exogenous enzymes. It is the purpose of this chapter to discuss the relevance of exogenous starch- and protein-degrading enzymes in the context of farm animal nutrition.

 Enzymes in Farm Animal Nutrition, 2nd Edition
(eds M.R. Bedford and G.G. Partridge)

Starch and Starch-degrading Enzymes

Starch

Starch consists of two polymers, amylose and amylopectin. Both polymers consist of glucose units (glucopyranosyl units) linked through α-1,4-glucosidic bonds. Amylose is essentially a linear polymer with a few branches linked by α-1,6-glycosidic bonds. The size of the amylose polymer varies considerably and can have a degree of polymerization (DP) of up to 600 glucose units (Perez *et al.*, 2009). Amylopectin, in contrast, is highly branched. It consists of chains of glucose linked together mainly by α-1,4-linkages and with α-1,6 bonds at the branch points. Amylopectin comprises three types of chains: short chains with a mean DP of 14–18, long chains with DP 45–55 and a few very long chains with DP >60. The side-chains of amylopectin orientate as α-helices, which arrange themselves into a dense, semi-crystalline structure. These amylopectin clusters form together with amylose starch granules, which differ in size and shape depending on the origin of the starch. More details on these aspects can be found in Buleon *et al.* (1998) and Donald (2004).

Starch can also be classified according to how easily it is digested: namely rapidly degraded starch; slowly digested starch; or resistant starch (Gordon *et al.*, 1997; Sajilata *et al.*, 2006). These fractions can be quantified *in vitro* (Englyst *et al.*, 1992). Resistant starch, in particular, is of interest in animal nutrition, as this is the fraction of starch that escapes digestion in the small intestine. Resistant starch is partly or totally degraded by fermentation by the microflora, to produce short-chain fatty acids and various gases. Resistant starches are further classified according to the reasons for resistance (Champ and Faisant, 1996; Haralampu, 2000): (i) physically inaccessible starch (RS1) due to its encapsulation in un-milled seed; (ii) raw starch (RS2) packed in granules that are so dense that the time taken for digestion is longer than the passage time in the gut; or (iii) retrograded starch (RS3), which is formed when gelatinized starch is cooled and, over time, forms un-degradable crystals. Gelatinized starch is formed when starch is heated to above 60°C in the presence of water (Colonna *et al.*, 1992). The temperature depends on the type of starch granules, but is generally between 65°C and 70°C for wheat and maize starch when excess water is present. When feed is processed during pelleting, both heat and moisture are added. During this process the water content is typically only around 20–30% while the temperature is increased up to a maximum of 100°C and, in some extreme cases, to 120°C. These physical conditions will not be sufficient to gelatinize much raw starch, as the water content will be too low (Colonna *et al.*, 1992), and only damaged starch (created during grinding of raw materials) will be gelatinized effectively under these conditions. In accordance with this, Svihus *et al.* (2005) showed that, at most, 5–20% of the total starch is gelatinized under standard pelleting conditions, and Eerlingen *et al.* (1993) have further shown that only a minor part of the gelatinized starch will retrograde during standard storage conditions.

Starch hydrolysed by enzymes in the small intestine (i.e. before the large intestine, where microbial degradation starts) yields glucose as the final product to be absorbed directly by the intestinal epithelium. However, of the starch degraded by microbes, only a fraction of the energy will be made available to the animal through the formation and absorption of short-chain fatty acids produced by microbial fermentation. This implies that easily degradable starch will be utilized more effectively than resistant starch, which is degraded by the microbial flora. De Schrijver *et al.* (1999) showed, for example, that both rats and pigs fed resistant starch showed a significantly lower apparent ileal energy digestibility compared with rats and pigs fed easily degradable starch, even when the amount of resistant starch comprised only around 6% of the total diet.

Starch-degrading enzymes

Several enzyme families have evolved to degrade starch. The amylolytic enzymes are structurally classified into families of glucoside hydrolases (GH), which are available on the CAZy internet site (Cantarel *et al.*, 2008). The most important family is GH 13, which includes the endo-specific α-amylases (EC 3.2.1.1) that hydrolyse internal 1,4-linkages in amylose/amylopectin chains and pullulanases (EC 3.2.1.41), which are able to hydrolyse the 1,6-branching points in amylopectin. GH 15 contains exo-specific amylo-glucosidases or glucoamylases (EC 3.2.1.3) that hydrolyse amylose/amylopectin chains from the non-reducing end and liberate one glucose unit at a time. Aside from these, there are different types of exo-amylases like β-amylases (EC 3.2.1.2, belonging to GH 14) and maltotetraohydrolases (EC 3.2.1.60, belonging to GH 13) that attack the non-reducing ends and release oligomers of two and four glucose units, respectively.

Several amylases are produced by the digestive system of animals (Tester *et al.*, 2004). Salivary α-amylases (GH 13, EC 3.2.1.1), secreted in the mouth, initiate the degradation of starch as soon as the feed is ingested. Pancreatic α-amylase (GH 13, EC 3.2.1.1) is produced in the exocrine pancreas and secreted into the duodenum, where accessible starch is degraded and glucose, glucose oligomers and dextrins (glucose units with and surrounding the α-1,6-glycosidic bonds) are produced. Glucose can be absorbed directly by the epithelial cells, whereas the other degradation products are further broken down to glucose by the action of maltase and isomaltase (EC 3.2.1.3 and 3.2.1.52) present in the epithelial brush border. Thereafter, the liberated glucose is absorbed.

Protein and Proteases

Protein consists of polymers of amino acids. All amino acids commonly consist of an amino and a carboxyl group, which interconnect the amino acids with

peptide bonds that comprise the backbone of the protein. Each amino acid has in addition a side-group, which has different chemical properties and is the basis for grouping the amino acids into hydrophobic, hydrophilic or aromatic groups. The specific composition and order of the amino acids in the protein, together with the three-dimensional structure, determines the properties of the final protein.

The enzymes that degrade proteins, the proteases, are characterized by their ability to hydrolyse bonds before or after specific amino acids. The proteases involved in degrading protein in the digestive system have been reviewed extensively, both for animals and humans (Whitcomb and Lowe, 2007). However, in the latter case, the pig is often used as a model for understanding human digestion. In general, activities from endogenous proteases are carefully regulated because their activity in the wrong location can lead to digestion of the animal's own tissues and/or may activate inflammatory pathways.

Cells in the gastric mucosa in pigs (and humans) and the proventriculus in poultry produce pepsinogen, a precursor for pepsin (EC 3.4.21.4). Pepsinogen is excreted into the digestive tract and activated by pepsin on exposure to the acidic environment. Pepsin is an endoprotease, which hydrolyses peptide bonds containing phenylalanine, tyrosine and leucine at a pH range of 1.8–3.5 (Piper and Fenton, 1965). Pepsin is especially useful in digesting muscle, tendons and other components of meat with a high collagen content. Chicken pepsin is active at less acidic conditions than pepsin from pigs and humans and is irreversibly inactivated at slightly alkaline pH (Bohak, 1969).

The pancreas is the major source of proteases in the gastrointestinal tract. Most of the proteases are synthesized as inactive pro-enzymes, as is the case with pepsinogen. These proteases include chymotrypsinogen, trypsinogen, proelastase and pro-carboxypeptidases. These pro-enzymes are activated by the protease trypsin. Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and elastase (EC 3.4.21.36) are endoproteases of the serine protease family. Trypsin hydrolyses peptides containing basic amino acids (lysine and arginine), chymotrypsin splits the protein backbone at bonds of aromatic amino acids (phenylalanine, tyrosine, tryptophan) and elastase hydrolyses at the site of uncharged small amino acids (such as alanine, glycine and serine) (Kraut, 1977). All these endoproteases release small oligopeptides, which are further degraded by carboxypeptidases, such as carboxypeptidase A (EC 3.4.17.1) and carboxypeptidase B (EC 3.4.17.2). These exopeptidases hydrolyse oligopeptides releasing free amino acids, which can be absorbed by the animal. Beside pepsin and the pancreatic proteases, the enterocytes of the small intestine produce several aminopeptidases (EC 3.4.11.1 and EC 3.4.11.2) and carboxypeptidases, which are most effective in digesting small peptides after the initial hydrolysis of complex proteins by gastric and pancreatic proteases.

Efficacy of Exogenous Starch-degrading Enzymes in Swine and Poultry

The principal amylase used in animal feed is the α-amylase from *Bacillus amyloliquefaciens* (BAA). It is highly liquefying, meaning that it rapidly fragments starch polymers into short oligomers. The primary hydrolysis products accumulated are maltotriose (DP 3) and maltohexaose (DP 6) (Robyt, 2009). This amylase also has relatively high thermostability, enabling a high degree of survival after feed pelleting. In contrast, when starch is hydrolysed by porcine pancreatic α-amylase (PPA), glucose to maltotetraose (DP 1–4) products are mainly formed, as well as so-called α-limit dextrins with one or two α-1-6 linkages (Robyt, 2009).

The initial hydrolysis of amylopectin by BAA and PPA is different, with BAA having a higher tendency than PPA to break the inner chain bonds (Goesaert *et al*., 2010). Therefore BBA is faster than PPA in fragmenting amylopectin to lower molecular sizes, whereas PPA trims down the chains of amylopectin in a more uniform manner. At a 10% degree of hydrolysis BAA was found to accumulate primarily DP 6–10, whereas PPA accumulated primarily DP 2–4 (Bijttebier *et al*., 2010). Based on these differences in mode of action, it is likely that BAA added to PPA increases the rate of amylopectin (as well as amylose) breakdown to short maltooligosaccharides that can readily be hydrolysed to glucose by maltase and isomaltase for absorption by the epithelial cells.

The usefulness of exogenous amylases in pig and poultry nutrition has not been unequivocally demonstrated. However, several theories persist suggesting that exogenous amylase may have a role in augmenting immature pancreatic production in neonates (Noy and Sklan, 1999a,b) or in assisting animals in instances when starches are recalcitrant to digestion. Gracia *et al.* (2003) demonstrated that exogenous amylase is capable of improving the performance of broiler chickens fed a maize/soy-based diet. Furthermore, supplemental amylase also improved the digestion of starch and organic matter, and was associated with improved AME (apparent metabolizable energy). These beneficial effects were independent of bird age (confirmed by factorial analysis), which suggests that it is not solely the neonate that may benefit from the use of starch-degrading enzymes. Although improved AME and starch digestibility was reported by Gracia and colleagues, the large improvements in performance (around 9% for body weight gain and 5% for feed conversion) cannot be explained solely via an improvement in the digestibility of dietary nutrients. Indeed, the effect of amylase on AME was a relatively modest 50–80 kcal kg^{-1} in this particular study (Gracia *et al*., 2003). The lack of interaction between age and amylase addition, and the apparent discrepancy between performance and digestibility improvements, suggest that exogenous amylase may have physiological effects not readily detected via conventional nutrient recovery assays. Instructively, the use of amylase significantly reduced the mass of the pancreas without influence on the other organs, suggesting that ingestion of amylase as part of the feed matrix may elicit important secretory effects (Gracia *et al*., 2003), perhaps a reduction in amylase production.

However, this contention is not unanimously supported in the literature. Ritz *et al.* (1995) showed clearly in turkeys that exogenous amylase was largely additive with endogenous amylase, suggesting limited secretory feedback. It is possible that the nature of the amylase fed, i.e. homology with pancreatic or brush border starch-degrading systems, the characteristics of the diet per se or the species or age of the animal are responsible for these conflicting responses. In fact the 'sparing' effect of exogenous amylase on endogenous production in broilers was recently confirmed by Jiang *et al.* (2008), where supplemental amylase reduced pancreatic mRNA expression for broilers fed a maize/soy-based diet.

Efficacy of Exogenous Proteases in Swine and Poultry

The effect of enzyme mixtures including protease has been extensively reported, but only a few trials have been published where the effect of supplemental protease has been established independently from an enzyme admixture. Yu *et al.* (2007) examined the effect of adding protease in a broiler trial, where both a conventional and a low-crude-protein maize–soy diet were used. *In vitro* the protease improved soy protein degradation in a model system that mimicked the digestive tract, whereas neither fishmeal nor maize was similarly influenced. These effects were confirmed in feeding trials, where broilers offered protease-supplemented diets showed numerical improvement in weight gain during the whole growth period (0–38 days) and a significant reduction in feed conversion rate (FCR). Despite this, no improvements in total tract apparent digestibility of protein and dry matter were observed. However, as the authors also concede, these latter data are of limited value due to the significant contribution of microflora to the faecal analysis. Thacker (2005) found significant improvements in FCR when protease was added to a wheat-based diet, and interestingly he also found no significant effect on dry matter digestibility, energy digestibility or nitrogen retention due to protease supplementation. Unfortunately, in this study only total tract digestibilities were measured. These two trials could indicate an effect other than simply improved degradation of protein in the gut – there may be a similar 'sparing' effect, as suggested for amylase addition, but this contention is not supported directly, partially due to the paucity of trials where protease has been used in isolation.

Peek *et al.* (2009) tested the effect of a protease-supplemented maize–wheat–soy diet in a trial with broilers challenged with *Eimeria* spp. and found that dietary supplementation with protease reduced the negative impact of a coccidiosis infection on body weight gain. The mechanisms for this effect remain unclear, although instructively coccidial lesions and oocyst excretion remained unaffected and the mucin layer was significantly thicker in the protease-treated broilers.

Finally, Ghazi *et al.* (2002) presented the effect of exogenous protease on the nutritional value of soybean meal for broilers and cockerels. In this case there were differences between proteases, with the most consistent effects observed when acid fungal protease was used compared with alkaline subtilisin.

These data suggest that there may be genuine differences between supplemental proteases on some occasions, though the data set is clearly too small to draw any meaningful general conclusions.

A number of potential modes of action have been suggested to explain the beneficial effects of proteases in the diets of poultry. Proteases may augment endogenous peptidase production, reducing the requirement for amino acids and energy or improve the digestibility of dietary protein. Additionally, proteases may hydrolyse protein-based anti-nutrients such as lectins or trypsin inhibitors (Huo *et al*., 1993; Marsman *et al*., 1997; Ghazi *et al*., 2002), improving the efficiency with which the bird utilizes amino acids and reducing protein turnover. However, considerable lack of knowledge persists about the mode of action of exogenous proteases, differences between different protease classes (e.g. optimal pH, kinetics and preferred substrate) and also their usefulness in animal feeding, either fed in isolation (which would be rare) or more likely as part of an enzyme admixture (e.g. xylanase, phytase, glucanase and amylase). Thus, in order to confirm previous reports which have suggested that exogenous protease may be a useful ally in animal nutrition, it is recommended that further work be done to elucidate mechanism of action, optimal dose, optimal protease types and preferred substrate, as well as to explore the interactions between protease and other supplemental and endogenous enzyme systems.

Mechanism of Action of Exogenous Amylase and Protease

The composition of the diet can influence the physiology of the digestive system. For example, Starck (1999) demonstrated a reversible, repeatable and rapid increase/decrease in the size of the digestive organs with changes in the fibre content of the diet in Japanese quail. This study was conducted in cages, but comparable changes have also been observed in wild birds, e.g. bar-tailed godwits (Piersma and Gill, 1998). Although farm animals are not exposed to such environmental and dietary changes, the potential for dietary adaptation may still be present. Corring demonstrated that diet influenced pancreatic output and composition among broilers (Corring, 1980). The ingestion of high concentrations of protein relative to carbohydrate biased pancreatic composition in favour of proteolytic enzymes, and this could rapidly be reversed if protein intake was decreased in favour of starch (Corring, 1980). Changes in pancreatic secretion with diet have also been shown in growing pigs, as reviewed by Makkink and Verstegen (1990) and Jakob *et al*. (1999). Interestingly, increased crude fibre concentration from addition of wheat bran in the diet resulted in an increased volume of secreted pancreatic juice, whereas the same effect was not observed when pure cellulose was added (Jakob *et al*., 1999).

These adaptive measures are entirely intuitive and suggest that the process of digestion is rather carefully regulated to ensure that gross overproduction of inappropriate digestive juices is avoided. This presents an opportunity where endogenous production may be minimized by feeding of various exogenous enzymes, improving performance not necessarily by increasing digestibility

coefficients but by minimizing secretory investment. This reduced output of, for example, mucins or digestive enzymes would translate to improved net utilization of ingested nutrients, but may not be associated with changes in ileal or total tract digestibility. In fact, Souffrant *et al.* (1993) demonstrated in pigs that the vast majority of endogenous nitrogen is recovered by the terminal ileum, and even more on a total-tract basis (> 80%), although the authors concede that nitrogen recovered in the large intestine is of limited immediate value to the animal. Nevertheless, it is possible that the true value of supplemental amylase and protease may in fact be in reducing maintenance energy requirements (and amino acid requirements) rather than in improving ileal digestible energy. If amylases and proteases do elicit a substantial part of their benefits indirectly, then it would be expected that the observed benefits would be most obvious for those nutrients involved in amylase and protease production, secretion and recovery. As poultry do not posses salivary amylase, these benefits would not be apparent until the pancreatic region of the small intestine and so gastric mucin and zymogen production may be unaffected. Furthermore, the benefits of amylase on, for example, ileal amino acid digestibility, may in fact be well correlated to pancreatic amylase (and/or brush border maltase/isomaltase) amino acid composition. Corring and Jung (1972) presented the amino acid composition of pig pancreatic amylase, and found it to be particularly rich in aspartic acid, glutamic acid, leucine and serine. Thus, it is possible that intervention with an exogenous amylase may confer particular benefits to the host for those amino acids in the same way that similar indirect benefits for pepsin and mucin have been demonstrated for phytases, i.e. beneficial effects that correlate with the composition of endogenous protein (Cowieson and Ravindran, 2007).

In reality, amylases and proteases are rarely fed in isolation and are more commonly found as part of an enzyme admixture, perhaps involving xylanases, glucanases, proteases and phytases. It has recently been demonstrated that the efficacy of such enzymes is inextricably linked to the digestibility of the diet to which they are added (Cowieson and Bedford, 2009; Cowieson, 2010). As theoretical (if not realistic) maximum ileal digestibility is 100%, digestibility-enhancing pro-nutrients constantly move digestibility towards that fixed asymptote, so opportunity for further improvement declines with each new addition. Indeed, this has been demonstrated recently for cooperativity between xylanase and glucanase (Cowieson *et al.*, 2010, in press) and the additivity of matrix values for xylanase and phytase (Cowieson and Bedford, 2009). Thus moderation is recommended when enzyme admixtures are assembled, and it is unlikely that the beneficial effects of amylase would remain entirely unchecked by the presence of other growth-promoting additives. Nevertheless, it is apparent from the (relatively scant) literature that exogenous amylases can be effective in improving performance and, as such, are a viable consideration when assembling enzyme admixtures for monogastrics. However, the fact that the benefits may be more 'net' than 'metabolizable' is a complexity currently not well addressed. Until poultry nutritionists formulate routinely on a 'net' basis, it may be difficult to appropriately credit these enzymes with meaningful nutrient matrices.

It can be concluded that exogenous amylases, and probably also proteases, are useful in poultry and swine nutrition, but how additive the effects are with other pro-nutrients such as phytases, xylanases, growth-promoting antibiotics, etc. remains unclear. Strategic intervention at a secretory level is a distinct possibility, and the benefits here may be of a magnitude larger than modest improvements in ileal energy recovery, but further research is necessary to understand how the animal responds to what it ingests.

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5 Phytases: Biochemistry, Enzymology and Characteristics Relevant to Animal Feed Use

R. Greiner and U. Konietzny

Introduction

Since the 1980s, phytases (*myo*-inositol(1,2,3,4,5,6)hexakisphosphate phosphohydrolases) have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology. Phytases represent a subgroup of phosphomonoesterases that are capable of initiating the stepwise dephosphorylation of phytate (*myo*-inositol(1,2,3,4,5,6)hexakisphosphate), the most abundant inositol phosphate in nature. They have been identified in plants, microorganisms and in some animal tissues (Konietzny and Greiner, 2002). In plant seeds and microorganisms phytases are even found in multiple forms (Ullah and Cummins, 1987; Baldi *et al.*, 1988; Greiner *et al.*, 1993; 2000b; Konietzny *et al.*, 1995; Moore *et al.*, 1995; Hübel and Beck, 1996; Maugenest *et al.*, 1999; Nakano *et al.*, 1999; Fujita *et al.*, 2000; Cottrill *et al.*, 2002; Greiner, 2002; Garchow *et al.*, 2006), and these may exhibit different stereospecificity of phytate dephosphorylation, be regulated in different ways, be directed to different localization within and outside the producing cell and thus may have different physiological functions.

The ability of phytases to hydrolyse phytate is well understood from *in vitro* assays, but their activity *in vivo* remains largely unknown. Therefore, some of the enzymes classified as phytases today may not be involved in phytate degradation *in vivo* but may have completely different functions. Thus far, only the germination-inducible phytases of plant seeds have been reported to participate in phytate breakdown to make phosphate, minerals and *myo*-inositol available for plant growth and development during germination (Greiner *et al.*, 2005). Because formation of extracellular phytases in moulds and yeast is triggered by phosphate starvation, these enzymes hydrolyse organic phosphorylated compounds, among others phytate, to provide the cell with phosphate from extracellular sources. These enzymes are therefore non-

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specific phosphatases that also exhibit phytate-degrading activity. The *in vivo* function of other enzymes with phytate-degrading activity is mainly speculative. As a result of the aforementioned function to provide the cell with phosphate, a role in stress response or bacterial pathogenesis has been postulated (Atlung and Brøndsted, 1994; Atlung *et al.*, 1997; DeVinney *et al.*, 2000; Zhou *et al.*, 2001; Chatterjee *et al.*, 2003).

Classification of Phytases

Phytases are a diverse group of enzymes that encompass a range of sizes, structures and catalytic mechanisms. Based on the catalytic mechanism, phytases can be referred to as histidine acid phytases (HAPhy), β-propeller phytases (BPPhy), cysteine phytases (CPhy) or purple acid phytases (PAPhy) (Mullaney and Ullah, 2003; Greiner, 2006). Depending on their pH optimum, phytases have been divided further into acid and alkaline phytases and also based on the carbon in the *myo*-inositol ring of phytate at which dephosphorylation is initiated into 3-phytases (E.C. 3.1.3.8), 6-phytases (E.C. 3.1.3.26) and 5-phytases (E.C. 3.1.3.72).

The majority of the phytases known to date belong to the subfamily of histidine acid phosphatases and do not need any co-factor for optimal activity. They have been identified in microorganisms, plants and animals (Wodzinski and Ullah, 1996; Mullaney *et al.*, 2000; Konietzny and Greiner, 2002; Lei and Porres, 2003). The structures of histidine acid phosphatases contain a conserved α/β-domain and a variable α-domain (Kostrewa *et al.*, 1997; Lim *et al.*, 2000). The active site is located at the interface between the two domains. Differences in substrate binding have been attributed to differences in the α-domain. The proposed structures also provide information about substrate binding and the catalytic mechanism at the molecular level. Histidine acid phosphatases share the highly conserved sequence motif RH(G/N)XRXP, considered to be the phosphate acceptor site near the N-terminus (van Etten *et al.*, 1991; Ostanin *et al.*, 1992; Lindqvist *et al.*, 1994). In addition, they contain a conserved HD-motif near the C-terminus where the aspartate is proposed to be the proton donor for the substrate leaving group (Lindqvist *et al.*, 1994; Porvari *et al.*, 1994). However, not all histidine acid phosphatases are able to act upon phytate. The most potent inhibitors of histidine acid phytases were found in Zn^{2+}, fluoride, molybdate, wolframate, vanadate and the hydrolysis product orthophosphate (Konietzny and Greiner, 2002). It is not clear whether metal ions modulate phytase activity by binding to the enzyme or by forming poorly soluble metal ion–phytate complexes. The appearance of a precipitate while adding Fe^{2+} or Fe^{3+} to assay mixtures suggests that the observed reduction in dephosphorylation rate is due to a decrease in active substrate concentration by the formation of poorly soluble ironphytate (Konietzny *et al.*, 1995). Fluoride, a well-known inhibitor of many acid phosphatases, inhibits histidine acid phytases competitively, with inhibitor constants ranging from 0.1 to 0.5 mM. Furthermore, the hydrolysis product orthophosphate and its structural analogues molybdate, wolframate and

vanadate were recognized as competitive inhibitors of enzymatic phytate degradation. It has been suggested that these transition metal oxo-anions exert their inhibitory effects by forming complexes that resemble the trigonal bipyramidal geometry of the transition state (Zhang *et al.*, 1997).

Besides the hydrolysis product phosphate, the substrate phytate was also reported to act as an inhibitor of many histidine acid phytases. The lowest phytate concentration necessary to inhibit phytase activity ranges from 300 μM for the maize root enzyme (Hübel and Beck, 1996) up to 20 mM for the soybean enzyme (Gibson and Ullah, 1988). With high substrate concentrations, the charge due to phosphate groups may affect the local environment of the catalytic domain of the enzyme. This might inhibit conversion of the enzyme–substrate complex to enzyme and product, although inhibition due to the formation of poorly soluble phytase phytate complexes cannot be ruled out. Substrate inhibition should be considered when determining phytase activity by the standard *in vitro* assay, because the activity of different phytases may be reduced to different degrees at the substrate concentration of the assay.

To date, only one alkaline phytase has been reported to contain the amino acid motifs characteristic for histidine acid phosphatase (Mehta *et al.*, 2006). This enzyme was identified in lily pollen, requires Ca^{2+} for full catalytic activity and is not inhibited by fluoride (Baldi *et al.*, 1988; Mehta *et al.*, 2006). Plant alkaline phosphatases whose activity is enhanced in the presence of Ca^{2+} were also found in cat's tail (*Typha latifolia* L.) pollen (Hara *et al.*, 1985) and a number of legumes (Mandel *et al.*, 1972; Scott, 1991; Greiner and Konietzny, 2006). Unfortunately, none of the corresponding genes has been cloned and no sequence data exist to confirm the presence of the signature motifs of histidine acid phosphatases.

The amino acid sequences of β-propeller phytases exhibit no homology to the sequences of any other known phosphatase (Kerovuo *et al.*, 1998; Kim *et al.*, 1998b; Ha *et al.*, 2000). Even the putative active site motifs RH(G/N)-XRXP and HD found in histidine acid phosphatases are absent. Initially, β-propeller phytases were reported from *Bacillus* species (Kerovuo *et al.*, 1998; Kim *et al.*, 1998a; Choi *et al.*, 2001; Tye *et al.*, 2002). Recently, β-propeller phytases were identified in *Xanthomonas oryzae* (Chatterjee *et al.*, 2003), a plant pathogen of rice, and the aquatic bacterium *Shewanella oneidensis* (Cheng and Lim, 2006). Furthermore, protein sequence identity suggests that β-propeller phytases are widespread in the aquatic environment (Cheng and Lim, 2006; Lim *et al.*, 2007). β-Propeller phytases have a six-bladed propeller folding architecture with six calcium-binding sites in each protein molecule (Shin *et al.*, 2001). Binding of three calcium ions to high-affinity calcium-binding sites results in a dramatic increase in thermal stability by joining loop segments remote in the amino acid sequence. Binding of three additional calcium ions to low-affinity calcium-binding sites at the top of the molecule turns on the catalytic activity of the enzyme by converting the highly negatively charged cleft into a favourable environment for the binding of phytate. Kinetic studies have established that β-propeller phytases could hydrolyse calcium phytate between pH 7.0 and 8.0 (Oh *et al.*, 2001). In contrast to histidine acid phytases, β-propeller phytases do not show any

reduction in activity in the presence of fluoride (Powar and Jagannathan, 1982; Shimizu 1992; Kerovuo *et al.*, 1998; Kim *et al.*, 1998a; Choi *et al.*, 2001; Tye *et al.*, 2002; Cheng and Lim, 2006).

Two further classes of phytases were reported to lack the RH(G/N)XRXP-motif (Hegeman and Grabau, 2001; Chu *et al.*, 2004). Representatives of both classes exhibit their optimal catalytic activity in an acidic environment. The first binuclear metal-containing phytase was reported in the cotyledons of a germinating soybean (*Glycine max* L. Merr.) seedling (Hegeman and Grabau, 2001). The gene encoding this soybean phytase has been cloned, and characterization of the gene product revealed that the enzyme contains motif characteristics of a large group of phosphoesterases, including purple acid phosphatases. Purple acid phosphatases have representatives in plants, mammals, fungi and bacteria (Schenk *et al.*, 2000) and contain binuclear Fe(III)–Me(II) centres where Me is Fe, Mn or Zn. Purple acid phosphatases with phytase activity were also reported in *Medicago tranculata* L. (Xiao *et al.*, 2005), wheat (*Triticum aestivum* L.; Nakano *et al.*, 1999; Dionisio *et al.*, 2007; Rasmussen *et al.*, 2007) and barley (*Hordeum vulgare* L.; Dionisio *et al.*, 2007). To date, purple acid phosphatases with phytase activity appear to be restricted to plants.

Another class of phytase has been reported from an anaerobic ruminal bacterium, *Selenomonas ruminantium* (Chu *et al.*, 2004; Puhl *et al.*, 2007). This enzyme does not need any co-factor for enzymatic activity. The phytase is believed to be distantly related to protein tyrosine phosphatases that are members of the cysteine phosphatase group. *S. ruminantium* phytase shares the active site motif HCXXGXXR(T/S) and other substantial similarities with cysteine phosphatases. The active site forms a loop that functions as a substrate-binding pocket unique to protein tyrosine phosphatases. This pocket is wider and deeper in *S. ruminantium* phytase and therefore able to accommodate the fully phosphorylated inositol group of phytate (Chu *et al.*, 2004). As with histidine acid phytases, enzymatic phytate dephosphorylation by *S. ruminantium* phytase is reduced in the presence of metal cations. The inhibitory effect of iron, copper, zinc and mercury cations was attributed to their ability to form complexes with phytate, but the stimulatory effect of lead cations remains unexplained (Yanke *et al.*, 1999). Very recently, protein tyrosine phosphatase-like phytases were reported to be present in the anaerobic bacteria *Selenomonas lacticifex* (Puhl *et al.*, 2008a), *S. ruminantium* subsp. *lactilytica* (Puhl *et al.*, 2008b) and *Megasphaera elsdenii* (Puhl *et al.*, 2009). So far, protein tyrosine phosphatase-like phytases appear to be restricted to anaerobic bacteria.

Phytase as an Animal Feed Additive

The increasing economic pressures currently being placed upon animal producers demand more efficient utilization of low-grade feed. Recent market trends have clearly shown that hydrolytic enzymes have emerged as feed supplements in order to improve the digestion and absorption of poorly

available nutrients from the animal diet. The first commercial phytase products were launched on to the market in 1991. Meanwhile, the market volume is in the range of €150 million (Haefner *et al.*, 2005). Even if potential applications of phytase in food processing or the production of pharmaceuticals were reported (Greiner and Konietzny, 2006), phytases have been mainly, if not solely, used as animal feed additives in diets largely for swine (Selle and Ravindran, 2008) and poultry (Selle and Ravindran, 2007), and to some extent for fish (Debnath *et al.*, 2005a).

The small intestine of monogastrics has only a very limited ability to hydrolyse phytate (Iqbal *et al.*, 1994) due to the lack of significant endogenous phytase activity and low microbial population in the upper part of the digestive tract. This fact also explains why phytate phosphorus is poorly available to monogastric animals (Walz and Pallauf, 2002). Phosphorus is absorbed as orthophosphate, and thus utilization of phytate phosphorus by monogastrics will largely depend on their capability to hydrolyse phytate. Numerous animal studies have shown the effectiveness of supplemental microbial phytase in improving the utilization of phosphate from phytate (Simons *et al.*, 1990; Augspurger *et al.*, 2003; Esteve-Garcia *et al.*, 2005; Adeola *et al.*, 2006). Therefore, including adequate amounts of dietary phytase for monogastric animals reduces the need for orthophosphate supplementation of the feed. As a result, excretion of phosphate can be reduced by as much as 50%, which is clearly beneficial from an environmental viewpoint. Thus, dietary supplementation with a microbial phytase has proved to be the most effective tool for the animal industry to reduce phosphate excretion from animal waste, enabling compliance with environmental regulations. In addition, phytase supplementation might improve amino acid availability. Phytate–protein interaction may induce changes in protein structure that can decrease enzymatic activity, protein solubility and proteolytic digestibility.

A negative effect of phytate on the nutritive value of protein, however, was not clearly confirmed in studies with monogastric animals (Sebastian *et al.*, 1998). While some have suggested that phytate does not affect protein digestibility (Peter and Baker, 2001), others have found improved amino acid availability with decreasing levels of phytate (Cowieson *et al.*, 2006). This difference may be at least partly due to the use of different protein sources. In addition, supplemental phytase was reported to improve utilization of minerals by animals (Lei *et al.*, 1993; Adeola *et al.*, 1995; Lei and Stahl, 2001; Debnath *et al.*, 2005b). Furthermore, it was hypothesized that phytase supplementation results in an increased energy utilization in monogastric animals (Selle and Ravindran, 2007).

Enzyme preparations with phytases from *Aspergillus niger*, *Peniophora lycii*, *Schizosaccharomyces pombe* and *Escherichia coli* are available commercially. In general, their large-scale production is based on the use of recombinant strains of filamentous fungi and yeasts. In addition, wild-type phytases are not the only forms produced: there are mutants exhibiting more favourable properties regarding their application as feed supplements. Today, all phytases used for animal feed application belong to the class of histidine acid phytases; β-propeller phytases have been advocated for several

applications. However, no commercial applications of β-propeller phytases are currently available. Furthermore, neither a cysteine phytase nor a purple acid phytase is currently being marketed, although they have been subjected to several studies. 'Ideal' phytases for animal feed applications should fulfil a series of quality criteria: they should be effective in releasing phytate phosphate in the digestive tract, stable to resist inactivation by heat from feed processing and storage as well as cheap to produce.

Phytate Hydrolysis in the Digestive Tract

The ability of a phytase to hydrolyse phytate in the digestive tract is determined by its enzymatic properties. With regard to phytate dephosphorylation in the gastrointestinal tract of animals, it is important to consider the low pH in the forestomach (crop) of poultry (pH 4.0–5.0) and in the proventriculus and gizzard of poultry and stomach of pigs and fish (pH 2.0–5.0) (Simon and Igbasan, 2002). On the other hand, the small intestine of animals presents a neutral pH environment (pH 6.5–7.5). Therefore, pH optima and pH activity profile of supplementary phytases generally determine their ability to develop catalytic activity in the afore-mentioned gastrointestinal compartments. To date, two main types of phytases have been identified: acid phytases showing maximal phytate dephosphorylation around pH 5.0 and alkaline phytases with a pH optimum of around pH 8.0 (Konietzny and Greiner 2002).

As mentioned above, all phytases used as animal feed supplements today belong to the class of histidine acid phytases. Therefore, they are expected to act most efficiently under the conditions present in the forestomach or stomach of the animal. Animal feeding studies have confirmed that the main functional site of supplemental phytase in pigs and fish is the stomach (Jongbloed *et al.*, 1992; Yi and Kornegay, 1996; Yan *et al.*, 2002). The site of phytase action in the gastrointestinal tract of poultry has received little attention. However, the crop was reported to be very probably the primary site of phytate dephosphorylation by supplementary phytase (Selle and Ravindran, 2007). A phytase that should be active in the small intestine requires a sufficiently high stability under the pH conditions in the stomach and intestine as well as a high resistance to proteolytic activities, mainly of pepsin in the stomach and the pancreatic proteases in the small intestine. To guarantee an efficient phytate dephosphorylation in the crop and stomach, stability in an acid environment and resistance to pepsin are properties that are reported also to be highly desirable for supplementary acid phytases.

Activity profile and stability of pH

With the exception of some bacterial phytases, especially those of the genera *Bacillus* and *Enterobacter* as well as some plant phytases, all phytases reported today exhibit a pH optimum in the range 4.0–6.0 (Table 5.1; Konietzny and Greiner, 2002).

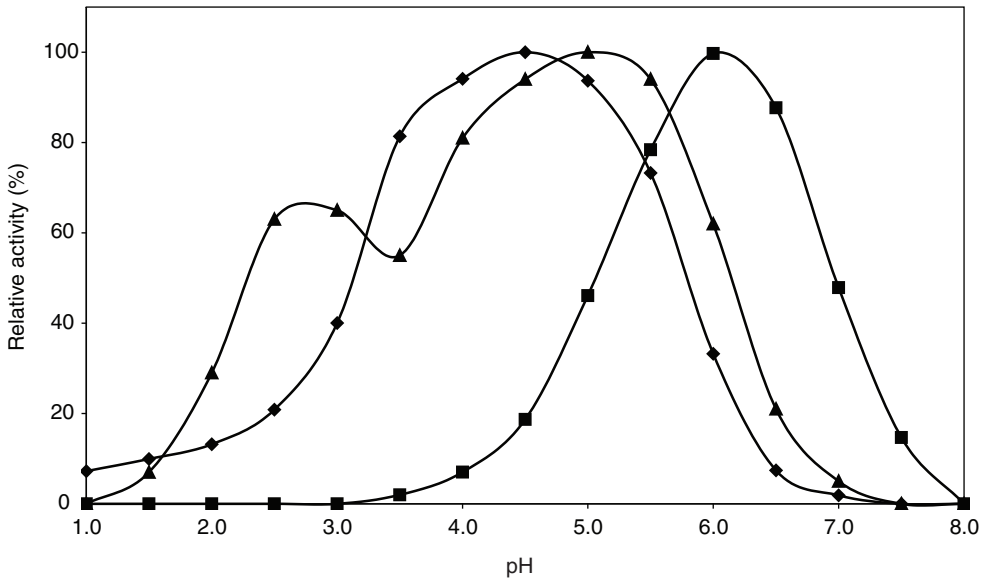
Table 5.1. Basic characteristics of selected phytases.

Phytase source	Optimal conditions		Specific activity	Phytase	Reference(s)
	pH	*T* (°C)	at 37°C (U mg^{-1})	classification	
Aspergillus niger	5.0–5.5	55–58	50–133	3-phytase	Ullah and Gibson (1987); Wyss *et al*. (1999a); Greiner *et al*. (2009)
Aspergillus terreus	5.0–5.5	70	142–196	3-phytase	Wyss *et al*. (1999a)
Aspergillus fumigatus	5.0–6.0	60	23–28	3-phytase	Wyss *et al*. (1999a); Rodriguez *et al*. (2000)
Thermomyces lanuginosus	6.0	65	110	–	Berka *et al*. (1998)
Penicillium simplicissimum	4.0	55	3	–	Tseng *et al*. (2000)
Periophora lycii	5.5	58	1080	6-phytase	Lassen *et al*. (2001); Ullah and Sethumadhavan (2003)
Candida krusei	4.6	40	1210	–	Quan *et al*. (2002)
Debaromyces castellii	4.0–4.5	55–60	–	3-phytase	Ragon *et al*. (2008)
Saccharomyces cerevisiae	4.5	45	135	3-phytase	Nayini and Markakis (1984); Greiner *et al*. (2001a)
Neurospora crassa	5.5	60	125	3-phytase	Zhou *et al*. (2006)
Escherichia coli	4.5	55–60	750–811	6-phytase	Greiner *et al*. (1993, 2000a); Golovan *et al*. (2000)
Selenomonas ruminantium	4.5–5.0	55	668	3-phytase	Puhl *et al*. (2007)
S. ruminantium subsp. *lactilytica*	4.5	55	16	5-phytase	Puhl *et al*. (2008b)
Selenomonas lacticifex	4.5	40	440	3-phytase	Puhl *et al*. (2008a)
Megasphaera elsdenii	5.0	60	269	3-phytase	Puhl *et al*. (2009)

Klebsiella terrigena	5.0	58	205	3-phytase	Greiner *et al*. (1997); Greiner and Carlsson (2006)
Pantoea agglomerans	4.5	60	23	3-phytase	Greiner (2004a,b)
Citrobacter braakii	4.0	50	3457	–	Kim *et al*. (2003)
Pseudomonas syringae	5.5	40	769	3-phytase	Cho *et al*. (2003)
Bacillus subtilis	6.5–7.5	55–60	9–15	3-phytase	Kerovuo *et al*. (1998); Greiner *et al*. (2007)
Bacillus amyloliquefaciens	7.0–8.0	70	20	3-phytase	Kim *et al*. (1998a); Greiner *et al*. (2007)
Wheat PHY1	6.0	45	127	4-phytase	Nakano *et al*. (1999, 2000)
Wheat PHY2	5.0	50	242	4-phytase	Nakano *et al*. (1999, 2000)
Spelt D21	6.0	45	262	4-phytase	Konietzny *et al*. (1995); Greiner and Larsson Alminger (2001)
Rye	6.0	45	517	4-phytase	Greiner *et al*. (1998); Greiner and Larsson Alminger (2001)
Oat	5.0	38	307	4-phytase	Greiner and Larsson Alminger (1999, 2001)
Barley P1	5.0	45	117	4-phytase	Greiner *et al*. (2000b); Greiner and Larsson Alminger (2001)
Barley P2	6.0	55	43	4-phytase	Greiner *et al*. (2000b); Greiner and Larsson Alminger (2001)
Faba bean	5.0	50	636	4-phytase	Greiner *et al*. (2001b, 2002)
Lupin L11	5.0	50	539	3-phytase	Greiner (2002); Greiner *et al*. (2002)
Lupin L12	5.0	50	607	3-phytase	Greiner (2002); Greiner *et al*. (2002)
Lupin L2	5.0	50	498	4-phytase	Greiner (2002); Greiner *et al*. (2002)
Lily pollen	8.0	55	0.2	5-phytase	Jog *et al*. (2005); Mehta *et al*. (2006)

Even though phytases show often maximal activity in the same pH range, their pH activity profiles may differ considerably. As an example, the phytases from rye, *Aspergillus niger* (*A. niger* 11T53A9) and a Malaysian waste-water bacterium (*Yersinia rhodei*) were compared, demonstrating three different pH activity profiles (Fig. 5.1a). The rye phytase had its optimum activity at pH 6.0 (Greiner *et al.*, 1998), whereas that from *A. niger* 11T53A9 showed maximum phytate-degrading activity at pH 5.0, with a second, lower optimum at pH 2.8 (Greiner *et al.*, 2009), and the optimum pH of the phytase from Malaysian waste water bacterium was determined to be pH 4.5 (Greiner and Farouk, 2007). The pH activity profiles of the two microbial phytases differed mainly in the pH range 1.5–3.5, whereas the rye phytase showed higher activity in the pH range 6–8 and lower activity below pH 5.5 when compared with the microbial phytases (Fig. 5.1a). Because phytases are in general supplemented according to their activity determined at standard conditions (pH 5.5, 37°C, sodium phytate 5 mmol l^{-1}; Engelen *et al.*, 1994), they will differ in their phytate-degrading activities at other pH conditions (Fig. 5.1b). Rye phytase clearly has an advantage over the microbial phytases at pH values above 5.5, whereas at pH values below 5.5 both microbial phytases have great advantages over rye phytase. In the pH range 3.5–5.5 and below 1.5, the phytase from Malaysian waste water bacterium exhibited a better phosphate release from phytate compared with that from *A. niger* 11T53A9. Therefore, differences in pH activity profiles may in part explain the difference in effectiveness of different phytases (plant, *A. niger*, *E. coli*, *P. lycii*) in diets for swine and poultry (Eeckhout and de Paepe, 1991; Zimmermann *et al.*, 2002; Applegate *et al.*, 2003; Augspurger *et al.*, 2003). Consequently, choosing another pH value for standard phytase activity determination might lead to a

(a)



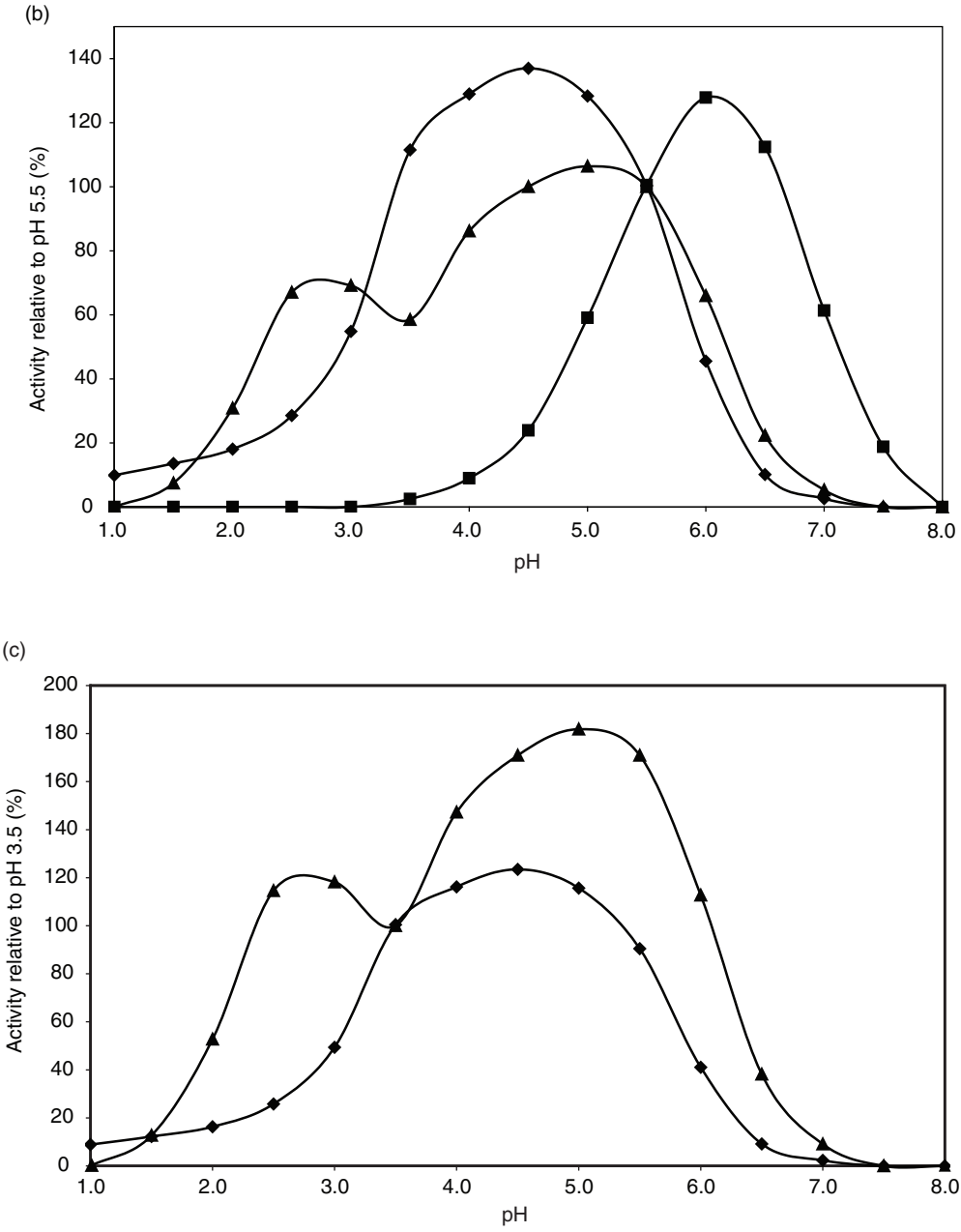


Fig. 5.1. (a) pH activity profiles of phytases from rye (■) (Greiner *et al*., 1998), *Aspergillus niger* 11T53A9 (▲) (Greiner *et al*., 2009) and a Malaysian waste-water bacterium (♦) (Greiner and Farouk, 2007), using sodium phytate as a substrate at 37°C. The activity at optimal pH was taken as 100%. Buffers: pH 1.0–3.5, glycine/HCl; pH 3.5–6.0, sodium acetate/NaOH; pH 6.0–7.0, Tris/H-acetate; pH 7.0–8.0, Tris/HCl (each 100 mM). (b) The same pH activity profiles shown as relative values compared with activity at pH 5.5 (the activity at pH 5.5 was taken as 100%). (c) pH activity profiles of microbial phytases shown as relative values compared with activity at pH 3.5 (the activity at pH 3.5 was taken as 100%).

completely different result in respect to ranking of phytases. If standard phytase activity determinations were conducted at pH 3.5, 37°C and sodium phytate 5 mmol l^{-1}, *A. niger* 11T53A9 phytase would be superior to that from Malaysian waste water bacterium over the complete pH range (Fig. 5.1c). However, it must be remembered that bioefficacy is determined not only by the pH activity profile of the phytase, but also by its stability under the pH conditions of the stomach or crop, its susceptibility to pepsin degradation and the electrostatic environment in the stomach. It was, for example, shown that the pH profiles of a fungal (*A. niger*) and a bacterial (*E. coli*) phytase could be modified by both the buffer and the introduction of salt (NaCl, $CaCl_2$; Ullah *et al.*, 2008).

In general, microbial acid phytases exhibit considerable enzymatic activity below pH 3.5, whereas plant acid phytases are almost inactive. It is obvious that a high phytate-degrading activity over the complete pH range at the site of action of the phytase is advantageous for efficient phytate dephosphorylation in the gastrointestinal tract of animals. Some phytases, for example those from *E. coli* (Greiner *et al.*, 1993; Golovan *et al.*, 2000), *Klebsiella terrigena* (Greiner *et al.*, 1997), rye (Greiner *et al.*, 1998), barley (Greiner *et al.*, 2000b) and oat (Greiner and Larsson Alminger, 1999), have a narrow pH activity profile, whereas other phytases were identified as having a very broad pH activity profile. It was shown, for instance, that the *Aspergillus fumigatus* phytase exerts activity between pH 2.5 and 8.5 and maintains 80% of its optimal activity within the pH range 4.0–7.3 (Wyss *et al.*, 1999a). Similar broad pH activity profiles were reported for phytases from *Thermomyces lanuginosus* (Berka *et al.*, 1998), *Aspergillus terreus* (Mitchell *et al.*, 1997; Wyss *et al.*, 1999a), *Myceliophthora thermophila* (Mitchell *et al.*, 1997) and *Yersinia rohdei* (Huang *et al.*, 2008). In addition, the pH stability of some microbial phytases below pH 3.0 and above pH 8.0 is remarkable, whereas the stability of most plant phytases decreases dramatically at pH values below pH 4 and above pH 7.5. The phytases from *E. coli* (Greiner *et al.*, 1993), *A. niger* 11T53A9 (Greiner *et al.*, 2009) and Malaysian waste water bacterium (Greiner and Farouk, 2007), for example, did not lose significant enzymatic activity even after exposure at pH 2.0 and 4°C for several hours. Phytases from rye (Greiner *et al.*, 1998), spelt (Konietzny *et al.*, 1995), barley (Greiner *et al.*, 2000b), oat (Greiner and Larsson Alminger, 1999), faba beans (Greiner *et al.*, 2001b) and lupin (Greiner, 2002), however, lost 63–83% of their initial activity within 24 h at pH 2.5 and 4°C.

Proteolytic stability

The effectiveness and limitations of feed supplementation with phytases may also depend on their susceptibility to proteolytic cleavage. By incubating phytases with pepsin at pH 2.0 and pancreatin at pH 7.0, differences in their ability to withstand degradation by these digestive proteases were observed. Bacterial histidine acid phytases have been shown to exhibit a greater pepsin and pancreatin resistance than fungal acid phytases (Rodriguez *et al.*, 1999; Igbasan *et al.*, 2000; Simon and Igbasan, 2002; Kim *et al.*, 2003; Elkhalil

et al., 2007; Greiner and Farouk, 2007; Huang *et al.*, 2008). The bacterial phytases (*E. coli*, *Klebsiella* spp. and Malaysian waste water bacterium) retained more than 80% of their initial activity after pepsin digestion, whereas phytases from *A. niger* and *P. lycii* retained only 26–42% and 2–20%, respectively. After incubation with pancreatin, phytases from *E. coli* and *Klebsiella* spp. retained more than 90% of their initial activity, whereas the *A. niger* phytase retained only 23–34% and the *P. lycii* phytase was completely inactivated. The consensus phytase was the only fungal phytase that was reported to have a pepsin and pancreatin tolerance similar to that of bacterial histidine acid phytases (Simon and Igbasan, 2002). In addition, the phytase from *Bacillus subtilis* showed a comparable pepsin resistance to *A. niger* phytase, whereas its susceptibility to pancreatin digestion was shown to be similar to the bacterial histidine acid phytases (Igbasan *et al.*, 2000; Simon and Igbasan, 2002). The high pancreatin resistance of *B. subtilis* phytase and its high susceptibility to pepsin digestion was also confirmed by Kerovuo *et al.* (2000). Furthermore, plant phytases are considered to be more susceptible to inactivation by gastrointestinal proteases. Wheat phytase was reported to be less resistant to pepsin and pancreatin than phytases of *A. niger* (Phillippy, 1999). It also has to be remembered that recombinant enzymes may differ in proteolytic resistance compared with their wild-type counterparts, as recently reported for *E. coli* and *A. niger* phytases produced in *Pichia pastoris* (Rodriguez *et al.*, 1999).

In addition, the proteolytic stability of phytases was studied in digesta supernatants from different gut segments of laying hens and broiler chickens (Igbasan *et al.*, 2000; Simon and Igbasan, 2002; Elkhalil *et al.*, 2007). Residual activities of bacterial acid phytases and consensus phytase in digesta supernatants of all gut segments and residual activities of *B. subtilis* phytase in digesta supernatants of intestinal gut segments were comparable to those obtained during direct incubation with the corresponding proteases. However, a much higher proteolytic stability of *B. subtilis* phytase in the digesta supernatant of the stomach (68%) and the phytases from *A. niger* (stomach, 60–70%; small intestine, 55–94%) and *P. lycii* (stomach, 59%; small intestine, 85–95%) in digesta supernatants of all gut segments was observed compared with direct incubation for corresponding proteases. Thus, phytases that have shown a high proteolytic susceptibility when incubated with pepsin at pH 2.0 or pancreatin at pH 7.0 were surprisingly stable in digesta supernatants. The cause for this increase in stability is not known. However, it can be speculated that the presence of the substrate phytate is capable of stabilizing phytases, or the greater tolerance might be due to the presence of additional proteins serving as substrates for the proteases. From these results it might be concluded that the intrinsic proteolytic resistance of a phytase is of minor importance for its *in vivo* performance.

Substrate specificity and end product of enzymatic phytate dephosphorylation

Substrate specificity may also have an effect on the *in vivo* performance of phytases. *In vitro* studies with purified phytases and sodium phytate as a

substrate revealed that phytases hydrolyse phytate via a pathway of stepwise dephosphorylations, to generate orthophosphate and a series of partially phosphorylated *myo*-inositol phosphates (Konietzny and Greiner, 2002). The reaction intermediates are released from the enzymes and serve as substrates for further hydrolysis. The different phosphate residues of phytate may be released at different rates and in different order. In general, however, phytases do not have the capacity to dephosphorylate phytate completely. The phosphate residue at position C-2 in the *myo*-inositol ring was shown to be resistant to dephosphorylation by phytases. Independent of their bacterial, fungal or plant origin, the majority of histidine acid phytases release five of the six phosphate residues of phytate, and the final degradation product was identified as *myo*-inositol(2)phosphate (Cosgrove, 1970; Lim and Tate, 1973; Hayakawa *et al.*, 1990; Wyss *et al.*, 1999b; Greiner *et al.*, 2000a, 2001a, 2002, 2007a, 2009; Nakano *et al.*, 2000; Greiner and Larsson Alminger, 2001; Greiner and Carlsson, 2006). Dephosphorylation of *myo*-inositol(2)-phosphate occurs only in the presence of high enzyme concentration during prolonged incubation. After removal of the first phosphate residue from phytate, histidine acid phytases continue dephosphorylation adjacent to a free hydroxyl group. In addition, acid phosphatases with phytate-degrading activity were identified in members of the Enterobacteriaceae family, such as *E. coli* (Cottrill *et al.*, 2002), *Pantoea agglomerans* (Greiner, 2004b) and *Enterobacter cloacae* (Herter *et al.*, 2006), which preferably degrade glucose-1-phosphate. These enzymes were shown to hydrolyse only the phosphate residue at the D-3 position of phytate, producing D-*myo*-inositol(1,2,4,5,6)-pentakisphosphate as the sole hydrolysis product. The alkaline phytases from cat's tail (Hara *et al.*, 1985), lily pollen (Mehta *et al.*, 2006), *B. subtilis* (Greiner *et al.*, 2007b), *B. amyloliquefaciens* (Greiner *et al.*, 2007b) and *S. oneidensis* (Greiner *et al.*, 2007b) yield *myo*-inositol trisphosphate as the final product of phytate dephosphorylation. With the exception of the phytase from lily pollen, alkaline phytases represent the class of β-propeller phytases and seem to prefer the hydrolysis of every second phosphate over that of adjacent ones, generating *myo*-inositol(2,4,6)trisphosphate as the final dephosphorylation product. The alkaline phytase from lily pollen possesses the conserved active site motifs characteristic for histidine acid phytases (Mehta *et al.*, 2006) and prefers removal of adjacent phosphate groups generating *myo*-inositol(1,2,3)trisphosphate as the end product of phytate dephosphorylation. In general, a marked decrease in hydrolysis rate was observed during phytate dephosphorylation by phytases. The decrease in the rate of phosphate release might be due to product inhibition by phosphate or a lower hydrolysis rate of the partially phosphorylated *myo*-inositol phosphates. Both factors probably play a role, but information about kinetic parameters of the different partially phosphorylated *myo*-inositol phosphates is almost entirely lacking, since most of the reaction intermediates are not available in pure form and sufficient quantities for kinetic studies.

In vitro feed experiments with microbial phytases suggest that enzymes with broad substrate specificity are better suited for animal nutrition purposes than enzymes with narrow substrate specificity (Wyss *et al.*, 1999a). In general,

phytases accept a variety of phosphorylated compounds as substrates (Konietzny and Greiner, 2002). Only a few phytases have been described as highly specific for phytate, such as the alkaline phytases from *B. subtilis* (Powar and Jagannathan, 1982; Shimizu, 1992), *B. amyloliquefaciens* (Kim *et al.*, 1998a), lily pollen (Baldi *et al.*, 1988) and cat's tail pollen (Hara *et al.*, 1985). In addition, the acid phytases from *E. coli* (Greiner *et al.*, 1993), *K. terrigena* (Greiner *et al.*, 1997), *A. niger* (Wyss *et al.*, 1999a) and *A. terreus* (Wyss *et al.*, 1999a) have been reported to be rather specific for phytate. Histidine acid phytases with broad substrate specificity readily degrade phytate to *myo*-inositol monophosphate, with no major accumulation of intermediates, whereas phytases with narrow substrate specificity result in *myo*-inositol tris- and bisphosphate accumulation during phytate degradation. Whether a similar accumulation of partially phosphorylated *myo*-inositol phosphate also occurs in the stomach of an animal is highly questionable. Due to the higher viscosity of stomach contents compared with the *in vitro* environment, it seems likely that reaction intermediates rather than phytate are preferentially dephosphorylated. Increasing the viscosity in *in vitro* studies has already demonstrated a reduction in the accumulation of *myo*-inositol tetrakis-, tris- and bisphosphates (Greiner, 2005, unpublished data). However, even if a major accumulation of partially phosphorylated *myo*-inositol phosphates occurs in the stomach, it might be without any consequence for phosphorus bioavailability, because partially phosphorylated *myo*-inositol phosphates with four or fewer phosphate residues are expected to be further dephosphorylated in the small intestine (Hu *et al.*, 1996), whereas phytate is a very poor substrate of phosphatases arising from the mucosa of the small intestine (Pointillart *et al.*, 1984, 1985). Therefore, a complete transformation of dietary phytate into *myo*-inositol tetra- and trisphosphates in the stomach seems to be much more important for the bioefficacy of supplementary phytase than complete dephosphorylation of single phytate molecules. Furthermore, phytases with broad substrate specificity do not act exclusively upon phytate and other *myo*-inositol phosphates, but also upon other phosphorylated compounds present in the stomach. Thus, a high affinity for phytate and *myo*-inositol pentakisphosphate, high turnover numbers with both compounds and narrow substrate specificity are concluded to be desirable properties for phytases used as feed additives.

However, this conclusion must be proved in animal feeding studies. The turnover numbers k_{cat} for hydrolysis of sodium phytate by phytases reported so far range from $<10\ s^{-1}$ (soybean, barley P2, maize; Gibson and Ullah, 1988; Laboure *et al.*, 1993; Greiner *et al.*, 2000b) to $10{,}325\ s^{-1}$ (*Yersinia intermedia*; Huang *et al.*, 2008). High affinity for sodium phytate is expressed by a low Michaelis–Menten constant K_M. K_M values of phytases studied range from <10 to 650 μM. Relatively low K_M values have been reported for phytases from *A. niger* (10–54 μM; Ullah, 1988; Wyss *et al.*, 1999a; Greiner *et al.*, 2009), *A. terreus* (11–23 μM; Wyss *et al.*, 1999a), *A. fumigatus* (<10 μM; Pasamontes *et al.*, 1997b; Wyss *et al.*, 1999a; Rodriguez *et al.*, 2000), *Schwanniomyces castellii* (38 μM; Segueilha *et al.*, 2002), *Klebsiella aerogenes* (62 μM; Tambe *et al.*, 1994), cat's tail pollen (17 μM; Hara *et al.*, 1985), maize root

(24–43 μM; Hübel and Beck, 1996), tomato root (38 μM; Li *et al.*, 1997), oat (30 μM; Greiner and Larsson Alminger, 1999), wheat bran (PHY1, 48 μM; PHY2, 77 μM; Nakano *et al.*, 1999), barley (P1, 72 μM; Greiner *et al.*, 2000b), soybean (48–61 μM; Gibson and Ullah, 1988; Hegeman and Grabau, 2001) and lupin (L11, 80 μM; Greiner, 2002). The kinetic efficiency of an enzyme is validated by means of the k_{cat}/K_M values for a given substrate. The phytases of *E. coli* (Golovan *et al.*, 2000; Konietzny and Greiner, 2002), *Citrobacter braakii* (Kim *et al.*, 2003) and *Yersinia* spp. (Huang *et al.*, 2008) exhibit k_{cat}/K_M values in the range of 1.03×10^7 M^{-1} s^{-1} to 8.2×10^7 M^{-1} s^{-1}, respectively, which are the highest values reported for phytases to date.

Initiation site of phytate dephosphorylation

Last, but not least, it was suggested that phytases with distinctly different initiation sites may show differences in bioefficacy. Today, three classes of phytases are recognized by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB): 3-phytase (EC 3.1.3.8) initially removes phosphate residue from the D-3 position of phytate, whereas 6-phytase (EC 3.1.3.26) preferentially initiates phytate dephosphorylation at the L-6 (D-4) position and 5-phytase (EC 3.1.3.72) at the 5 position in the *myo*-inositol ring. To date, only 3- and 6-phytases have been extensively used in animal feeding studies, and these studies do not give any clear indication that differences in bioefficacy are based on the position of initiating phytate dephosphorylation, especially since supplementary phytases also differ in other enzymatic properties such as pH activity profile, pH stability and pepsin tolerance. Initially, microbial phytases were considered to be 3-phytases, and 6-phytases were said to be characteristic for seeds of higher plants. Most phytase studies so far with regard to their phytate degradation pathway fit into this pattern (Table 5.1). However, this is not a general rule, as exemplified by the indication of 3-phytase activity in lupin (Greiner, 2002) and soybean seeds (Greiner, 2000, unpublished data) and 6-phytase activity in *Paramecium* (van der Kaay and van Haastert, 1995), *E. coli* (Greiner *et al.*, 2000a), *P. lycii* (Lassen *et al.*, 2001) and Malaysian waste water bacterium (Greiner *et al.*, 2007a). It is worth mentioning that the 6-phytases of plant seeds initially hydrolyse the L-6 (D-4) phosphate residue from phytate (Hayakawa *et al.*, 1990; Nakano *et al.*, 2000; Greiner and Larsson Alminger, 2001; Greiner *et al.*, 2002), whereas microbial 6-phytases initially remove the phosphate residue attached to the D-6 (L-4) position (van der Kaay and van Haastert, 1995; Greiner *et al.*, 2000a, 2007a; Lassen *et al.*, 2001).

To bring some clarification to biochemical pathway interpretation, the current rule is to number the *myo*-inositol phosphates in the D configuration (counter-clockwise). Thus, the above-mentioned 6-phytases of plant origin have to be classified as 4-phytases (Table 5.1); it is exceptionally important to distinguish those from the microbial 6-phytases. 5-Phytase activity was discovered in lily pollen (Barrientos *et al.*, 1994; Mehta *et al.*, 2006) and *S. ruminantium* subsp. *lactilytica* (Puhl *et al.*, 2008b). Phytases preferentially

initiating phytate dephosphorylation at position 2 of the *myo*-inositol ring have to be present, for example, within animal cells, because intracellular phytate shows a high turnover, and intracellularly occurring partially phosphorylated *myo*-inositol phosphates are dephosphorylated at the C-2 position in the *myo*-inositol ring. Thus, it could be suggested that all six possibilities of initiating phytate dephosphorylation are realised in nature (Fig. 5.2), even though the existence of a 1-phytase has not been reported to date.

Furthermore, it has been argued that a combination of phytases with distinctly different initiation sites would result in linearly additive responses, or even synergistic effects, in respect to phosphate release. Zimmermann *et al.* (2003) concluded from their studies on growing pigs that intrinsic cereal phytase (rye, wheat) and supplemental *A. niger* phytase exhibit linear additivity in their response on apparent phosphorus absorption. This result implies that both types of phytase degrade phytate independently from each other. Synergistic effects have so far not been observed from the combination of various phytases (Augspurger *et al.*, 2003; Gentile *et al.*, 2003; Stahl *et al.*, 2004).

A prerequisite for more efficient phosphate release from phytate is that reaction intermediates generated by one of the phytases are dephosphorylated faster than they are produced by the other phytase. However, different phytases may exhibit different phytate degradation pathways and therefore lead to the generation and accumulation of different *myo*-inositol phosphate intermediates (Fig. 5.3). It is unlikely that a particular phytase accepts all theoretically possible *myo*-inositol phosphate esters as a substrate. Therefore, some reaction intermediates generated by a certain phytase may be slowly dephosphorylated by a different phytase or may even act as a competitive inhibitor, while

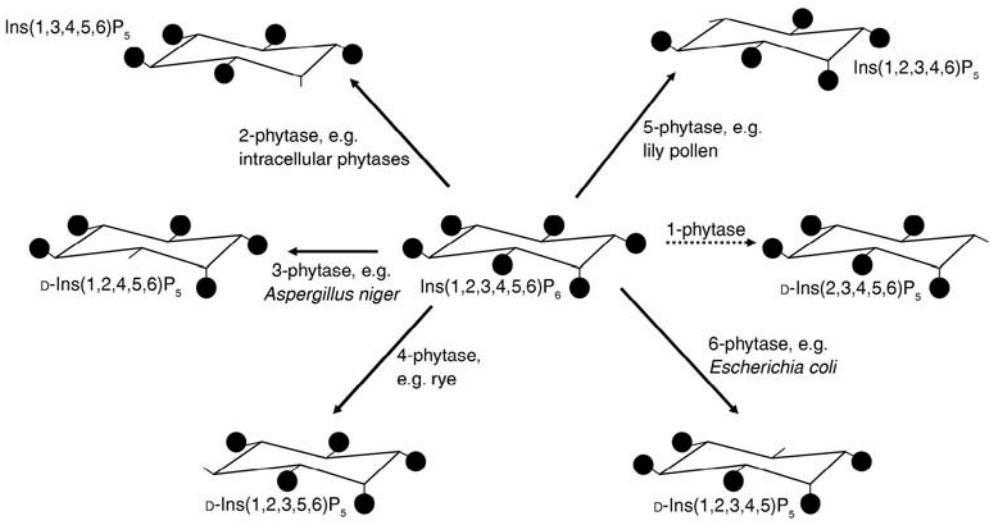


Fig. 5.2. Classification of phytases based on the carbon in the *myo*-inositol ring of phytate at which dephosphorylation is initiated (●, phosphate residue).

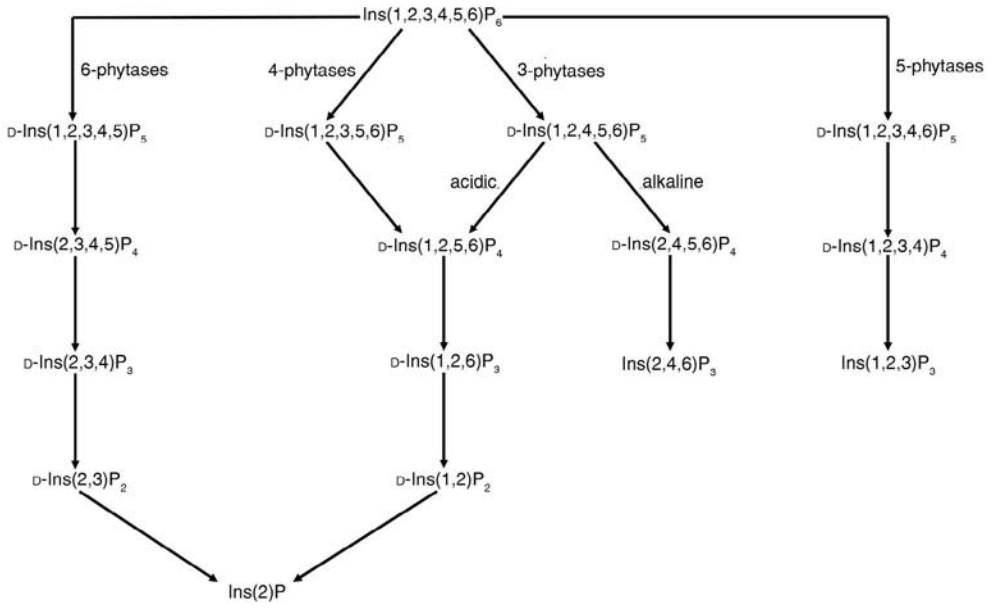


Fig. 5.3. Major phytate degradation pathways for the four classes of phytase (from Hayakawa *et al.*, 1990; Greiner *et al.*, 2000a, 2001a, 2002, 2007a,b, 2009; Nakano *et al.*, 2000; Greiner and Larsson Alminger, 2001; Mehta *et al.*, 2006).

binding to the active site without being hydrolysed. Thus, phytases that are planned to be used in combination have to be well tuned to achieve synergistic effects with respect to phosphate release from phytate in the gastrointestinal tract of an animal.

Specific activity

Specific activity is one key factor in commercial exploitation of phytases, in particular because they are supplemented according to their enzymatic activity and not according to their mass. The higher the specific activity of a phytase, the more phosphate is released from phytate by a given mass of phytase in a defined time period. Specific activities of phytases range from <10 U mg^{-1} (lily pollen, mung bean, soybean, maize, *Penicillium simplicissimum*) to >1000 U mg^{-1} (*C. braakii*, *Candida krusei*, *P. lycii*, *Yersinia* spp.) at 37°C and their individual optimum pH (Greiner and Konietzny, 2006; Huang *et al.*, 2008). In general, microbial phytases seem to exhibit higher specific activities than their plant counterparts (Table 5.1). The highest specific activities were reported for *C. braakii* (3457 U mg^{-1}; Kim *et al.*, 2003), *Yersinia* spp. (2344–3960 U mg^{-1}; Huang *et al.*, 2008), *C. krusei* (1210 U mg^{-1}; Quan *et al.*, 2002) and *P. lycii* (1080 U g^{-1}; Lassen *et al.*, 2001; Ullah and Sethumadhavan, 2003). Commercially available phytases from *A. niger* (Ullah and Gibson,

1987; Wyss *et al.*, 1999a; Greiner *et al.*, 2009) and *E. coli* (Golovan *et al.*, 2000; Konietzny and Greiner, 2002) were reported to exhibit specific activities in the range of 50–133 U mg^{-1} and 750–811 U mg^{-1}, respectively.

Thermostability

Thermostability is a particularly important issue, since feed pelleting is commonly performed at temperatures between 60 and 95°C. Depending on the subsequent cooling system, the phytase is exposed to pelleting temperature for a time period in the range of seconds to minutes. Although phytase inclusion using an after-spray apparatus for pelleted diets and/or chemical coating of phytase may help bypass or overcome heat destruction of the enzyme, thermostable phytases will no doubt prove to be more suitable candidates for feed supplements. Likewise, an enzyme that can tolerate long-term storage or transport at ambient temperatures is undisputedly attractive. In purified form, most phytases from plants will have been irreversibly inactivated at temperatures above 70°C within minutes, whereas most corresponding microbial enzymes retain significant activity even after prolonged incubation.

Thermal stability of commercialized phytases was determined by Simon and Igbasan (2002) at 70°C in aqueous solution. They reported the phytase from *A. niger* to be slightly more stable under the conditions applied than that from *P. lycii*, and the phytase from *E. coli* was shown to be even less stable than that from *P. lycii*. With regard to thermostability, the same ranking of phytases was observed in pelleting experiments (Simon and Igbasan, 2002). The phytases most resistant to high temperatures reported so far have been isolated from *Pichia anomala* (Vohra and Satyanarayana, 2002), *S. castellii* (Segueilha *et al.*, 1992), *A. fumigatus* (Pasamontes *et al.*, 1997b) and *Lactobacillus sanfranciscensis* (De Angelis *et al.*, 2003). Incubation of these enzymes at 70°C for 10 min did not result in a significant loss of activity, and the phytase of *P. anomala* was reported even to tolerate 30 h of treatment at 70°C without any loss of activity. The *A. fumigatus* enzyme lost only 10% of its initial activity after exposure for 20 min at 90°C; however, it was shown not to be thermostable, but had the remarkable property of being able to refold completely into native-like, fully active conformation after heat denaturation (Wyss *et al.*, 1998). Thermostability of the *B. subtilis* phytase is also due to its capacity to partially refold after heat treatment (Kerovuo *et al.*, 2000). However, the stability of this enzyme is strongly dependent on the presence of Ca^{2+}.

Phytases with more Favourable Properties for Feed Applications

Phytases with all the required properties for animal feed applications have not been found in nature to date. Thus, screening nature for phytases with more favourable properties for feed applications and engineering phytases in order to optimize their catalytic and stability features are suitable approaches to produce better candidates for use as feed supplements.

Screening nature for phytases with more favourable properties for feed applications

Screening microorganisms for phytase production is not a trivial exercise. In microorganisms, expression of phytases is subject to complex regulation, but their formation is not controlled uniformly across classes (Konietzny and Greiner, 2004). A tight regulatory inhibition of the formation of phytases by phosphate levels is generally observed in microorganisms, including moulds, yeasts and bacteria. With the majority of microorganisms, however, it was demonstrated that phosphate concentration is not the only factor affecting phytase production. Depending on the microorganism under investigation, phytate (Powar and Jagannathan, 1982; Lambrechts *et al.*, 1993; Tambe *et al.*, 1994; Greiner *et al.*, 1997; Kerovuo *et al.*, 1998; Kim *et al.*, 1998a), phytate dephosphorylation products (Greiner, 2009, unpublished data), anaerobiosis (Greiner *et al.*, 1993; Lambrechts *et al.*, 1993), aeration (Nair *et al.*, 1991), carbon starvation (Greiner *et al.*, 1997), glucose (Sreeramulu *et al.*, 1996; De Angelis *et al.*, 2003), pH and temperature (Lambrechts *et al.*, 1993; Kim *et al.*, 1999; Andlid *et al.*, 2004) were all shown to modulate phytase formation. Therefore, failure to detect phytase activity does not necessarily imply that the microorganism under investigation is not a phytase producer at all, but perhaps that the culture conditions are disadvantageous for expression. In addition, fast and easy screening methods depend upon the phytase being secreted. However, most microorganisms produce only intracellular phytases. Extracellular phytase activity was observed almost exclusively in filamentous fungi and yeasts (Konietzny and Greiner, 2002). The only bacteria showing extracellular phytase activity were those of the genera *Bacillus* (Powar and Jagannathan, 1982; Shimizu, 1992; Kerovuo *et al.*, 1998; Kim *et al*., 1998a) and *Enterobacter* (Yoon *et al.*, 1996).

Today, strategies such as: (i) exploiting databases obtained from genome projects on microorganisms through a BLAST search using representative genes from the four classes of phytases (Cheng and Lim, 2006; Lim *et al.*, 2007); and (ii) identifying putative phytase-encoding genes by PCR using degenerate primers based on conserved amino acid sequences of each of the four classes of phytases (Mitchell *et al.*, 1997; Pasamontes *et al.*, 1997a,b) are seen as an alternative to successfully identifying phytase-producing micoorganisms. The disadvantage of these strategies is that it is impossible to find new types of phytase with novel catalytic mechanisms, since the search depends upon known sequences.

Engineering phytases in order to optimize their catalytic and stability features

Tailor-made biocatalysts can be created from wild-type enzymes by either protein engineering or directed evolution techniques. The use of the term 'engineering' implies that there is some precise understanding of the system that is being modified. Thus, determinants for the property of an enzyme to be improved must be known and, therefore, rational enzyme design usually

requires both the availability of the structure of the enzyme and knowledge about the relationships between sequence, structure and catalytic mechanism to make the desired changes. Since site-directed mutagenesis techniques are well developed, the introduction of directed mutations is easy and relatively inexpensive. The major drawback in rational protein design is that detailed structural knowledge of an enzyme is often unavailable. Therefore, optimization of catalytic properties has been approached in the past mostly on a trial-and-error basis by random mutagenesis. However, rapid progress in solving protein structures by NMR spectroscopy (instead of by X-ray diffraction of crystals) and the enormously increasing number of sequences stored in public databases have significantly improved access to data and structures. Even if there are no structural data available, the structure of a homologous enzyme could be used as a model to select amino acid substitutions to increase selectivity, activity or stability of a given enzyme. Computer-aided molecular modelling seeks to identify the effect of amino acid alterations on enzyme folding and substrate recognition. However, it can be extremely difficult to predict the effects of a mutation, because even minor sequence changes by a single-point mutation may cause significant structural disturbance. Thus, even if one trait is successfully designed, it is virtually impossible to predict its effect on another.

One powerful tool for the development of biocatalysts with novel properties with no requirement of knowledge of enzyme structures or catalytic mechanisms is provided by a collection of methods mimicking the natural process of enzyme evolution in the test-tube by using modern molecular biology methods of mutation and recombination. This collection of methods has been termed 'directed evolution' (Chirumamilla *et al.*, 2001). Furthermore, directed evolution provides the possibility of exploring enzyme functions never required in the natural environment and for which the molecular basis is poorly understood. Thus, this bottom-up design approach contrasts with the more conventional, previously mentioned top-down one in which proteins are tamed rationally using computer-based modelling and site-directed mutagenesis. Protein engineering, as well as direct evolution techniques, have been applied to improve phytate hydrolysis at low pH values, enhance thermal tolerance of phytases and increase their specific activity in order to optimize phytases for animal feed applications.

Detailed inspection of both amino acid sequence alignments and experimentally determined or homology-modelled three-dimensional structures has been used to identify active-site amino acids that were considered to correlate with activity maxima at low pH in fungal phytases. Site-directed mutagenesis experiments were used to confirm such predictions. Replacement of glycine at position 297 in *A. fumigatus* wild-type phytase by lysine gave rise to a second pH optimum shift, from 2.8 to 3.4 (Tomschy *et al.*, 2000b). In addition, the Lys68Ala single mutation and the Ser140Tyr and Asp141Gly double mutation decreased the pH optimum by 0.5 to 1.0 units, with either no change or even a slight increase in maximum specific activity (Tomschy *et al.*, 2002). Increased phytase activity for *A. niger* NRRL 3135 phytase at intermediate pH levels (3.0–5.0) was achieved by replacement of lysine at position 300 by glutamic acid (Mullaney *et al.*, 2002). This single mutation

resulted in an increase of phytate hydrolysis of 56% and 19% at pH 4.0 and 5.0, respectively, at 37°C. The Glu228Lys mutation in *A. niger* phytase resulted in a shift of pH optimum from 5.0–5.5 to 3.8 and 266% greater phytate hydrolysis at pH 3.5 than the wild-type enzyme (Kim *et al.*, 2006). The improved efficacy of the mutant was confirmed in an animal feed trial.

Naturally occurring phytases having the required level of thermostability for application in animal feeding have, to date, not been found in nature. The poor thermostability of phytases is therefore still a major concern for animal feed applications. Several strategies have been used to obtain an enzyme capable of withstanding higher temperatures. A shift in temperature optimum of *E. coli* phytase from 55°C to 65°C and a significant enhancement in its thermal stability at 80°C and 90°C were achieved by expression of the enzyme in the yeast *P. pastoris* after introduction of three glycosylation sites into the amino acid sequence by site-directed mutagenesis (Rodriguez *et al.*, 2000). Gene site saturation mutagenesis technology was a further approach used to optimize the performance of *E. coli* phytase (Garrett *et al.*, 2004). A library of clones incorporating all 19 possible amino acid changes in the 431 residues of the sequence of the *E. coli* phytase was generated and screened for mutants exhibiting improved thermal tolerance. The most suitable mutant showed no loss of activity when exposed to 62°C for 1 h and 27% of its initial activity after 10 min at 85°C, which is a significant improvement over the parental phytase. In addition, a 3.5-fold enhancement in gastric stability was observed. Recently directed evolution has been applied to improve thermostability of *E. coli* phytase (Kim and Lei, 2008). This approach involved the generation of a vast library of the gene of interest by random mutagenesis (error-prone PCR), followed by screening of mutants for the desired property. Compared with the wild-type enzyme, two mutants (Lys46Glu and Lys65Glu/Lys97Met/Ser209Gly) showed over 20% improvement in thermostability when determined at 80°C for 10 min. In addition, overall catalytic efficiency (k_{cat}/K_M) of Lys46Glu and Lys65Glu/Lys97Met/Ser209Gly was improved by 56% and 152%, respectively, compared with that of the wild type at pH 3.5. Thus, the catalytic efficiency of these enzymes was not inversely related to their thermostability.

By using the consensus approach, which is based on the comparison of amino acid sequences of homologous proteins and subsequent calculation of a consensus amino acid sequence using one of the available standard programmes, a fully synthetic phytase was generated, which exhibited a 21–42°C increase in intrinsic thermal stability compared with the 19 parent fungal phytases used in its design (Lehmann *et al.*, 2002). The consensus phytase was found to be stable in aqueous solutions at 70°C and in feed at pelleting temperatures of 80–90°C (Simon and Igbasan, 2002). Furthermore, by replacing a considerable part of the active site of the generated enzyme with the corresponding residues of the phytase of *A. niger* NRRL 3135, a shift in catalytic properties was observed, demonstrating that rational transfer of favourable catalytic properties from one phytase to another is possible by using this approach (Lehmann *et al.*, 2000). By substituting the glutamic acid residue located in position 27 by leucine, as in *A. terreus* phytase, Tomschy *et al.* (2000a) improved the specific activity threefold without changing its substrate

specificity. A similar increase in specific activity of the phytase from *A. niger* T213 was achieved by substituting the arginine residue in position 297 by glutamine (Tomschy *et al.*, 2000b). In both cases it was suggested that the replaced amino acid residue (Glu27, Arg297) interacted with one of the phosphate residues of phytate and that release of the reaction product *myo*-inositol(1,2,4,5,6)pentakisphosphate was the rate-limiting step in the enzymatic reaction.

A single amino acid substitution in *Yersinia frederiksenii* phytase (Ser51Thr) was shown to improve almost all properties relevant for an application as a feed supplement (Fu *et al.*, 2009). The amino acid replacement shifted the pH optimum from 2.5 to 4.5. The mutant enzyme was shown to be more stable at acidic pH conditions. It retained more than 60% of its initial activity at pH 1.0–2.0, whereas the wild-type phytase was completely inactivated under these conditions. Furthermore, thermal stability was improved by the amino acid replacement. After incubation at 60°C for 2 min, the wild-type phytase was completely inactivated whereas the mutant enzyme retained 45% of its initial activity. Last but not least, the v_{max} values at pH 2.5 and 4.5 for the mutant enzyme were twofold and fivefold higher, respectively, when compared with the wild-type enzyme.

Phytase Production Systems

Finally, a phytase will not be competitive if it cannot be produced at high yield and purity by a relatively inexpensive system. Because wild-type organisms tend to produce low levels of phytase and since purification is both tedious and cost intensive, wild-type organisms are not suitable for industrial applications. Therefore, highly efficient and cost-effective processes for phytase production by recombinant microorganisms have been developed. The fact that most of the phytases characterized to date are monomeric proteins (Konietzny and Greiner, 2002) facilitates their overexpression in microbial and plant, as well as in animal systems. High levels of phytase activity accumulating in the fermentation medium have been described using economically competitive expression/secretion systems for *E. coli* (Miksch *et al.*, 2002) as well as for the yeasts *Hansenula polymorpha* (Mayer *et al.*, 1999) and *P. pastoris* (Yao *et al.*, 1998).

Inclusion of phytase activity in the plant seed itself is an alternative strategy for improving nutrient management in animal production. Increased phytase activity in the plant seed by heterologous expression of fungal and bacterial phytases has already been achieved, and it was shown that only limited amounts of transgenic seeds are required in compound feeds to ensure proper degradation of phytate (Pen *et al.*, 1993). A different strategy to overcome the problems encountered in using phytase as a feed additive, such as cost, inactivation at the high temperatures required for pelleting feed and loss of activity during storage, might be to add those enzymes to the repertoire of digestive enzymes produced endogenously by swine and poultry. In the meantime, swine were generated with a gene from *E. coli* for the production

of a phytase in the saliva (Golovan *et al.*, 2001). It was shown that provision of salivary phytase activity enabled essentially complete digestion of dietary phytate, largely removing the requirement for phosphate supplementation, and reduced faecal phosphate output by up to 75%. This reduction even exceeded the 40% reduction reported for pigs fed phytase supplements.

Summary and Future Directions

Numerous feeding studies with poultry, swine and fish have demonstrated the efficacy of phytase supplementation for improving phosphorus and mineral availability. In particular, microbial phytases offer technical and economical feasibility for their production and application. The greater pH- and thermostability, higher protease tolerance and specific activities of microbial compared with plant phytases make the former more favourable for animal feed applications. However, it is important to realize that no single phytase may ever be able to meet all the diverse needs of its commercial application. Thus, screening nature for phytases with more favourable properties for that application, coupled with engineering them to optimize their catalytic and stability features, is a rational approach to deliver a phytase more suited to animal feed applications. Predictably, the quest for more effective phytases will continue, with emphasis on thermal tolerance, a broad pH activity profile and enhanced stability under the pH conditions of the intestinal tract. In addition to the repeatedly discussed features of an 'ideal' supplementary phytase, a high level of activity on *myo*-inositol pentakisphosphate seems to be desirable. A complete transformation of dietary phytate into *myo*-inositol tetra- and trisphosphates in the stomach seems to be much more important for the bioefficacy of supplementary phytase than a complete dephosphorylation of single phytate molecules, because dietary phosphatases that do not accept phytate as substrate and phosphatases arising from the mucosa of the animal's small intestine are expected to dephosphorylate *myo*-inositol phosphates with up to four phosphate residues sufficiently well.

Furthermore, combined supplementation of phytase with other feed enzymes such as carbohydrases, proteases or phosphatases (inclusive of those accepting phytate as a substrate) should be exploited as a strategy to improve overall nutrient utilization of animal feeds. The combination of xylanase and fungal acid protease with phytase showed additive effects on phytate dephosphorylation *in vitro* (Zyła *et al.*, 1995, 1999). From a further *in vitro* study it was concluded that a combination of *Bacillus* and *Aspergillus* phytase might induce a more efficient phosphate release from phytate in the intestinal tract of animals (Park *et al.* 1999). However, *Bacillus* phytases act effectively only in the small intestine. Due to their susceptibility to pepsin, gastrointestinal carriers might be useful in protecting *Bacillus* phytases from pepsin in the stomach or crop. Aside from the physico-chemical properties of a supplementary phytase, its economic large-scale production is a further aspect that must be considered. Therefore, there is still interest in developing highly efficient and cost-effective processes for phytase production.

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6 Effect of Digestive Tract Conditions, Feed Processing and Ingredients on Response to NSP Enzymes

B. SVIHUS

Introduction

Non-starch polysaccharide-degrading enzymes (NSP-ases) have become an integral part of the feed industry, and are now routinely added to diets for poultry, and to a lesser extent for pigs, throughout the world. A number of fibre-degrading enzymes have been studied but currently the β-glucanases, which degrade β-(1-3)(1-4)-glucans, and the xylanases, which degrade arabinoxylans, are those enjoying the most widespread use and having the best-documented effects. This chapter will therefore be limited to these two classes of enzyme.

Although the beneficial effect of β-glucanases and xylanases on nutrient availability for diets containing wheat, barley, oats or rye is documented beyond doubt, the responses obtained are variable and sometimes lacking. There are a number of possible causes for this, from the obvious ones – that the content of fibre is too low to have any negative effects in the first place – to more sophisticated causes such as those discussed in this chapter. A number of relevant factors implicated in the variable response to NSP-ases are outside the scope and limitations of this chapter. Interaction with the microflora in the digestive tract is one such factor. Variation between different enzyme sources and the optimal dosage of these enzymes is another topic that may have a large influence on the results, but which will not be dealt with here.

The topics discussed in this chapter have been selected not only due to their assumed importance for understanding variation in response to NSP-ases, but also due to the large number of data published on these topics, and therefore the presumably useful mechanistic understanding that can be extracted from these vast sources of scientific data.

 Enzymes in Farm Animal Nutrition, 2nd Edition
(eds M.R. Bedford and G.G. Partridge)

Influence of Digestive Tract Conditions on Effect of Enzymes

Exogenous enzymes added to the diet must exert their effect during the short time from when the feed is moistened in the anterior digestive tract up to the point that feed residues have passed the small intestine. In addition, the range of pH encountered in the digestive tract must be relevant for their activity and must not threaten their stability. Furthermore, the enzyme must be able to withstand the digestive processes in order to function, not the least activity of host digestive proteases. This complicated matrix of conditions will determine the scale and variation of activity of an enzyme added to the diet and thus its biological effects. It is therefore essential to understand these digestive conditions and how they may vary in order to be able to predict the beneficial potential of added enzymes.

Most exogenous NSP enzymes have a pH optimum between 4.0 and 5.0, but great variation may exist between different sources of enzymes, which results in catalytic activity at both lower and higher pH. Xylanases usually have a pH optimum between 4.0 and 6.0 (de Vries and Visser, 2001), but Ding *et al.* (2008) showed that, between pH 3.0 and 7.0, the specific xylanase studied maintained more than 50% of its maximum activity, which occurred at pH 6.0. Similar results were found by Wu *et al.* (2005). This contrasts with Thacker and Baas (1996), who found very low activity of ten different commercial xylanase enzyme preparations when incubated at pH 6.5 or 3.5, but high activity at pH 4.5 and 5.5. In a study of commercial feed enzymes, Ao *et al.* (2008) found very little activity of a xylanase at pH 3.0, but activity was still 64% of maximum activity at pH 7.0. The same authors found a commercial β-glucanase that was reported to have an optimum pH of 5.0, to have similar catalytic activity at pH 3.0 and more than 50% of its maximum activity when pH was raised to 7.0. Baas and Thacker (1996), on the other hand, found a number of commercial β-glucanases of optimum pH 5.5, very low activity at pH 2.5 and 3.5 and a considerably lower activity at pH 6.5. Vahjen and Simon (1999) found similar low activities at pH 6.5 or above for a β-glucanase from *Aspergillus niger* and *Trichoderma reesei*, while activity for a β-glucanase from *Humicola insolens* was still significant at pH 7. Only the β-glucanase from *T. reesei* had any level of enzymatic activity at pH 3.5 or lower. These data highlight that simply noting that a xylanase or glucanase has been employed in an animal trial does not provide any information with regard to the potential activity in the intestine. This is true even if the units of activity added are declared, since the assay (usually pH 5.0–5.5) bears little relationship to the pH range encountered in the intestine.

In addition to pH, enzyme activity is affected by temperature. Most enzymes used today have a temperature optimum between 45 and 65°C (Vahjen and Simon, 1999; Igbasan *et al.*, 2000; de Vries and Visser, 2001; Simon and Igbasan, 2002; Garrett *et al.*, 2004; Wu *et al.*, 2005; Ding *et al.*, 2008), and only small changes in enzyme activity have been observed when temperature increases from 40 to 50°C (Wu *et al.*, 2005; Ding *et al.*, 2008).Thus, body temperature does not appear to be a critical factor in pigs and poultry.

Enzymes are dependent on an aqueous environment to exert their activity. The amount of water needed for optimum activity does not seem to have been studied to a large extent. Denstadli *et al.* (2006) observed that activity of an exogenous phytase was very low both at 25 and 35% moisture. At 45% moisture, however, activity increased dramatically (Denstadli *et al.*, 2006, 2007). Although the effect of higher moisture levels was not tested, the fact that the phytase was able to degrade 50% of the inositol 6-phosphate after only 10 min incubation at 45% moisture indicates that moisture was no longer a critical factor. Whether moisture is essential for mobility of the enzyme, solubility of the substrate and enzyme, or both, is still unclear.

The minimum time needed for an effective degradation of the substrate is another factor that needs to be taken into account. Again, this is something that has not been studied extensively. Under optimal conditions, indications of considerable fibre degradation such as release of degradation products or reduced viscosity have been observed after incubation times of 1.0–2.5 h (Meng *et al.*, 2005; Sørensen *et al.*, 2007). However, Sørensen *et al.* (2007) showed that degradation continued for more than 24 h, which indicates that a considerable time is needed for more complete degradation of non-starch polysaccharides. As NSP-ases are added to the diet primarily to break soluble fibres into smaller fractions with less anti-nutritive properties, complete degradation is probably not needed, although the optimal extent of degradation is unknown.

Although monogastric animals employ similar digestion principles, considerable variation in retention time, moisture content and pH in the different portions of the gastrointestinal tract can be observed, not only between individuals but also between species. Thus, the efficiency of enzymes must be discussed separately for each animal species. Here, discussion will be limited to poultry and pigs.

Poultry

In poultry, passage of ingesta has been shown to be rather fast for both the growing chicken and the laying hen, with most studies showing that a marker added to the feed will appear in the faeces within 2.0–2.5 h after feeding, and most of the marker will have been excreted within 12 h (Tuckey *et al.*, 1958). A typical cumulative passage curve is shown in Fig. 6.1. Marker can be detected up to 72 h after feeding (Duke *et al.*, 1968), but this is due to the fact that a portion of the ingesta may enter the caecum. Although enzymes may effect caecal fermentation through their effect on the amount of substrate and production of oligosaccharides, it is not likely that caecal retention of an enzyme will affect its anti-nutrient-alleviating effect. The effect of retention time in the caecum will therefore not be discussed in this chapter. More recent experiments with broiler chickens have shown that average retention time in the digestive tract, excluding the caecum, is 4–8 h (Shires *et al.*, 1987; van der Klis *et al.*, 1990; Almirall and Esteve-Garcia, 1994; Dänicke *et al.*, 1999; Hetland and Svihus, 2001). For broiler chickens in particular, it is generally

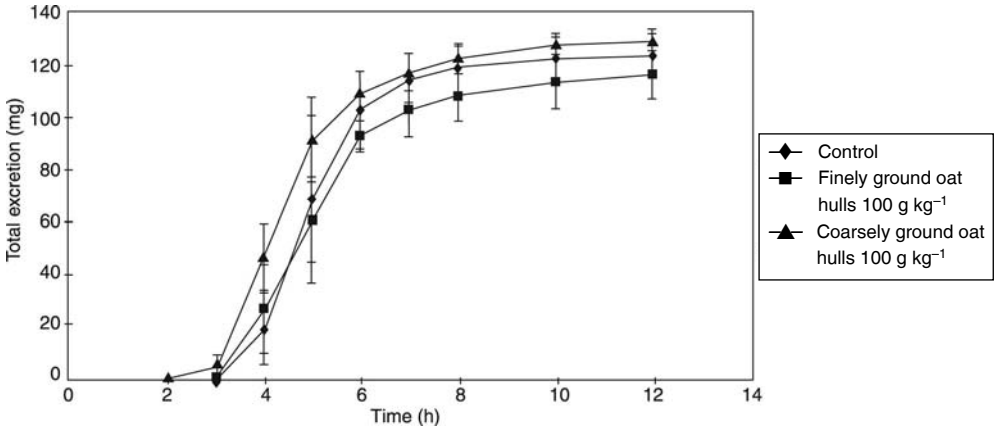

Fig. 6.1. Cumulative excretion rates for broiler chickens fed wheat diets supplemented with oat hulls and without supplementation. Bars indicate standard deviation (n = 4). (From Hetland and Svihus, 2001.)

accepted that the holding capacity of the digestive tract is a major limiting factor to feed intake, at least when pelleted diets are fed (Bokkers and Koene, 2003). A high passage rate would therefore facilitate a high feed intake, which is a significant factor in selection programmes, and this may explain why passage is so fast for broilers and why it may actually be increasing with time.

Even more relevant than total retention is retention time in the different portions of the digestive tract, as specific conditions in different portions may be of major importance for enzyme activity and/or survivability. Retention time in the different segments will be a rather complicated product of flow rate of feed, holding capacity of the different segments and absorption and secretion of material in that segment. In addition, bulk density and variations in bulk density due to, for example, water absorption may also affect retention time, as well as anti-peristaltic movements. In addition, different fractions of the feed may pass through segments at different rates, for example due to the fact that fluids pass more quickly than solids, as shown very clearly for the gizzard by Vergara *et al*. (1989).

Under the assumption that feed is able to absorb water without any considerable swelling, the retention time in the segments anterior to the small intestine will to a large extent be a product of passage rate and holding capacity of these segments. In addition, passage rate has been shown to be dependent on feeding patterns, in particular length of the preprandial fast. It is now well established that feed will pass without entering the crop if the gizzard is empty (Chaplin *et al*., 1992). Jackson and Duke (1995) showed the same to hold true for the gizzard. In an experiment where growing turkeys were fed a finely ground diet after a 10 h fast, the small intestine was filled with feed within 25 min of commencement of feeding. Although the extent to which feed entered the crop varied greatly among individual birds, only 50% of the diet eaten in the morning after an overnight fast and in the afternoon prior to darkness on

average entered the crop. Observations of commercial broilers on *ad libitum* feeding have shown that they eat in a semi-continuous way (Nielsen, 2004), and that the crop is not used to its maximal capacity under such conditions (Denbow, 1994). In fact, the crop is thought mainly to have a role as a storage organ for birds under situations of discontinuous feeding, and is not involved in feed intake regulation (Jackson and Duke, 1995). *Ad libitum* feeding will thus probably result in even less use of the crop. Boa-Amponsem *et al*. (1991) found negligible amounts of feed materials in the crop of *ad libitum*-fed fast- and slow-growing broilers, while intermittent feeding resulted in significantly increased crop contents. Although large variations among individual birds were observed, recent experiments have confirmed that *ad libitum*-fed broiler chickens do not use the crop to any significant extent (Svihus *et al*., 2010). Although more data are needed, this indicates that *ad libitum*-fed birds will adapt a habit of letting feed bypass the crop. When birds are trained to intermittent feeding, however, feed intake changes to the meal type of feeding, which involves transient storage of large quantities of feed in the crop (Svihus *et al*., 2010). Dänicke *et al.* (1999) found average retention time in the crop to be approximately 50 min but, as discussed above, it is obvious that retention time in the crop may vary substantially.

Storage capacity of the anterior digestive tract may increase substantially over time when birds are adapted to intermittent feed availability. Barash *et al.* (1992) showed that birds adapted to two meals per day were able to consume approximately 40% of the daily intake of *ad libitum*-fed birds during each meal. It has been shown that broiler chickens use both the crop and the proventriculus/gizzard as storage organs for food when adapted to long periods of food deprivation (Buyse *et al.*, 1993). Barash *et al.* (1993) observed a significant increase in weight and feed-holding capacity of both crop and gizzard when chicks were fed meals one or two times per day instead of *ad libitum*. Thus, Buyse *et al.* (1993) still found considerable amounts of feed in the crop of broiler chickens 5 h following the previous feed. Studies where broiler chickens had access to feed only every fourth hour have also confirmed that birds store feed in the crop and that feed can be found in the crop at least 3 h following feeding (Svihus *et al.*, 2002). The contents of the crop are gradually moistened, reaching 50% moisture within 90 min, as shown in a recent unpublished experiment (Fig. 6.2). In free-range village hens in Tanzania, crop contents at dawn were found to contain 57% water on average (Mwalusanya *et al.*, 2002). Interestingly enough, Bolton (1965) found that the contents of the crop contained 66% moisture after 1 h when mash feed was given, while when pelleted diets were given the contents still contained less than 50% moisture after 2.5 h. Since the crop is the only segment of the digestive tract where water content may be a limiting factor for enzyme activity, the time needed for soaking may be a critical factor in determining the efficacy of an exogenous enzyme, provided that the crop is indeed a major site of enzyme activity.

Mean retention time in the proventriculus and gizzard has been estimated to vary between 30 and 60 min (Shires *et al.*, 1987; van der Klis *et al.*, 1990; Dänicke *et al.,* 1999). This seems to be in accordance with the results of

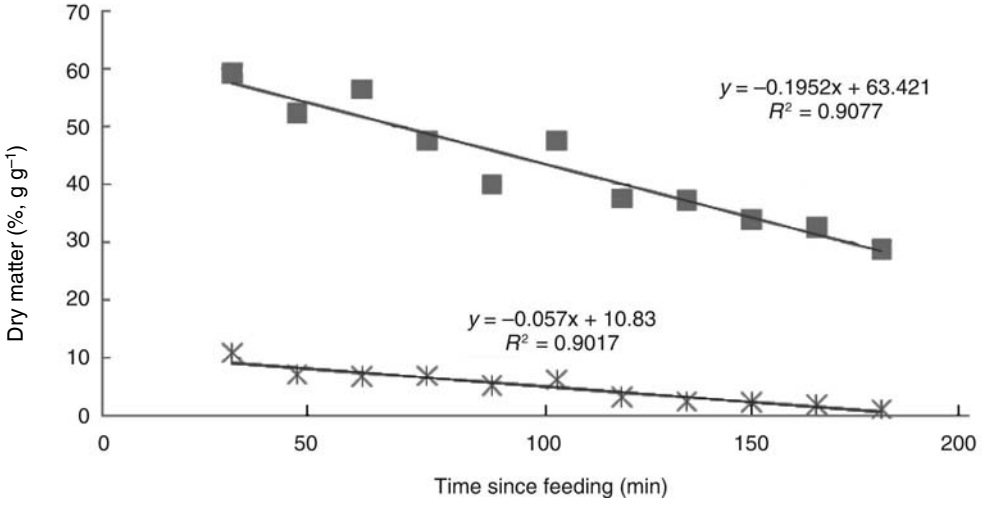

Fig. 6.2. Dry matter percentage (grey squares) and content (asterisks) in crop of meal-fed 20-day-old broiler chickens at different times after having had access to feed for 15 min.

Svihus *et al.* (2002), where 50% of the feed had passed this region within 2 h. It has been shown that the volume of the gizzard may increase substantially when structural components are added to the diet, sometimes to more than double the original size (Amerah *et al.*, 2008, 2009). Although it has been shown that larger particles are selectively retained in the gizzard (Hetland *et al.*, 2003), and that passage rate of a non-structural marker such as titanium oxide is the same independent of diet structure (Svihus *et al.*, 2002), it is obvious that mean retention time of feed particles will increase substantially with increasing diet structure. If retention time is close to 1 h when a standard commercial diet with few structural components is fed, mean retention time can be assumed to approach 2 h if gizzard development is stimulated by added structural components. Selective retention in the gizzard will also result in some fine particles having an extremely short gizzard retention time. Svihus *et al.* (2002) showed that considerable amounts of feed had passed the gizzard within 30 min of feeding. The divergence in retention time for feed particles of different size/characteristics clearly has significant implications for the opportunity for enzyme application on specific components of the diet.

Retention time in the small intestine was calculated to be approximately 220 min by Dänicke *et al.* (1999), while others found retention time in the jejunum and ileum to vary between 136 and 206 min (Shires *et al.*, 1987; van der Klis *et al.*, 1990; Gutierrez del Alamo *et al.*, 2009a,b). This appears to fit with the observation of Svihus *et al.* (2002), where retention time was around 120 min in the segment anterior to the small intestine and where only 20–30% of the marker had passed the ileo-caeco-colonic junction 180 min after feeding. A retention time in the small intestine of 3–4 h is also in accordance with a total tract retention time of 4–8 h, as mentioned above.

From the above, it is obvious that time may be a limiting factor for enzyme activity, particularly in the crop and the gizzard. It is also clear, however, that retention time in the crop and gizzard may be manipulated by dietary structure and feeding management. In addition to the challenge of short retention time, the exogenous enzymes may have an optimum pH that corresponds only to some parts of the digestive tract.

In the crop, large variations in pH have been observed, as summarized in Table 6.1. In a number of experiments, pH has been found to be above >6.0 (Bolton, 1965; Riley and Austic, 1984; Boros *et al.*, 1998; Ao *et al.*, 2008), while a pH between 4.5 and 5.9 has been observed in other experiments (Mahagna and Nir, 1996; Gordon and Roland, 1997; Hinton *et al.*, 2000; Andrys *et al.*, 2003; Huang *et al.*, 2006; Jozefiak *et al.*, 2007; Garcia *et al.*, 2008; Smulikowska *et al.*, 2009). Feeds for monogastrics are usually reported to have a pH varying between 5.5 and 6.5 (Bolton, 1965; Yi and Kornegay, 1996; Carlson and Poulsen, 2003; Partanen *et al.*, 2007; Ao *et al.*, 2008). It is thus reasonable to assume that once feed enters the crop, pH will be similar to that of the feed. However, a prolonged retention time in the crop is associated with a considerable fermentation activity dominated by lactic acid-producing bacteria (Hilmi *et al.*, 2007), with considerable quantities of other short-chain fatty acids also being produced (Huang *et al.*, 2006). Thus, different retention times and therefore different extents of fermentation may explain pH variance between experiments. In accordance with this, Bolton (1965) observed that the pH dropped as retention time increased, but only for chick feed and not for layer feeds, the latter having a higher initial pH and a much higher buffering capacity, presumably due to higher calcium carbonate content. Also, pH was found to be around 4.0 for crops characterized by having large quantities of feed that had remained there for a prolonged time, so-called sour crops. Similarly, Bayer *et al.* (1978) observed that the pH of crop contents collected 2.5 h following meal-feeding dropped from 5.1 to 4.5 during 2 h of incubation *ex vivo*. Mahagna and Nir (1996) found the pH of crop contents to increase from 4.0 to nearly 6.0 from 7 to 21 days of age. The cause for this is probably that retention time decreases with age due to increased feed intake.

The gastric juice secreted from the proventriculus has been reported to have a pH of around 2.0 (Duke, 1986). However, the amount, retention time and chemical characteristics of the feed in the gizzard/proventriculus area will result in a more variable and usually higher pH. In a recent experiment at our laboratory, for example, the pH of gizzard contents from broiler chickens varied between 1.9 and 4.5, with an average value of 3.5. As summarized in Table 6.1, most of the average values recorded in recent years for broiler chickens are reported to be between 3.0 and 4.0 for normal pelleted diets (Steenfeldt, 2001; Andrys *et al.*, 2003; Gabriel *et al.*, 2003; Engberg *et al.*, 2004; Bjerrum *et al.*, 2005; Huang *et al.*, 2006; Jozefiak *et al.*, 2007; Ao *et al.*, 2008; Gonzales-Alvarado *et al.*, 2008; Frikha *et al.*, 2009; Jimenez-Moreno *et al.*, 2009; Shakouri *et al.*, 2009), with average pH values as high as 4.2 and even 5.7 reported in a couple of cases (Smulikowska *et al.,* 2009; Boros *et al.,* 1998, respectively). Older data, however, seem to report pH values between 2.0 and 3.0 (Farner, 1960; McLelland, 1979; Riley and Austic, 1984; Mahagna *et al.*,

Table 6.1. Overview of published data showing pH at different segments of the digestive tract of poultry (broiler chickens, unless otherwise stated).

Crop	Gizzard[a]	Intestine	Comments[b]	Reference
6.3–6.7	–	–	Layer diet	Bolton (1965)
4.5–6.1	–		Chick diet	Bolton (1965)
5.1–5.2	–	–	High-fibre diets	Bayer *et al*. (1978)
6.3–6.9	1.6–2.3	7.3–7.7		Riley and Austic (1984)
–	2.8–3.1	6.2–6.9		Mahagna *et al*. (1995)
3.8–5.8	2.3–3.2	5.5–6.4	7–21 days of age	Mahagna and Nir (1996)
4.6–4.7	3.4–4.1	6.0–6.1	Layers, jejunal samples	Gordon and Roland (1997)
6.3–6.5	4.8–5.7	6.5–7.3		Boros *et al*. (1998)
5.5	–	–	Immediately after feeding	Hinton *et al*. (2000)
–	2.8–3.9	6.4–7.1	Ileal samples	Steenfeldt (2001)
–	2.0–2.6	–	Whole wheat added	Hetland *et al*. (2002)
4.7–5.1	2.2–3.4	5.7–5.8	Duodenal samples, acids added	Andrys *et al*. (2003)
–	3.3–4.0	6.0–6.8	Whole wheat added	Gabriel *et al*. (2003)
–	2.9–3.6	5.8–6.0	Ileal samples, whole wheat added	Engberg *et al*. (2004)
–	2.0–3.6	6.2–7.9	Ileal samples, whole wheat added	Bjerrum *et al*. (2005)
4.9–5.1	3.3–3.8	–	Mash and pellets, coarse and fine	Huang *et al*. (2006)
–	4.1–5.2	–	Layers	Hetland and Svihus (2007)
4.6–5.3	3.0–3.7	5.8–6.3	Ileal samples	Jozefiak *et al*. (2007)
–	3.9–4.8	5.9–7.5	Layers	Steenfeldt *et al*. (2007)
6.5	3.0	7.0–7.5		Ao *et al*. (2008)
5.6–6.2	–	–		Garcia *et al*. (2008)
–	3.2–3.3	5.7–7.4		Gonzales-Alvarado *et al*. (2008)
–	3.5–4.0	–	Pullets	Frikha *et al*. (2009)
–	3.1–3.5	–	Hulls added	Jimenez-Moreno *et al*. (2009)
–	4.3–4.7	6.0–6.4	Layers, whole wheat added	Senkoylu *et al*. (2009)
–	3.3–3.7	6.2–7.4	Ileal samples	Shakouri *et al*. (2009)
4.9–5.2	4.0–4.4	6.1–6.7	Acids added	Smulikowska *et al*. (2009)

[a]Proventriculus and/or gizzard.

[b]Jejunal and ileal samples unless otherwise mentioned.

1995; Mahagna and Nir, 1996), although a similarly low pH has also been reported more recently (Hetland *et al*., 2002). Due to a high calcium carbonate content in the diet, pH values for gizzard contents are commonly between 4.0 and 5.0 for layer hens (Hetland and Svihus, 2007; Steenfeldt *et al*., 2007; Senkoylu *et al*., 2009), although a pH around 3.5 has also been reported for laying hens (Gordon and Roland, 1997).

It has been shown repeatedly that when structural components such as whole or coarsely ground cereals or fibre materials such as hulls or wood shavings are added, the pH of the gizzard content decreases by 0.2–1.2 units (Gabriel *et al.*, 2003; Engberg *et al.*, 2004; Bjerrum *et al.*, 2005; Huang *et al.*, 2006; Gonzales-Alvarado *et al.*, 2008; Jimenez-Moreno *et al.*, 2009; Senkoylu *et al.*, 2009). The logical explanation for this is the increased gizzard volume and thus a longer retention time, which allows for more hydrochloric acid secretion. It must be borne in mind that during grinding contractions in the gizzard, material is returned to the proventriculus, and thus the proventriculus and gizzard must be considerd as one compartment with regard to retention time and pH (McLelland, 1979). Since feed usually has a pH close to neutral, high feed intake can be expected to result in an elevated gizzard pH, unless gastric juice secretion is able to increase in accordance with intake. This is probably the main reason why gizzard pH is reported to be higher with pelleted diets as compared with mash diets (Huang *et al.*, 2006; Frikha *et al.*, 2009), although reduced structure due to the grinding effect of pelleting will also contribute to this effect (Svihus *et al.*, 2004).

In the small intestine, pH is less variable than in the crop and the gizzard (Table 6.1). The acidic contents from the gizzard are rapidly neutralized by the alkaline secretions from the pancreas and intestinal wall, resulting in average pH values most commonly varying between 6.5 and 7.5, although average values as low as 5.5 and as high as 7.9 have been reported (Riley and Austic, 1984; Mahagna *et al.*, 1995; Mahagna and Nir, 1996; Boros *et al.*, 1998; Steenfeldt, 2001; Andrys *et al.*, 2003; Gabriel *et al.*, 2003; Engberg *et al.*, 2004; Bjerrum *et al.*, 2005; Ao *et al.*, 2008; Gonzales-Alvarado *et al.*, 2008; Shakouri *et al.*, 2009; Smulikowska *et al.*, 2009).

From the foregoing, it is obvious that both pH and retention time following moistening are limiting factors for breakdown of anti-nutritive NSPs in the avian digestive tract. Under commercial *ad libitum* feeding conditions and under the assumption that in such a case the feed does not have significant retention time in the crop, it seems clear that moistening of the feed becomes a critical factor in the anterior digestive tract. This is particularly so for pelleted diets, which have been shown to moisten more slowly than mash diets. Adding to the limitations of the anterior digestive tract as a site for NSP-ase action is the fact most diets used today have very little structure, which reduces retention time in the gizzard. The pH seems to be within an acceptable range for the crop, except possibly for layers, where the pH has been shown to remain high even after prolonged retention time (Bolton, 1965). The pH in the gizzard, however, may often be too low for any appreciable NSP-ase activity. Considering all these factors together, it can be concluded that, for modern poultry fed a pelleted diet with few structural components *ad libitum*, the anterior digestive tract may not be an important site for NSP-ase action. This conclusion is in accordance with experimental data showing small or no reduction in viscosity in the anterior digestive tract after NSP-ase addition (Boros *et al.*, 1998; Lazaro *et al.*, 2004; Senkoylu *et al.*, 2009). By the time the ingested material enters the small intestine it will be well moistened, and the acceptable pH and the rather long retention time in this segment favours

activity of the β-glucanases and xylanases added to the diet, although the pH may be too high for some enzymes. As discussed above, data from the literature seem to be conflicting in this area, but results such as those from Baas and Thacker (1996) and Thacker and Baas (1996) indicate that activity will be low at a pH of 6.5 or above, a value often reported, particularly in the lower digestive tract.

A further question is whether enzymes will survive the proteolytic activity of the gastric region. This seems not to have been studied extensively, but the few data that exist specifically for poultry seem to indicate that, although enzyme activity is reduced after incubation in gastric juices, the majority remains. Almirall and Esteve-Garcia (1995) found that after incubation at pH 3.2 with pepsin present, a β-glucanase retained its activity even after 90 min. Vahjen and Simon (1999) found the activity of a number of xylanases to be between 60 and 95% of their original activity after a 30 min incubation in avian gastric digesta. Also, a high activity level of enzymes in the small intestine of broiler chickens fed diets containing β-glucanase or xylanase has been reported, indicating that these enzymes can withstand gastric degradation (Annison, 1992; Inborr and Bedford, 1994). Similarly, Boros *et al.* (1998) observed no viscosity reduction in the gizzard but a large reduction in the small intestine, indicating that enzymes were active in this segment. Stability may vary between enzyme sources, however, since Annison (1992) found no ileal xylanase activity for several of the enzyme preparations tested. A small to moderate reduction in activity after retention in the gastric region is in accordance with results obtained with pigs sampled 2 h after feeding (Inborr *et al.*, 1999) and with *in vitro* experiments (Hristov *et al.*, 1998; Morgavi *et al.*, 2001).

From the foregoing it may be postulated that, under current commercial conditions, the majority of enzyme activity takes place in the small intestine, where survival during passage through the gastric region and the small intestine, coupled with pH higher than optimum in the small intestine, creates the major limitations to efficacy. These limitations are difficult to overcome, since pH in the small intestine is closely regulated and conditions in the gizzard are difficult to change without precipitating major negative effects such as lower diet digestibility or increased risk of pathogens entering the small intestine. Coating of the enzyme such that it bypasses the gizzard may be a possibility, but a risk of reduced activity due to lag of release and hence activity of the enzyme in the small intestine is inherent in this strategy.

Manipulation of retention time in the anterior digestive tract is probably a more feasible strategy. As shown above, a change from *ad libitum* to intermittent feeding would train birds to use the crop as an intermediate storage organ for feed, and thus would increase retention time considerably. Feeding only every fourth hour has been shown to give similar weight gain to *ad libitum* feeding, and would result in an average retention time in the crop of 2 h, as compared with an assumed negligible retention time under *ad libitum* feeding. Even if it is assumed that 1 h is needed to moisten the feed sufficiently for enzymes to exert their activity, such a feeding strategy would still allow 1 h retention time in the crop under close to optimal moisture, temperature and

pH conditions for NSP-ases. In addition, enzymatic degradation can be assumed to continue as the moistened feed material enters the gizzard, until the pH drops too low for activity. Due to the higher buffering capacity of layer diets, this strategy may be limited to meat-producing birds. Retention time in the gizzard could also be increased by feeding a diet with more structural components such as coarse cereals or hulls, which would further increase retention time through increased volume of the gizzard. However, the more acidic conditions created in the gizzard due to increased dietary structure would reduce the activity of the enzymes in the gizzard and would also increase the risk of their inactivation, both through low pH and proteolytic destruction. The net effect of this is therefore uncertain for meat-producing birds. An increased retention time in the gizzard would possibly be particularly effective for improving enzymatic degradation for layers, since the higher pH of the diet probably would result in prolonged favourable conditions in this segment.

Pigs

In pigs, passage through the digestive tract is much slower than for poultry, with mean retention time reported to vary between 32 and 85 h (Freire *et al.*, 2000; Partanen *et al.*, 2007; van Leeuwen and Jansman, 2007; Wilfart *et al.*, 2007). As with poultry, the digestive tract anterior to the large intestine is the relevant segment in relation to effect of enzymes. Since the bulk of total retention time is in the large intestine, usually reported to be between 26 and 73 h (Partanen *et al.*, 2007; van Leeuwen and Jansman, 2007; Wilfart *et al.*, 2007), it is obvious that feed spends proportionately much less time in the small intestine and the stomach. Partanen *et al.* (2007) found total retention time in the stomach and small intestine to vary between 7.2 and 11.2 h. Large variations in retention time in the stomach have been reported. Van Leeuven and Jansman (2007) found retention time in the stomach to vary between 3 and 6 h, while Wilfart *et al.* (2007) found it to be around 1 h. The cause for this large difference is probably that van Leeuven and Jansman (2007) fed the pigs only twice daily, while the pigs in the Wilfart *et al.* (2007) experiment were fed every fourth hour.

With a limited number of meals per day it is logical that larger quantities are stored in the stomach, and assuming that feed is metered into the small intestine at a constant rate, limiting access to feed will therefore increase gastric retention time. This is in accordance with findings of Rapp *et al.* (2001), who also studied passage of material from the stomach of pigs fed twice per day. It was found that only about one-third of the ingested material had passed the stomach after 1 h, and that more than 20% of ingested material still remained in the stomach after 6 h. An average gastric retention time of 2.5–6.0 h has been reported in a number of experiments where feeding has been restricted to two or three times per day (Gregory *et al.*, 1990; Potkins *et al.*, 1991; Johansen *et al.*, 1996; Snoeck *et al.*, 2004). Also, Gregory *et al.* (1990) showed that retention time increased with size of the meal, thus demonstrating that both size and number of meals per day have a large influence on retention time.

Retention time in the small intestine is usually reported to be between 4 and 10 h (Potkins *et al.*, 1991; Partanen *et al.*, 2007; van Leeuwen and Jansman, 2007; Wilfart *et al.*, 2007), although up to 20 h has been reported for diets with a high water-holding capacity (van Leeuwen and Jansman, 2007).

The pH of the stomach contents of pigs will necessarily vary with time after feeding, nature of the feed and amount of feed in the stomach, as for poultry. Average pH values are usually reported to be between 3.0 and 5.0 (Potkins *et al.*, 1991; Baas and Thacker, 1996; Yi and Kornegay, 1996; Kemme *et al.*, 1998; Inborr *et al.*, 1999; Medel *et al.*, 1999; Ange *et al.*, 2000; Mikkelsen *et al.*, 2004). It has been shown that pH in the proximal stomach increases slightly after feeding, and then gradually decreases after prolonged feed withdrawal (Ange *et al.*, 2000). Potkins *et al.* (1991) also observed that pH decreased from 5.0 30 min after feeding to 3.7 after 4 h and 2.8 after 7.5 h. This is in accordance with results from Baas and Thacker (1996), who found the pH falling from 4.8 to 4.0 after 4 h retention time in the stomach. To conclude, an average pH of stomach contents of around 4.0 seems to be a good estimate, with a higher pH during the first hours following feeding and a lower pH after a long time of feed withdrawal.

In the small intestine, a similar range of pH values as for poultry has been reported. Inborr *et al.* (1999) found pH values between 7.8 and 8.3 in ileal contents, while Partanen *et al.* (2007) found the pH values in contents from the same segment to vary between 5.7 and 6.0. A pH between 6.0 and 7.5 seems to be the most common, however (Mathew *et al.*, 1996; Cuche and Malbert, 1998; Franklin *et al.*, 2002; Nyachoti *et al.*, 2006).

Despite some early concerns that exogenous enzymes are not effective for pigs, there have been a number of experiments published recently showing that exogenous enzymes are in fact able to function in the digestive tract of the pig, although this has primarily been shown in experiments with phytase (Rapp *et al.*, 2001; Oryschak *et al.*, 2002; Kemme *et al.*, 2006). Thus, the lack of effect in the early experiments with NSP-ase added to the diet could be due to the fact that pigs are less sensitive to the anti-nutritive properties of soluble fibres (Bedford and Schulze, 1998), rather than to the concern that the digestive tract of the pig is inhospitable to exogenous enzymes. Nevertheless, the question still remains as to whether conditions are sufficient to allow NSP-ases to exert a meaningful biological response.

Based on the above discussion, it appears that retention time in the stomach and the small intestine are not limiting factors, with a possible exception for retention time in the stomach under conditions of *ad libitum* feeding. When it comes to pH, the value is somewhat too low in the stomach and somewhat too high in the small intestine, although the stomach values do not deviate as much from the optimal value as is the case for poultry. The considerably longer retention time in the stomach and the initial higher pH value after a meal will potentially create a favourable environment for high enzyme activity.

Due to the difficulty in measuring NSP-ase activity, very few experiments appear to have been carried out to assess activity of xylanase and β-glucanase *in situ* in the stomach. However, a number of experiments have been carried

out to test the activity of phytases in the stomach of pigs. Phytases are usually reported to have a pH optimum of between 4.5 and 5.5, and although there is a considerable activity still remaining at pH 3.5, this falls rapidly with decreasing pH in a similar way to NSP-ases (Igbasan *et al.*, 2000; Simon and Igbasan, 2002; Tomschy *et al.*, 2002). Despite this, data have shown that 52% of inositol 6-phosphate is degraded in the pig's stomach, and that this value increased to only 65% in the ileum (Kemme *et al.*, 2006). This indicates that the stomach is the most important site for enzymatic phytate degradation. Kemme *et al.* (1998) similarly concluded that almost all phytate could potentially be degraded during 8 h retention time in the stomach, given that enough phytase is present. Although phytase appears to be somewhat more acid tolerant than xylanases and β-glucanases, these results indicate that the stomach may be a site for considerable NSP-ase activity in the pig. This conclusion is supported by the results of Inborr *et al.* (1999), who studied xylanase and β-glucanase activity in the stomach of pigs, and mimicked these conditions *in vitro* using both optimal pH conditions and those as found in the stomach. There was a considerable activity after 2 h, during which time pH decreased from 4.9 to 4.3, although activity for xylanase was halved when the analysis was done under the conditions of the stomach. After 4 h, when pH had fallen to 2.9, activity was considerably reduced.

The potential for enzymes to exert their effect in the small intestine depends on the conditions in the small intestine and the extent to which the enzymes are degraded during exposure to low pH and pepsin in the stomach. It is worth noting that Morgavi *et al.* (2001) concluded that NSP-ases were only modestly susceptible to degradation at low pH with pepsin present, and that Hristov *et al.* (1998) found similar results when the pH was 3.0 or higher. A number of experiments have been carried out to address this issue. Baas and Thacker (1996) and Thacker and Baas (1996) studied the survivability of five different commercial sources of β-glucanase and xylanase, respectively, when incubated at different pH levels and incubation times to simulate the pig's stomach. For xylanase, incubation at pH 3.5 for 1 h or more with pepsin resulted in significantly reduced activity when the pH was subsequently raised to optimal levels, while the loss in activity generally was small when this pre-incubation was at pH 4.5. Also, enzyme activity was not dramatically reduced, even after 4 h in the stomach at a pH that varied between 4.0 and 4.8. β-Glucanase activity was not as susceptible as most xylanase preparations after incubation at pH 3.5, with more than 50% of the maximum activity still remaining after 2 h. In the stomach, however, a considerable reduction in β-glucanase activity was seen already after 1 h, and after 4 h the enzyme had lost around two-thirds of its activity, suggesting that the *in vitro* model may not be a true representation of *in vivo* conditions. As mentioned above, Inborr *et al.* (1999) found enzyme activity to be largely intact after 2 h in the stomach at a pH between 4.3 and 4.9, while activity had fallen considerably after 4 h when pH in the stomach had dropped to 2.9.

A study of survivability of phytases has resulted in a similar conclusion, i.e. that a considerable proportion of the enzymes will be degraded in the stomach

(Yi and Kornegay, 1996). In this context, it is interesting to note that Wyss *et al.* (1999) found that there was a considerable difference between different phytases with regard to their resistance towards proteases. From this it can be concluded that the environment in the stomach will to some extent inactivate exogenous enzymes, and that the gravity of this effect will be determined largely by pH and retention time, and by the characteristics of the enzyme itself. Likewise, it can be concluded that some enzyme activity will still remain in the material entering the small intestine.

Despite the fact that enzyme activity is still apparent in the digesta entering the small intestine, it has been reported to decrease with passage of material down the small intestine (Yi and Kornegay, 1996). This could be due to digestion by endogenous proteases and microbial activity in the posterior small intestine. Despite the fact that Yi and Kornegay (1996) concluded that the small intestine is not an important site of action for phytases, this matter does not appear to have been investigated in sufficient detail to draw such conclusions with regard to NSP-ases. Since retention time in the small intestine may be so much longer than in the stomach, this may well make up for any loss in activity during transit. The pH of small intestinal contents does vary, but there remains considerable opportunity for NSP-ase activity in those segments of the upper small intestine where pH is often below 6.5.

From the foregoing it can be postulated that the digestive tract of the pig is largely favourable for catalytic activity of NSP-ases, and that within the tract the stomach appears to be the segment with the greatest potential. However, from the data discussed above it is also reasonable to assume that an increased retention time in the stomach, as facilitated by only two or three meals per day, would further increase the potential for enzyme activity. Since pH in the stomach appears to be a critical factor for enzyme activity in this segment, the use of ingredients or additives that increase the pH of the diet could also possibly facilitate enzymatic degradation. Ange *et al.* (2000), for example, showed that pH of stomach contents from pigs increased from around 3.5 to 4.7 when 200 mOsm bicarbonate salts were added to the drinking water. It is probably less relevant to influence the conditions of the small intestine; retention time is less easily influenced in the small intestine than in the stomach, and in addition it has been shown that intestinal pH is not easily manipulated through use of components added to the diet (Riley and Austic, 1984; Andrys *et al.*, 2003; Partanen *et al.*, 2007). Thus, it is probably not a feasible strategy to try to manipulate conditions in the small intestine through dietary composition or feeding management.

Conclusion

As an overall summary of the discussion of the interaction between gut conditions and exogenous enzyme addition, it is clear that for both pigs and poultry, conditions in many parts of the digestive tract are amenable to the activity of exogenous enzymes. Retention time in the anterior digestive tract appears to be a limiting factor for poultry, while this is less of a problem with

the pig, except possibly under *ad libitum* feeding conditions. Also, pH in the stomach appears to be somewhat higher in the pig than in poultry. This could partly be due to the fact that the stomach of the pig has a storage function, where large quantities are deposited during feeding, which increases pH for a considerable time due to the buffering capacity of the neutral feed. Based on this, and the fact that retention time in the small intestine is longer in pigs than in poultry, it can be postulated that the digestive tract of the pig is more favourable for catalytic activity of exogenous enzymes than that of the bird. Yet the majority of the data suggest that efficacy in poultry is superior and more consistent: this may be a result of the lower moisture content of the digesta in poultry compared with pigs, which by definition would effectively concentrate the viscous anti-nutrients that are the targets of NSP enzymes. Thus, although conditions in the digestive trace of swine may favour enzyme activity, this very activity is required far more in the avian gastrointestinal tract.

Efficacy of exogenous enzymes in poultry could possibly be increased by facilitating a longer retention time in the anterior digestive tract, through intermittent feeding and an increased content of structural components in the diet.

Ingredient Factors

Feeds for poultry and pigs are composed of a large number of different ingredients. Numerous interactions between ingredients, processing and the effect of enzymes can therefore be envisaged. In this section, the discussion will be limited to ingredients affecting pH and buffering capacity of the diet and to variation in fibre content and properties of cereals. Other relevant topics, such as the effects of minerals on enzyme activity or the effect of variation in plant protein sources, are outside the limits of this chapter.

Effect of ingredients that alter pH in the digestive tract

Ingredients that alter pH are most likely to influence enzyme activity through changes in pH during retention in the anterior digestive tract. As discussed above, pH during retention in the crop may be too high for optimal enzyme activity, while pH during retention in the gizzard of the bird or stomach of the pig may be too low. The influence of altering the pH of the diet on the effect of enzymes has not been studied extensively, but data indicate that pH of both the crop and the gizzard/stomach can be affected by ingredients such as acids or calcium and, since most enzymes are sensitive to pH and pH may be marginal in these segments, it is possible that such ingredients would have a positive effect.

For pigs, increasing dietary pH by adding limestone would potentially counteract the reduction in pH from increased retention time in the stomach, and thus enhance degradation activity of added enzymes. For poultry, however, the situation is more complex, since extended retention in the crop would

benefit from addition of acids to reduce pH, while extended retention in the gizzard would benefit from addition of limestone or other basic ingredients to increase pH. In addition, layer diets already contain large quantities of limestone, resulting in a favourable pH in the gizzard. Thus, for layers the most suitable strategy would possibly be to improve dietary structure such that retention time in the gizzard increases. As discussed earlier, there is great potential for increase in retention time in the avian crop. Since the crop does not actively adjust the pH of the contents, reducing pH by addition of acids would have great potential, at least for broiler chickens. A reduction of pH from around 6.0 to 5.0 would increase activities of most exogenous enzymes significantly. However, Smulikowska *et al.* (2009) did not observe any synergistic effect of enzyme and organic acid addition in broiler chickens. In this experiment, crop pH was reduced from 5.2 to 4.9 due to the addition of organic acids. The low pH of the control in this experiment and the small effect of the additive could thus be the cause for the lack of beneficial effects. Also, there was no estimate in this study of the retention time of feed in the crop.

For pigs, while addition of limestone or other basic ingredients would potentially increase efficacy of exogenous enzymes, such an effect would be counteracted as a result of increased hydrochloric acid secretion. Also, if an increased pH of the stomach was achieved it might have other disadvantageous effects, such as reduced diet degradation rate and thus lower digestibility. In conclusion, adding acids appears to be an interesting option when optimizing enzyme efficiency for broilers under feeding management regimes that allow for a long retention time in the crop, while the effect of adjusting pH for layers and pigs is less certain.

Variation in cereals affecting response to enzymes

Although enzymes with new target substrates are continuously being developed, β-glucanases that target β-(1-3)(1-4)-glucans and xylanases that target arabinoxylans still dominate the market. In addition, these are the enzymes where the mechanism of action is best documented. Of all ingredients utilized in feed manufacture, cereals are the principal source of β-glucans and arabinoxylans, and thus this discussion will be limited to cereals. Maize, wheat and barley and are the most important feed cereals globally. Since maize has the lowest fibre content of these, with negligible amounts of β-glucans and only 0.5% soluble arabinoxylans (Knudsen, 1997), this cereal has not been considered to be significantly responsive to NSP-ase supplementation. Although experiments have frequently failed to demonstrate any beneficial effect of NSP-ase supplementation (Persia *et al.*, 2002; Palander *et al.*, 2005; Yu *et al.*, 2007; Olukosi *et al.*, 2008; Shakouri *et al.*, 2009), some experiments have shown significant improvements in performance and/or nutrient digestibility when xylanase was been added to maize-based diets (Zanella *et al.*, 1999; Cowieson and Ravindran, 2008a,b; Gracia *et al.*, 2009). Although the soluble arabinoxylan content is low, maize contains approximately 5% insoluble

arabinoxylans (Knudsen, 1997). A beneficial effect of xylanase could therefore be mediated through destruction of endosperm cell wall integrity and thus release of proteins and starch entrapped in these cells. However, the enzyme cocktail used included other enzymes such as proteases and amylases, thus making it difficult to ascribe effects such as an increased starch (Zanella *et al.*, 1999; Gracia *et al.*, 2003) and/or protein (Zanella *et al.*, 1999; Cowieson and Ravindran, 2008a,b; Gracia *et al.*, 2009) digestibility to NSP-ase addition. In addition, adding amylase (Gracia *et al.*, 2003) or protease (Yu *et al.*, 2007) alone has been demonstrated to improve performance. It is therefore difficult to conclude whether the beneficial effects observed are due to the amylase and protease in the enzyme cocktail or to NSP-ase.

The variation in nutritional value of barley and wheat, and the beneficial effects of enzyme addition, have been extensively studied in poultry, but with fewer experiments published and less conclusive effects found for pigs. Therefore, the discussion of the interaction between variation in these cereals and enzyme addition will be carried out using the broiler chicken as a model. Although the magnitude of the response will possibly be less for the pig, it is reasonable to assume that the fundamental mechanisms will be the same.

It has been observed frequently that different varieties and batches of wheat and barley may vary considerably in nutritional value when fed to broiler chickens (Mollah *et al.*, 1983; Rogel *et al.*, 1987; Choct *et al.*, 1999; Steenfeldt, 2001; Svihus and Gullord, 2002; Scott *et al.*, 2003; Choct *et al.*, 2006; Maisonnier-Grenier *et al.*, 2006; Gutierrez del Alamo *et al.*, 2008). This variation, summarized in Table 6.2, has been linked to the content and properties of fibres in the cereal (Annison, 1991; Choct *et al.*, 1995, 1999, 2006; Carré *et al.*, 2002), although exactly how these fibres affect nutritive value is still not fully understood.

Viscosity has been shown to be one important factor determining anti-nutritive effects (Choct *et al.*, 1995; Carré *et al.*, 2002; Svihus and Gullord, 2002; Gutierrez del Alamo *et al.*, 2008), but a clear relationship between viscosity and nutritional value has not always been observed for wheat (McCracken *et al.*, 2001; Svihus and Gullord, 2002), particularly when the study tests large numbers of batches. In addition to a direct viscosity effect, it has been shown that soluble fibres may also have negative effects through stimulation of bacterial proliferation in the small intestine (Choct *et al.*, 1996). An alternative explanation for the negative effect of fibres and the beneficial effect of enzymes is that fibres, being a part of the cell wall, may entrap nutrients. Although conclusive evidence for the accuracy of this theory is lacking, Maisonnier-Grenier *et al.* (2006) and Cowieson *et al.* (2005) did observe that fibres were solubilized when enzymes were added, in accordance with the mechanisms inherent in this theory. Similarly, Bedford (2002) observed that small-intestinal material contained particles with intact cell walls apparently containing entrapped nutrients. Pelleting, and in particular extrusion, can be assumed to cause rupture of cell walls, and has been shown to cause considerable reduction in particle size (Svihus *et al.*, 2004). If the theory of cellular nutrient entrapment is correct, the effect of NSP-ases on pelleted or extruded diets would be expected to be reduced. This has not been the case for either pelleted

Table 6.2. Overview of published data showing variation in nutritive value (apparent metabolizable energy (AME), in MJ kg^{-1}) of wheat and barley, and the effect of enzyme addition.

AME[a]	AME (diet with added NSP-ase)	Comments	Reference
11.0–15.9	–	80% wheat, cold-pelleted diets	Mollah *et al.* (1983)
10.4–14.8	–	82% wheat, cold-pelleted diets	Rogel *et al.* (1987)
12.0–14.5	14.8–14.9	80% wheat, cold-pelleted diets	Choct *et al.* (1995)
13.7–15.3	15.1–15.8	80% wheat, mash diets	Scott *et al.* (1998)
11.7–13.9	13.6–14.9	80% barley, mash diets	Scott *et al.* (1998)
9.2–15.0	–	82% wheat	Choct *et al.* (1999)
13.4–14.4[b]	0.6 units higher[c]	79% wheat, pelleted diets	McCracken *et al.* (2001)
12.7–14.7	–	81.5% wheat, mash diets	Steenfeldt (2001)
11.1–13.3	11.6–14.4	77% wheat, cold-pelleted diets	Svihus and Gullord (2002)
10.5–13.3	12.7–13.7	77% barley, cold-pelleted diets	Svihus and Gullord (2002)
9.1–13.1	10.5–13.6	80% wheat, mash diets	Scott *et al.* (2003)
11.5–13.6	0.6 units higher[c]	82% wheat, cold-pelleted diets	Choct *et al.* (2006)
12.2–13.4	12.9–13.8	59.7% wheat, pelleted diets	Maisonnier-Grenier *et al.* (2006)
12.2–12.8	12.1–13.0	70% wheat, mash diets	Gutierrez del Alamo *et al.* (2008)

[a]Values are for the complete diet.
[b]Values are calculated for the wheat fraction only.
[c]Average value for all batches tested.

or extruded diets (Vranjes *et al.*, 1996; Scott *et al.*, 2003, respectively). As will be discussed below, the process of pelleting and extrusion not only disrupts cell wall structure but also results in a greater proportion of the soluble fibre becoming viscous. Since soluble fibre content and viscosity have been shown to increase during these processes, it cannot be ruled out that this effect has overruled the beneficial effect of cell wall rupture.

Independently of the mechanisms discussed above, fibre-degrading enzymes have been shown consistently to improve nutrient utilization in diets containing different batches of wheat and barley (Choct *et al.*, 1995, 2006; Scott *et al.*, 1998, 2003; McCracken and Quintin *et al.*, 2000; McCracken *et al.*, 2001; Svihus and Gullord, 2002; Maisonnier-Grenier *et al.*, 2006; Gutierrez del Alamo *et al.*, 2008; Table 6.2). In addition, several studies have shown that the improvement in nutritional value with enzyme addition is particularly large for batches of cereals with low nutritional value (Choct *et al.*, 1995, 2006; Scott *et al.*, 1998; Svihus and Gullord, 2002; Gutierrez del Alamo *et al.*, 2008). First, this indicates that fibre content and anti-nutritive properties are major causes for variation in the nutritional value of batches of wheat and barley. Second, it indicates that enzyme addition is particularly desirable when the cereal used has a low nutritional value. However, fibre is not the sole determinant of wheat or barley quality. Some batches of wheat and barley do not respond significantly to enzymes despite a determined high

viscosity and low nutritive value. One possible cause could be a high content of enzyme inhibitors in the cereal that negate the efficacy of the NSP-ase, as discussed by Cowieson *et al.* (2006). Alternatively, nutritive value may be compromised by low nutrient content, for example due to low starch content in the endosperm caused by unfavourable conditions such as drought during the latter part of plant growth. Svihus and Gullord (2002) found a significant correlation between starch content and nutritional value of wheat. Such problems would not respond to an enzyme targeting the fibre of cereals.

Conclusion

It is clear from this review that ingredients can have a major influence on effect of enzymes. Altering dietary pH will alter the pH of the anterior digestive tract, with potential ramifications for enzyme efficacy. Wheat and barley may vary considerably in nutritional value and, in many cases but not always, the efficacy of the addition of xylanase and β-glucanase will be particularly noticeable for batches with a low nutritional value.

Influence of Processing on Effect of Enzymes

With a few exceptions, such as the α-amylase isolated from a hyperthermophilic bacterium and having a temperature optimum of 100°C (Leuschner and Antranikian, 1995), most enzymes will lose catalytic capability when exposed to high temperatures. The three-dimensional structure of the protein, held together by covalent and non-covalent bonds and which is a prerequisite for catalytic activity, is destroyed as the temperature rises to the point where the protein unfolds and becomes denatured. This process can be considered as a two-stage process, where the first modification, usually through breakage of non-covalent bonds, is reversible, while the second step causes irreversible changes due to breakage of covalent bonds such as disulfide bridges (Weijers and van't Riet, 1992). This denaturation process is facilitated by high water content, under which denaturation commences when the temperature exceeds the temperature for maximum enzyme activity.

The general mechanisms have been extensively reviewed by Adams (1991) and Ludikhuyze *et al.* (2003), and will be only briefly outlined here. Water molecules interact with the enzyme through non-covalent van der Waals bonds, and may even contribute to conformational stability by forming a membrane around the enzyme (Adams, 1991). As temperature increases and water molecules reach a higher energetic state, however, water molecules will destabilize enzymes in a concentration-dependent manner. Due to this interaction, enzymes can withstand severe heat treatments at very low water concentrations (Ludikhuyze *et al.*, 2003). With excess water content, most feed enzymes will start to denature at temperatures between 60 and 70°C, although some enzymes may be inactivated already at temperatures above 40°C while others may be stable at 80°C or higher (Adams, 1991). The heat

stability of enzymes is determined mainly by the extent to which the enzyme is stabilized by either covalent bonds or prosthetic groups, with disulfide bonds and calcium ions, respectively, being good examples of these. Pressure has been shown to facilitate enzyme denaturation mainly through breakage of non-covalent bonds, although sulfydryl groups and disulfide bonds may also be affected (Ludikhuyze *et al.*, 2003). Since hydrogen bonds are less affected, pressure inactivates enzymes mainly through changes at the tertiary and quaternary levels. A synergy between pressure and temperature has been observed for many enzymes, although an antagonistic relationship has also been observed for some enzymes at some temperature–pressure combinations (Ludikhuyze *et al.*, 2003).

Feeds for pigs and poultry are exposed to heat mainly during the pelleting process, in which the ground ingredients are moulded into macro-particles. In some cases, feeds will be exposed to elevated temperatures through other processes such as expansion, extrusion and dry heating, but these processes are not commonly used in diets for pigs and poultry, and will therefore not be the main focus of this chapter. In the pelleting process, the dry feed ingredients are conditioned in a process where saturated steam is injected into the feed while it is being mixed in a paddle mixer. This process, which usually takes less than 1 min, results in a temperature rise to around 75°C, and at the same time moisture level increases from 12 to 15–16%. Immediately following this conditioning process, the feed enters the pellet press, where it is forced through cylindrical holes in a die and is shaped into pellets. Due to the friction caused by the rolls that force the material into the holes and the friction in these holes, the temperature rises further to around 80–85°C (Svihus *et al.*, 2004), although this increment is very much dependent upon the formulation of the diet and processing conditions. Thus, the process of shaping feeds into pellets exposes most enzymes added to the feed to temperatures above their denaturation temperature. In addition, the pressure incurred by the process will also facilitate enzyme degradation. Conversely, the low water content and the short exposure time are factors limiting enzyme denaturation. Thus, predicting the extent to which enzymes are inactivated when added to the diet prior to conditioning/pelleting is not straightforward.

Studies carried out to assess the stability of NSP-ases during pelleting indicate that the combination of pressure and heat applied during the process may inactivate enzymes, despite the low water content, but that conditioning and pelleting under conditions of low temperature may spare enzymes from inactivation (Inborr and Bedford, 1994; Spring *et al.*, 1996; Silversides and Bedford, 1999; Vahjen and Simon, 1999; Samarasinghe *et al.*, 2000; Cowieson *et al.*, 2005). Since Spring *et al*. (1996), Samarasinghe *et al.* (2000) and Cowieson *et al.* (2005) found that pelleting temperature had to reach 90°C before any NSP-ase inactivation was observed, while Inborr and Bedford (1994) and Silversides and Bedford (1999) observed reduction in enzyme activity when the pelleting temperature reached 80°C, these results all show that the pelleting process is not a constant between mills, or the enzymes employed in each study differ in stability, or both. It does suggest, however, that in many cases the pelleting process operates at the threshold of enzyme

inactivation conditions. Similar results have been observed with phytase, although this enzyme appears to be even more sensitive to conditions during pelleting, with more than 50% of the activity being lost even at pelleting temperatures not exceeding 70°C (Slominski *et al.*, 2007).

The negative effect of soluble fibre, which exogenous enzymes are supposed to degrade, is at least partly due to its role in increasing intestinal viscosity, an effect which acts as a barrier to digestion and absorption of nutrients. It therefore adds to the problem of enzyme inactivation that several experiments have shown, i.e. that the process of heat treatment through pelleting, extrusion, expansion or micronization increases diet viscosity per se (Graham *et al.*, 1989; Pettersson *et al.*, 1991; Inborr and Bedford, 1994; Spring *et al.*, 1996; Medel *et al.*, 1999; Silversides and Bedford, 1999; Samarasinghe *et al.*, 2000; Cowieson *et al.*, 2005; Garcia *et al.*, 2008; Zimonja *et al.*, 2008). This effect is probably related to soluble fibres, as indicated by the fact that addition of fibre-degrading enzymes alleviates this effect (Silversides and Bedford, 1999; Cowieson *et al.*, 2005). Graham *et al.* (1989) also showed that a small fraction of starch was solubilized during pelleting, and it was suggested that this component may also contribute to increased viscosity, although the magnitude of the dissolution was proportionately so small that this effect is likely to be of minor importance. Since published data indicate that the amount of soluble fibres does not increase with processing (Petterson *et al.*, 1991; Inborr and Bedford, 1994; Cowieson *et al.*, 2005; Garcia *et al.*, 2008), it is possible that it is the viscous properties of soluble fibres that change during processing. Thus, heat treatment through processing of diets containing specific types of fibres may affect nutrient availability negatively, through both increased viscosity and reduced activity of the enzymes added to alleviate this problem. Conversely, the beneficial effect of enzymes will be particularly large for processed diets if precautions are taken so that enzymes are active post-processing, as demonstrated by Vranjes and Wenk (1995).

Based on the aforementioned, means to avoid inactivation of enzymes during processing would in many cases be beneficial. There are a number of ways this can be done, from spraying the enzyme as a liquid on to the pellets after pelleting to modifications to the enzyme or enzyme preparation added to the diet. Spraying enzyme on to pellets post-pelleting obviously results in no enzyme loss during processing, but requires special equipment installed in the feed factory and care in assuring that the liquid is added evenly (Edens *et al.*, 2002). The latter is particularly challenging, since only small quantities of liquid can be added due to the limits in absorption capacity of the pellets and the need to keep water content of the feed as low as possible. Making the enzyme preparation more resistant to the heat applied during processing can be achieved by coating enzyme components such as starch, fibre, protein and/or fat (Gibbs *et al.*, 1999). Few experiments appear to be published documenting the protective effect on enzymes of coating, although a number of patents can be found. From these patents and basic mechanisms by which enzymes are protected by these methods, it is clear that coating of enzymes will have a protective effect, although data from Kirkpinar and Basmacioglu (2006) showed that even a coated phytase was significantly inactivated when pelleted

at 85°C. As already discussed, however, the window of time in the digestive tract where conditions are suitable for enzyme activity is short. Thus, a potential problem with these coating techniques is that they will also delay dissolution and activation of the enzyme in the digestive tract and, due to this, potentially result in a less efficient substrate breakdown.

A more interesting and promising alternative is to make the enzyme more thermostable by altering the enzyme itself. Such stabilization can take place by protein engineering where, for example, disulfide bonds are introduced into the enzyme structure or components such as metal ions are bound to the enzyme (Weijers and van't Riet, 1992). Using protein engineering, Ding *et al.* (2008) were able to increase the heat stability of a xylanase. Similarly, Garrett *et al.* (2004) used gene site saturation mutagenesis technology to create a large number of phytase mutants that were screened for heat stability. The result was selection of a phytase with considerably improved heat stability compared with the original. Alternatively, a number of different enzymes with similar substrate specificities from different fungal or bacterial sources can be screened, with selection of the most thermostable.

Thermophilic (growth temperature 65–85°C) and hyperthermophilic (growth temperature 85–110°C) microorganisms isolated from hot springs and volcanic areas have been shown to contain a number of heat-stable carbohydrate-degrading enzymes (Leuschner and Antranikian, 1995). Screening of thermophilic fungi or bacteria has therefore been shown to be particularly effective (Maheswari *et al.*, 2000), as shown recently by Maalej *et al.* (2009), who were able to isolate a thermostable xylanase through this type of screening. Since no experiments appear to have been published demonstrating the thermostability of coated or modified NSP-ases, it is uncertain whether such modifications have resulted in sufficient thermal stability under pelleting conditions. However, since current pelleting conditions only partially denature exogenous enzymes, it is reasonable to assume that even a modest improvement in thermal stability would result in significantly improved enzyme recovery from standard pelleting conditions. Timmons *et al.* (2008) found that a heat-stable phytase was able to withstand pelleting temperatures exceeding 90°C, thus demonstrating the potential for genetic engineering to stabilize enzymes.

Although expansion, extrusion and micronization are not commonly used for feed destined for poultry and pigs, there is a growing interest in these processes. As temperature and/or moisture content increases, the extent of enzyme degradation will also increase. Vranjes *et al.* (1996), for example, found that extrusion abolished all β-glucanase activity in a poultry diet with a commercial enzyme preparation added. It is therefore likely that more extensive processing procedures such as extrusion will not be compatible with enzyme addition prior to processing.

General Conclusion

This chapter clearly demonstrates that dietary ingredients, their form, the husbandry conditions under which the animal is grown and individual variation

in digestive tract conditions of the animal and in the composition of the ingredients offered means that the conditions to which the enzyme is exposed are rarely constant. The animal scientist can maximize the response to feed enzymes by understanding these sources of variation that contribute to mitigating or accentuating the effect of an enzyme and, as a result, optimize economic return.

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7 Phytate and Phytase

P.H. Selle, V. Ravindran, A.J. Cowieson and M.R. Bedford

Introduction

A century ago, phytase activity was detected in rice bran (Suzucki *et al*., 1907). Phytase is the requisite enzyme for degradation of phytate (*myo*-inositol hexaphosphate, IP_6) and liberation of phytate-bound phosphorus (phytate-P). This stepwise hydrolysis yields inorganic phosphorus (P), lesser *myo*-inositol phosphate esters with diminished chelating capacities and, ultimately, in theory, inositol. Phytase has the potential both to enhance P digestibility and counteract the anti-nutritive properties of phytate, the so-called 'extra-phosphoric' effects of phytase (Ravindran, 1995).

The global harvest of maize, wheat, barley, sorghum, soybean, rapeseed/canola and cottonseed, all major feedstuffs for pigs and poultry, represents an estimated 16 million t of phytate (Lott *et al*., 2000). Phytate, the mixed salt of phytic acid, contains 282 g P kg^{-1}, this total representing approximately 4.5 million t of phytate-P. Phytate is invariably present in practical pig and poultry diets at concentrations in the order of 10 g kg^{-1}, but monogastric species are only able to partially utilize phytate-P. Therefore, it is necessary to supplement diets with inorganic P sources such as dicalcium phosphate to meet P requirements. Moreover, phytate is a polyanionic molecule, with a marked capacity to chelate positively charged nutrients, and this capacity is probably fundamental to its anti-nutritive effects. Phytate has been accurately described as 'both an anti-nutritional factor and an indigestible nutrient' (Swick and Ivey, 1992).

Attempts to develop a phytase feed enzyme for inclusion in pig and poultry diets were initiated in the early 1960s, as reported by Wodzinski and Ullah (1996). This was mainly in response to the capacity of phytate to limit Ca and P availability in poultry, and this interest is reflected in several early studies (Warden and Schaible, 1962; Nelson *et al*., 1968b; Rojas and Scott, 1969).

However, it was not until 1991 that a fungal-derived (*Aspergillus niger*) phytase (Natuphos®) was commercially introduced in the Netherlands. This development was largely driven by concerns about the negative ecological impact of P in effluent from pig and poultry units. In a landmark study, Simons *et al*. (1990) demonstrated that, in association with dietary manipulations, phytase activity of 1000 FTU (phytase units) kg^{-1} reduced P excretion by 35% in pigs and by 47% in broilers.

Legislation designed to curb environmental P pollution fuelled acceptance of phytase in the Netherlands. Initially, it was considered that the use of exogenous phytases would be confined to countries in which financial penalties were imposed on excessive levels of P generated by pigs and poultry (Chesson, 1993). However, contrary to these expectations, phytase inclusion in pig and poultry diets escalated rapidly on a global scale, but only after a considerable lag phase. Given sales of phytase feed enzymes with an estimated value of US$500 million at the turn of the century (Abelson, 1999), this delayed product acceptance is possibly without precedent. The introduction of an increasing number of commercial phytase products, declining inclusion costs, increasing prices for P supplements and feed ingredients in general, prohibition of the use of meat-and-bone meal in several countries, coupled with growing concerns about P pollution, were all factors. In addition, the development of a better scientific understanding of the phytate–phytase axis in pig and poultry nutrition and increasing experience and confidence in the practical application of phytase feed enzymes have also contributed to the 'heady pace' in the growth of their use (Bedford, 2003).

Several reviews of the roles of dietary phytate and microbial phytase have been completed and the reader is encouraged to refer to these papers, including Bedford (1995), Ravindran *et al*. (1995), Bedford and Schulze (1998), Selle *et al*. (2000, 2006, 2009a), Cowieson *et al*. (2006a,b, 2009) and Selle and Ravindran (2007, 2008), in the interest of greater detail. However, focus on phytate and phytase is not confined to pig and poultry nutrition, because the multifaceted properties of phytate are also of great interest in human nutrition, medical science, food technology, plant physiology and plant breeding (Feil, 2001).

Nevertheless, despite the scientific endeavour, numerous aspects of the phytate–phytase axis have not been properly elucidated. One fundamental obstacle is that rapid, accurate analysis of phytate concentrations in feedstuffs is not straightforward, and this problem is amplified in determinations of phytate in complete diets, ileal digesta and excreta. This is reflected in the paucity of studies where the end products of phytate degradation by exogenous phytase have been determined at the level of the ileum or on a total-tract basis in pigs and poultry. The extent of phytate degradation is obviously fundamental to the quantity of phytate-P released and the 'P equivalence' of phytase. However, the pattern of degradation and the particular *myo*-inositol phosphate esters generated by phytase may also hold relevance. As discussed below, some studies indicate that phytase has the capacity to increase ileal digestibility of protein/amino acids and to enhance energy utilization. However, the effects of phytase addition on ileal amino acid digestibility are not consistent and the extra-phosphoric effects of

phytase are more pronounced in broiler chickens than in pigs. The precise extent to which phytase increases amino acid availability and energy utilization across a range of dietary contexts is of immense practical importance, so considerable scope remains for further research to define these impacts.

The Substrate: Phytate

The substrate, phytate, is found in feedstuffs of plant origin where the P component serves as a P reservoir during seed germination and the intact phytate acts as a protectant against oxidative stress during the life of the seed (Doria *et al*., 2009).

Phytate, the mixed salt of phytic acid (*myo*-inositol hexaphosphate, IP_6), has a molecular weight of 660, a P concentration of 282 g kg^{-1} and consists of six P moieties located on a six-carbon *myo*-inositol ring ($C_6H_{18}O_{24}P_6$). The P moieties are aligned equatorially, apart from the axially aligned P in the C_2 position. Phytate is usually present in feedstuffs as a mineral–phytate complex in which magnesium and potassium are coupled to the IP_6 inositol phosphate ester. The model proposed by Lott *et al*. (2000) is represented in Fig. 7.1, where IP_6 is complexed with three Mg^{2+} and six K^+ ions.

Phytate concentrations in feedstuffs

Phytate and phytate-P are both nutritionally and ecologically important. The concentrations of total P and phytate-P concentrations in major feed ingredients

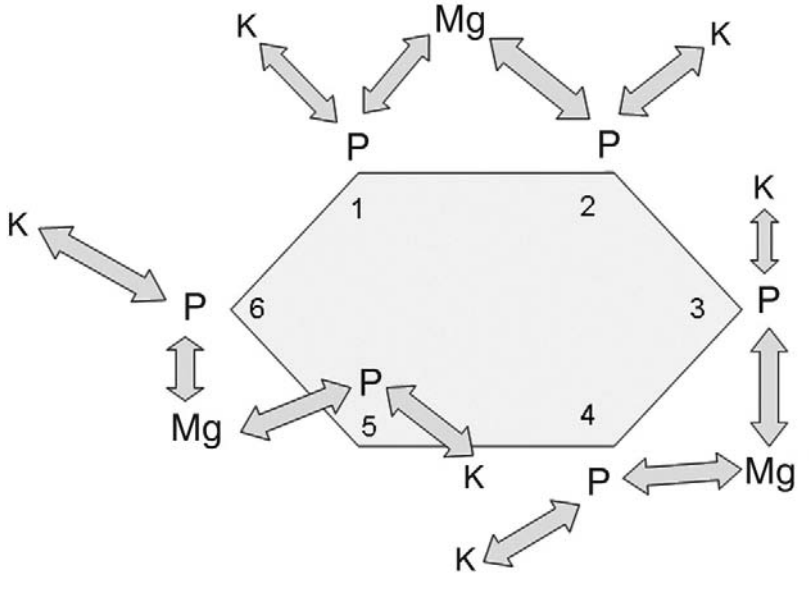

Fig. 7.1. Schematic diagram of the Mg–K–phytate axis, as proposed by Lott *et al*. (2000).

from nine surveys are summarized in Table 7.1. The majority of P in feedstuffs of plant origin is present as phytate-P and the phytate-P proportion of total P ranges from 60% (soybean meal) to 80% (rice bran). That phytate-P is only partially utilized by monogastrics is reflected in the poor bioavailability of total P for pigs in these feed ingredients, as reported by Cromwell (1992).

Practical diets typically contain approximately 10 g phytate kg^{-1}, but substrate concentrations are prone to variation depending on phytate levels in constituent feedstuffs. Phytate-P levels in wheat, for example, varied from 1.20 to 3.26 g kg^{-1} around a mean value of 2.00 g phytate-P kg^{-1} in 73 wheat samples from two Australian surveys (Kim *et al*., 2002; Selle *et al*., 2003b). This corresponds to a range of 4.3–11.6 g phytate kg^{-1} in wheat, which would clearly influence total phytate levels in wheat-based diets.

Ideally, given this potential variability, dietary phytate levels should be established. It follows that the magnitude of responses to added phytase, including P equivalence, will be governed by dietary substrate levels, and this could also provide an indication as to the appropriate phytase inclusion rate in a given pig or poultry diet.

It is possible that near-infrared spectroscopy (NIRS) calibrations could be developed (De Boever *et al*., 1994; Smith *et al*., 2001) so that dietary phytate levels could be rapidly determined and formulations of phytase-supplemented diets adjusted accordingly.

Ecological importance of phytate

The excretion of excess and undigested P by pigs and poultry and entry into the environment in effluent from production units is of serious ecological concern, as P contributes to the eutrophication of freshwater reserves, which may become apparent as 'algal blooms' and lead to death of fish. As overviewed by Daniel *et al*. (1998), the causal relationship between P derived from agriculture, including pig and poultry production, and eutrophication has been the subject of considerable research. This topic is not considered in detail in this chapter, but the ecological hazards posed by P have been integral to the development and acceptance of phytase feed enzymes, as they have contributed to the amelioration of P pollution of the environment.

Nutritional importance of phytate

Phosphorus is an imperative nutrient for numerous biochemical pathways, physiological processes and skeletal integrity, but due to the partial availability of phytate-P, diets are supplemented with P sources such as dicalcium phosphate or, where permitted, meat-and-bone meal to meet P requirements. However, it may be argued that P requirements have been neither consistently nor accurately defined, and are presently further complicated by the dietary inclusion of microbial phytases. The dependence on inorganic P supplements is a challenge, because global reserves of rock phosphate are not renewable (Abelson, 1999) and the price of phosphates has escalated in recent years.

Table 7.1. Mean (and range) of total P and phytate-P concentrations, proportion of phytate-P in total P and bioavailability of total P for pigs in common feed ingredients.

Feed ingredient	Data sets/ samples[a] (*n*)	Total P[a] (g kg^{-1}) (range in parentheses)	Phytate-P[a] (g kg^{-1}) (range in parentheses)	Phytate-P[a] (proportion of total P) (%)	P bioavailabity for pigs[b] (%)
Barley	4/41	3.21 (2.73–3.70)	1.96 (1.86–2.20)	61.0	30.0
Maize	7/45	2.62 (2.30–2.90)	1.88 (1.70–2.20)	71.6	13.0
Sorghum	6/64	3.42 (2.71–3.80)	2.66 (1.90–3.26)	77.8	20.0
Wheat	6/97	3.07 (2.90–4.09)	2.19 (1.80–2.89)	71.6	49.0
Canola meal	4/28	9.72 (8.79–11.50)	6.45 (4.00–7.78)	66.4	21.0
Cottonseed meal	3/21	10.02 (6.40–11.36)	7.72 (4.9–9.11)	77.1	1.0
Soybean meal	6/89	6.49 (5.70–6.94)	3.88 (3.54–4.53)	59.9	27.0
Rice bran	6/37	17.82 (13.40–27.19)	14.17 (7.90–24.20)	79.5	25.0
Wheat bran	6/25	10.96 (8.02–13.71)	8.36 (7.00–9.60)	76.3	41.0

[a]Weighted means derived from Nelson *et al.* (1968a); Doherty *et al.* (1982); Kirby and Nelson (1988); Eeckhout and de Paepe (1994); Ravindran *et al.* (1994); Mahgoub and Elhag (1998); Viveros *et al.* (2000); Selle *et al.* (2003b); Godoy *et al.* (2005).
[b]From Cromwell (1992).

However, the nutritional importance of phytate is not limited to P availability. Notionally, the polyanionic phytate molecule may carry 12 negative charges that confer a tremendous capacity for IP_6 to chelate divalent cations, including Ca^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+}, and the availability of these complexed minerals is reduced. The formation of insoluble Ca–phytate complexes in the small intestine, probably as Ca_5-K_2-phytate (Evans and Pierce, 1981), reduces the availability of both Ca and P and, for this reason, phytate is considered to be an aetiological factor in 'rickets' or osteomalacia (Mellanby, 1949). Similarly, because of its particular affinity for Zn, phytate limits Zn availability and phytate is considered to be a causative factor of parakeratosis, which is a manifestation of Zn deficiency in swine (Oberleas *et al*., 1962). Indeed, much of our knowledge of phytate stems from the development of procedures to extract phytate from soy protein concentrates because of concerns about phytate reducing the availability of Zn in diets for humans (Sandstead, 1992; Wise, 1995).

Phytate extraction from protein concentrates has revealed that phytate has the capacity to bind protein either as binary or ternary protein–phytate complexes (Cosgrove, 1966). Binary complexes are more important because they have the potential to bind more protein than ternary protein–phytate complexes (Champagne *et al*., 1990). Given the capacity of phytate to bind directly with protein, it follows that phytate may depress amino acid digestibility (Officer and Batterham, 1992a,b). In all likelihood this is the case, but outcomes of phytase ileal amino acid digestibility assays in pigs, in particular, and poultry do not consistently support this proposal. The reasons for these inconsistencies and the mechanisms whereby phytate may depress amino acid digestibility are discussed later, but the likelihood is that *de novo* formation of binary protein–phytate complexes at acidic pH in the gut are fundamental to the 'protein effect' of phytate and phytase.

Also, on the basis of responses to dietary inclusions of phytase, phytate appears to depress energy utilization, which is more evident in broilers than in pigs. Again, the 'energy effect' of phytate and phytase is discussed later. However, graded inclusion levels of *A. niger* phytase in diets based on wheat-sorghum blends have illustrated the negative effects of phytate on protein and energy utilization in broilers. As shown in Fig. 7.2, Ravindran *et al.* (2001) reported that increasing phytase inclusion levels improved both average ileal amino acid digestibilities and dietary available metabolizable energy (AME) values in an essentially linear manner. The peak responses recorded were at 1000 FTU kg^{-1}, where phytase increased average apparent ileal digestibility (AID) coefficients of 15 amino acids by 5.7%, from 0.775 to 0.819, and at 750 FTU kg^{-1}, where phytase increased AME by 0.50 MJ, or 3.5%, from 14.22 to 14.72 MJ kg^{-1}.

Dephytinization

The pre-feeding elimination of phytate from a feed ingredient, or dephytinization, is an interesting approach in overcoming the anti-nutritive properties of phytate that may have application in practice, particularly in aquaculture. Also,

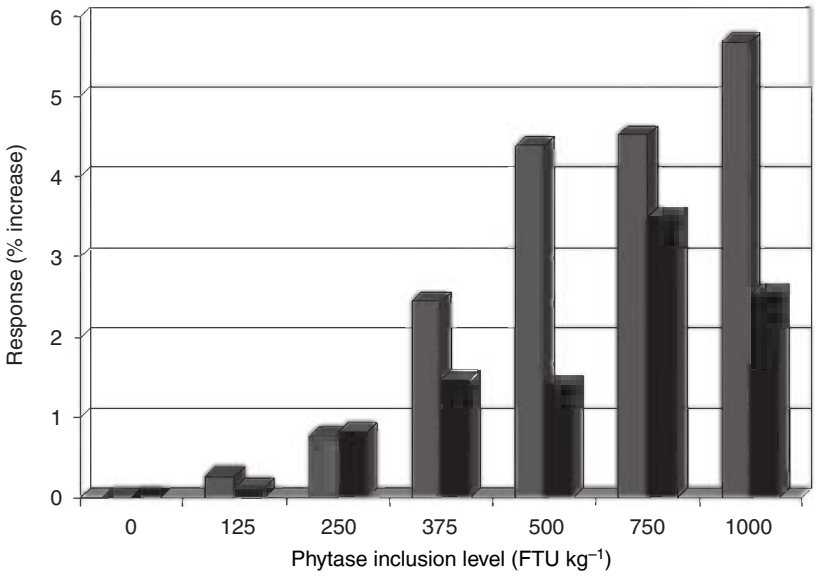

Fig. 7.2. Responses (% increase) in average apparent ileal digestibility of 15 amino acids (grey) and available metabolizable energy (black) to graded phytase inclusion levels (FTU kg^{-1}) in broilers. FTU, phytase units. (From Ravindran *et al.*, 2001.)

theoretically, dephytinization should be a means of defining the anti-nutritive properties of phytate. Canola meal contains relatively high phytate levels; in one survey, canola meal contained averages of 8.76 g total P kg^{-1} and 6.69 g phytate-P kg^{-1}, or 76.4% of total P (Selle *et al.*, 2003b). Newkirk and Classen (1998, 2001) dephytinized canola meal and incorporated untreated, sham-treated and dephytinized canola meal into maize–soy broiler diets at 300 g kg^{-1}. In comparison with the sham-treatment, dephytinization of canola meal with a purified phytase increased average AID coefficients of 17 amino acids by 16.0%, from 0.648 to 0.752. Individual increases in amino acid digestibility ranged from 39.8% (proline) to 0.2% (methionine), and the majority of responses were statistically significant. Given that approximately half the dietary protein was derived from canola meal, the implication is that dephytinization substantially increased the digestibility of amino acids in canola meal. This raises the question as to the actual extent by which phytate depresses protein/amino acid digestibility. Therefore, the anti-nutritive properties of phytate may be potent but they are not fully declared by phytase supplementation, because degradation of phytate is incomplete (Selle and Ravindran, 2007). However, though dephytinization via industrial processes is possible, it may lead to inimical changes other than the removal of phytate, e.g. Maillard complexing of lysine following heat treatment, and so a cost–benefit analysis is warranted.

Determination of phytate concentrations

A fundamental issue, as emphasized by Lasztity and Lasztity (1990), is that the determination of phytate concentrations is not a straightforward procedure. In the majority of cases, phytate-P concentrations are determined by methods based on the ferric chloride-precipitation principle of Heubner and Stadler (1914). Phytate is precipitated by ferric chloride (Fe_3Cl_2) at acidic pH, and concentrations of P or Fe are determined in the supernatant or the precipitate from which the phytate-P concentration is calculated. Given complete phytate extractions, these methods are satisfactory for phytate determinations in individual feedstuffs, but they do not differentiate between the various *myo*-inositol phosphate esters present. However, in more complex samples (e.g. complete diets, ileal digesta), ferric chloride-precipitation methods are not satisfactory due to co-precipitation of P from other sources.

An important limitation is that basic methods of determining phytate-P do not have the capacity to identify the various *myo*-inositol phosphate esters of phytate. However, it is possible to differentiate phytate esters with high-performance liquid chromatography, anion exchange chromatography and nuclear magnetic resonance spectroscopy (Phillippy and Johnston, 1985; Rounds and Nielsen, 1993; Skoglund *et al*., 1998; Kemme *et al*., 1999). However, these advanced methods require sophisticated equipment and may be expensive and time consuming (Kwanyuen and Burton, 2005; Gao *et al*., 2007). Arguably, the current difficulties associated with accurate phytate analysis have been an important constraint on scientific progress in this area.

The Enzyme: Phytase

Notionally, phytases have the capacity to degrade IP_6 phytate completely to inositol and to liberate six P moieties. However, because the P moiety axially located at C_2 is not readily released, complete dephosphorylation of phytate by phytase probably does not occur in pigs and poultry. By contrast, there is a possibility that endogenous phosphatases (associated with the brush border) do provide some inositol, particularly in the more distal regions of the small intestine. Thus the role of inositol genesis by microbial phytase and phosphatases in the overall efficacy of such products is obscure and warrants further study. Phytases and phosphatases exist widely in nature, but four sources of phytase activity are relevant in target species.

Sources of phytase activity

Intrinsic 'plant' phytase

Certain feed ingredients, particularly wheat and its by-products (Peers, 1953), possess intrinsic phytase activity. However, the importance of plant phytase in standard diets is questionable because it is less effective than microbial phytases

at gastrointestinal pH and may be inactivated by acidic pH levels in the gut. Moreover, the practical importance of plant phytase is diminished because it will be reduced or even eliminated by steam-pelleting of pig and poultry diets. Plant phytases are heat labile and, in purified form, most are destroyed at temperatures above 70°C within minutes (Konietzny and Greiner, 2002). As reported by Jongbloed and Kemme (1990), steam-pelleting a diet based on wheat, maize and soybean meal at 80°C eliminated wheat phytase activity and reduced total-tract P digestibility by 37% in pigs. It follows that responses to microbial phytases may be compromised by the presence of plant phytase activity in the diet, so wheat may be 'pre-pelleted' in feeding studies to eliminate intrinsic phytase and avoid this potential confounding factor. However, robust responses to microbial phytase have been reported despite the dietary presence of wheat phytase in weaner pigs (Campbell *et al*., 1995). This suggests that the presence of plant phytase may not necessarily compromise responses to microbial phytases. Indeed, Zimmermann *et al*. (2002) reported that *in vivo* efficacy of plant-derived phytases was only 40% of microbial phytase on a unit for unit basis, suggesting that plant-derived phytases do not possess characteristics optimal for efficacy in the gastrointestinal tract.

Endogenous mucosal phytase

Patwardhan (1937) first detected the presence of mucosal phytase activity in rats, and it has been identified in the small intestine of pigs (Hu *et al*., 1996) and poultry (Maenz and Classen, 1998). Nevertheless, the importance of mucosal phytase is usually dismissed, but its activity may be governed by dietary non-phytate P levels. However, dietary Ca levels appear critical, as Tamim *et al*. (2004) reported an ileal degradation coefficient for phytate of 0.692 in maize–soy broiler diets containing 2.8 g phytate-P kg^{-1} at a dietary Ca level of only 2.0 g kg^{-1}. However, when Ca was increased to 7.0 g kg^{-1}, the coefficient was noticeably reduced to 0.254. Clearly, Ca has a substantial impact on the efficacy of mucosal phytase and, presumably, this is largely a consequence of the formation of insoluble Ca–phytate complexes at pH approaching neutrality in the small intestine (Wise, 1983). Consequently, the extent of phytate degradation generated by mucosal phytase will be limited by the Ca levels in practical pig and poultry diets.

Gut microfloral phytase

The microfloral population in the gastrointestinal tract, especially in the hindgut, is known to generate phytase activity, although degradation of phytate in the hind gut is of relatively little importance. It is, however, often assumed that undigested phytate-P is excreted by pigs and poultry, but the amount may be markedly reduced by hindgut fermentation, particularly in pigs. While hindgut fermentation of phytate-P may be of value to coprophagic animals, this confounds total-tract assessments of phytate degradation since P released post-ileum appears to be of little value to the animal (Zimmerman *et al*. 2002).

Exogenous microbial phytase (feed enzymes)

Presently, the majority of phytases are derived from fungi (e.g. *A. niger*) or, more recently, bacteria (e.g. *Escherichia coli*), and the fermentative production processes depend on genetically modified organisms. However, it should be noted that genetically modified material is not found in preparations of phytase feed enzymes. It is also probable that the purity, or the lack of enzymic side-activities, and the yield of phytase activity have increased over time with the refinement of production processes. A corollary of this development is the possibility that the microbial phytases evaluated in early studies (Simons at al., 1990; Beers and Jongbloed, 1992; Ketaren *et al*., 1993) are not identical to the feed enzymes presently available.

Enzymatic dephosphorylation of phytate in pigs and poultry

The main sites of phytate degradation by microbial phytases are the stomach in pigs and the forestomach (crop, proventriculus and gizzard) in poultry, with relatively little degradation in the distal gastrointestinal tract. The extent and rapidity of dephosphorylation is critical to both the destruction of phytate (and so removal of the associated anti-nutritive effect) and the P equivalence of phytase. Arguably, the P equivalence of phytase is a simple function of dietary phytate levels and the degree to which it is hydrolysed. Equally, the amelioration of the anti-nutritive properties of phytate should be governed by the extent and timing of its degradation.

However, surprisingly few studies have investigated the dephosphorylation of phytate along the gastrointestinal tract. Taken together, two broiler studies suggest that degradation of phytate by 500 FTU *A. niger* phytase kg^{-1} does not exceed 35% at the level of the ileum (Camden *et al*., 2001; Tamim *et al*., 2004). In layers, van der Klis *et al*. (1997) reported that 500 FTU *A. niger* phytase kg^{-1} increased ileal degradation of phytate (0.661 versus 0.081), which indicates that microbial phytase degraded 58% of dietary phytate. This comparison suggests that phytase is more effective in laying hens than in broiler chickens, which may be due to longer digesta retention times in the forestomach and is reflected in lower recommended phytase inclusion rates for layer diets than broiler diets.

More relevant studies have been completed in pigs (Jongbloed *et al*., 1992; Mroz *et al*., 1994; Rapp *et al*., 2001; Kemme *et al*., 2006); collectively, these reports indicate that in the order of 50% of dietary phytate is degraded by microbial phytase at the ileal level. Assuming all dietary phytate is present as IP_6, the uniform hydrolysis of all IP_6 to IP_3, with the release of three P moieties per IP_6, would correspond to 50% phytate P destruction. However, dephosphorylation of phytate is a stepwise reaction and a considerable proportion of undegraded phytate remains intact as IP_6 at the ileal level. For example, in the study of Rapp *et al*. (2001), 60% of phytate remained intact as IP_6. Thus the following equation illustrates phytate degradation by microbial phytase where, at the level of the ileum, 50% of phytate-P has been liberated

and the balance is present as either IP_6 or a range of lesser *myo*-inositol phosphate esters:

$$IP_6\ (100\%\ P) \Rightarrow IP_6\ (30\%) + [IP_3, IP_2, IP_1]\ (20\%) + \text{inorganic P}\ (50\%).$$

The likelihood is that little, if any, IP_6 is completely dephosphorylated to inorganic P and inositol, essentially because microbial phytases do not release P located at the axial C_2 position of the *myo*-inositol ring. Alternatively, if the majority of undegraded phytate remains intact as IP_6, then this has consequences. This is because the chelating capacity and anti-nutritive properties of phytate are disproportionately diminished as IP_6 is degraded to lesser *myo*-inositol esters, which are relatively innocuous (Luttrell, 1993). Recent advances in the understanding of the stepwise dephosphorylation of IP_6 have indicated that there is a considerable difference between 6-phytases and 3-phytases in this regard (Wyss *et al*. 1999; Greiner *et al*., 2000, 2001). While the commercially employed 3-phytases effectively tend to continue their attack on a selected IP_6 molecule until it is reduced to IP_1, the 6-phytases seem to halt their assault, momentarily, on IP_4 and lower esters (due to an apparently higher K_M for these substrates). As a result, for the provision of similar quantities of P as determined by FTU assay, there is considerably more destruction of IP_6 by a 6- compared with a 3-phytase. This will clearly influence the relative extra-phosphoric effects of the 3- versus the 6-phytases.

Phosphorus and Calcium Equivalence of Phytase

Effectively, phytase is a source of P and Ca following the enzymatic degradation of phytate and the liberation of P inherent in the substrate and Ca bound to the phytate. In the formulation of phytase-supplemented diets, P and Ca levels are usually reduced to accommodate this release of macro-minerals on the basis of P and Ca equivalency values for phytase. This adjustment in dietary P levels contributes to the reduction in P excretion, which is a prime objective of phytase supplementation.

Hoppe and Schwarz (1993) concluded that 500 FTU phytase was equivalent to 1 g P as monocalcium phosphate in maize–soy pig diets and, essentially, this precedent remains accepted. For example, the recommendation of the relevant manufacturer is that 500 FTU *A. niger* phytase kg^{-1} is equivalent to 1.15 g P kg^{-1} and 1.00 g Ca kg^{-1} in diets for pigs and broiler chickens, and broadly similar recommendations are made by other manufacturers. Interestingly, that phytase liberates somewhat less Ca than P is a concept that is still accepted, although it may be questioned from a theoretical viewpoint.

Phosphorus equivalence

Up to this point, P equivalence values have been established by incorporating graded quantities of either an inorganic P source or microbial phytase into a P-deficient basal diet. P replacement values are calculated from regression equations that best describe responses in selected parameters generated by

additional P and microbial phytase; usually, the parameters are weight gain and a measurement of bone mineralization.

In broiler chickens, body weight gain and percentage toe ash are sensitive indicators of P availability (Potter, 1988) and are usually selected as the response criteria in P equivalence studies. Selle and Ravindran (2007) reviewed nine P equivalency studies in broilers in which P-deficient basal diets contained an average of 2.00 g non-phytate P kg^{-1} and 2.37 g phytate-P kg^{-1}, with a Ca:P ratio of 1.84:1.00. Collectively these studies indicated that approximately 766 FTU phytase kg^{-1} is equivalent to 1.0 g P kg^{-1} in broilers, which implies 42% phytate degradation. This phytase equivalency value is less than standard recommendations, which may indicate that commercial diets contain a surplus of P as a safeguard, which is arguably the case. However, the accuracy and relevance of P equivalency studies are questionable because the basal diet, by definition, contains inadequate levels of non-phytate P. As a consequence the Ca:P ratios may be higher than in standard diets. This could negatively influence the extent of phytate degradation and the P equivalency value. Alternatively, phytase may positively influence weight gain quite independently of phytate-bound P release, which would tend to inflate P equivalency values (Wu *et al*., 2004).

Reservations in relation to P equivalency studies have been expressed by other workers (Angel *et al*., 2002; Driver *et al*., 2005). However, it appears that nutritionists are electing to use higher phytase inclusion rates in practice, which would be expected to increase P equivalency values and permit greater reductions in dietary P levels and, in turn, amounts of P excreted. This emphasizes the need to develop more accurate P equivalence values based on the extent of phytate degradation induced by phytase, coupled with established dietary phytate concentrations in preference to values derived from 'classic' P equivalency studies.

From basic principles, if phytase degrades 40% of phytate in a broiler diet containing 2.8 g phytate-P kg^{-1}, then there is a generation of 1.12 g P kg^{-1}. The P equivalency value of phytase is clearly a function of the dietary substrate level and the extent of phytate degradation, which are both variables. It is also clear that the susceptibility to or availability of phytate to phytase hydrolysis may be ingredient dependent. Leske and Coon (1999) demonstrated that, although canola meal contained almost twice as much phytate-P as soybean meal, the subsequent P equivalency of 600 FTU of an *Aspergillus* phytase was three times higher in soybean meal compared with canola. Ideally, therefore, P equivalence values should be based on determined dietary phytate concentrations and a prediction of phytase-induced substrate degradation. The development of such an approach should be a future objective to permit more appropriate manipulations to dietary formulations.

Calcium equivalence

Calcium equivalency studies follow the same principle, where graded levels of Ca as limestone or microbial phytase are added to Ca-deficient basal diets. Few

Ca equivalency studies have been completed, but it is accepted that for an *Aspergillus* phytase 500 FTU kg^{-1} is equivalent to about 1.00 g Ca kg^{-1}, and the formulation of phytase-supplemented diets is usually adjusted accordingly. In early studies, Schöner *et al*. (1994) reported that a Ca equivalency of 500 FTU phytase kg^{-1} was approximately 0.44 g Ca kg^{-1} in broilers, and Kornegay *et al*. (1996) found that a Ca equivalency of 500 FTU phytase kg^{-1} ranged from 0.38 to 1.08 g Ca kg^{-1} in pigs. Further conflicting results have been recorded. Augspurger and Baker (2004) reported that 500 FTU *E. coli* phytase kg^{-1} released 0.90 g Ca kg^{-1} on the basis of tibia ash in maize-soy broiler diets; however, Mitchell and Edwards (1996) and Yan *et al*. (2006) concluded that phytase had little impact on Ca release in broilers. Ca levels in the basal diet of equivalency studies are intentionally low. However, Farkvam *et al*. (1989) found that increasing dietary Ca concentrations in broiler diets increased the amount of Ca bound by phytate. Therefore, it follows that Ca-deficient basal diets reduce the amount of Ca bound by phytate, which may explain the inconsistent results and generally low values recorded in calcium equivalency studies.

The likelihood is that the Ca equivalence of phytase is governed by the extent of *de novo* Ca–phytate complex formation in the small intestine. One phytate molecule may bind up to five Ca atoms as Ca_5-K_2-phytate; if so, in a diet containing both phytate (IP_6) and Ca at 10 g kg^{-1}, phytate would have the capacity to bind 3.0 g Ca kg^{-1} or approximately one-third of dietary Ca. Simplistically, this suggests that phytase has an equivalency value of 1.5 g Ca kg^{-1} assuming a 50% degradation of phytate. However, the capacity of phytate to complex Ca declines at a disproportionately greater rate as IP_6 phytate is degraded into lesser inositol phosphate esters. Indeed, Luttrell (1993) found the *in vitro* Ca-binding affinity of IP_4 to be 32% in comparison with that of IP_6, and the Ca-binding affinities of IP_3, IP_2 and IP_1 were negligible.

Consequently, it seems likely that, rather than being in parallel, the liberation of phytate-bound P and Ca by phytase is 'uncoupled'. The liberation of P is directly proportional to the extent of phytate degradation, but the liberation of Ca may exceed this rate and it may be that the enzymatic hydrolysis of dietary phytate by microbial phytase generates more Ca than P in the initial phase. This would be particularly so for 6-phytases, which seem to prefer IP_6, IP_5 and IP_4 as substrates over IP_3 and IP_2, in contrast to 3-phytases which seem to have equal affinity for all. If so, this is not reflected in matrix values applied to phytase-supplemented diets.

Calcium is a critical nutrient but, as discussed later, relatively high Ca levels in pig and poultry diets, particularly as limestone, may have a negative influence on phytase efficacy. Consequently, Ca levels in phytase-supplemented diets should be kept to an acceptable minimum and, for this reason alone, more accurate assessments of the Ca equivalency of phytase are required. It is even likely that larger, more appropriate, Ca reductions in phytase-supplemented diets will enhance the efficacy of the feed enzyme.

Release of phosphorus and calcium

Several experiments have been completed where increasing phytase inclusion rates, at times to apparently high levels, have been evaluated in pigs and poultry. In several studies where the highest inclusion rate employed was not excessive (e.g. less than 2500 FTU kg^{-1}), it is not unusual that responses to phytase observed appeared to plateau or even decline at higher inclusions (Ravindran *et al*., 2001). However, in studies where 'mega-doses' of phytases have been investigated (Rosen, 2001; Veum *et al*. 2006), the data clearly indicate a log-linear relationship between dose and response, suggesting that much of the research is conducted at well below the 'optimum' inclusion rate of this enzyme. As a result, the apparent 750–1000 FTU kg^{-1} optima determined in studies where dosages do not exceed 2500 FTU kg^{-1} are often an artifact of the design of the study.

Of interest is that Nelson *et al*. (1980) altered the cation–anion balance of a maize–soy broiler diet with P as mono–dicalcium phosphate and Ca as limestone. They reported that net increases in cation levels were negatively correlated with nitrogen-corrected AME ($r = -0.72$; $P < 0.01$) and digestibility of 17 amino acids ($r = -0.79$; $P < 0.01$). Microbial phytase induces the release of P and Ca with the potential to impact on the effective cation–anion balance. If, in fact, phytase liberates more Ca than P this would generate a net increase in dietary cationic levels, which would be detrimental on the basis of the Nelson *et al*. (1980) study. This point is made because, fundamentally, the greatest impact following the dietary inclusion of phytase is on P and Ca availability. Arguably, the consequences of effectively increasing the dietary levels and altering the balance of these two macro-minerals have not received proper consideration, nor have dietary formulations been appropriately adjusted. Therefore, assessments of P and Ca phytase equivalence values at both standard and elevated inclusion levels merit more accurate definitions.

'Protein Effect' of Phytate and Phytase

Officer and Batterham (1992a) were probably the first to suggest that microbial phytase has a 'protein effect'. In grower pigs offered diets based on linola meal as the only protein source, phytase significantly increased the ileal digestibility of nitrogen (22.6%) and lysine (20.3%), and it was suggested that these responses may be 'due to the release of amino acids bound in phytate linkages'. It is established that phytate can bind protein to form protein–phytate complexes (Cosgrove, 1966; Anderson, 1985), and it follows that the prior hydrolysis of phytate by phytase in the gut would reduce the extent of *de novo* protein–phytate complex formation (Selle *et al*., 2000). Phytate is capable of binding up to ten times its weight of protein under *in vitro* conditions (Kies *et al*., 2006), which implies that in a diet with 10 g phytate kg^{-1} and 200 g protein kg^{-1}, half the protein present may be complexed by phytate. It could be anticipated that phytase enhances ileal digestibility of amino acids in pigs and poultry via reductions in protein–phytate complex formation. However,

the outcomes of phytase amino acid digestibility assays are inconsistent, particularly in pigs where responses to phytase have often been negligible. Indeed, Adeola and Sands (2003) were inclined to the view that phytase does not have a positive effect on protein utilization in pigs. Nevertheless, regardless of the conflicting data arising from phytase amino acid digestibility studies, some practical nutritionists elect to confer amino acid matrix values to microbial phytase in pig and poultry diet formulations. The protein effect of phytate and phytase is still an open question and, given that microbial phytases have been commercial entities for nearly two decades, it is not acceptable that this fundamental issue remains unresolved.

Microbial phytase amino acid digestibility assays in broilers

Despite its recognized limitations as a dietary marker (Jagger *et al*., 1992), chromic oxide has been used in the majority of phytase amino acid digestibility assays. However, in broiler chickens, amino acid digestibility responses to phytase using acid-insoluble ash or titanium oxide have been consistently more pronounced than those involving chromic oxide (Selle *et al*., 2006; Selle and Ravindran, 2007). It is recognized that ileal digestibility of amino acids is more meaningful than assessments made on a total-tract basis (Ravindran *et al*., 1999b). Nevertheless, Hassanabadi *et al*. (2008a,b) determined the influence of microbial phytase on total-tract digestibility of amino acids by quantitative excreta collection, which did not involve a dietary marker. *Aspergillus niger* phytase (500 FTU kg^{-1}) increased mean AID coefficients of 13 amino acids by 5.1% (0.902 versus 0.858) in female chicks and by 4.2% (0.889 versus 0.853) in male chicks. The magnitude of these responses is very similar to ileal digestibility assays in which acid-insoluble ash or titanium oxide were used as markers.

Eight assays are identified in which the effect of microbial phytase on AID of amino acids was determined in broilers with either acid-insoluble ash or titanium oxide as dietary marker (Table 7.2). In the eight studies, phytase

Table 7.2. Phytase amino acid digestibility assays in broilers offered complete diets in which either acid-insoluble ash (AIA) or titanium oxide (TiO_2) was used as dietary marker.

Phytase	Inclusion (FTU kg^{-1})	Diet type	Marker	Reference
Aspergillus niger	500	Wheat–casein	AIA	Ravindran *et al*. (1999a)
A. niger	800	Wheat–sorghum	AIA	Ravindran *et al*. (2000)
A. niger	500	Wheat–sorghum	AIA	Ravindran *et al*. (2001)
A. niger	500	Wheat–sorghum	AIA	Selle *et al*. (2003b)
Peniophora lycii	500	Maize	TiO_2	Rutherfurd *et al*. (2004)
Escherichia coli	1000	Maize	TiO_2	Ravindran *et al*. (2006)
E. coli	500	Maize	TiO_2	Ravindran *et al*. (2008)[a]
E. coli	500	Wheat	TiO_2	Selle *et al*. (2009b)

[a]Effect of phytase at a dietary electrolyte balance (DEB) of 225 mEq kg^{-1}.

increased average AID coefficients of 18 amino acids by 4.7%, from 0.787 to 0.824, over 123 observations (Table 7.3). Among individual amino acids, percentage increases ranged from 1.8% (methionine) to 7.1% (threonine, cystine, serine), and this response pattern reflects the relatively higher inherent digestibility of methionine (0.894). There was a significant negative relationship ($r = -0.972$; $P < 0.001$) in the tabulated mean values between the response (percentage increase) to phytase and the inherent digestibility of amino acids in the control diets. Indeed, the apparently poor response to phytase when chromic oxide has been used may be associated with an overestimation, compared with alternative markers, of the digestibility of amino acids in the control diet (Cowieson and Bedford, 2009).

Impact of phytate on protein/amino acid digestibility

On the basis of acid-insoluble ash/titanium oxide broiler assays, microbial phytase has a positive influence on ileal amino acid digestibility and, axiomatically, phytate has a negative impact. The *de novo* formation of binary

Table 7.3. Overall effects of microbial phytase on apparent ileal digestibility (AID) of amino acids from eight assays[a] where broilers were offered complete diets with acid-insoluble ash or titanium oxide as dietary marker.

Amino acid	Number of observations	Average AID coefficients		Response (%)
		Nil	Phytase	
Essential				
Arginine	8	0.846	0.870	2.8
Histidine	8	0.778	0.821	5.5
Isoleucine	8	0.784	0.824	5.1
Leucine	8	0.801	0.838	4.6
Lysine	8	0.829	0.862	4.0
Methionine	5	0.894	0.910	1.8
Phenylalanine	8	0.804	0.844	5.0
Threonine	8	0.731	0.783	7.1
Tryptophan	4	0.783	0.818	4.5
Valine	8	0.777	0.814	4.8
Non-essential				
Alanine	7	0.774	0.809	4.5
Aspartic acid	7	0.767	0.814	6.1
Cystine	4	0.673	0.721	7.1
Glutamic acid	7	0.838	0.874	4.3
Glycine	7	0.746	0.788	5.6
Proline	4	0.794	0.835	5.2
Serine	7	0.751	0.804	7.1
Tyrosine	7	0.789	0.808	2.4
Mean	123 (total)	0.787	0.824	4.7

[a]Ravindran *et al.* (1999a, 2000, 2001, 2006, 2008); Rutherfurd *et al.* (2002); Selle *et al.* (2003b, 2009b).

protein–phytate complexes at acidic pH in the stomach of pigs and forestomach of poultry is probably fundamental to the negative impact of phytate.

The capacity of phytate to bind protein as both binary and ternary complexes is established and, as described by Rajendran and Prakash (1993), binary complex formation is a biphasic reaction. The polyanionic phytate molecule electrostatically binds with basic arginine, histidine and lysine residues and this initial, rapid step is followed by a slower aggregation of protein and may result in precipitation of the complex. Binary complex formation occurs at a pH less than the isoelectric point of a given protein, and in the case of sodium phytate and α-globulin the reaction was maximal at pH 2.3 and dependent upon phytate to protein ratios. Similarly, sodium phytate interacts with gossypulin, a globulin cottonseed protein, at pH 2.0–3.0 (Yunusova and Moiseeva, 1987).

Pivotally, several studies have found that complexed protein is refractory to pepsin hydrolysis (Barré and Nguyen-van-Hout, 1965; Camus and Laporte, 1976; Kanaya *et al.*, 1976; Inagawa *et al.*, 1987; Knuckles *et al.*, 1989). Moreover, Vaintraub and Bulmaga (1991) reported that phytate retarded pepsin hydrolysis of soy protein by 60% at pH 2.0–3.0 under *in vitro* conditions, but this did not occur at pH 4.0–4.5. These workers concluded that phytate retards pepsin digestion only when phytate is bound to the protein, which makes the important distinction that phytate binds with the substrate (protein) and not the enzyme (pepsin). Indeed, the paucity of basic amino acids in pepsin (Blumenfeld and Perlmann, 1958; Tang *et al.*, 1973) probably precludes phytate from binding with the enzyme. However, although phytate and pepsin may not interact directly, the activation fragment of pepsinogen is heavily basic (13/44 amino acid residues) and so phytate may compromise activation of the zymogen (Dykes and Kay, 1977; Dunn *et al.*, 1978). Alterations in protein solubility and structure induced by aggregation with phytate presumably render the substrate less susceptible to pepsin activity, and thus phytate impedes the initiation of the protein digestive process. Additionally, pepsin-generated peptides are regulators of protein digestion processes (Krehbiel and Matthews, 2003), so it follows that pepsin-refractory complex formation may disrupt these regulatory functions.

Although protein–phytate complexes dissociate once gut pH exceeds protein isoelectric points, proteins still may be less readily digested in the small intestine due to structural changes pursuant to their aggregation with phytate. Furthermore, the dissociated complexes release proteins that have escaped pepsin processing and, as a result, are not optimally processed for digestion by trypsin, chymotrypsin, elastase and additional small-intestinal proteases. As a result the rate of protein digestion and absorption is reduced, and if transit rates remain largely unchanged this could result in delivery of excess nitrogen to the fermentative bacteria in the large intestine, with the concomitant risk of multiplication of putrefactive bacteria.

Low (1990) concluded that physicochemical properties of foodstuffs are dominant determinants of gastric function and, although speculative, the refractory nature of insoluble protein–phytate complexes may prompt gastric hypersecretion of pepsin and HCl as a compensatory mechanism. Decuypere

et al. (1981) investigated the effects of diets containing water-soluble or insoluble soy protein isolates (140 g kg^{-1}) in pigs fitted with gastric fistulae. It was concluded that the physical properties of the protein sources were important in regulating pepsin and HCl secretions, as there were marked differences between diets in the 3 h postprandial interval. For example, pepsin secretion with insoluble soy protein was about 88% higher than with soluble soy protein 150 min following feed intake. Zebrowska *et al*. (1983) reported that pepsin activity in digesta from the proximal duodenum of pigs offered a barley–soybean meal diet was 93% higher than those fed on 'phytate-free' diets containing wheat starch, casein and sucrose. The barley–soybean meal diet contained a retrospectively estimated 9.8 g phytate kg^{-1}. Korczynski *et al*. (1997) offered isonitrogenous, low- (wheat–casein) and high-fibre (wheat bran–wheat–casein) diets to pigs with denervated gastric pouches. However, the increase in dietary fibre was associated with an estimated increase in phytate levels from approximately 6.9 to 16.6 g kg^{-1}, and the dietary transition increased pepsin secretion by 70%. Like phytate, condensed tannin also has the capacity to bind protein; therefore, it is relevant that tannin has been shown to increase pepsin and HCl secretion in rats (Mitjavila *et al*., 1973). It is possible the secretion of the regulatory peptide, gastrin (Burhol, 1982; Furuse, 1999) triggers the compensatory outputs of pepsin and HCl in response to the gastric presence of refractory, phytate-bound protein.

As pepsin and HCl are 'endogenous aggressors' (Allen and Flemstrom, 2005), their increased outputs would be countered by protective mucin and sodium bicarbonate ($NaHCO_3$) secretions. Importantly, therefore, phytate has been shown to increase excretion of mucin and Na in broilers, which was ameliorated by microbial phytase (Cowieson *et al*., 2004). As mucin remains largely undigested in the small intestine, any increase in mucin secretion would exacerbate flows of endogenous amino acids derived from its protein component. Moreover, it has been demonstrated that phytate increases, and phytase decreases, endogenous amino acid flows in broilers (Cowieson and Ravindran, 2007; Cowieson *et al*., 2008). The amino acid profiles of pepsin (Blumenfeld and Perlmann, 1958; Tang *et al*., 1973) and mucin (Lien *et al*., 1997) have been documented and, instructively, the phytase-induced percentage increases in amino acid digestibility in broilers (Table 7.3) are correlated with amino acid profiles of pepsin ($r = 0.54$; $P < 0.05$) and mucin ($r = 0.70$; $P < 0.01$). These significant relationships indicate that microbial phytase enhances the digestibility of amino acids that are abundant in pepsin and mucin, presumably via stemming endogenous amino acid flows.

Ravindran *et al*. (2006) demonstrated that increasing dietary phytate levels decreased ileal Na digestibility (–0.38 versus –0.24; $P < 0.05$) and, conversely, microbial phytase increased Na digestibility (–0.18 versus –0.52; $P < 0.001$). Also, 500 FTU *E. coli* phytase kg^{-1} increased ileal digestibility coefficients of Na from –0.52 to –0.04 in broilers offered wheat-based diets containing 11.0 g phytate kg^{-1} (Selle *et al*., 2009b). Thus phytate has the capacity to pull Na into the small intestinal lumen, but this depletion of Na is counteracted by phytase. This phytate-induced transition of Na into the gut lumen may be in the form of $NaHCO_3$ to buffer excess HCl secretion. In addition, $NaHCO_3$ has

been shown to enhance intestinal alkaline phosphatase activity in rats (Akiba *et al*., 2007), which may be another reason for the movement of Na into the gut in response to dietary phytate. Furthermore, work by Mothes *et al.* (1990) demonstrated that the rate of formation of protein–phytate complexes could be reduced significantly through the addition of increasing levels of Na, with levels equivalent to those found in a 0.2% Na diet being sufficient to break up more than 65% of these complexes. It is possible, therefore, that the current 'requirements' for Na, which were generated prior to the use of phytases, may encompass a need to have adequate gastric Na concentrations to displace protein–phytate complex formation. In the presence of increasing phytase dosage, such complex-disrupting activities become more and more superfluous and, as a result, the Na requirement of the animal may well need to be reviewed in this era of ubiquitous phytase usage.

The absence of Na^+ in the medium has been shown to inhibit arginine, glutamic acid, glycine, leucine and valine transport in avian intestinal tissue (Lerner, 1984). Also, Ravindran *et al.* (2008) found that phytase increased ileal amino acid digestibility in maize–soy broiler diets at low dietary Na levels, but that this effect was diminished with increasing $NaHCO_3$ inclusion. It follows that phytate-induced Na depletion in the small intestine may disrupt Na-dependent transport systems and sodium pump (Na^+-K^+-ATPase) activity and this, in turn, could lead to diminished intestinal uptakes of amino acids and other nutrients. Phytate, as sweet potato extracts or Na phytate, has markedly reduced jejunal and ileal Na^+-K^+-ATPase activity in rats (Dilworth *et al*., 2005). Alternatively, 1000 FTU *E. coli* phytase kg^{-1} increased Na^+-K^+-ATPase activity in the duodenum and jejunum of broilers offered maize–soy diets by nearly 20% (Liu *et al.*, 2008). Also, phytate has been shown to reduce intestinal uptakes of glutamic acid and leucine as free amino acids in chickens (Onyango *et al*., 2008).

In summary, the likelihood is that phytate decreases protein digestibility, exacerbates endogenous amino acid flows and depresses intestinal uptakes of dietary and endogenous amino acids. The amelioration of these influences by phytase may be expressed as increased ileal amino acid digestibility in broilers.

Microbial phytase amino acid digestibility assays in swine

While there is the possibility that phytase increases ileal amino acid digestibility in pigs, the majority of assays indicate that this is not the case. This is curious because some studies have suggested that phytase enhances protein utilization in pigs (Beers and Jongbloed, 1992; Ketaran *et al*., 1993; Campbell *et al*., 1995; Biehl and Baker, 1996; Selle *et al*., 2003a). The Ketaren *et al*. (1993) study is of particular relevance because *A. niger* phytase increased protein deposition by 13.9% (123 versus 108 g day^{-1}) and protein retention by 9.1% (0.36 versus 0.33) in grower pigs. However, these improvements may have been secondary to phytase enhancing skeletal development rather than solely a primary 'protein effect'.

Interestingly, Just *et al*. (1985) offered a range of diets with an average protein level of 161 g kg^{-1} to 50 kg female pigs and determined protein deposition rates, which averaged 85 g day^{-1}. From retrospective estimates, 21 diets had average phytate contents of 9.4 g kg^{-1} and phytate:protein ratios of 0.063:1.0. However, dietary phytate:protein ratios were negatively correlated to protein deposition rates ($r = -0.48$; $P < 0.05$). The linear regression equation is as follows:

Protein deposition (g day^{-1}) = 99.8 – (243 × phytate:protein ratio).

The Just *et al*. (1985) study therefore suggests that increases in phytate levels relative to dietary protein have a deleterious impact on protein deposition. Also, the above equation predicts that a reduction in the phytate:protein ratio, via phytase degrading 50% of dietary phytate, would increase protein deposition by 8.3%, from 85.6 to 92.7 g day^{-1}.

Nevertheless, in general microbial phytases have not generated ileal amino acid digestibility responses of corresponding magnitudes. Only three studies (Officer and Batterham, 1992a,b; Barnett *et al*., 1993; Kornegay *et al*. 1998) have been reported where phytase has tangibly enhanced ileal amino acid digestibility. Coincidentally or not, in these three studies ileal digesta samples were taken from 'intact' (slaughtered or anaesthetized) pigs rather than cannulated animals. The method for taking ileal digest samples, either by various cannulation procedures or directly from intact pigs, appears to be pivotal. As reviewed by Selle and Ravindran (2008), in five assays involving 61 observations, phytase increased AID coefficients of amino acids by an average of 6.5% (0.767 versus 0.723) at a mean inclusion rate of 590 FTU kg^{-1} in intact pigs. In contrast, in cannulated pigs, 905 FTU phytase kg^{-1} increased AID of amino acids by only 1.7% (0.798 versus 0.785) from 281 observations in 11 studies. It is noteworthy that chromic oxide was used as the dietary marker in all 16 studies.

Curious outcomes have arisen from phytase amino acid digestibility assays in cannulated pigs. For example, Mroz *et al*. (1994) reported that 800 FTU *A. niger* phytase kg^{-1} significantly increased AID of methionine by 5.1% (0.806 versus 0.767), but numerically depressed threonine digestibility by 2.4% (0.720 versus 0.738). This response pattern is quite unusual as, among essential amino acids, threonine is usually the most, and methionine the least, phytase responsive. A more typical pattern was reported by Kornegay *et al*. (1998) in intact pigs, where phytase improved threonine digestibility by 16.2%, but methionine digestibility by 9.3% (Table 7.4).

In intact pigs, Officer and Batterham (1992a,b) reported that microbial phytase substantially increased the ileal digestibility of ten amino acids in linola meal (400 g kg^{-1}) by an average of 14.5% (0.715 versus 0.627), as shown in Table 7.4. Linola meal, a variant of linseed meal, was the sole protein source and the grower pigs were fed on a once-daily basis. Increasing Ca concentrations, relative to dietary phytate and protein levels, may depress amino acid digestibility responses to phytase (Selle *et al*., 2009a). In the studies of Officer and Batterham (1992a,b), the basal diet contained approximately 8.8 g Ca kg^{-1} and 9.0 g total P kg^{-1}; thus the 'inverse' Ca:P ratio of 0.98 coupled with

Table 7.4. Impact of microbial phytase on apparent ileal digestibility (AID) coefficients of amino acids where ileal digesta samples were taken from euthanized pigs (adapted from Officer and Batterham, 1992a,b; Kornegay *et al.*, 1998).

	Officer and Batterham (1992a,b)			Kornegay *et al.* (1998)		
Amino acid	Control	(1000 FTU) Phytase	Response (%)	Control	(500 FTU) Phytase	Response (%)
Essential						
Arginine	–	–	–	0.816	0.879	7.7
Histidine	0.57	0.69	21.1	0.790	0.850	7.6
Isoleucine	0.65	0.72	10.8	0.724	0.819	13.1
Leucine	0.64	0.72	12.5	0.787	0.828	5.2
Lysine	0.59	0.71	20.3	0.720	0.840	16.7
Methionine	0.71	0.75	5.6	0.761	0.832	9.3
Phenylalanine	0.67	0.74	10.5	0.770	0.837	8.7
Threonine	0.50	0.62	24.0	0.648	0.753	16.2
Valine	0.63	0.70	11.1	0.715	0.803	12.3
Non-essential						
Alanine	–	–	–	0.729	0.795	9.1
Aspartic acid	–	–	–	0.745	0.833	11.8
Cystine	0.68	0.81	19.1	0.688	0.772	12.2
Glutamic acid	–	–	–	0.806	0.847	5.1
Glycine	–	–	–	0.603	0.660	9.5
Proline	–	–	–	0.770	0.798	3.6
Serine	–	–	–	0.750	0.819	9.2
Tyrosine	0.63	0.69	9.5	0.719	0.797	10.9

the poor inherent amino acid digestibility of linola meal may have contributed to the pronounced responses generated by 1000 FTU phytase kg^{-1}.

In a subsequent Wollongbar study, Barnett *et al.* (1993) reported that 1000 FTU phytase kg^{-1} significantly improved ileal N digestibility by 7.6% (0.71 versus 0.66) using a slaughter technique in weaner pigs offered P-adequate, conventional diets *ad libitum* from 28 to 49 days of age. Phytase also numerically increased ileal lysine digestibility by 5.5% (0.77 versus 0.73), crude protein deposition by 7.6% (59.5 versus 55.3 g day^{-1}) and feed efficiency by 4.0% (1.43 versus 1.49). In fact, this is one of a series of a weaner pig studies which indicate that the magnitude of phytase feed efficiency responses is governed by dietary phytate levels (Selle *et al.*, 2003a).

Segments of the Kornegay *et al.* (1998) study have subsequently been published in refereed journals (Radcliffe *et al.*, 1999, 2006; Zhang and Kornegay, 1999). This study is instructive, as 500 FTU *A. niger* phytase kg^{-1} increased AID coefficients of 17 amino acids by an average of 3.8% (0.779 versus 0.751) in cannulated pigs offered low-protein, maize–soy diets. In contrast, phytase increased amino acid digestibility by 9.5% (0.806 versus 0.738) when ileal digesta samples were taken from intact pigs (Table 7.5). These data therefore indicate that the impact of microbial phytase on amino acid digestibility is more pronounced when ileal digesta samples are taken from intact pigs as opposed to cannulated animals.

Table 7.5. Effects of *Aspergillus niger* phytase 500 FTU kg^{-1} on apparent ileal digestibility (AID) of amino acids assessed in cannulated and intact pigs offered low-protein (100 g kg^{-1}) maize–soy diets (adapted from Kornegay *et al*., 1998).

	Cannulated pigs			Intact pigs		
Amino acid	Control (AID)	Phytase (AID)	Response (%)	Control (AID)	Phytase (AID)	Response (%)
Essential						
Arginine	0.840	0.859	2.26	0.816	0.879	7.72
Histidine	0.804	0.817	1.62	0.790	0.850	7.59
Isoleucine	0.730	0.759	3.97	0.724	0.819	13.12
Leucine	0.799	0.816	2.13	0.787	0.828	5.21
Lysine	0.727	0.760	4.54	0.720	0.840	16.67
Methionine	0.758	0.780	2.90	0.761	0.832	9.33
Phenylalanine	0.786	0.808	2.80	0.770	0.837	8.70
Threonine	0.660	0.700	6.06	0.648	0.753	16.20
Valine	0.700	0.735	5.00	0.715	0.803	12.31
Non-essential						
Alanine	0.714	0.743	4.06	0.729	0.795	9.05
Aspartic acid	0.744	0.776	4.30	0.745	0.833	11.81
Cystine	0.747	0.778	4.15	0.688	0.772	12.21
Glutamic acid	0.823	0.847	2.92	0.806	0.847	5.09
Glycine	0.629	0.6897	9.22	0.603	0.660	9.45
Proline	0.789	0.810	2.66	0.770	0.798	3.64
Serine	0.780	0.804	3.08	0.750	0.819	9.20
Tyrosine	0.738	0.764	3.52	0.719	0.797	10.85
Mean	0.751	0.779	3.83	0.738	0.810	9.89

It is noteworthy that chromic oxide has been used as the marker in phytase amino acid digestibility assays in pigs given its general, and perhaps specific, shortcomings (Selle and Ravindran, 2008). However, Nitrayova *et al*. (2009) compared chromic oxide and acid-insoluble ash as dietary markers in a phytase amino acid digestibility assay in cannulated pigs. While responses to phytase in ileal amino acid digestibility were slightly higher with acid-insoluble ash than with chromic oxide, the authors concluded that marker selection was not the main factor responsible for the ambiguous outcomes recorded in the literature.

Apart from the surgical intervention, cannulated pigs are usually fed on a restricted, twice-daily basis in contrast to the normal situation where pigs have unrestricted access to feed. Conceivably, this difference in feeding regimen may cause variations in retention of digesta in the stomach and gastric pH. Presumably, longer gastric retention times would facilitate phytate hydrolysis by microbial phytase, and more acidic gastric contents would promote protein–phytate complex formation. However, there is a lack of compelling evidence that differences in feeding regimen would greatly influence gastric emptying (Gregory *et al*., 1990) and gastric pH (Babouris *et al*., 1965; Lawrence, 1972; Laplace, 1974).

Bryden and Bluett (1968) reported that gut microflora made up 12% of ileal contents on a dry matter basis in chicks. Thus, amino acids in ileal digesta

may be derived from gut microbes as well as being of dietary and endogenous origin. However, it is probable that greater concentrations of amino acids from gut microflora are present in ileal digesta of pigs than broilers. Indeed, Jorgensen and Just (1988) concluded that microbial activity at the terminal ileum in cannulated pigs was equivalent to that in the mid-large intestine. Rowan *et al*. (1992) also judged that there is a substantial microfloral population in the small intestine of ileostomized pigs, which may have implications for nutrient digestibility determinations in surgically modified animals. Brand *et al*. (1990) compared endogenous secretions of protein/amino acids in intact and ileo-rectal anastomosed pigs offered protein-free diets. Surgical intervention doubled crude protein secretions from 5.79 to 12.11 g day^{-1} and, among amino acids, absolute increases were most pronounced for glycine, histidine, leucine and threonine. It is likely that the surgical intervention created an inflammatory response, part of which is evident as an increase in villus turnover and mucin secretion, particularly in the vicinity of the wound. This, coupled with increased microbial interconversions of this 'extra amino acid bounty' post-cannula, is the likely reason for these confusing results and suggests that data from cannulated animals need to be interpreted with care.

Miner-Williams *et al*. (2009) offered cannulated pigs casein-based diets on the premise that casein amino acids would be absorbed proximal to the terminal ileum. On this basis, 19.8% of amino acids in ileal digesta flows were of microbial origin, with the balance consisting of endogenous amino acids. Thus there may be a greater concentration of amino acids of microbial origin at the terminal ileum of cannulated, as opposed to intact, pigs. This raises the possibility that microbial proliferation in the small intestine of cannulated pigs converts sufficient amino acids from dietary and endogenous origin to bacterial amino acids in the terminal ileal digesta, thereby masking the beneficial effects of phytase on dietary and endogenous amino acids.

In summary, it appears that investigations into the influence of phytase on ileal amino acid digestibility in cannulated pigs are unlikely to be rewarding. Consideration could be given to alternative methods to elucidate the possible 'protein effect' of microbial phytase in pigs. For example, Gagne *et al*. (2002) found that phytase increased postprandial plasma concentrations of α-amino N in growing pigs, and suggested this was indicative of phytase enhancing amino acid absorption. Jansman *et al*. (1997) described a procedure to define the postprandial time interval at which absorption of amino acids was maximal in pigs. These workers used this procedure to compare amino acid absorption rates from various protein sources (e.g. soy concentrate versus soybean meal), and found significant differences. Presumably, this procedure could be used to evaluate the influence of phytase on amino acid absorption rates.

A further possibility would be to adopt the indicator amino acid oxidation technique to determine the effects of phytase on the availability of amino acids (Kim and Bayley, 1983; Moehn *et al*., 2005; Elango *et al*., 2009). Using this approach, Moehn *et al* (2007) reported that the metabolic activity of lysine varied between soybean meal (87.5%), cottonseed meal (75.1%) and canola meal (71.4%) as opposed to free lysine (100%) in pigs. They also found that heating peas reduced lysine metabolic availability from 75.8 to 68.3%. The

determination of the impact of microbial phytase on metabolic availability of amino acids in relevant feedstuffs may prove highly instructive.

Nevertheless, the fundamental differences between pigs and poultry in this respect cannot be dismissed. For example, as reviewed by Cowieson *et al*. (2009), phytate has consistently been shown to exacerbate endogenous amino acid flows in broiler chicks, which were attenuated by microbial phytase. In contrast, the study by Woyengo *et al*. (2009) indicates that phytate, as Na phytate, does not influence endogenous amino acid losses in weaner pigs offered casein–maize starch diets.

Factors influencing protein–phytate complex formation

Factors that influence protein–phytate complex formation are considered because the extent of their formation is probably critical to the 'protein effect' of phytate and phytase. The results from the *in vitro* study of Vaintraub and Bulmaga (1991) emphasize the critical importance of pH on the pepsin-refractory nature of complexed protein. At pH 2.5, pepsin digestion of phytate-bound casein was retarded by 50% but the digestion of casein was not impeded at pH 4.0. Gizzard fluid taken from 22 non-anaesthetized birds had an average pH of 2.05 (Farner, 1943), which would be conducive to phytate binding protein and reducing its vulnerability to pepsin digestion. Phytate has an affinity for casein, as Na phytate has been shown to reduce *in vitro* casein solubility from 99 to 1% at pH 2.0 (Kies *et al*., 2006). Shan and Davis (1994) added 20 g Na phytate kg^{-1} to an atypical broiler diet containing 150 g casein kg^{-1}, which depressed weight gain (44%), feed intake (22%) and feed efficiency (29%) from 28 to 46 days post-hatch. Presumably the profoundly depressed growth performance was pursuant to reduced protein digestibility following the binding of casein by Na phytate. Given the importance of pH in the stomach or proventriculus, it is noteworthy that limestone, a key source of Ca, has a very high acid-binding capacity of capacity of 15,044 meq kg^{-1} at pH 3.0 (Lawlor *et al*., 2005). Thus Ca, as limestone, will tend to increase gut pH and high dietary limestone levels may depress the formation of protein–phytate complexes.

The propensity of proteins to be bound by phytate is variable, which may be dependent on their structure and the accessibility of basic amino acid residues (Champagne, 1988). For example, Kies *et al*. (2006) found that the affinity of phytate for canola meal protein was relatively low. At pH 2.0, Na phytate reduced the solubility of canola meal protein solubility from 100 to 63% but phytate had little influence as pH increased. This is consistent with the relatively modest average increase of 2.7% (0.799 versus 0.778) in AID coefficients of 14 amino acids following the addition of *A. niger* phytase 1200 FTU kg^{-1} to a broiler diet containing 526 g canola meal kg^{-1} (Ravindran *et al*., 1999a). Alternatively, Kies *et al*. (2006) reported that Na phytate reduced soybean meal protein solubility from 91 to 2% at pH 2.0 and from 60 to 23% at pH 3.0. In keeping, Ravindran *et al*. (1999a) reported that phytase increased amino acid AID coefficients by a more robust 4.2% (0.850 versus 0.816) in broiler diets containing 438 g soybean meal kg^{-1}.

Ravindran *et al*. (1999a) also reported that phytase enhanced AID of amino acids in wheat (9.3%) to a greater extent than in maize (3.4%) in broilers, and this difference in response to phytase was subsequently confirmed (Rutherfurd *et al*., 2002). These findings are consistent with the formation of protein–phytate complexes in wheat reported by Hill and Tyler (1954b), whereas O'Dell and De Boland (1976) did not detect protein–phytase complex formation in maize. However, Kies *et al*. (2006) reported that Na phytate reduced the solubility of maize protein from 100 to 28% at pH 2.0, with a more modest reduction at pH 3.0 and little influence at pH 4.0–5.0. However, the negative finding by O'Dell and De Boland (1976) was made following gel filtration at pH 4.4, which does not preclude phytate complexing maize proteins at a more acidic pH. It seems reasonable to conclude that, if the pH that prevailed in the forestomach of broilers offered maize-based diets in the studies of Ravindran *et al*. (1999a) and Rutherfurd *et al*. (2002) had been more acidic, both complex formation and amino acid digestibility responses to added phytase might have been greater.

Broilers may be fed diets containing a proportion of whole grains, which stimulates gizzard function (Cumming, 1994). In one experiment, it was shown that feeding whole grains significantly increased gizzard weight by 28% (37.5 versus 29.2 g) and reduced the pH of gizzard digesta from 3.6 to 2.9 (Rutkowski and Wiaz, 2001). On the one hand, this reduction in pH would tend to increase the solubility of Mg–phytate complexes (Cheryan *et al*., 1983) and presumably increase the extent of phytate degradation by exogenous phytase, particularly if digesta are retained for longer intervals in a more active gizzard. On the other, the formation of insoluble protein–phytase complexes would be favoured by such a pH reduction. Therefore, microbial phytase may be more effective in a context of feeding whole grains as opposed to diets in which the entire grain component is ground.

'Energy Effect' of Phytase

The possibility that phytate depresses energy digestion and utilization and that phytase has a reciprocal, positive effect is clearly an increasingly important issue. Microbial phytase consistently enhances metabolizable energy (ME) of broiler diets, but the impact of phytase on digestible energy (DE) of pig diets is not as pronounced. One example where a DE effect has been reported in pigs (from 20–107 kg liveweight) is that of Johnston *et al*. (2009). The effect of phytase on digestibility and subsequent utilization of energy suggests that net energy (NE) studies may provide clarification. The formulation of pig diets on the basis of NE (de Lange and Birkett, 2005) is an increasing practice. It seems possible that the phytate content of relevant feedstuffs contributes to the differential between the NE of a diet and the DE of swine diets and the ME of poultry diets. Certainly, the work of Pirgozliev *et al.* (in press) suggests that, in poultry, in some cases the use of phytase has little effect on ME but significant effects on NE, suggesting there may be a post-absorptive partitioning effect of this enzyme. On the other hand, the consistent beneficial effect that phytase

addition has on feed intake of birds fed P-deficient diets will clearly increase daily energy intake. The use of phytase under these circumstances will increase the proportion of AME intake that is in excess of maintenance requirements. Thus NE studies, or AME studies coupled with data on intake effects of this enzyme, would be most appropriate. The vast majority of studies, however, have focused on the DE and AME effects of this enzyme in isolation.

Early studies in poultry, involving dephytinized feed ingredients, suggested that phytate negatively influences energy utilization in broilers (Rojas and Scott, 1969; Miles and Nelson, 1974). As reviewed by Selle and Ravindran (2007) in a series of 12 studies, phytase activity of 662 FTU kg^{-1} increased the AME of broiler diets by an average of 0.36 MJ (13.64 versus 13.27 MJ kg^{-1} dry matter) where the percentage responses in AME were negatively correlated ($r = -0.562$; $P < 0.02$) to the energy density of the control diets.

Baker (1998) suggested that the positive impact of phytase on energy utilization stems from an accumulation of increased protein, fat and starch digestibilities; essentially, this proposition was confirmed by Camden *et al*. (2001). These workers evaluated two phytase feed enzymes (*Bacillus subtilis* at 250, 500 and 1000 FTU kg^{-1}; *A. niger* at 500 FTU kg^{-1}) in broilers offered maize–soy diets and, overall, phytase increased ileal digestibility coefficients of protein by 2.6% (0.866 versus 0.844), fat by 3.5% (0.954 versus 0.921) and starch by 1.4% (0.978 versus 0.964). This was associated with phytase-induced increases in AME of 0.17 MJ (15.29 versus 15.12 MJ kg^{-1}) and apparent ileal digestibility of energy of 0.26 MJ (16.34 versus 16.08 MJ kg^{-1}).

Axiomatically, enhanced digestibility of amino acids would increase the utilization of energy derived from proteins, and the roles of phytate and phytase have been discussed in this connection. In respect of fats, there is evidence that phytate interacts with lipids via the formation of 'lipophytins', which are complexes of Ca–/Mg–phytate, lipids and peptides (Cosgrove, 1966). Therefore, it is likely that Ca–phytate and lipids are involved in the formation of metallic soaps in the gut lumen of poultry, which are major constraints on utilization of energy derived from lipids, particularly saturated fats (Atteh and Leeson, 1984; Leeson, 1993). Matyka *et al*. (1990) found that beef tallow reduced phytate-P utilization in young chicks and increased the percentage of fat excreted as soap fatty acids. Also, Ravindran *et al*. (2000) reported more pronounced AME responses to phytase in diets with higher Ca levels, which is consistent with the involvement of Ca–phytate complexes in the formation of insoluble metallic soaps. If Ca–phytate is a component of metallic soaps in broilers, it follows that phytase would partially prevent their formation by prior hydrolysis of phytate in more proximal parts of the gut.

It has been suggested that phytate may bind with starch either directly, via hydrogen bonds, or indirectly, via proteins associated with starch (Thompson, 1988a,b; Rickard and Thompson, 1997). This would provide a rationale for phytase increasing energy utilization from this source; however, there is a paucity of *in vitro* evidence to support the existence of starch–phytate complexes (Selle *et al*., 2000). Phytate has been shown to reduce blood glycaemic indices in humans (Thompson *et al*., 1987). However, as discussed by Rickard and Thompson (1997), this may be related to depressed intestinal

glucose uptakes rather than to impaired starch digestion, as phytate addition to a glucose test meal has been shown to reduce glucose absorption (Demjen and Thompson, 1991). Alternatively, phytate is a potent inhibitor of α-amylase activity (Cawley and Mitchell, 1968). This has been confirmed frequently in subsequent studies; indeed, Desphande and Cheryan (1984) proposed that phytate inhibition of α-amylase might regulate starch reserves during seed germination. While Martin *et al*. (1998) reported that phytase supplementation reduced amylase activity in the small intestine of ducks, it is not clear whether phytate inhibition of α-amylase in the gastrointestinal tract of poultry limits starch digestion, although responses to α-amylase supplementation have been reported in broilers (Gracia *et al*., 2003) and turkeys (Ritz *et al*., 1995). Phytate may have the capacity to inhibit α-amylase *in vivo*, but whether or not this is a limiting factor on starch digestion, which could be counteracted by phytase, is questionable.

Tangible evidence of a corresponding energy effect of phytase in pigs is lacking and, anecdotally, it was thought that any effect would be limited to that derived from enhanced protein digestibility (Selle *et al*., 2000). However, Brady *et al*. (2003) reported that graded inclusions of *Peniophora lycii* phytase linearly increased DE in pigs. Phytase (1000 FTU kg^{-1}) increased the DE of diets based on barley, maize and soybean meal by 0.9 MJ (15.2 versus 14.3 MJ kg^{-1}). Phytase also increased back-fat depth measurements and decreased lean carcass yield, and this adverse influence on carcass traits was attributed to phytase-induced, increased energy utilization.

Ostensibly, the findings of Brady *et al*. (2003) suggest that phytase has an energy effect in pigs; however, at the standard rate of 500 FTU kg^{-1}, phytase had a negligible impact on DE (14.5 versus 14.3 MJ kg^{-1}). Also, the experimental diets contained low levels of available P (1.3 g kg^{-1}) and phytase markedly increased total-tract P digestibility. It is possible that, in this context, these phytase-induced increases in P availability were reflected in enhanced energy utilization. In an earlier study, O'Quinn *et al*. (1997) reported that *A. niger* phytase addition (300 and 500 FTU kg^{-1}) to sorghum–soybean meal diets did not alter apparent ileal or total-tract energy digestibility, dressing percentage or back-fat depth. Similarly, Harper *et al*. (1997) found that phytase addition (250 and 500 FTU kg^{-1}) to low-P, maize–soy diets did not influence carcass yield or back-fat measurements. Therefore, it may be that investigations of phytase at higher than standard inclusion rates are merited in respect of energy effects in pigs.

It should be appreciated from the above that some of the anti-nutritive effects of phytate are immediate and reduce digestibility of nutrients, whereas others take several days to develop and, as a result in reduced efficiency, in partitioning of ME to NE. As a result, simple true ME, AME or DE assays may not capture neither the full anti-nutritive effect of phytate, nor axiomatically the full benefit of a phytase when fed commercially.

Factors Influencing Phytase Efficacy

Numerous factors have been identified that influence the efficacy of exogenous phytases, which is partially reflected in the inconsistent responses to phytase that have been reported in the literature. An exhaustive consideration of all potential factors is simply impractical. To take one example, Leslie *et al*. (2006) reported that reducing the lighting programme for broilers from 24 to 12 h increased dephosphorylation of IP_6 by phytase. Presumably, this is a consequence of longer digesta retention times in the crop, which would facilitate phytase activity. Dietary phytate levels and their sources, the particular type of phytase added and its inclusion rate are clearly important factors. Nevertheless, dietary Ca levels, usually provided as limestone, have a considerable influence on phytase efficacy.

Calcium

The impact of Ca on phytase efficacy was specifically considered in a review by Selle *et al*. (2009a). The concept that high dietary Ca levels and/or 'wide' Ca:P ratios diminish responses to exogenous phytases is well accepted; the likely genesis of this concept was a weaner pig study reported by Lei *et al*. (1994). The addition of phytase 1200 FTU kg^{-1} to P-inadequate diets containing vitamin D 660 IU kg^{-1} was associated with markedly enhanced growth performance with 4.0 g Ca kg^{-1} as compared with 8.0 g Ca kg^{-1}. For example, from 21 to 30 days of age, weaners on the higher-Ca diets had a daily gain of 303 g, a daily feed intake of 840 g and a gain:feed ratio of 367. In contrast, the corresponding figures for the lower-Ca diets were 573 g, 1192 g and 480, which represents improvements of 89%, 42% and 31%, respectively. The authors concluded that higher Ca levels, and wider Ca:P ratios, depressed exogenous phytase efficacy, which was attributed to Ca progressively precipitating phytate in 'extremely insoluble' Ca–phytate complexes in the intestine. However, a superior trial design would have included non-phytase-supplemented diets to determine the impact of Ca per se in this context.

However, Driver *et al*. (2005) subsequently reported conflicting results in broilers, as 1200 FTU *A. niger* phytase kg^{-1} was more effective in maize-soy diets containing 8.6 g Ca kg^{-1} than 4.7 g Ca kg^{-1}. Predictably, these authors concluded that much of the published data concerning the efficacy of phytase at different Ca:P ratios was misleading, that phytase efficacy is a complex function of dietary Ca, total P and phytate-P concentrations, and that Ca reactions with inorganic P, which may lead to the flocculent precipitation of calcium orthophosphate ($Ca_3(PO_4)_2$), merit more attention. While the Lei *et al*. (1994) study (and similar studies) is open to criticism, the influence of Ca on phytate degradation by phytase in pigs and poultry is an issue that has been raised.

Thus Ca–phytate complex formation along the gastrointestinal tract, where one phytate (IP_6) molecule binds up to five Ca atoms, assumes importance

since approximately one-third of dietary Ca may be bound to phytate in digesta. Consequently, phytate limits the availability of both P and Ca as a result of insoluble Ca–phytate complex formation, the extent of which is driven by gut pH and molar ratios of the two components. It is accepted that Ca–phytate complexes are mainly formed in the small intestine, where they have a substantial negative influence on the efficacy of mucosal phytase. However, exogenous phytases are mainly active in more proximal segments of the gut and at lower pH levels, so their efficacy should not be influenced by Ca–phytate complexes in the small intestine. There are, however, data to indicate that Ca and phytate interactions occur under acidic conditions with the formation of soluble and insoluble Ca–phytate species, which could negatively impact on exogenous phytase efficacy. Also, limestone has a high acid-binding capacity, which may raise the pH of the gastric phase. For example, McDonald and Solvyns (1964) increased dietary Ca levels from 9 to 13 g kg^{-1} with limestone, which elevated digesta pH from 5.6 to 6.1 in the small intestine of chickens. Given that pepsin-refractory, protein–phytase complexes are formed in a narrow pH range of 2.0–3.0 (Vaintraub and Bulmaga, 1991), any limestone-induced increase in gut pH could reduce complex formation and mute the negative impact of phytate on protein digestibility.

Indeed, Pontoppidan *et al*. (2007) suggested that increasing Ca:phytate ratios will counteract the precipitation of protein by phytate, and these workers reported that Ca modestly increased the solubility of phytate and protein between pH 2.0 and 5.0. Moreover, Prattley *et al*. (1982) reported that additional Ca reduced the amount of bovine serum albumin bound to sodium phytate by approximately 40% over the same pH range. Similarly, Hill and Tyler (1954a) found that high Ca:phytate molar ratios from limestone addition substantially increased the solubility of wheat gluten–sodium phytate complexes and formed insoluble protein–phytase complexes at pH 3.0. Okubo *et al*. (1974a,b, 1976) investigated the binding of glycinin by phytate where, at pH levels below the isoelectric point of glycinin (pH 4.9), Ca decreased the stability of protein–phytase complexes. These researchers found that sufficient Ca was able to dissociate glycinin–phytate complexes at pH 3.0, which was attributed to Ca directly competing with basic protein residues for the negatively charged P moieties of phytate. In fact, the capacity of Ca to release protein from binary complexes at acidic pH has been adopted to prepare phytate-free soy protein isolates (Okubo *et al*., 1975).

There is also evidence that Ca may react with soy protein directly, even under conditions of acidic pH (Kroll, 1984; Gifford and Clydesdale, 1990). The suggestion is that high dietary concentrations of Ca (relative to phytate and protein) may reduce the extent of protein–phytase complex formation in the stomach by reacting with phytate and/or protein at acidic pH. As a result, increasing Ca concentrations may have the capacity to diminish binary protein–phytase complex formation.

In this context, the study by Ravindran *et al*. (2000) is relevant, where *A. niger* phytase 800 FTU kg^{-1} increased the AID of eight amino acids in broiler diets based on wheat–sorghum blends by an average of 3.75%, with responses ranging from 1.06 to 7.45%. However, this assay embraced a range of dietary

concentrations of Ca (8.7–13.9 g kg^{-1}), phytate (12.06–22.34 g kg^{-1}) and protein (213–221 g kg^{-1}). It may be deduced from the analysed values that there were significant, negative correlations between phytase-induced percentage increases in amino acid digestibility and both Ca:phytate ratios and Ca:protein ratios. Taken together, the multiple linear regression equation (r = 0.74; P <0.001) is as follows:

Mean percentage phytase response = 15.0 – (10.1 × Ca:phytate) – (78.6 × Ca:protein).

This equation predicts that, as dietary Ca levels increase relative to phytate and protein contents, amino acid digestibility responses to phytase diminish. Interestingly, Agbede *et al*. (2009) subsequently determined the effects of *P. lycii* phytase on amino acid digestibility in caecectomized layers on maize-based diets with adequate (44.9 g kg^{-1}) and low (38.5 g kg^{-1}) Ca levels. At adequate Ca levels, phytase increased the average digestibility coefficient of 13 amino acids by 0.70% (0.862 versus 0.856). However, at low levels of Ca, the phytase response was a more robust increase of 2.25% (0.864 versus 0.845). For example, phytase increased the digestibility of threonine by 2.7% in adequate-Ca diets, but by 4.1% in low-Ca diets. The outcome prompted the authors to conclude that interactions between dietary Ca and phytase may be responsible for the variations reported in phytase amino acid digestibility assays.

Phosphorus

Microbial phytase increases dietary non-phytate P levels, and it is to be expected that the addition of phytase to diets that are inadequate in this respect drives growth performance responses. Alternatively, the supplementation of diets that are adequate or even contain a surplus of non-phytate P may generate different outcomes, although there is the argument that high levels of inorganic P will have a negative influence on phytase efficacy (Lei and Stahl, 2000). For example, Atteh and Leeson (1983) investigated the effects of increasing available P levels in maize–soy broiler diets from 7 to 10 g kg^{-1}. This increase in available P significantly depressed weight gain by 11.3% (468 versus 528 g per bird), feed efficiency by 3.4% (1.51 versus 1.48) and tended to increase leg deformities in chicks to 21 days of age. Clearly, the implication is that the addition of phytase to diets already containing relatively high non-phytate P levels could generate a counterproductive P excess. This emphasizes the importance of applying appropriate phytase matrix values to supplemented diets with identified non-phytate and phytate-P concentrations.

Feed processing

In recent years increasing attention is being paid to the effects of feed processing on pig and poultry performance, with emphasis on grain particle size and

temperatures at which diets are steam-pelleted. There are some initial indications that these procedures may influence responses to microbial phytase. Kasim and Edwards (2000) offered maize–soy diets to broilers in which the grain component (532 g kg^{-1}) was ground to three different sizes with geometric mean diameters of 484 μm (fine), 573 μm (medium) and 894 μm (coarse). Determined on a total-tract basis, retention of phytate-P increased (P <0.05) with particle size from 0.389 (fine) to 0.426 (medium) to 0.457 (coarse). The addition of 600 FTU phytase kg^{-1} further increased (P <0.01) phytate-P retention to 0.558, 0.585 and 0.628, respectively, and there was no treatment interaction. Similar findings have been reported by Berwal *et al*. (2008), in that increasing particle size of a maize-based diet was associated with higher total P retention. Subsequently, Amerah and Ravindran (2009) offered broilers maize–soy diets in which the grain was ground to medium (611 μm) and coarse (849 μm) particle sizes. In this study, 500 FTU phytase kg^{-1} increased toe ash of broilers offered medium-ground maize diets (11.65 versus 10.41%; P <0.05). However, there was a treatment interaction (P <0.01) because phytase did not significantly influence bone mineralization (11.78 versus 11.42%) in coarse maize diets. The authors suggested that coarsely grinding maize had beneficial effects on P bioavailability. Therefore, it is interesting that Gabriel *et al*. (2008) reported that offering broilers diets containing whole wheat (200–400 g kg^{-1}) significantly increased alkaline phosphatase activity in the duodenum and jejunum by approximately 16.5%. It may be that stimulation of gizzard function by feeding whole or coarsely ground grain in turn stimulates the development of small intestinal mucosa and alkaline phosphatase secretion, which could enhance P bioavailability.

The addition of microbial phytase to broiler diets based on either 'raw' wheat or the same wheat that had been pre-pelleted (90°C) was compared (Selle *et al*., 2007). More robust AME and growth performance responses were observed following the addition of phytase to 'raw' wheat diets, but treatment interactions were not significant. However, phytase increased N retention in broiler diets based on 'raw' wheat but depressed N retention with pre-pelleted wheat, so that there was a significant (P <0.01) treatment interaction. There is some evidence to suggest that heat-treating wheat reduces phytate and protein solubility (Ummadi *et al*., 1995) and, if so, it follows that phytate may be less readily enzymatically degraded and the extent of protein–phytase complex formation may be reduced. This suggests that high pelleting temperatures of diets may depress responses to phytase supplementation.

Other enzymes

It has recently been reported that the beneficial effects of exogenous xylanase in poultry and swine diets are inextricably linked to the size of the undigested portion of fat, protein and starch that leaves the ileum (Cowieson and Bedford, 2009). This observation, supported by some 19 peer-reviewed papers published between 1998 and 2009, rules out, by definition, full additivity between pro-nutrients. As phytase (whether credited or not) improves ileal

protein, fat and starch digestibility by reducing endogenous loss and improving dietary nutrient solubility, it thereby reduces the undigested fraction. Thus, in this situation the energy matrix for xylanase should be reduced (by around 20%) in the presence of phytase to acknowledge the now reduced undigested fraction. By definition then, only the first additive of choice can carry its full matrix when added to a 'virgin' diet, but subsequent additives should have their matrices discounted to accommodate the influence of the current incumbents. As theoretical (if not realistic) maximum ileal digestibility is 100%, digestibility-enhancing pro-nutrients constantly move digestibility toward that fixed asymptote, so opportunity for further improvement declines with each new addition. It is therefore recommended that, if the matrix values that a supplier promotes were established in diets that do not contain phytase, antibiotic growth promoters, coccidiostats and other commonly used additives, the matrix be discounted proportionate to the benefits of the incumbents. For example, an energy matrix of 100 kcal kg^{-1} for a xylanase may end up being 50–60 kcal kg^{-1} in a diet containing an array of performance- and digestibility-enhancing therapeutics and enzymes.

Energy matrices and added fat

Conventionally, the energetic benefits conferred by exogenous enzymes are captured by a reduction in the lipid concentration in the diet, i.e. removal of vegetable or animal fat sources. However, it is important to note that enzymes are not necessarily a suitable direct replacement for fats and oils, as extra-caloric effects of lipids will not be delivered through the use of enzyme technology. Examples of extra-caloric benefits of fat include pellet quality, essential fatty acids, fat-soluble vitamins (A, D, E and K), balancing gastric emptying with protein and carbohydrate digestion, mill efficiency (energy use and throughput) and perhaps even heat increment. Clearly, xylanases and phytases are not direct replacements for these important effects and so the removal of fat to accommodate the energy matrices of enzymes should be done with care. In a recent study (Cowieson, 2010), the removal of 2% soy oil from a maize soy-based broiler diet resulted in a significant decrease (~3%) in ileal amino acid digestibility at day 21. Interestingly, this effect was not observed by day 42 (change from PC to NC = approximately 0.4%), and furthermore not all amino acids were similarly influenced. This observation supports a previous report in piglets (Li and Sauer, 1994), where the removal of canola oil resulted in a significant reduction in amino acid digestibility. Presumably these effects are mediated by changes in gastric empyting, which is driven in part by dietary fat concentrations (Stacher *et al*., 1990; Gentilcore *et al*., 2006), i.e. low-fat diets may reduce residency of feed in the proventriculus/gizzard, or even residency of food in the intestinal tract *per se* (Mateos *et al*., 1982).

It is interesting that the amino acids most detrimentally influenced by the removal of added fat are those that have been shown to be released last from the sequence of endogenous proteolytic mechanisms (Low, 1990). Thus, the removal of oil to accommodate the metabolizable energy advantages that

enzymes confer may be unwise in young animals, as this strategy may inadvertently compromise ileal amino acid digestibility, especially for threonine which tends to be the last dietary amino acid to be exposed to exopeptidase activity. Additionally, removal of fat may compromise the digestibility of non-lipid energy sources such as glucose and fructose (Mateos and Sell, 1980), another cause for constraint in application of bullish energy matrices in young animals. It may be wise to employ moderation in fat removal in starter diets and to capture the economic value of energy matrices in the grower and finisher phases, when fat concentrations are higher and the animal is less susceptible to gastric digestion constraints. Instructively, rapid gastric emptying caused by the ingestion of diets with a low fat density does not persist, as compensatory mechanisms are activated over time (Covasa and Ritter, 2000). These deleterious effects may be transitory and restricted to neonates, a contention that is supported by a previous report (Cowieson, 2010).

A further unforeseen consequence of reduced gastric residence time is that the efficacy of a phytase, if present, will also be compromised, since the proventriculus/gizzard is thought to be the most relevant for phytase activity. Thus a dietary modification made in order to profit from the energy-sparing benefit observed when a xylanase is used may result not only in direct losses in amino acid and starch digestibility but also in phytate hydrolysis, with the ensuing further losses in mineral, energy and amino acid benefits that were attributed to phytate hydrolysis.

Conclusions

The gastrointestinal tracts of pigs and poultry differ structurally, physiologically and functionally; therefore, it is not surprising that responses to the dietary inclusion of phytases differ between the species. Somewhat paradoxically, phytases appear to degrade phytate to a greater extent and liberate more phytate-bound P in pigs than in broiler chickens, but the 'extra-phosphoric effects' of phytases appear to be pronounced in broiler chickens. In a parallel situation, growth performance and nutrient utilization responses to non-starch polysaccharide (NSP)-degrading enzymes are typically of a greater magnitude in broiler chickens than in pigs. Perhaps this is because grower-finisher pigs are better able to tolerate the anti-nutritive effects of either phytate or NSP than broilers. However, weaner pigs are probably more vulnerable to phytate, as reflected in feed efficiency responses to phytase in relation to dietary phytate levels (Selle *et al*., 2003a), which may reflect the relative immaturity of their gut development.

While microbial phytases have been used in practice for nearly two decades, many advances could be made in their application in apparently fundamental areas. The rapid and accurate determination of dietary phytate levels is one example. Another is to establish the quantity of Ca actually released by phytase, as it seems that this may be understated at present and further reductions in dietary Ca levels are feasible, and that such reductions would enhance enzyme efficacy. The extent to which phytase increases ileal amino acid digestibility

and/or protein availability in pigs and poultry still requires clarification so that full advantage of the 'protein effect' of phytase may be taken. This situation is at least equally true for the possible phytase-induced enhancement of energy utilization.

The likelihood remains that more effective exogenous phytases and/or combinations with other facilitative enzymes will be developed. In this regard, inherent phytate-degrading capacity, a broad pH spectrum of activity, resistance to endogenous proteolytic enzymes, thermostability and the feasibility of higher inclusion rates are all key factors. In this event, a better appreciation of how best to manipulate diet formulations to take full advantage of higher phytate degradation rates will be needed. Assuming that these advances take place, exogenous phytases will be added to an even larger majority of pig and poultry diets on a global basis. The growth in acceptance of feed enzymes in pig and poultry production over the last two decades has been an extraordinary development, as inclusions of NSP-degrading enzymes in wheat- and barley-based poultry diets have already reached saturation point (Bedford, 2003). The acceptance of exogenous phytases will also approach this point, with appropriate scientific advances to the benefit of sustainable pig and poultry production.

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8 Developments in Enzyme Usage in Ruminants

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Introduction

Commercial use of feed enzymes in beef and dairy cattle diets is still very limited, although increasing feed costs and declining enzyme costs continue to fuel research efforts to develop and evaluate ruminant enzyme additives. Enzyme additives that supply cellulases, hemicellulases, proteases and ferulic acid esterase activity are of primary interest for ruminant applications. Enzyme additives have significant potential to improve fibre digestion and animal performance and, consequently, their commercial use in beef and dairy diets is expected to increase over the next few years. This chapter reviews the research on enzyme additives for ruminants and attempts to provide a rationale for their effective use in beef and dairy diets, with emphasis on future research needs and opportunities.

Why Use Feed Enzymes in Ruminant Diets?

The primary objective of using feed enzyme additives in ruminant diets is to decrease the cost of producing meat and milk. The cost of forages and feed grains has risen sharply in recent years and, consequently, beef and dairy producers are now, more than ever, seeking ways of improving feed conversion efficiency (i.e. reducing the amount of feed required per kilogram of weight gain or milk produced) and animal performance (increased weight gain or milk production per day). Most of the research on ruminant enzymes has focused on fibrolytic enzymes to improve fibre digestibility, because increasing fibre digestibility can increase the intake of digestible energy by the animal. As a result, less feed is required to produce 1 kg of milk or liveweight gain or, alternatively, more milk or weight gain results per kilogram of feed consumed by the animal.

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Feed enzyme additives target mainly the fibre fraction of forages, although some limited work has also been done using amylases to improve starch utilization (Hristov *et al.*, 2008; Tricarico *et al.*, 2008; Klingerman *et al.*, 2009). Forages contain about 30–70% neutral detergent fibre (NDF) on a dry matter (DM) basis. Even under ideal feeding conditions, NDF digestibility in the digestive tract of ruminants is generally less than 65% (Van Soest, 1994), and NDF digestibility in the rumen (degradability) is often less than 50%. Improvements in ruminal fibre degradability can increase total-tract digestibility, but this is not always the case. However, improvements in ruminal fibre degradability allow cattle to consume more feed (Dado and Allen, 1995) by reducing physical fill in the rumen. Higher DM intakes (DMI) are especially beneficial for dairy cows, where milk production is limited by digestible energy intake. For example, a one percentage unit increase in forage NDF degradability in the rumen has been reported to increase DMI by 0.17 kg day^{-1} and fat-corrected milk yield by 0.25 kg day^{-1} (Oba and Allen, 1999). Similarly, a one percentage unit increase in NDF degradability of maize silage increased DMI by 0.12 kg day^{-1} and fat-corrected milk yield by 0.14 kg day^{-1} (Jung *et al.*, 2004). Increased NDF degradability in the rumen also stimulates microbial nitrogen synthesis (Oba and Allen, 2000), which increases the supply of metabolizable protein to the cow. Thus, enzyme additives that increase NDF degradability have the potential to substantially improve the productivity and feed conversion efficiency of dairy cows and other high-producing ruminants.

Proposed Mode of Action

The mode of action of ruminant enzymes is still relatively unknown, because of the complexity of the ruminal microbial ecosystem and the process of fibre digestion. The need for further research in this area is evident. A more lengthy discussion of the possible mode of action of enzyme additives in ruminant diets is given elsewhere (Beauchemin *et al.*, 2004). Our interpretation of the most critical factors accounting for animal responses to enzymes is as follows.

Enzyme additives are relatively stable in the ruminal environment, particularly when administered via the feed (Hristov *et al.*, 1998a; Morgavi *et al.*, 2000b, 2001). Conditions in the rumen after feeding, such as reduced proteolytic activity and lower pH, help increase the stability of feed enzymes (Morgavi *et al.*, 2001). Furthermore, the presence of feed substrate helps decrease the sensitivity of enzymes to inactivation (Fontes *et al.*, 1995). Exogenous enzymes have the ability to increase enzymic activity within the ruminal environment (Morgavi *et al.*, 2000b; Colombatto *et al.*, 2003c). Increased hydrolytic capacity of the rumen will, however, depend upon the amount of enzyme applied to the feed and the activity of the exogenous enzymes under ruminal conditions (i.e. pH range 5.5–6.8, temperature of 39 ± 1°C). For example, most enzymes from *Trichoderma* are optimal at higher temperature and lower pH than typically found in the rumen. While some products may have high enzymic activities when assayed at optimal conditions for that enzyme, activity may be much lower when conditions of the

assay reflect those in the rumen. Lowered activity in the rumen will affect the animal's response to enzyme supplementation of the diet. For example, Vicini *et al*. (2003) observed no improvement in milk production when one of two enzymes was added to the diet of dairy cows. The lack of response was attributed to the fact that two-thirds of enzyme activities was lost when enzymes were assayed at ruminal pH, and a further two-thirds of the remainder was lost at ruminal temperatures.

Wallace *et al*. (2001) estimated that, at the levels typically used in feeding studies, enzyme additives supply about 5–15% of enzymic activities normally present in the rumen. A greater increase in ruminal enzymic activity (up to 56%) was reported by Hristov *et al*. (2000), but the dose used in that study was much higher than that typically fed to cattle. The true increase in enzymic activity in the rumen due to feeding enzymes is, however, difficult to quantify. Exogenous enzymes and ruminal microbial enzymes act cooperatively, and the net effect is a substantial increase in overall hydrolytic capacity, exceeding the additive effects of each of the individual components (Morgavi *et al.*, 2000a). These synergistic effects were not accounted for in the studies by Hristov *et al.* (2000) or Wallace *et al.* (2001).

In addition to increasing enzymic activity in the ruminal environment, applying enzymes to feed initiates hydrolysis of the fibre (Nsereko *et al*., 2000b). This hydrolysis alters the structure of the feed, in a manner that increases the surface area. It is well documented that ruminal bacteria start their initial adhesion mainly on cut or macerated surfaces of forage particles (Miron *et al*., 2001). Thus, changes in feed surface area due to initial hydrolysis by exogenous enzymes may account for the observation that enzyme additives stimulate adhesion to fibre and colonization of ruminal microbes (Yang *et al*., 1999; Wang *et al*., 2001; Morgavi *et al*., 2004). Bacterial adhesion is essential for subsequent fibre cell wall degradation (Miron *et al*., 2001). However, it has also been noted that feed enzymes compete with fibrolytic bacteria in the rumen for available binding sites on feed (Morgavi *et al*., 2004). Thus, although adherence to plant substrates such as alfalfa hay and maize silage is stimulated by low concentrations of enzymes, a competing effect is observed at higher concentrations. This effect may provide an explanation for lack of effect of feed enzymes when used at higher dose rates. There is also evidence that adding feed enzymes to the diet increases bacterial numbers in the rumen (Wang *et al*., 2001). Although most of the benefits of using enzyme additives in ruminant diets are attributed to ruminal effects, the possibility of post-ruminal effects cannot be discounted, although post-ruminal effects are probably minor.

Animal Responses to Enzyme Additives

There are numerous studies in which enzyme additives have been fed to ruminants, and a comprehensive review of the literature is presented by Beauchemin *et al*. (2003). Animal feeding studies have been conducted using numerous enzyme products applied at various dose rates, and experimental

conditions of these studies have varied widely. Different animal types (i.e. sheep, goats, beef cattle, dairy cows) at various stages of production (i.e. dairy cows in early, mid- and late lactation; sheep fed maintenance energy requirements; growing and finishing beef cattle) have been used. Various types of forages (grasses, legumes, whole-crop cereal silage, maize silage, etc.) have been fed, and the enzyme products in those studies were provided to the animals in a variety of ways (sprayed on to forage, added to concentrate or sprayed on to the total mixed ration (TMR), dry powder added to feed, ruminally infused). Furthermore, information on enzyme products and their activity units were not often provided or, when activity units were provided, conditions of enzyme assays were not specified. Together, these factors make the interpretation of results difficult.

A range of effects of using fibrolytic enzymes in ruminant diets has been reported. Some enzyme formulations increased DMI (Lewis *et al*., 1999; Beauchemin *et al*., 2000; Kung *et al*., 2000; Pinos-Rodrıguez *et al*., 2002), *in vivo* fibre digestibility (Feng *et al*., 1996; Rode *et al*., 1999; Bowman *et al*., 2002; Pinos-Rodrıguez *et al*., 2002; Kreuger *et al*., 2008b), average daily gain (ADG) of beef cattle (Beauchemin *et al*., 1995, 1997, 1999a), milk production of dairy cows (Lewis *et al*., 1999; Rode *et al*., 1999; Schingoethe *et al*., 1999; Yang *et al*., 2000; Adesogan *et al*., 2007) and feed efficiency of beef (Beauchemin *et al*., 1997) and dairy (Adesogan *et al*., 2007) cattle. However, many other studies reported no effects of enzyme additives on milk production of dairy cows (Kung *et al*., 2000; Knowlton *et al*., 2002; Sutton *et al*., 2003; Vicini *et al*., 2003; Elwakeel *et al*., 2007; Miller *et al.,* 2008b) or the ADG of growing beef cattle (McAllister *et al*., 1999; Miller *et al*., 2008a). Thus, when viewed across a spectrum of enzyme products and experimental conditions, the variability in animal response to enzyme additives is high. Long-term viability of using feed enzymes in ruminant diets depends on developing an understanding of the reasons for this variability.

It appears that enzymes are most effective when added to diets fed to high-producing ruminants with high energy requirements. For dairy cows, stage of lactation appears to be critical in terms of ensuring a response to enzyme additives. For example, Schingoethe *et al.* (1999) applied increasing dose rates of an enzyme additive (FinnFeeds Int., Marlborough, UK) to the forage portion (60% maize silage and 40% alfalfa hay) of a TMR. Cows in early lactation (<100 days in milk at the onset of the study) responded with 10–30% higher feed conversion efficiency (measured as kilograms of 3.5% fat-corrected milk per kilogram of DMI) and 18–24% higher fat-corrected milk yield, depending upon the dose rate applied (Table 8.1). However, cows in mid-lactation did not respond to enzyme supplementation. Differences in the response of early- and mid-lactation cows to enzyme supplementation were also reported in other studies (Zheng *et al*., 2000; Knowlton *et al*., 2002).

Rapidly growing beef cattle (Beauchemin *et al*., 1995) and sheep (Cruywagen and van Zyl, 2008) have also shown improvements in animal performance due to the use of enzyme additives. Beauchemin *et al*. (1995) added incremental levels of an enzyme blend (Spezyme CP, Genencor, Rochester, New York and Xylanase B, Biovance Technologies Inc., Omaha, Nebraska) to alfalfa cubes

Table 8.1. Effects of supplementing lactating dairy cow diets with fibrolytic enzymes.

	Schingoethe *et al*. (1999) (ml enzyme product kg^{-1} forage DM)[a]								Lewis *et al*. (1999) (ml enzyme product kg^{-1} forage DM)[b]			
	Early-lactation cows				Mid-lactation cows							
Parameter	0	0.7	1.0	1.5	0	0.7	1.0	1.5	0	1.25	2.05	5.0
DMI (kg day^{-1})	20.9	22.1	20.4	22.1	20.6	21.2	20.7	23.3	24.4^{e}	26.2^{d}	26.2^{d}	26.6^{d}
FCM/DMI (kg kg^{-1})[c]	1.21	1.33	1.46	1.41	1.12	1.07	1.16	1.09	1.77	1.64	1.89	1.62
3.5% FCM (kg day^{-1})	25.2^{d}	29.5^{e}	29.7^{e}	31.2^{e}	23.1	22.6	24.0	25.3	43.1^{e}	43.0^{e}	49.4^{d}	43.2^{e}
Milk yield (kg day^{-1})	26.4^{f}	29.1^{g}	28.7^{g}	30.4^{g}	23.6	23.0	23.3	25.2	39.6^{e}	40.8^{e}	45.9^{d}	41.2^{e}
Milk fat (%)	3.67^{d}	3.81^{e}	3.94^{e}	3.83^{e}	3.75	3.78	3.94	3.86	3.99^{d}	3.83^{d,e}	4.00^{d}	3.75^{e}
Milk protein (%)	3.14^{d}	3.33^{e}	3.42^{e}	3.36^{e}	3.40	3.41	3.46	3.33	2.95^{d}	2.87^{e}	2.88^{e}	2.85^{e}

DM, dry matter; DMI, dry matter intake; FCM, 3.5% fat-corrected milk.

[a]Polysaccharidase enzyme product from FinnFeeds Int., Marlborough, UK; activity units not given.

[b]Polysaccharidase enzyme product from FinnFeeds Int., Marlborough, UK; the enzyme product contained 1800 carboxymethylcellulase units and 7300 xylanase units ml^{-1}. Conditions of the enzyme assays were not given.

[c]Not statistically analysed.

[d,e]Within a row and a study, means with different superscripts differ (P <0.05).

[f,g]Within a row and a study, means with different superscripts tend to differ (P <0.10).

during manufacturing. Compared with the control diet (no enzyme added), ADG was increased by 23–30% with low to moderate application rates (0.25–1.0 l t^{-1} DM), but higher levels (2 and 4 l t^{-1}) were not effective (Fig. 8.1). Dose-dependent responses have also been reported for dairy cows. Lewis *et al*. (1999) applied increasing doses of an enzyme formulation (FinnFeeds Int., Marlborough, UK) to the forage portion of the TMR fed to dairy cows in early lactation. Cows receiving the medium enzyme dose level (2.5 ml kg^{-1} forage DM) recorded a 15% increase in 3.5% fat-corrected milk production (Table 8.1), but there was no improvement in milk yield at lower and higher dose rates. However, DMI was increased at all dose rates.

Although fibrolytic enzymes are expected to benefit mainly ruminants fed high-forage diets, some enzyme additives have also proved effective for feedlot

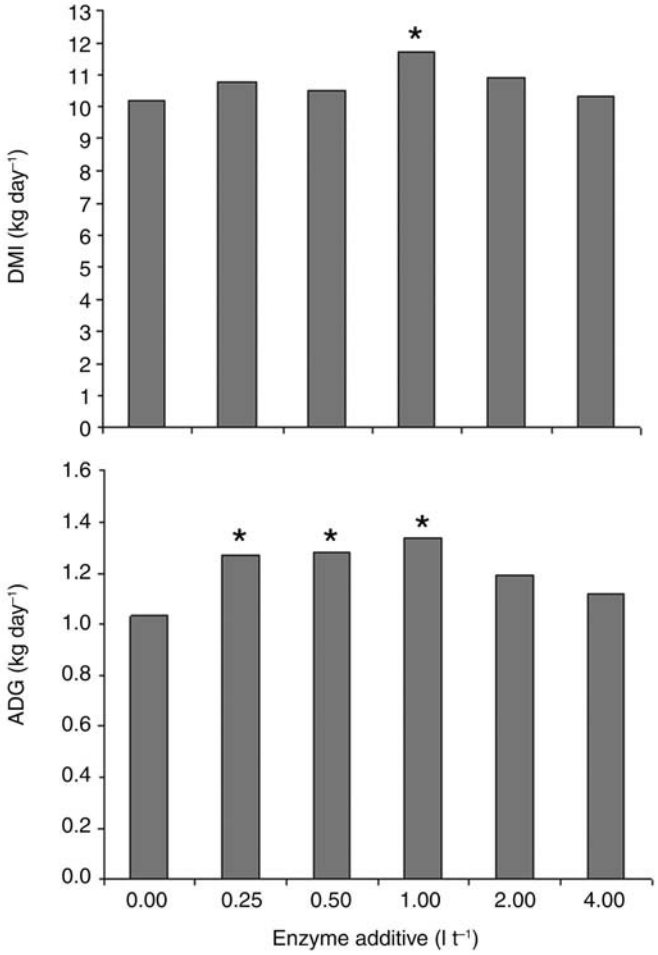

Fig. 8.1. Effects of adding a fibrolytic enzyme additive to alfalfa cubes fed to growing beef cattle (Beauchemin *et al*., 1995). Conditions of the enzyme assays were not given. Asterisk denotes treatment was different from control (P <0.10). DMI, dry matter intake; ADG, average daily gain.

finishing cattle fed high-grain diets. Positive results have mainly been reported for diets high in barley grain which, compared with maize, is high in fibre. Beauchemin *et al*. (1997) fed high-concentrate (95%, DM basis) diets containing either barley grain or maize grain to steers. The diets contained either no enzyme or one of two enzyme mixtures differing in endoglucanase:xylanase ratio. Feed conversion ratio of cattle fed barley containing the high-xylanase enzyme was improved by 11% (Table 8.2). In contrast, enzyme treatments had no effect for cattle fed the maize diets. In a subsequent study with barley-based rations, ADG was increased by 9% and feed conversion efficiency by 10% (Table 8.2). In these high-grain diets, it is not clear whether the response to enzymes was due entirely to improvements in fibre digestion. More complete digestion of barley aleurone and endosperm cell walls may have enhanced access to starch granules by ruminal and intestinal endogenous enzymes, thereby improving starch digestion.

Cattle fed *ad libitum* are likely to respond better to feed enzymes than animals fed for restricted intake. Fibre digestibility tends to be lower when cattle are fed *ad libitum*, because residence time in the rumen is relatively short and ruminal pH usually drops below the optimum for fibre digestion (NRC, 2001). Enzyme additives tend to increase the rate of fibre degradation in the rumen (i.e. degradation after short incubation times) rather than the extent of degradation (i.e. degradation after long incubation times) (Colombatto *et al*., 2007; Ranilla *et al*., 2008). Increased rate of fibre degradation in the rumen is most likely to improve total-tract digestibility when residence time of

Table 8.2. Effects of fibrolytic enzyme supplementation of high-grain diets on the performance of feedlot cattle.

	Beauchemin *et al*. (1997)						Beauchemin *et al*. (1999)	
	Barley diet			Maize diet			Barley diet	
Parameter	Control	Enz 1[c]	Enz 2[c]	Control	Enz 1[c]	Enz 2[c]	Control	Enz 3[d]
ADG	1.43	1.52	1.40	1.33	1.19	1.33	1.40[b]	1.53[a]
DMI	9.99	9.53	9.86	9.55	9.29	9.10	10.73	10.62
Feed:gain	7.11[b]	6.33[a]	7.13[b]	7.26[a,b]	7.83[b]	6.95[a]	7.72	6.95

ADG, average daily gain; DMI, dry matter intake.

[a,b]Within a diet, means with different superscripts differ (P <0.01).

[c]Enzymes 1 and 2 (Enz 1, Enz 2) were prepared by combining Spezyme CP (Genencor, Rochester, New York; 90 FPU ml^{-1}) and Xylanase B (Biovance Technologies Inc., Omaha, Nebraska; 4200 IU of xylanase and 32 FPU g^{-1}). Stock solutions (l^{-1}) consisted of, for Enz 1, 371 ml of Spezyme and 298 g of Xylanase B and, for Enz 2, 927 ml of Spezyme and 119 g of Xylanase B. Enzyme activities per kg of dietary DM were, for Enz 1, 4567 IU of xylanase and 155 FPU of cellulase, and for Enz 2, 1823 IU of xylanase and 316 FPU of cellulase. Conditions of enzyme assays were not given.

[d]Enzyme 3 (Enz 3) (Promote, Biovance Technologies) contained 25.9 IU (mmol reducing sugars ml^{-1} min^{-1}) of endoglucanase (carboxymethyl cellulose) and 51.4 IU of xylanase (oat spelts xylan). Assays were conducted at pH 6.5 and 39°C. The enzyme was applied at 1.40 l t^{-1} and activities per kg of DM were: 66.3 IU for cellulase and 33.4 IU for xylanase.

feed in the rumen is short, as is the case for animals fed *ad libitum*. This effect is demonstrated in the study reported by Yang *et al.* (2000) in which the total-tract digestibility of DM was 17% lower when measured in dairy cows compared with sheep (Table 8.3). Consequently, supplementing the diet with enzymes improved total-tract digestibility in dairy cows, but not in sheep. Thus, enzyme technology is less likely to benefit ruminants fed to meet maintenance energy requirements; the greatest responses are expected to occur when ruminants are fed for maximum productivity.

From the existing body of literature, it can be concluded that feed enzyme additives can be a highly effective means of improving performance in ruminants. However, positive responses are not always obtained and, given the cost of this technology, the uncertainty of response is a major limitation. The key is to develop a better understanding of the mode of action and to identify the key enzyme activities and dose rates required, thereby ensuring cost-effective use of these additives.

Table 8.3. Effects of method of adding an enzyme product to diets fed to either dairy cows in early lactation or lambs (Yang *et al.*, 2000).

Parameter	No enzyme	Enzyme applied to total mixed ration[a]	Enzyme applied to concentrate[a]
Dairy cows			
DMI (kg day^{-1})	19.4	20.4	19.8
FCM/DMI (kg kg^{-1})	1.62	1.64	1.66
4% FCM (kg day^{-1})	31.5	30.5	32.5
Milk yield (kg day^{1})	35.3[c]	35.2[c]	37.4[b]
Milk fat (%)	3.34	3.14	3.19
Milk protein (%)	3.18	3.13	3.13
Digestibility (%)			
DM	63.9[c]	65.7[b,c]	66.6[b]
NDF	42.6	45.9	44.3
ADF	31.8	35.5	33.7
Lambs			
DMI (kg day^{-1})	1.07	1.18	1.05
Digestibility (%)			
DM	75.6	74.8	74.5
NDF	55.4	56.8	56.9
ADF	45.9	48.7	49.5

DMI, dry matter intake; FCM, fat-corrected milk; DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre.

[a]The enzyme product was supplied by Biovance Technologies Inc., Omaha, Nebraska, was produced from *Trichoderma longibrachiatum* and contained 1168 ± 17 IU (nmol reducing sugars mg^{-1} min^{-1}, pH 6.5 and 39°C) of xylanase (oat spelts xylan substrate) and 138 ± 13 IU of endoglucanase (carboxymethyl cellulose). The product was added to either the concentrate or the total mixed ration at the rate of 50 mg kg^{-1} total mixed ration.

[b,c]Means within the same row with different superscripts differ (P <0.05).

Enzyme Formulation

Main enzymic activities involved in digesting fibre

Most ruminant feed enzymes contain cellulases and hemicellulases, because cellulose and hemicellulose are the major structural polysaccharides in plants (Van Soest, 1994). The types of cellulases and hemicellulases can differ substantially among commercial enzyme products depending on the source organism and how that organism is grown (Considine and Coughlan, 1989; Gashe, 1992). Enzyme activities expressed by the source organism will greatly influence the effectiveness of enzyme additives.

The major enzymes involved in cellulose hydrolysis are endoglucanase, exoglucanase and β-glucosidase (Bhat and Hazlewood, 2001). Endoglucanases hydrolyse cellulose chains at random to produce cellulose oligomers; exoglucanases hydrolyse the cellulose chain from the non-reducing end, producing cellobiose; and β-glucosidases release glucose from cellobiose and hydrolyse short cellulose chains from both reducing and non-reducing ends. All three enzymes are necessary for complete hydrolsis of cellulose.

The main enzymes involved in degrading the xylan core of hemicellulose to soluble sugars are endo β-1,4-xylanase and β-1,4-xylosidase, which yield short xylan chains and xylose, respectively (Bhat and Hazlewood, 2001). Many other hemicellulase enzymes are involved in the digestion of side chains, including mannosidase, arabinofuranosidase, glucuronidase, galactosidase, acetyl-xylan esterase and ferulic acid esterase.

Key activities required in feed enzyme additives

Commercial enzyme products contain many enzymic activities, and it has been a challenge to identify the key activities and optimum dose rates needed for ruminant applications. Part of the difficulty is that exogenous enzymes act synergistically with microbial enzymes in the rumen, and thus the key activities required may vary depending upon the endogenous microflora (Morgavi *et al*., 2000a). In addition, the key enzymic activities required depend on the chemical composition of the feed on which the enzyme is expected to act. Thus, a particular enzyme formulation will not be effective for all diets, and optimum dose rates will differ among feeds. For example, Beauchemin *et al*. (1995) supplemented an enzyme product for growing cattle fed alfalfa hay, timothy hay or barley silage. Average daily gain of the cattle increased when moderate levels of enzyme were added to alfalfa hay and when a high level of enzyme was added to timothy hay, but no response occurred for cattle fed barley silage, regardless of enzyme level. Colombatto *et al.* (2003b) evaluated 26 enzyme products *in vitro*, and only one product was effective for both alfalfa hay and maize silage. Thus, enzyme additives need to be formulated for specific types of forage, and response may also depend on forage quality.

Recently, Eun and Beauchemin (2008) conducted a meta-analysis to identify the key enzymic activities in feed enzyme additives responsible for

improving *in vitro* forage NDF degradability. For alfalfa hay, data from eight studies with 83 enzyme treatments using 45 enzyme additives were evaluated. For maize silage, the data were from six studies with 61 enzyme treatments using 23 enzyme additives. All studies were conducted using the same batch culture *in vitro* procedure and all enzyme assays were performed by the same laboratory using the same pH (6.0), temperature (39°C) and substrate conditions, which helped minimize variation due to the methodology of assaying enzyme units. The definition of a unit of enzyme activity is methodology dependent, and variable among laboratories (Colombatto and Beauchemin, 2003).

The increase in NDF degradability achieved for alfalfa hay averaged 12.3%, ranging from –32.1 to 82.3% (Table 8.4). Similarly, the increase in NDF degradability achieved for maize silage averaged 14.3%, ranging from –23.3 to 60.5%. Thus, sizeable increases in NDF degradability were obtained for both forages with some enzyme additives. However, the range in degradability shows the importance of product formulation. It is also clear that enzyme additives can have detrimental effects on fibre digestion when enzyme activities and dose rates are not optimized. Exoglucanase was the main enzymic activity associated with increased NDF degradability, accounting for 75% of the improvement for alfalfa hay and 55% of the improvement for maize silage.

However, for maize silage, the same amount of improvement could also be accounted for by endoglucanase activity, which supports the conclusion from other studies (Wallace *et al*., 2001; Eun *et al*., 2007b) that endoglucanase activity is a good indicator of the ability of an enzyme additive to stimulate *in vitro* fermentation of maize silage. A smaller portion of the improvement was explained by protease or the endoglucanase:xylanase ratio. For maize silage, there was substantial overlap in the effects of the various activities, but the other activities explained no more of the improvement in NDF degradation

Table 8.4. Relationship between added enzymic activities from feed enzyme additives and *in vitro* fermentation responses for alfalfa hay and maize silage[a] (Eun and Beauchemin, 2008).

Forage	Endo-glucanase (E)	Xylanase (X)	Ratio (E:X)	Exo-glucanase	Protease	Change in *in vitro* NDF degradation (%)
Alfalfa hay						
Mean	307	1,351	0.47	23.0	0.005	+12.7
Minimum	0	0	0	0	0	–32.1
Maximum	1,613	12,990	2.91	84.0	0.077	+82.3
Maize silage						
Mean	441	2,046	0.43	33.4	0.005	+14.3
Minimum	0	0	0	1.47	0	–23.3
Maximum	1,613	12,990	1.97	84.0	0.063	+60.5

NDF, neutral detergent fibre.

[a]Endoglucanase, nmol glucose released min^{-1}; xylanase, nmol xylose released min^{-1}; exoglucanase, nmol glucose released min^{-1}; protease, mg azocasein hydrolysed min^{-1}. All enzymic activities were expressed as if they were added to 1 g of forage dry matter.

than did exoglucanase alone. These results suggest that, over a range of forage types, exoglucanase shows the strongest relationship with increased NDF degradability.

For xylanase, the type and characteristics of the enzymes appear to be more important than activity units. Eun and Beauchemin (2007a) evaluated recombinant, single-activity enzyme products (13 endoglucanases and ten xylanases) for their potential to improve *in vitro* ruminal degradation of alfalfa hay. Six of the endoglucanases and five of the xylanases increased organic matter (OM) degradation; up to 20% increased OM degradation was observed for both types of enzyme product. The correlation between added endoglucanase activity (determined at ruminal conditions) and OM degradation was moderate (r^2 = 0.50), whereas for xylanase the response was not a direct function of the activity added. In that study, xylanase activity was determined using arabinoxylan from wheat grain at pH 5.4 and 37°C, and again using birchwood xylan at pH 6.0 and 39°C. The fact that improvements in forage degradation occurred with some single-activity xylanases indicates that xylanases are important, but that the response could not be predicted using standard assays of activity, confirming findings from a previous study (Eun *et al*., 2007b).

Thus, the research to date indicates that both cellulase and xylanase activities have a beneficial effect on fibre degradation of forages. While the concentration of cellulase activity appears to be important in improving forage degradation, for xylanase the type and characteristics of the enzymes seem to be more important than activity. It seems that the assay used for cellulase is simply more biologically relevant than that for xylanase, which is an area that needs to be addressed in subsequent research.

Other activities

Ferulic acid esterase

For ruminants, the focus to date has been on xylanases and cellulases. However, it is well known that lignin and phenolic acids are inhibitory to the biodegradation of plant cell wall polysaccharides. The cross-linking of lignin with cell wall polysaccharides through ferulic acid bridges limits microbial access to the digestible xylans in the plant cell wall (Jung and Allen, 1995). Microbial esterases 'shave off' some of the side-chains and break the cross-linkages of plant polymers (Williamson *et al*., 1998), and therefore it is reasonable to expect that enzyme products containing ferulic acid esterases may be effective in increasing forage digestion. Many of the fibrolytic enzyme products used in previous animal studies may have contained ferulic acid esterase activity, but the activity is not routinely measured because of the complexity of the assay.

However, there is evidence that supplementation of diets with enzymes containing ferulic acid esterase may improve DM and NDF degradability of various forages (Yu *et al*., 2005). For example, Krueger *et al.* (2008a) recently reported that the *in vitro* NDF degradability of some poor-quality forages was enhanced using a polysaccharidase product that also contained relatively high

ferulic acid esterase activity. The study indicated that the enzyme hydrolysed cell wall polysaccharides and released phenolic acids and consequently enhanced digestibility, but responses differed among the forages tested.

Nsereko *et al*. (2008) explored the possibility of feeding silage inoculated with lactic acid bacteria as a unique approach to delivering ferulic acid esterases to the rumen. Among 10,000 lactic acid bacteria screened, 500 contained ferulic acid esterase activity. Perennial ryegrass was then inoculated and ensiled with some of the bacteria that produced ferulic acid esterase. Forage NDF degradability *in vitro* generally increased by 9–11% after the ensiling process, but if the lactobacilli were inoculated into the silage immediately prior to feeding, then no effect on NDF degradability was observed. This indicates that most if not all of the beneficial effect of these enzymes takes place during the ensilage process. It would be worthwhile to investigate whether lactic acid bacteria that produce ferulic acid esterases would be effective if offered directly to the animals at the time of feeding as a direct-fed microbial. However, feeding the bacteria themselves as a means of supplying ferulic acid esterases assumes that the lactobacilli would integrate fully into the microbial population in the rumen, which may not occur. Integration of bacterial direct-fed microbials into the highly competitive ruminal environment presents significant challenges.

Protease

Protease activity may also be important for some forages. Studies performed in our laboratory reported increases in *in vitro* NDF degradability of alfalfa hay (Colombatto *et al*., 2003a,b) and rice straw (Eun *et al*., 2006) as a result of supplementation with a product containing only serine protease with no measureable cellulase or xylanase activity. Eun and Beauchemin (2005) fed this same protease product to dairy cows using a dose rate (1.25 ml kg^{-1} diet DM, 533 mg azocasein hydrolysed ml^{-1}) similar to that used in previous *in vitro* studies (Colombatto *et al*., 2003a,b; Eun *et al*., 2006). When added to a low-forage diet (18.2% barley silage, 16.0% alfalfa hay and 65.8% concentrate on a DM basis), NDF digestibility in the total tract increased by 26%; when added to a high-forage diet (44.5% barley silage, 16.0% alfalfa hay and 39.5% concentrate on a DM basis), there was no effect on NDF digestibility. Lack of effect of the enzyme in the higher-forage diet may have reflected the higher concentration of barley silage in the ration, as this product was shown not to be effective for barley silage (McGinn *et al*., 2004). Using a different proteolytic enzyme product, *in vitro* NDF degradability of alfalfa hay was improved by 19% (dose of 0.25 mg g^{-1} DM) and NDF degradability of maize silage by 17% (dose of 0.5 mg g^{-1} DM) (Eun and Beauchemin, 2007b).

It has been suggested that proteases enhance fibre degradation by attacking some of the cell wall nitrogen-containing components that are physical barriers to degradation (Colombatto *et al*., 2003a). Tyrosine residues may play a role in the cross-linking of dicotyledonous plants (Jung, 1997), such as alfalfa. Alkaline proteases have been shown to be more effective in increasing forage fibre degradability than acidic proteases (Eun *et al*., 2007a). However, this observation

may be confounded by differences in source organisms, as alkaline proteases tend to be from *Bacillus* spp. and acid proteases tend to be from *Aspergillus* spp. Thus, the relationship between protease activity and improvement in fibre degradation appears to depend upon the type of protease.

Amylase

Few studies have examined the potential use of amylases for ruminants. Most of these studies used a powdered *Aspergillus oryzae* extract containing amylase activity (Amaize, Alltech Inc., Nicholasville, Kentucky). It is also possible that some of the fibrolytic enzyme products evaluated previously in other studies also contained significant amylase activity, but this activity was not reported in most cases. In addition, some amylase products also supply significant levels of cell wall-hydrolysing enzymes, and thus it can be difficult to pinpoint the mechanism of response to amylases, particularly given that total-tract starch digestibility is typically not improved using amylases (Tricarico *et al*., 2008).

Starch comprises a significant portion of the carbohydrates fed to feedlot and dairy cattle. Apparent total-tract digestibility of starch from processed grains is generally over 90% in dairy and feedlot cattle (Firkins *et al*., 2001; Zinn *et al*., 2007). Thus, it is generally thought that exogenous amylases would not be useful for ruminants. However, there are situations in which starch digestion in ruminants is lower than expected, especially for dry, cracked maize, steam-rolled maize and minimally processed barley grain. Furthermore, the site of starch digestion in the gastrointestinal tract is variable, and this can affect the efficiency of starch utilization by the animal (Firkins *et al*., 2001). These factors may contribute to the possibility that amylases would aid starch digestion in ruminants.

Tricarico *et al*. (2008) recently reviewed the limited information on amylases from *A. oryzae* extract. Some studies reported increased DMI and weight gain in feedlot cattle and increased milk yield with reduced milk fat content in dairy cattle when diets were supplemented with amylase extract, but the results have been inconsistent. In a very recent study by Klingerman *et al*. (2009), dairy cows were fed diets supplemented with one of three amylase products, and these authors reported higher DMI and increased milk production, although starch digestion in the total tract was not increased. Tricarico *et al*. (2008) concluded that amylases from *A. oryzae* do not increase ruminal starch digestion, but rather they shift ruminal fermentation to increase the molar proportions of butyrate and acetate at the expense of propionate. It was proposed that exogenous amylases increase production of oligosaccharides from amylose and amylopectin, and that these would be used by amylolytic and non-amylolytic bacteria in cross-feeding mechanisms that modify the resulting products of fermentation in the rumen. Thus, it appears that there may be opportunity for amylase inclusion in ruminant diets in some circumstances, but further research is required before amylases can be recommended. Pure sources of amylase, with no contaminating cell wall hydrolase activity, will be vital in this regard.

Phytase

Phytases have increasingly been used in poultry and swine diets to solve nutritional and environmental problems associated with phytate. Phytate, the principal form of phosphorus in plants, is not fully utilized by non-ruminants, and the resulting excretion of phosphorus contributes to phosphorus pollution. In contrast, the rumen is a source of highly active phytases, and thus ruminants can use phytate as a source of phosphorus (Guyton *et al*., 2003; Nakashima *et al*., 2007). However, despite the presence of phytase activity in the rumen, there is evidence that phytate may not be fully utilized by ruminants, especially when ruminants are fed high-concentrate diets. For example, phytate has been detected in the manure of cattle fed grain, although minimal amounts were detected in the manure of pasture-fed cattle (Benjamin and Leytem, 2004). Furthermore, Bravo *et al*. (2002) reported that adding phytase to a high-concentrate diet increased the solubilization of phosphorus in the rumen, although that was not the case for a high-forage diet. Differences between grain and forage diets may be explained by the higher proportion of phosphorus present as phytate in grains compared with that in leafy plants (Ravindran *et al*., 1994).

Apparent total-tract digestibility of dietary phosphorus in dairy cows is variable (10–50%), and can be low for some diets, particularly those containing barley grain (Knowlton and Herbein, 2002; Kincaid *et al*., 2005). About half the dietary phosphorus fed to dairy cows is from phytic acid, although the proportion would vary with the forage:concentrate ratio of the diet. It has been suggested that exogenous dietary phytases might improve phosphorus utilization in beef cattle and dairy cows in some dietary situations. Improved phosphorus availability from feed would allow the animal's requirement to be met with reduced phosphorus intake, thus reducing the phosphorus content of manure.

There has been limited research to evaluate the impact of phytase supplementation of ruminant diets. Adding phytase to dairy cow diets containing barley or maize grain decreased the excretion of phytate phosphorus and increased the concentration of serum inorganic phosphorus (Kincaid *et al*., 2005). Knowlton *et al*. (2007) reported that adding a cellulase-phytase enzyme additive (Cattle-Ase-P, Animal Feed Technologies, Greeley, Colorado) to the diet of dairy cows lowered phosphorus excretion. In another study, the same enzyme additive increased apparent phosphorus digestibility of diets fed to lactating cows from 40.5 to 50.1% (Knowlton *et al*., 2005). Combining polysaccharidases and phytase can be effective with barley diets, and to a lesser extent wheat diets, because the phytate in these grains is in the aleurone layer surrounding the endosperm. In contrast, the phytate in maize is concentrated in the germ, which is highly susceptible to hydrolysis. Hurley *et al*. (2002) reported higher phosphorus digestibility in feedlot cattle fed mainly whole maize supplemented with phytase. However, there have also been studies in which no effects of feeding phytase were reported. For example, in the research reported by Kincaid *et al*. (2005), cows in a second study failed to respond to phytase supplementation. It is not clear whether the lack of response in the

second experiment was due to the higher milk production of the cows, the higher concentration of phosphorus in the diet, the higher phosphorus digestibility or to other factors. The composition of the diet, and hence the proportion of phosphorus as phytate, may also play a role.

It appears that there may be some advantage to supplementing dairy and feedlot cattle diets with phytase, but the effects are likely to vary with the composition of the basal diet, the type of feed processing and the level of intake of the animal. Additionally, most commercial phytases have been developed to function in the monogastric stomach and may not possess the appropriate pH optima to function to any extent within the rumen (Simon and Igbasan, 2002). Diets fed to lactating dairy cows or feedlot cattle that comprise substantial quantities of grains, especially grains that are not extensively processed before feeding, or barley and wheat grain where the phytate is located in the aleurone, are likely to benefit most from phytase supplementation. In those situations, limited ruminal retention time and the physical barriers of the grain that prevent microbial access to feed in the rumen may contribute to the animal's response to phytase. However, further research is needed before phytase supplementation of diets can be recommended with certainty for ruminants.

Predicting Efficacy of Enzyme Products for Ruminants

Because the response to enzymes from enzymic activities cannot be predicted with accuracy, there is a need to screen enzyme additives using a bioassay that mimics their effects in the rumen. Use of an *in vitro* batch culture incubation in buffered ruminal fluid can be a powerful screening tool for selecting enzyme additives that improve fibre degradation (Eun and Beauchemin, 2008). *In vitro* methods are less expensive, less time consuming and allow more control of experimental conditions than *in vivo* experiments. Furthermore, *in vitro* systems can accommodate a large number of enzyme candidates. But, ultimately, conducting animal feeding studies using rapidly growing cattle or dairy cows in early lactation is the best way to assess whether an enzyme product enhances feed utilization.

Selection of enzyme additives based on *in vitro* response: an example

Using a 24 h *in vitro* batch culture to screen a range of enzyme additives, we identified two products (from Dyadic International, Jupiter, Florida) that improved NDF degradability of maize silage (Eun and Beauchemin, 2007b). Both products contained endoglucanases, exoglucanases and xylanases. In a subsequent experiment, the two products were combined and investigated for effects on maize silage degradation compared with individual-component enzyme treatments and a control (no enzyme). The combination treatment improved degradability of maize silage NDF by 31% and acid detergent fibre (ADF) by 47%. The substantial increase in fibre degradability due to the

combination exceeded that obtained by the component enzymes. When the component enzymes were used individually, fibre degradability was increased by up to 13% for NDF and 19% for ADF. Therefore, synergistic effects were observed by combining the individual enzyme components.

Based on the positive results observed *in vitro*, the combination product was then evaluated in a feeding study by Adesogan *et al*. (2007). The enzyme product was added to maize silage-based diets containing a high or low level of concentrate. The enzyme solution was sprayed on to the TMR to supply the same enzyme units per kilogram of feed as was used in the *in vitro* screening study of Eun and Beauchemin (2007b). The diets were fed to 60 lactating cows in early lactation. For cows fed the high-concentrate diet, enzyme supplementation increased milk yield by 3 kg day^{-1} (9% increase), without changing DMI (Table 8.5). For cows fed the low-concentrate diet, enzyme supplementation improved feed efficiency by 15%, indicating that more milk was produced per unit of feed consumed. Thus, for both diets the enzyme additive improved animal performance, but whether improved fibre degradation increased milk production or improved feed efficiency differed for high- and low-concentrate diets. This difference is probably related to the dose rate of the enzyme used expressed on the basis of the fibre content of the diet (7.6 versus 10.3 mg g^{-1} forage NDF for low-concentrate and high-concentrate diets, respectively). Because the same amount of enzyme product was used in both diets, the amount of enzyme per unit of forage fibre was lower for the low-concentrate diet. The results from this feeding study are compelling evidence of the beneficial effects obtainable for enzyme additives that have been specifically developed for ruminants using a rigorous *in vitro* screening process.

Using Enzymes in Ruminant Diets: Practical Considerations

Method of supplementation

Several methods of adding enzymes to the diet have been used across studies. Enzymes have been dosed directly into the rumen (Lewis *et al*., 1996; Hristov *et al*., 1998b, 2008); powdered enzymes have been added directly to a

Table 8.5. Effect of an enzyme additive on the performance of dairy cows fed diets containing a low or high level of concentrate[a] (adapted from Adesogan *et al*., 2007).

	Low concentrate		High concentrate		
Parameter	Control	Enzyme	Control	Enzyme	SE
Dose rate (mg g^{-1} forage NDF)	–	7.56	–	10.3	
DMI (kg day^{-1})	22.9	21.2	25.6	25.3	1.1
Milk yield (kg day^{-1})	32.0	32.9	33.5[b]	36.5[c]	1.0
Milk/DMI (kg kg^{-1})	1.40[b]	1.62[c]	1.32	1.48	0.07

NDF, neutral detergent fibre; DMI, dry matter intake.
[a]Enzyme additive from Dyadic International, Jupiter, Florida.
[b,c]Within a diet, means with different superscripts differ (P <0.05).

component of the TMR (Knowlton *et al*., 2002; Titi and Tabbaa, 2004; Elwakeel *et al*. 2007); and liquid enzyme products have been applied either to the TMR (Higginbotham *et al*., 1996; Beauchemin *et al*., 1999b; Yang *et al*., 2000; Sutton *et al*., 2003, Vicini *et al*., 2003) or to a component of the ration, including hay (Beauchemin *et al*., 1995; Yang *et al*., 1999; Lewis *et al*., 1996), ensiled forages (Beauchemin *et al*., 1995), a blend of hay and silage (Schingoethe *et al*., 1999; Kung *et al*., 2000; Zheng *et al*., 2000; Dhiman *et al*., 2002), concentrate (Rode *et al*., 1999; Yang *et al*., 2000; Sutton *et al*., 2003), supplement (Bowman *et al*., 2002) or premix (Bowman *et al*., 2002). There has also been some interest in applying enzymes at the time of harvesting of the forage (Feng *et al*., 1996; Krueger *et al*., 2008b), which decreases the need to add the enzyme product daily to the ration.

The method of providing the enzyme additive to the animal appears to be an important consideration affecting animal response. However, it must be emphasized that this area needs further research, because few studies provide direct comparisons of the method of providing enzymes to animals. Furthermore, in some studies it is difficult to assess the effects of method of delivery, because the lack of animal response may have been due to an ineffective enzyme formulation or to an inappropriate dose rate.

From the existing body of literature, it seems that dosing enzymes directly into the rumen has generally not been an effective means of enzyme supplementation (Lewis *et al*., 1996; Hristov *et al*., 1998b, 2008). Likewise, adding powdered enzymes to the diet has generally not been effective (Knowlton *et al*. 2002; Elwakeel *et al*., 2007). However, the latter method of delivery cannot be completely excluded, because there has not been a direct comparison of the effects of adding powdered enzymes to the diet versus spraying a diluted solution of the same enzyme product on to feed. However, applying liquid enzyme to feed is thought to be important because the enzyme is then permitted to bind to substrate, enhancing enzyme stability in the rumen (Morgavi *et al*., 2000b, 2001). In addition, applying enzymes to feed causes a pre-ingestive attack of the enzymes upon the plant fibre, which alters the structure of the feed thereby making it more amenable to microbial attachment (Morgavi *et al*., 2004). As such, spraying or pouring enzymes on to the feed prior to feeding, particularly the drier portion of the ration, such as the concentrate, hay or silage mixed with hay, can be an effective means of enzyme delivery (Lewis *et al*. 1999; Rode *et al*., 1999; Schingoethe *et al*., 1999; Zheng *et al*., 2000).

However, direct application of enzymes to very moist feeds and silages may be undesirable. Feng *et al*. (1996) observed no effect when an enzyme additive was added to fresh or wilted forage but, when applied to dried grass, enzymes increased fibre digestibility. Similarly, Yang *et al*. (2000) reported increased digestibility of the diet and higher milk production when enzymes were added to the concentrate portion of a dairy cow ration, but there was no increase in milk production when they were added directly to the TMR (Table 8.3). The reduced efficacy of some enzyme additives when applied to moist, ensiled feeds may be due to inhibitory compounds in fermented feeds (Nesereko *et al*., 2000a) or to decreased binding of the enzyme to the substrate.

The application of enzymes to silages can also accelerate aerobic deterioration, which could lead to decreased nutritive value of the silage if not consumed immediately by the animals (Wang *et al*., 2002).

It may be surprising to some that enzyme additives can be effective when applied to the concentrate portion of a ration (Rode *et al*., 1999; Yang *et al*., 2000), even though the forage, and not the concentrate, is the target substrate. Although pre-ingestive effects occur when enzymes are applied to feed, these effects may not be as important as the increased hydrolytic capacity of the rumen due to the synergistic effects of exogenous and endogenous enzymes. This concept is supported by the results of an *in vitro* batch culture study in which we used ruminal fluid either from control cows (Control RF) or cows fed a diet supplemented with enzymes (Enzyme RF; Eun and Beauchemin, unpublished results). Samples of TMR with (TMR + Enzyme) or without (TMR Control) enzyme additive were then incubated *in vitro* using either sources of ruminal fluid. Thus, the design of the experiment allowed us to determine the effects of adding enzyme directly to the substrate (TMR Control versus TMR + Enzyme) during *in vitro* incubation, as well as the effects of the ruminal fluid (Control RF versus Enzyme RF). Adding enzyme to the TMR during *in vitro* incubation increased NDF degradation, regardless of the source of ruminal fluid (Fig. 8.2). In addition, using ruminal fluid from cows fed enzymes resulted in higher NDF degradation, regardless of whether the TMR was incubated with enzymes.

The fact that the degradability of the control TMR was enhanced when incubated with RF + enzyme indicates that feeding enzymes boosts the hydrolytic capacity of the ruminal fluid. These results indicate that feeding enzymes to cows increases the digestibility of the feed to which the enzymes

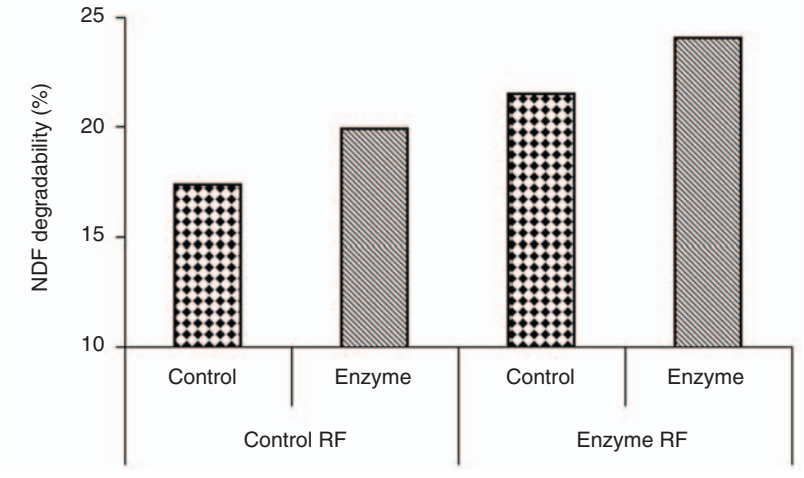

Fig. 8.2. Effects of adding enzyme during *in vitro* incubation of a total mixed ration on degradability (%) of neutral detergent fibre (NDF) at 24 h. The rumen fluid (RF) was from cows fed a ration with (enzyme) or without (control) enzyme additive. All treatments differ significantly (P <0.05); SE, 0.92. Control, total mixed ration without added enzymes (Eun and Beauchemin, unpublished results).

are applied, as well as the digestibility of the feed that is not directly treated with enzymes. Thus, the response to enzymes is not limited to the portion of the feed to which the enzymes are applied. The effects of direct application of enzyme to feed are additive to the effects of the ruminal fluid, and thus the most effective application of enzyme is to the target forage if the forage is dry. Applying the enzyme to the concentrate is less desirable than applying the enzyme to dry forage, but avoids the potential negative effects of applying the enzyme to ensiled feeds and minimizes the labour associated with the daily spraying of enzyme on to forages or TMR.

Diet formulation

Enzyme additives increase the rate of fibre digestion, which can provide more digestible energy to the animal for growth or milk production. However, higher productivity increases the animal's requirement for metabolizable protein. Thus, it is necessary to ensure that the metabolizable protein content of the diet does not limit production when using enzyme additives.

Furthermore, increasing the rate of digestion of the fibre fraction in the rumen using enzymes may increase the risk of ruminal acidosis, particularly if the diet is already highly fermentable. Ruminal fermentability of feeds can have a major impact on ruminal pH, and thus a further increase in diet fermentability can cause ruminal pH to drop if the diet is high in starch and does not contain sufficient long-particle forage. For example, Eun and Beauchemin (2005) fed a high-concentrate diet containing only 34% forage (DM basis) to dairy cows and reported a mean ruminal pH of 5.6. Adding a proteolytic enzyme to the ration increased total-tract NDF digestibility by 26% (from 39.9 to 50.2%), causing a further drop in mean ruminal pH to 5.5, which is undesirable from the standpoint of avoiding acidosis. There are other studies in which signs of ruminal acidosis occurred as a result of feeding enzymes, such as lower ruminal pH after feeding (Lewis *et al*., 1996; Sutton *et al*., 2003) and milk fat depression (Rode *et al*., 1999). Thus, care must be exercised when adding feed enzymes to diets that are low in physically effective fibre. To avoid ruminal acidosis, it may be advantageous to increase the proportion of forage in the diet (or lower the starch content) when using enzyme additives. Thus, enzymes provide cattle producers with the opportunity to feed higher-fibre diets, thereby maintaining productive performance while minimizing digestive upsets.

Conclusions

The use of feed enzyme additives is an emerging technology that shows promise in terms of improving the use of forages by ruminants. Responses to fibrolytic feed enzymes are expected to be greatest in situations in which digestible energy is the first limiting nutrient in the diet. Although positive responses in milk production, weight gain and feed conversion efficiency have been observed for some enzyme additives, results have been inconsistent,

particularly when viewed across all products. Designing enzyme additives that deliver the key enzymic activities needed to enhance the degradability of the target forage substrate will improve the effectiveness of this technology. As the mode of action becomes better understood and the critical enzymic activities continue to be identified for a range of forages, product formulations and application methods and rates can be tailored to elicit the desired response at minimal cost. *In vitro* bioassays that reflect the conditions of the rumen can be useful for selecting enzyme candidates for subsequent evaluation in animal feeding studies.

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9 Other Enzyme Applications Relevant to the Animal Feed Industry

A. Péron and G.G. Partridge

Introduction

This chapter examines the potential of enzyme technology to add value, either during production processes (e.g. in the bioethanol industry) or by pre-treatment of raw materials, to reduce certain anti-nutritional factors and/or increase nutrient availability (e.g. by dephytinization, mycotoxin reduction, glucosinolate reduction or production of protein hydrolysates). It also examines the potential role of enzymes in nutritionally upgrading raw materials that arise from some of these processes, e.g. fibrous co-products from the bioethanol industry.

Bioethanol Production and its Implications for the Use of Exogenous Enzymes

The increasing drive since the early 2000s to replace fossil fuels with renewable fuel sources, such as biodiesel and ethanol, has created multiple opportunities for the use of enzyme technology. The production of ethanol by the enzymatic breakdown of starch and sugars, followed by a yeast-driven fermentation of glucose to ethanol, is a good example of how enzyme technology can potentially be used as part of a two-step application process – both outside and, subsequently, within the animal – first, to be added at the fermentation plant to increase ethanol yield and, secondly, to be added as a feed supplement in animal diets that use co-products derived from the ethanol production process, e.g. distillers' dried grains with solubles (DDGS). The extent to which enzymes are used in the ethanol process is one determinant of the characteristics of the co-product, but the process conditions themselves will also be highly influential on the feeding value of the resultant co-product and its subsequent potential for upgrading by enzymes when fed to the animal.

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Use of Enzymes in the Ethanol Production Process

A variety of agricultural crops are being used globally as primary fermentation feedstocks in the ethanol fermentation process (Shetty *et al*., 2008; EDC, 2009). These include maize (corn) and sorghum (milo) in the USA and eastern Canada and wheat, rye, triticale and barley in Europe and western Canada. Blends of grains are also used in some ethanol production plants, depending on the availability of local grain sources. In Brazil, in contrast, sugarcane has been used to produce ethanol for their fuel market.

In its simplest, least capital-intensive form the bioethanol production process takes the whole grain (e.g. maize) and hammer-mills the material. This ground grain then goes through a process of liquefaction involving fresh and recycled water addition, coupled with the addition of appropriate enzymes (Fig. 9.1). Increasingly, most of the fermentation alcohol being distilled is produced from maize starch that has been removed from other components of the grain, e.g. gluten, germ and fibre. Either way, the starch is then usually pressure-cooked at around 105°C in the presence of a thermostable α-amylase and then liquefied further at 85°C. The dextrinized mash is then cooled and saccharified at either 60°C with glucoamylase, or simply cooled to 32°C and simultaneously saccharified with glucoamylase and fermented with yeast. The fermentation usually takes 1–3 days. The fermented 'beer' normally contains about 18% ethanol before being distilled and processed into a nearly 100% anhydrous form (EDC, 2009).

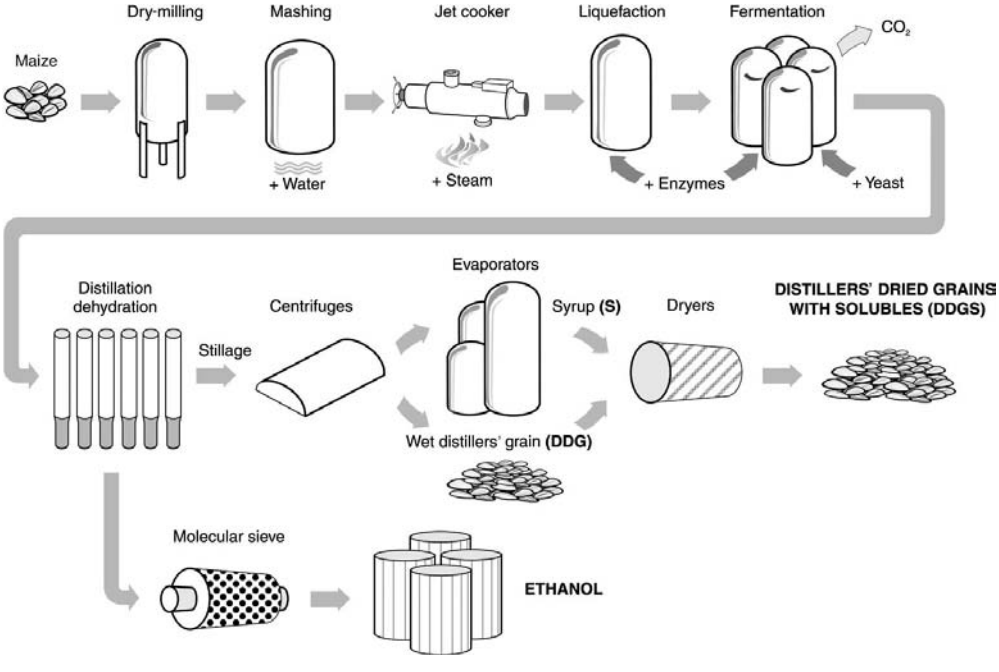

Fig. 9.1. Diagrammatic representation of the bioethanol production process.

Several enzyme companies are working on eliminating the need to cook the grain or starch before fermentation, by finding and developing enzymes that will work at lower fermentation temperatures. This will improve ethanol yield and further reduce levels of organic acids and glycerol.

A further refinement in the pre-processing of maize includes the use of a thermostable phytase as part of the ethanol fermentation process, as described by Shetty *et al.* (2008). This system is known as 'PALS' (phytase amylase liquefaction system), and offers advantages in terms of increasing alcohol yield without major process modifications at the ethanol plant, together with producing DDGS and thin stillage with low levels of phytic acid as 'value-added' co-products.

In the European production of bioethanol using wheat, rye or barley as a feedstock, there is usually a pre-treatment step where the mash is incubated with viscosity-reducing enzymes (e.g. cellulases, hemicellulases) at a temperature below 60°C for 1–2 h prior to liquefaction at 85°C. This results in a DDGS fraction with reduced phytic acid content due to the hydrolysis of phytic acid by endogenous phytases present in these particular grains. Maize and sorghum, in contrast, have very low levels of endogenous phytase, so the PALS process described by Shetty *et al.* (2008) offers good opportunities in this respect.

Kim *et al.* (2008) describe the use of wet distillers' grains themselves as a potential feedstock for bioethanol production, to increase yield in current dry grind ethanol facilities. Wet distillers' grains contain around 20% total glucan (including cellulose and residual starch), which can be hydrolysed to glucose monomers. These authors describe the use of an enzyme system comprising cellulase, β-glucosidase, xylanase and feruloyl esterase, followed by yeast fermentation, both to increase ethanol yield and produce a protein-enhanced distillers' grains co-product.

Currently, the US Department of Energy is actively encouraging industry and others to develop ethanol production from cellulosic biomass, e.g. switchgrass, rice straw and maize stover. Although inherently more challenging substrates for bioethanol production, these feedstocks have the advantage of high productivity and can be grown in many areas of the world without intensive agricultural inputs. They are therefore seen as a much more sustainable way forward than the use of grains.

Bioavailability of Nutrients in Co-products from the Bioethanol Production Process

The main co-product available from the bioethanol industry is DDGS. Following the yeast fermentation and distillation process to produce ethanol, the remaining 'whole stillage' (Fig. 9.1) is centrifuged to produce a wet grain fraction (DDG) and a thin stillage fraction, which is then subsequently evaporated to produce syrup (condensed distillers' solubles, CDS). The syrup is then added back into the wet DDG, in varying concentrations from one production plant to another, before being drum-dried to give the final product (DDGS). The amount of solubles added back and the drying process itself have major effects

on the composition and nutritional value of the final co-product (Zijlstra and Beltranena, 2009). For maize DDGS the solubles portion contains more oils/fats and phosphorus versus the grain, while the grain contains more of the protein. As a consequence, energy feeding values rise with increasing solubles content in the DDGS (Noll, 2007).

The starting grain sources used will be another influence on the ultimate nutritional value of the co-product to the animal. Maize contains more starch and oil and less protein and fibre than wheat, and the co-product from fermentation of these grains (or blends of them) will reflect these differences (Table 9.1). Following fermentation of starch to ethanol and carbon dioxide, the resultant DDGS will obviously represent the original grain feedstock in a more concentrated form. As a 'rule of thumb', many fractions will be concentrated by a factor of three versus the starting grain or grain blend (Zijlstra and Beltranena, 2009).

Like other chemical constituents, the phosphorus in maize and wheat DDGS is more concentrated in the co-product. Due to transformations in the fermentation and drying process, some intact phytate (IP_6) is converted into a range of phytate esters, from IP_2 to IP_5 (Table 9.1). This could have implications for the 'anti-nutritive' properties of the phytate in DDGS versus feedstock grains (e.g. probably less detrimental) and, consequently, for the responses to the subsequent use of exogenous phytase in the animal. Mineral bioavailability appears to be generally increased in DDGS versus grain sources, which could

Table 9.1. Some chemical characteristics of wheat, maize (corn), wheat/maize and wheat distillers' dried grains with solubles (g kg^{-1} dry matter) (adapted from Zijlstra and Beltranena, 2009).

		Distillers' dried grains with solubles (DDGS)		
Chemical	Wheat	Maize	Wheat/maize	Wheat
Crude protein	198	303	424	445
Crude fat	18	128	47	29
Ash	21	48	50	53
Acid-detergent fibre	27	146	195	211
Neutral-detergent fibre	94	312	306	303
Total NSPs	97	192	219	229
Xylose	34	62	81	81
Arabinose	23	43	47	49
Total phosphorus	4.0	8.6	10.2	11.0
Inositol diphosphate (IP_2)	0.0	0.0	0.0	0.8
Inositol triphosphate (IP_3)	0.0	0.9	0.9	0.9
Inositol quadraphosphate (IP_4)	0.0	1.9	1.8	2.8
Inositol pentaphosphate (IP_5)	0.0	4.5	3.3	6.4
Phytate (IP_6)	13.9	9.2	6.2	8.1

NSPs, non-starch polysaccharides.

be related to the removal or reduction of phytate-bound ions (Pedersen *et al.*, 2007; Widyaratne and Zijlstra, 2007).

Cell wall (fibre) fractions are also similarly concentrated, with arabinoxylan content two to three times higher than levels in grain feedstock (Table 9.1). This concentration of the fibre fraction could have positive implications for responses to exogenous carbohydrase enzymes in the animal (e.g. xylanase addition), although the fermentation process and subsequent drying of the product could potentially be modifying influences on xylanase response. In wheat DDGS, soluble arabinoxylans from the grain can enter the final product via the addition of CDS prior to drying. Again, this could magnify the response to xylanase, especially in poultry, which are particularly vulnerable to viscosity negatively influencing nutrient digestion in the gut.

A major concern with DDGS as a raw material for monogastrics is its protein quality and amino acid availability due to: (i) the characteristics of the fermentation process, e.g. batch or continuous, and its duration; (ii) the varying quantities of CDS ('stillage') added back during the manufacturing process; and (iii) the subsequent drying of the DDGS material in terms of temperature and duration. Processing plants for DDGS would prefer to maximize the addition of stillage, but the resultant lumping can be a problem during subsequent storage (e.g. caking in storage bins). Over-drying of the material can be a consequence of trying to avoid these risks during storage.

The amount of intact (bioavailable) lysine per unit of crude protein, measured using reactive lysine analysis (Fontaine *et al.*, 2007), is generally regarded as a more critical measure of protein quality than DDGS sample colour per se. However, in general terms, over-drying of DDGS does lead to a darker product colour and is increasingly associated with protein damage – principally through the formation of Maillard reactions between sugars and amino groups, which render some of the lysine (in particular) unavailable to the animal.

A further challenge to the use of DDGS in monogastric feeds is that the fermentation process does not destroy mycotoxins. So, as with other components, these are concentrated around three times versus their equivalent levels in grain (Applegate *et al.*, 2008). In bad harvest years where there are high levels of damaged and/or moist grains this can be a considerable threat to animal performance, and nutritionists often limit inclusions of DDGS for this reason. Later in this chapter we will examine possible enzymatic solutions for some of these mycotoxin issues.

Effects of Exogenous Enzymes on the Feeding Value of Bioethanol Co-products

The huge rise in production and availability of co-products from the bioethanol industry in recent years has sparked increases in research work to examine the potential of exogenous enzymes in upgrading their nutritional value. Table 9.2 summarizes some recent studies where varying levels and types of enzymes have been used in either broiler or swine rations containing DDGS, at varying levels.

Table 9.2. Recent studies examining the effects of exogenous enzymes in diets containing DDGS for broilers, piglets or grower-finisher pigs.

Effects of enzymes observed, co-product used and level (%)	Enzyme(s) used	Reference
Broilers		
Bodyweight gain: significant improvements (P <0.01) at 10 days (+4%) and 21 days (+4%); AME: significant improvements (P <0.01) at 10 days (+7%) and 21 days (+6%); maize or wheat DDGS used at 10% level	Endo-1,4-β-xylanase; 6-phytase	Pérez Vendrell *et al*. (2009)
Bodyweight gain: significant improvements (P <0.05) at 56 days in 'high energy' diets (+5%) and 'low energy' diets (+12%); femur break strength: significant improvements (P <0.05) in both diets; maize DDGS used at 10% level	Endo-1,4-β-xylanase; α-amylase; subtilisin (protease); 6-phytase	Péron *et al.* (2009)
Piglets		
Bodyweight gain: improvements (P <0.08) in maize DDGS; gain:feed ratio: improvements (P <0.08) in both maize and sorghum DDGS; dry matter digestibility: significant improvements (P <0.05) in both maize and sorghum DDGS; maize or sorghum DDGS used at 30% level	Endo-1,4-β-xylanase; α-amylase; subtilisin (protease); endo-1,3(4)-β-glucanase	Feoli (2008)
Grower-finisher pigs		
Bodyweight gain: no significant improvements; dry matter, nitrogen and gross energy digestibility: significant (P <0.01) improvements in both maize and sorghum DDGS; maize or sorghum DDGS used at 40% level	Endo-1,4-β-xylanase; α-amylase; subtilisin (protease); endo-1,3(4)-β-glucanase	Feoli (2008)
Bodyweight gain and gain:feed ratio: no significant improvements; ileal and faecal digestibility: no significant improvements; wheat DDGS at 25% or 40% level	Endo-1,4-β-xylanase	Widyaratne *et al*. (2009)
Ileal protein and amino acid digestibility: significant (P <0.05) improvements (4–8%); faecal gross energy digestibility: significant (P <0.05) improvements (6%); maize DDGS used at 20% level	Endo-1,4-β-xylanase; 6-phytase	Péron and Plumstead (2009)
Bodyweight gain and gain:feed: no significant improvements in four experiments with four different commercial enzyme products; experiments 1 and 2: maize DDGS used at 15% level; experiment 3: maize DDGS used at 45 and 60% levels; experiment 4: maize DDGS used at 30% level	1. β-Mannanase 2. β-Glucanase, cellulase and protease 3. Proprietary blends (no activity details) 4. Bacterial endo-1,4-β xylanase	Jacela *et al*. (2009)
Bodyweight gain and gain:feed ratio: significant (P <0.05) improvements by 8–10% and 14–16%, respectively; faecal dry matter, nitrogen and energy digestibility: some significant (P <0.05) effects at 4 weeks into the trial, none at 8 weeks; maize DDGS used at 6% level	1. β-Mannanase 2. β-1,4-Mannanase; α-1,6-β-galactosidase; β-1,4-mannosidase	Wang *et al*. (2009)

AME, apparent metabolizable energy; DDGS, distillers' dried grains with solubles.

The benefits from the addition of exogenous enzymes illustrated in Table 9.2 appear quite inconsistent, but it should equally be recognized that a variety of different enzyme activities have been tested in these studies (some of them relatively ill defined), and both the content and source of the DDGS used in diets has varied widely, particularly in swine studies (e.g. 6–60% DDGS content coming from maize, wheat and/or sorghum sources). The future research focus should therefore be on further studies in both poultry and pigs where the DDGS material is initially very well defined, and the sequential effects of different enzyme combinations systematically studied. The description of the enzyme product in terms of the major activities present should also be well defined to facilitate interpretation of the data and its commercial relevance.

Biotransformation of Mycotoxins

Mycotoxins are secondary metabolic products from fungal growth. They are found as natural contaminants in many feedstuffs of plant origin, especially in cereal grains. It has been estimated that over a quarter of the world's grain crops are contaminated above detectable levels by some mycotoxic compound (Fink-Gremmels, 1999). These small and quite stable molecules are extremely difficult to remove or eradicate, and can enter the feed chain while maintaining their toxic properties. Among the numerous mycotoxins that exist, the most relevant ones to the feed industry (Table 9.3) are produced by moulds belonging to the genera *Fusarium*, *Aspergillus* and *Penicillium*. Two groups of mycotoxin-producing fungi can be distinguished. The first one consists of fungi that grow on their substrate and produce mycotoxins on the growing plants before harvesting. This category of field (preharvest) toxins includes

Table 9.3. Mycotoxins most relevant in animal production (adapted from Binder, 2007).

Mycotoxin class	Most relevant representatives in grains and feed	Examples of mycotoxin-producing fungi
Aflatoxins	Aflatoxins B1, B2, G1, G2	*Aspergillus flavus, Aspergillus parasiticus*
Trichothecenes	Deoxynivalenol, 3- or 15-acetyl-deoxynivalenol, nivalenol, T-2 toxin, diacetoxyscirpenol, HT-2 toxin (type A trichothecenes), fusarenon X (type B trichothecenes)	*Fusarium graminearum, Fusarium sporotrichoides, Fusarium poae, Fusarium equiseti*
Zearalenone	Zearalenone	*F. graminearum*
Ochratoxins	Ochratoxin A	*Aspergillus ochraceus, Penicillium verrucosum, Penicillium viridicatum*
Ergot alkaloids	Ergometrine, ergotamine, ergosine, clavines	*Claviceps purpurea, Claviceps paspaspali, Claviceps fusiformis*
Fumonisins	Fumonisins B1, B2, B3	*Fusarium verticilloides, Fusarium proliferatum*

aflatoxins and *Fusarium* toxins. The other group contains fungi that produce toxins after harvesting and during crop storage and transportation. These toxins are named storage (or postharvest) toxins, and ochratoxin A belongs to this group (EFSA, 2009).

Mycotoxins have become a concern in animal production systems as they can affect performance, health, reproductive fitness and product quality of most species. While research has been conducted on techniques to reduce the production of mycotoxins in both the field and storage, the application of feed additives fed concurrently with contaminated feeds has received the most attention. There are many different forms of feed additives used, ranging from enzymes to promote biotransformation and compounds to alleviate the symptoms themselves, to the addition of adsorbents to the diet that bind to the toxin and prevent absorption of the toxin by the animal. Several microbes, yeasts and fungi have been identified and considered able to degrade/detoxify mycotoxins due to the production of specific enzyme activities.

In vitro studies

Varga *et al*. (2005) screened more than 50 filamentous fungi for their ability to degrade mycotoxins. None of the tested fungi exhibited aflatoxin-degrading activity, but several *Rhizopus* isolates were shown successfully to break down ochratoxin A and zearalenone. Similarly, biological degradation of aflatoxin B1 was achieved by the use of white rot fungi (Alberts *et al*., 2009). In this latest work, laccase activity was identified as the enzyme involved in the degradation process. Recent studies have also investigated the possibility to use mycotoxin-degrading bacteria and yeasts, isolated from the digestive tract of animals, in order to transform mycotoxins into less toxic compounds. Molnar *et al*. (2004) showed that a yeast strain of the genera *Trichosporon*, isolated from the hindgut of a termite, exhibited potential deactivation of ochratoxin A and zearalenone in animal feeds. Under optimum conditions, a microbial isolate obtained from tapir faeces reduced the presence of aflatoxin B1 in the culture medium by more than 80% (Guan, 2009). The same author also studied microbial populations present in fish gut and demonstrated that some microbes found in the digestive tract of catfish were able to degrade trichothecene mycotoxins (Guan *et al*., 2009). Finally, other studies have revealed that de-epoxidation reactions by ruminal and intestinal flora significantly reduced the toxicity of trichothecene mycotoxins *in vitro* (He *et al*., 1992; Kollarczik *et al*., 1994). This enzymatic reaction leads to the cleavage of the toxic 12,13-epoxy group of the trichothecene molecule.

In vivo studies

A strain of *Eubacterium*, isolated from the bovine rumen and referred to as BBSH 797, was found to have trichothecene-detoxifying activity (Binder *et al*., 2001). The metabolism of this microorganism was shown to produce

de-epoxidase enzymes (see previous paragraph). This bacterial strain was then used in order to develop the first mycotoxin-deactivating commercial feed additive. Product efficacy was first confirmed in monogastric *in vitro* models, and then confirmed in feeding trials with broiler chickens (Heidler and Schatzmayr, 2003; Diaz *et al*., 2005). Direct feeding of microorganisms does not constitute the only option that has been considered by researchers. The effect of an enzyme product (named 'MDE') in pigs fed diets contaminated with *Fusarium* mycotoxins was investigated by Chen *et al*. (2006). The study revealed that the mycotoxin deactivator MDE provided a partial or complete toxic-sparing effect from mycotoxins as measured by different criteria including growth performance, serum biochemistry and immune parameters, as well as by histo-pathological observations.

Other potential strategies

In a review paper about minimizing the effects of mycotoxins, Jouany (2007) described a method relying on GMO (genetically modified organism) technology. A fungus growing on maize was shown to produce enzymes capable of degrading fumonisins. The genes encoding for these enzyme activities were cloned and transferred into transgenic maize. However, as mentioned by the author, this novel microbial approach may have some limitations, such as the concentration and the reaction time of the enzymes within the digestive tract of animals.

Creating Feedstuffs of Greater Nutritional Value

Most livestock feed ingredients contain one or more anti-nutritive factors. Amongst the most common are non-starch polysaccharides (NSPs), phytate, protein inhibitors, polyphenolics (e.g. tannins), lectins and alkaloids. Different strategies can be used in order to reduce or remove the negative effects of these components. One of these is the pre-treatment of the feed, or the raw materials, with enzymes. With increasing concerns about the preservation of feed resources and the protection of the environment, the feed industry is looking at opportunities to create new feedstuffs with greater nutritional value (e.g. dephytinized grains) and also to safely recycle animal by-products that would otherwise need disposal (e.g. feather meal). Enzyme-mediated improvement of feed digestibility has a direct effect on the amount of feedstuffs needed to satisfy nutritional requirements. It also reduces manure output (Péron and Partridge, 2009).

Dephytinization of feedstuffs

Removal of phytate in plant materials, such as cereals and legumes, can be achieved by washing (extraction with water or acidic solvents), autolysis

(activation of endogenous phytases) or application of microbial phytases. The last of these options is discussed below.

Pre-treatment of ingredients before feed manufacture

Several authors have investigated the possibility of using microbial phytases to eliminate phytate from raw materials, before inclusion into feed. As long ago as the late 1960s, scientists considered using enzyme pre-treatments to increase the nutritional value of feedstuffs. Nelson *et al*. (1968) used mould phytase to reduce phytate levels in soybean meal. Rojas and Scott (1969) demonstrated that phytase treatment could improve the metabolizable energy value of cottonseed meal fed to chickens. Although beneficial effects of the enzyme were evident, the high cost of enzyme production was a limiting factor. With the development of commercial enzymes during the early 1990s, new strategies were implemented. Due to their relatively high level of phytate, oilseed meals were an ingredient of choice for researchers. Solid-state fermentation of canola meal was shown successfully to reduce phytic acid content in the tested material (Nair and Duvnjal, 1990). However, with buffered systems and greater inoculum concentration, *in vitro* systems have proved to be faster and more efficient in reducing phytate levels. Zyla and Koreleski (1993) reported the complete hydrolysis of phytate in canola meal, using a crude phytase during pre-treatment. The use of a purified enzyme resulted in lower efficacy for degrading phytate molecules. Similar observations were made by Newkirk and Classen (1998), suggesting that non-phytase enzymes may facilitate the action of phytase. Since plant phytate is associated closely with other cellular components (e.g. the fibre fraction), it is believed that a combination of phytase with other enzyme activities may enhance phytate hydrolysis. While studying the effects of enzyme pre-treatment on the dephytinization of soybean and cottonseed meals, Han (1988) showed that the extent of phytate hydrolysis was further increased when phytase was combined with cellulase.

In aqua feeds the high temperatures usually applied during processing (e.g. >100°C during extrusion) will destroy in-feed phytase. Equally, for some species, the rearing water temperature will be too low for optimum phytase efficacy. Consequently, the removal or reduction of phytate in cereal grains and oilseeds has been of particular interest for the fish production industry. Pre-treatment of soybean meal and soy proteins with phytase resulted in better growth and nutrient utilization in rainbow trout (Cain and Garling, 1995; Vielma *et al*., 2006; Wang *et al*., 2009), carp (Nwanna *et al*., 2007) and catfish (Van Weerd *et al*., 1999). Similar observations were made when pre-treating all-plant-based diets fed to tilapia (Cao *et al*., 2008).

Online pre-treatment

Pre-treatment of feedstuffs with phytase is generally achieved using solid-state fermentation or wet incubation methods (see previous paragraph). However,

these methods involve several procedures before the ingredients can be included in the feed mixture. Ultimately, these additional steps in the feed production line cost both time and energy. An alternative strategy is to include the phytase pre-treatment as a part of the feed manufacture itself, before the high-temperature processing steps (e.g. extrusion). This method has been developed by Denstadli *et al*. (2006): using a mixture of wheat and soybean meal, the authors tested different conditioning options (various temperatures, moisture contents, retention times, etc.) in the presence of formic acid and *Escherichia coli* phytase, and assessed the efficiency of phytate hydrolysis in the feed mixture. After identifying the optimum conditions, it was shown that up to 86% of phytate was hydrolysed. Then, following successful confirmation on a larger scale, the authors concluded that the method was suitable to become an integral part of the online processing of compound feed, and a patent application was submitted. This new concept was later compared with the more common method of phytase coating (liquid application) after processing. Results showed that, unlike phytase coating, online pre-treatment of vegetable feed ingredients resulted in greater mineral utilization in Atlantic salmon (Denstadli *et al*., 2007).

Improving the digestibility of protein sources used in the livestock industry

The use of enzymes to design a 'perfect protein source' for animals has been reviewed by Woodgate (1994). The concept is based on the idea of manufacturing protein sources containing a range of amino acids that are both essential and balanced in a specific manner, and would be adapted to the requirements of each species and various physiological stages. However, one of the major challenges is to define very precisely the amino acid requirements of farmed animals. The author describes several strategies that have been implemented by the biotechnology and feed industries. So far, most of these methods have been based on the biotransformation of traditional feedstuffs and animal by-products. However, as discussed by Woodgate (1994), these materials have specific deficiencies and it is unlikely that processing alone will ever lead to a 'perfect' ingredient. Nevertheless, described below are enzymatic pre-treatment methods aimed at significantly improving the nutritive value of protein sources commonly used in the feed industry.

Pre-treatment of oilseed meals

Oilseed meals are major protein sources for animal feeds. However, they contain several anti-nutritional factors (e.g. NSPs and protease inhibitors) that can affect their utilization. The degradation or inactivation of these components represents a major opportunity for enzyme producers. Processing of soybean or rapeseed for oil extraction usually involves steps in which solvents are used (e.g. n-hexane). In order to reduce the use of these hazardous/pollutant

compounds, the industry has turned to new technological approaches. One of these is described as aqueous extraction processing. It relies on the use of water as a separation medium. The addition of enzymes can increase the yield of the process and produce higher-quality co-products. For example, the use of cell wall-degrading enzymes during the aqueous extraction of rapeseed was shown to produce a protein meal of greater nutritive value for piglets (Danielsen *et al*., 1994).

Supplementation of soybean-containing feeds with exogenous enzymes such as protease and α-galactosidase activities has received a lot of attention from researchers, but with inconsistent results. Pre-incubation of soybean meal with enzymes has also been investigated. Caine *et al*. (1998) showed that protease (*Bacillus subtilis* subtilisin) pre-treatment of soybean meal had the potential to improve the availability and digestion of soy proteins for monogastrics. In a broiler feeding trial, Ghazi *et al*. (2003) reported positive effects of enzyme pre-treatment (protease and α-galactosidase) on the nutritive value of soybean meal. In aqua feeds, pre-treatment of soybean meal has been quite extensively studied, with most work focusing on dephytinization (see above). However, the literature contains some more unusual enzyme applications such as the exposure of a soybean residue to papain, with successful results in terms of fish growth (Wong *et al*., 1996). The low cost and high availability of palm kernel cake in many tropical countries where aquaculture is practised has generated much interest in its use in fish feeds. Unfortunately, the use of this ingredient can be limited due to its low protein content and the presence of high levels of NSPs. Studies from Ng *et al*. (2002) have shown that pre-treatment of palm kernel cake with commercial feed enzymes resulted in better growth and improved feed efficiency in tilapia.

Pre-treatment of animal by-products

Feathers are produced in huge amounts as a waste by-product from the poultry processing industry. A large quantity of these feathers is available for use in animal feeds; however the variability in nutrient composition and protein quality remains a major concern for nutritionists. Limited digestibility of feather protein has been related to the high degree of cross-linking and compacted structure within the keratin molecule. As a consequence, production of feather hydrolysates by microbial degradation has been considered as a viable alternative (Grazziotin *et al*., 2008). Most studies have investigated the efficacy of proteolytic enzymes isolated from *Bacillus* bacteria, and belonging to the group of serine endopeptidases. Papadopoulos (1985) showed that a treatment using a commercial protease named 'maxatase', isolated from *B. subtilis*, cleaved cystine disulfide bonds and improved feather solubility and susceptibility to digestive enzymes. Williams *et al*. (1991) studied the nutritive value of feathers treated with *Bacillus licheniformis*, a bacterium exhibiting keratinase activity. Results indicated that broilers fed feather meal treated with the bacterium had better growth response than birds fed untreated feather meal.

Similarly, enzymatic treatment of feathers prior to inclusion in the diet was shown to improve performance and/or energy utilization in poultry (Tadtiyanant *et al*., 1993; Kim and Patterson, 2000; Woodgate and Leary, 2009) and fish (Fasakin *et al*., 2005; Woodgate, 2007).

The volume of by-products from the seafood industry is significant. In the important research to find alternatives for fishmeal in aqua feeds, and with the difficulty of carnivorous species in utilizing plant protein sources, the use of fish waste has stimulated a strong interest. Enzymatic hydrolysis of fish and shrimp body parts (by-products) produces protein hydrolysates rich in low-molecular-weight peptides and free amino acids. These hydrolysates can be used in fish feeds as protein supplements (Rebeca *et al*., 1991). They have successfully been used for partial replacement of fishmeal in diets fed to turbot (Olivia-Teles *et al*., 1999), cod (Aksnes *et al*., 2006) and salmon (Refstie *et al*., 2004). Fish protein hydrolysates have also been shown to stimulate feed intake (Refstie *et al*., 2004) and to be beneficial for the development of fish larvae (Carvalho *et al*., 1997; Cahu *et al*., 1999). Finally, recent research on protein hydrolysates prepared from by-products of shrimp-processing operations has indicated a good potential for utilization in animal diets (Bueno-Solano *et al*., 2009).

Other examples of applications

As discussed above, enzymatic transformation of feed ingredients can be used to degrade mycotoxins. A similar strategy has also been applied to target other toxic components present in feed ingredients. This section describes two other methods that have been developed to reduce the negative effects of endogenous toxins in certain feedstuffs.

Degradation of glucosinolates in rapeseed meal

The utilization of rapeseed meal as a protein source in animal feed is limited by the presence of glucosinolates. Major deleterious effects of the ingestion of glucosinolates in animals include reduced palatability and decreased growth and production. Several methods have been tested to improve the nutritional value of rapeseed meal for livestock production. Ultimately, microbial breakdown of glucosinolates and their degradation products appeared to be one of the most interesting and economical strategies. Vig and Walia (2001) demonstrated that solid-state fermentation of rapeseed meal significantly reduced the level of glucosinolates in the ingredient. The benefits were greater for longer fermentation times (>2 days). Similar results were achieved with mustard meal, with complete hydrolysis of glucosinolates after 60–96 h (Rakariyatham and Sakorn, 2002). Direct supplementation of feeds containing rapeseed meal with thioglucosidase activity was also studied in pigs. Results showed that the addition of enzyme gave very small and inconsistent effects (Lawrence *et al*., 1995).

Feeding microorganisms that produce high levels of enzymes

Pre-treatment does not represent the only option for improving the nutritional value of plant materials. Another interesting area of research deals with the concept of delivering enzymes directly into the digestive tract of animals, using microorganisms. Early work from Jones and Lowry (1984) and Jones and Megarrity (1986) demonstrated this strategy perfectly. These researchers showed that the transfer of ruminal microflora from Indonesian and Hawaiian goats, resistant to the poisonous legume *Leucaena*, was able to transfer toxin resistance to Australian livestock. A relatively similar idea was then developed by Cooper *et al*. (1995) in order to protect domestic ruminants against the toxicity of fluoroacetate present in several pasture species. However, in this case, scientists did not transfer a microorganism that was present in the digestive tract of other ruminants, but had to genetically modify a bacterial strain already present in the rumen of the animal, by inserting a gene isolated from a soil bacterium that allows it to degrade the toxin. Although this approach is very promising and may be used to solve numerous toxicity issues related to plant feeding, the fact that it was based on GMO technology is likely to generate potential concern from the public and may ultimately affect its development.

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10 Thermostability of Feed Enzymes and their Practical Application in the Feed Mill

C. Gilbert and G. Cooney

Introduction: The Challenges of Feed Processing for Enzymes

The majority of feeds for monogastrics undergo some form of thermal processing (Bedford *et al.*, 2001), which can include conditioning, pelleting or extrusion/expansion. In the first decade of the 21st century several factors have contributed to an overall increase in feed conditioning and pelleting temperatures applied in feed mills. Some of the factors are not new – for example, the need to ensure good pellet quality in order to maximize production efficiencies. However, other issues have assumed greater importance as consumers become more concerned with the quality of the food they buy. Food safety has become a paramount concern for both the consumer and the animal feed industry, in particular levels of *Salmonella* and *Campylobacter*. One of the consequences of this is an increased focus on the production of 'hygienic' feed for livestock. In response to these food safety concerns, pelleting temperatures are more often being pushed to 90–95°C or higher, and even traditional mash feeds (e.g. for laying hens) are now undergoing some steam conditioning treatments to improve hygiene levels. In addition, the ban on feed ingredients of animal origin in the EU towards the end of the 1990s resulted in increased processing temperatures and longer conditioner retention times being needed to maintain pellet quality.

In efforts to meet these demands, feed mills have had to make changes to the feed manufacturing process. These changes include the introduction of increased conditioning times and conditioning temperatures, double pelleting and conditioning, and the use of expanders and hygienizers in some markets. Increased pelleting temperature has been shown positively to influence factors in the feed, such as the degree of starch gelatinization as well as feed throughput and pellet quality (Ravindran and Amerah, 2008). However, many of the factors involved in the conditioning and pelleting process such as pressure, heat, retention time and steam quality can all result in denaturation of exogenous

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enzymes (Thomas *et al.*, 1998). The susceptibility to heat varies between different enzyme types, with phytases widely accepted as being less inherently thermotolerant than many fibre-degrading enzymes. Phytases have been shown to lose significant amounts of activity when pelleting temperatures exceed 70°C, whereas carbohydrases typically lose significant activity only if temperatures exceed 80°C (Gill, 1997).

Production of pelleted feeds basically involves the feed first passing through a conditioner, where steam is applied, and then through the pellet die before passing through a pellet cooler. In the next sections these processes will be examined in more detail, including the implications for the use of feed enzymes in either dry or liquid form.

Conditioning

Feed is passed through a conditioner prior to pelleting to improve pellet durability and decrease the amount of fines (fine dust) in the final feed. Conditioning also increases mill production rates and reduces the energy consumption of the pellet mill.

There are several types of conditioner used in feed mills, including:

- small-diameter, short-length, high-speed;
- large-diameter, medium-length, slow-speed;
- large-diameter, long-length, slow-speed;
- double;
- hygienizer (super-conditioner); and
- vertical.

Retention times in conditioners can vary from 10 to 90 s, and steam pressures can vary from 15 psi (1 bar) to 75 psi (5 bar), depending on the type of conditioner being used and the temperatures that the feed miller wants to achieve. In addition, there is wide variation in the mixing speeds of conditioners, varying from 60 to 400 rpm (1–7 rps). Therefore, the type of conditioner being used in the mill can affect the recovery of the enzyme product, and this is one of the challenges facing enzyme producers, i.e. how to test the enzyme product in conditions that are representative for all feed mills.

Inside a conditioner, steam enters under pressure and latches on to anything cold (i.e. the feed). The steam enters at temperatures up to 150°C (300°F), and is then cooled when it comes into contact with the feed during the process of heat transfer. Therefore, some of the feed in the conditioner will be exposed to temperatures much higher than the 90–95°C often quoted (Table 10.1).

Conditioners can also be classified as follows:

- Fluidizing conditioner: this is an older design of conditioner but is still abundant in the feed industry. This conditioner tends to be approximately 400 mm in diameter by 2 m long, having on average 48 paddles. This is the best conditioner type for pellet quality, as it maximizes contact

Table 10.1. How high steam pressures in the conditioner increase steam temperatures.

Steam pressure (psi)	Steam pressure (bar)	Steam temperature (°F)	Steam temperature (°C)
75	5.0	320	160
55	3.3	302	149
35	2.3	280	138
25	1.5	265	130

between feed particles and the steam entering the conditioner. However, it tends to be harsher on feed additives due to the high pressures involved in the process. Retention time is typically 10–15 s.

- Stirring conditioner: this conditioner is a more recent design, having been introduced in the 1990s. It is usually 2–4 m long, and the paddles rotate at 120–140 rpm. This type has a slow, stirring motion, and therefore pressures are lower and feed additives are not exposed to such harsh conditions. Retention times can be 30–60 s.

Conditioners that use high steam pressure and high-speed mixing are the most damaging for feed enzyme recovery. For feed enzyme applications it is therefore very important to find out the type of conditioner being used in the mill, and its properties (Table 10.1).

Pelleting

During pelleting the feed is forced through a pellet die under pressure. In a standard pellet mill operating at ~240 rpm (~4 rps) it takes on average eight compressions per second to produce a feed pellet. The pelleting process creates die friction, which results in an increase in temperature of the pellets across the die. This temperature rise is usually in the range of 3–6°C (5–10°F) higher than the targeted pelleting temperature. Factors such as excessive equipment age and wear can increase die friction, which can have negative consequences for post-pellet enzyme recovery.

Application and Use of Liquid Enzyme Products

A wide range of steam conditioning and pelleting conditions can exist between different feed mills. Given the challenges of feed processing mentioned previously, it is advised that where these conditions exceed a feed enzyme's maximum temperature recommendations, as specified by the manufacturer, then liquid enzymes should be used. The liquid enzyme is usually sprayed on to the feed after pelleting, and this approach ensures that the enzyme is not exposed to high processing temperatures.

Applying liquid enzymes accurately after pelleting tends to be complex and costly. Accurate application of the liquid enzyme, as with some other critical liquid micro-ingredients, requires specialized spraying equipment and, even then, consistency of accurate enzyme application can be an issue (Bedford and Cowieson, 2009). This equipment usually needs to be specially designed for a specific feed mill, such that expert engineering advice should always be sought before installing a system. Factors that should be considered in the design of a liquid enzyme application system include:

- control of the dry pellet and liquid flow rates;
- uniform exposure of the feed pellets to a fan spray of the liquid;
- continuous enzyme/feed pellet flow to maintain feed throughput;
- flexibility of the system to adjust flow rates if needed, without compromising accuracy of dosing;
- minimizing the creation of fines, or spraying after fines have been removed;
- reliability of the system;
- particular specifications dictated by the feed mill;
- hygiene and safety; and
- cost of the system.

Critical to the accurate application of a liquid enzyme is the monitoring and control of liquid and dry flow rates, with the dry flow of feed being metered along with the liquid enzyme as described by Steen (1998) and Cooney (1999). The system needs to ensure that every pellet or particle has the chance of being coated by the enzyme. The flow of feed should be controlled, otherwise fluctuations can occur as a result of the upstream equipment. Control of the flow of feed can be achieved through installation of a hopper that delivers a constant volume of feed through the dry-flow meter and the liquid-dosing equipment. Measurement and control of liquid flow is achieved through the use of a flow meter that gives a process signal that is proportional to the application rate needed, and also to the dry-flow capacity. This signal can then be used to vary the speed of the dosing pump.

The most accurate dosing can be achieved using a gravimetric dosing system. This type of system weighs both the liquid and dry elements gravimetrically (mass/time) using a mass-flow meter, and adjusts the dosing accordingly. The system is able to evaluate the gravimetric capacity of the conveying device, the speed at which it is running and also the weight of the material on the conveyer. This information is then used to adjust the flow rate (Cooney, 1999).

When considering liquid-dosing systems, the viscosity, temperature and specific gravity of the product should be known. In addition, inclusion rates of some enzyme products (for example, phytases) can be as low as 25–50g t^{-1} and therefore, to aid accurate application and increase the chances of all pellets receiving some of the enzyme, it is necessary to add water to the products. This is typically done at the spray point and means there is more liquid to be sprayed on to the pellets so that the coating efficiencies of the system are increased. If the feed is to be screened to remove fines prior to being packed,

then it is vital that the liquid enzyme is added after this screening process. Research has shown that as much as 30% of the enzyme activity can be found in the pellet fines, and therefore adding the enzyme before screening would result in a lower than expected dosage in the final feed and wastage of the enzyme product (Engelen, 1998).

Application and Use of Dry Enzyme Products

From a practical perspective, where conditioning and processing conditions allow, applying dry enzymes is the preferred option for many feed mills. A major advantage is the ease with which the dry enzyme product can be added, either via the existing manual (hand-tip) area or in an automated micro-dosing system. With dry enzyme products the more heat stable the better, as it is vital for accurate dosing and maximum bioefficacy that the enzyme survives the feed-pelleting and conditioning process. Most commercial enzymes can withstand a high temperature range of 85–90°C for only a short period of time before they start to denature. For this reason, feed additive companies have made recent developments in both coating technologies and in the production of intrinsically heat-stable products. This now means that addition of dry products pre-pelleting is possible.

Thermostability of the feed enzyme is a key issue if it is going to be used in a dry form and therefore be added to the feed before pelleting. This is one of the ideal characteristics for a feed enzyme recommended by Selle and Ravindran (2007). The full list includes:

1. A high specific activity per unit of protein.
2. Good thermostability during feed processing.
3. High activity in the biologically relevant pH range of the gut.
4. Resistance to the animal's endogenous proteases (e.g. pepsin).
5. Good stability under ambient temperatures.

For most of the major enzyme activities used in animal feeds, attaining acceptable levels of thermostability remains the greatest challenge. Phytases are one good example. The so-called 'second generation' *Escherichia coli* phytases have been shown to be superior to traditional fungal phytases (e.g. from *Aspergillus* and *Peniophora*) in terms of their higher specific activities (Wyss *et al.*, 1999, Leeson *et al.*, 2000), wider pH profiles and improved pepsin resistance (Wyss *et al.*, 1999; Igbasan *et al.*, 2000, 2001, 2002; Bedford and Cowieson, 2009). However, many of these second-generation *E. coli* phytases still have deficiencies in terms of their thermostability, like their first-generation counterparts.

As the animal feed industry moves towards harsher processing conditions for feed hygiene, so the feed enzyme industry has had to adapt to this new set of challenges. Three principal strategies have been used by the feed enzyme industry to help solve the problem of pre-pelleting addition of feed enzymes and how to ensure the survival of the enzyme through the pelleting process (Graham and Bedford, 2007; Bedford and Cowieson, 2009):

1. Applying a coating to dry enzyme products to protect the enzyme from the heat and moisture used in feed manufacture.
2. Genetic manipulation of the enzyme product to make it more inherently thermostable.
3. Discovery of wild-type enzymes that are intrinsically thermostable.

To date, the most widely applied of these options are the application of protective coatings and the use of genetic manipulation. There are potential concerns with both strategies that need to be carefully considered.

Genetic manipulation of an enzyme to improve its thermostability is usually achieved through changes to the amino acid structure of the enzyme. This change in structure can be through the substitution of surface amino acids in the enzyme by more hydrophobic amino acids, as well as via an increase in the number of specific amino acids capable of forming cross-bonds within the enzyme molecule. Genetic manipulation of the amino acid structure must be carried out carefully to avoid altering the geography of the active site (Graham and Bedford, 2007). A change to the structure of the active site could reduce the affinity of the enzyme for its target substrate and therefore result in a reduction in efficacy of the enzyme. Also, while enhancing the thermotolerance of the molecule, a high activity needs to be maintained in the biologically relevant temperature range for the target animal, which is 37–40°C (Bedford, 2008). Similarly, the optimum pH of the enzyme needs to be maintained for it to be functional in the gut of the animal.

In recent years there has been a lot of work concerning the genetic manipulation of xylanases and phytases, and this has now resulted in several intrinsically thermostable feed enzyme products being launched. However, due to the variety of feed-processing conditions used commercially, these genetically modified products will still not be stable for all pelleted feeds, but are purported to be suitable for the majority (Bedford and Cowieson, 2009). As previously discussed, no two feed mills will be the same due to variations in the equipment used, conditioning times, die friction and individual feed formulations. Cowieson *et al*. (2005) studied the effects of pelleting temperature on post-pelleting recovery and *in vivo* efficacy of a xylanase that had been genetically modified to improve its thermostability. These authors found that this product could be used in diets pelleted at up to 90°C without compromising broiler performance. Birds fed diets containing xylanase gave consistent performance regardless of pelleting temperature, which ranged from 80°C to 90°C. In the past, xylanases have also been shown to be difficult to assay accurately, this being mainly attributed to the binding of the enzyme to the pelleted feed matrix. New xylanases have now been developed that have intrinsic thermostability and are also easier to assay (Bedford, 2008). These developments allow enzyme users more confidence about consistency of response when using enzymes in pelleted feeds.

A method of protecting enzymes from heat that is widely used in the textile and detergent industries is the encapsulation or application of a protective coating to the enzyme. The feed enzyme industry has now also used this principle. An ideal enzyme coating for animal feed needs to:

1. Protect the enzyme through steam conditioning (typically 85–90°C or higher) and through subsequent pelleting.
2. Release the enzyme from the coating quickly in the gastrointestinal tract of the target animal, to ensure optimum efficacy.

Taking the example of a coated phytase, the enzyme needs to be active in the crop of the bird where the target substrate (phytic acid) is in a soluble form. The phytase consequently needs to be released quickly from its coating in the animal's gut. Any delay to the release of the phytase can lead to reduced efficacy, with negative effects on phosphorus nutrition, skeletal health and the welfare of the animal. Achieving this balance poses a challenge, with the characteristics needed to repel water and heat during the pelleting process seeming to be in contradiction to those needed for quick release in the animal. There are now commercial feed enzyme products on the market where this balance has been struck.

Several studies have demonstrated good post-pelleting recoveries of coated phytases across a range of pelleting conditions. Ward and Wilson (2001) measured post-pellet recoveries of a *Peniophora lycii* phytase and reported an average 68% recovery following pelleting at 93°C, and a recovery range of 68–90% over pelleting temperatures from 73 to 99°C. Angel *et al*. (2006) also investigated post-pellet recoveries of a coated *P. lycii* phytase and showed 77.2, 67.1 and 57.7% retained phytase activity following pelleting at 70, 80 and 90°C, respectively. More recently, Timmons *et al*. (2008) compared post-pellet recoveries (average pelleting temperature, 93.3°C) of a coated *P. lycii* phytase and a coated *E. coli* phytase, and found recovery ranges of 64–80% and 69.5–81.0%, respectively.

There have been some studies that suggest that coating of enzymes can reduce the efficacy of the product when directly compared with an uncoated version of the same product. Kwakkel *et al*. (2000) tested an uncoated and a fat-coated fungal phytase, and observed that weight gains and tibia ash of broilers were reduced by 40% when fed diets containing the fat-coated compared with the uncoated product. This was attributed by the authors to a delayed release of the phytase from the coated product in the digestive tract of the animal. However, in contrast, a coated *E. coli* phytase has been shown to release quickly *in vivo*. A recent study demonstrated that when a coated *E. coli* phytase product was compared with an uncoated version of the same enzyme (on a wheat carrier), the presence of the coating had no detrimental effect on the efficacy of the product, with both products giving similar performance results (Owusu-Asiedu *et al*., 2007). Since these diets were fed as mash, it demonstrates that the coated product did not need to have been through the pelleting process to release in the feed.

Checking the Addition of Enzymes in the Feed Mill

In order to determine whether an enzyme product has been added at the correct dosage and has been correctly distributed throughout the feed there

needs to be good test procedures, in addition to a sensitive and reliable assay methodology. Key parameters that should be monitored when considering both liquid and dry enzyme additions are:

- homogeneity of the enzyme in the feed;
- concentration of the enzyme in the feed; and
- batch-to-batch carry-over of the enzyme.

As previously mentioned, it is a challenge for the feed enzyme manufacturer to find a test that can replicate all conditions used commercially for the manufacture of feed. Model situations can be used to help identify any limitations of the product, but ultimately products should be tested to ensure that they perform under the specific commercial mill conditions a customer is using. There are different conditioner types, as previously discussed, as well as numerous designs of pellet mills, hygienizers, extruders, mixers, etc. Add in factors such as equipment age and wear, and no two feed mills will have exactly the same conditions.

When running a test to check the homogeneity and recovery of the enzyme in a feed, multiple feed samples should be taken from throughout the batch of feed containing the enzyme, whether it has been added as a dry or a liquid product. When running a test to check the thermostability of an enzyme it is important to collect both mash and pelleted feed samples. These should be collected from the same batch of feed, meaning that the tests must be well monitored and carefully timed. Collection of pelleted feed samples too early or too late may result in an inaccurate measure of thermostability. In addition, when collecting pelleted feed samples it is important that samples are collected after the pellet cooler. Collection of hot pellets will essentially result in the pellets continuing to cook after collection and, again, this can bias results and mean that the feeds tested will not be truly representative of the feed offered to the animals.

When running a test in a commercial mill where enzyme products are already being used (i.e. to prove that an enzyme survives a potential customer's mill processes), it is important to ensure that the current enzyme product has been removed from the formulation before conducting the test. This is vital if the enzyme already routinely being used has the same activities as the enzyme being tested. Failure to do this could confound the results and result in an overestimation of thermostability. To ensure that there is no carry-over or contamination of the feed samples with another similar enzyme product, it is advisable to run multiple batches of feed for the test. Samples should only be taken once the mill is at operating temperature (according to the mill computer) and, ideally, from the second or third batch manufactured. This minimizes any chances of cross-contamination of the samples. The final objective is that the test results will be more accurate and reliable.

Following collection of samples, it is essential that they are assayed using a proven and accurate in-feed assay that is suitable for the enzyme activity in question.

The criteria for a good enzyme assay are:

- The enzyme assay should measure the enzyme activity using a suitable parameter to assess functionality.
- The pH, temperature and substrate for the assay should be optimal for the enzyme being measured.
- The assay method should be reproducible both within and across different laboratories, and have been validated.

Pellet-testing protocol

When conducting a mill test to check thermostability the timing of sample collection is vital, and therefore a strict protocol should be followed to ensure success. Many enzyme producers claiming thermostability follow a similar protocol, an example of which is as follows:

1. Remove any existing enzyme product containing the same activity as the test enzyme from the formulation.
2. Add the test enzyme to each mixer batch at an appropriate dose for the product being used. Ideally, there would be five mixer batches for the test, especially if the mill routinely uses a similar product.
3. From the batch of feed containing the test enzyme take five mixer (mash) feed samples.
4. A check will be made with the mill staff when the identified mixer batch will exit the pelleter and cooler. Their knowledge of mill capacity (tonnes of feed produced per hour) will help in this decision.
5. When the pellet mill has reached its operating capacity and steam heat target (as indicated on the mill computer), record the temperature of the feed pellets that are exiting the pellet mill using a digital temperature probe, in conjunction with an insulated cup or thermos flask.
6. Take ten pelleted and cooled samples from a suitable location immediately after the pellet cooler. It is a good idea to check with the mill staff when the mixer batch that was sampled will start to exit the cooler. Sample consecutively and every 20–30 s to ensure that representative samples from throughout the feed batch are obtained.
7. All samples (both mash and pellet) should then be assayed using a suitable assay method, and the average enzyme activity and homogeneity (coefficient of variation %) calculated.

Conclusion

There is a trend developing within the feed industry to push feed-processing temperatures higher in a bid to minimize microbial loads and ease the public's and regulator's concerns about food safety. These increased temperatures and harsher processing conditions are harmful to a number of additives and

nutrients in the feed including, but not limited to, vitamins, proteins and feed enzymes. Traditionally, where harsh feed-processing conditions have been used, mills have opted for post-pellet application of liquid enzymes, but this is a costly process requiring specialist equipment. Due to the challenges being faced by the industry, the feed enzyme manufacturers have over the last decade increased their efforts to develop more thermostable dry enzyme products, either through genetic manipulation or, more often, through the use of protective coatings. These developments have been of great benefit to feed manufacturers and animal producers alike. The development of these dry thermostable enzymes allows more confidence in the consistency of performance seen. However, the ultimate goal for enzyme manufacturers, researchers and the industry remains to find, through screening for heat resistance, relevant enzyme activities for animal feed that are naturally thermostable and would require neither coatings nor genetic manipulation.

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11 Analysis of Enzymes, Principles and Problems: Developments in Enzyme Analysis

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Introduction

Since the 1980s, the addition of enzymes to monogastric diets has become commonplace throughout the world. The three most widely used enzymes are phytase and the two principal non-starch polysaccharide (NSP)-degrading enzymes, xylanase and β-glucanase. Other enzymes that are also used in animal feeding include protease, pectinase, mannanase, α-amylase and α-galactosidase. Enzymes are proteins and rely on their three-dimensional structure for activity. This three-dimensional structure can be disrupted by the heat applied to feed during processing, and so it has become increasingly important through subsequent feed analysis to ensure that the feed that becomes the animal's diet contains the active enzyme. The routine analysis of enzymes in animal feed samples has become an essential quality control step in feed production and the feed manufacturing process (Aehle, 2007). Issues addressed in this chapter will include some discussion of the methods of detection and, in particular, their advantages and disadvantages in the analysis of enzyme activities in feed samples, strategies for ensuring reliable sub-sampling of feeds in the laboratory, problems with analysis due to interaction with the feed matrix (and methods of limiting this effect) and future trends.

Principles of Analysis of Activity of Enzymes Used in the Feed Industry

Enzyme reactions generally follow the simple principle of enzyme + substrate = reaction product(s). The enzyme activity may be detected by the disappearance of substrate or by the formation of reaction product that is catalysed by the enzyme activity. For the enzymes that are most often used in the feed industry

it is usually the latter, especially when it comes to quality control of the enzyme products and other premixes, prior to mixing with the feed. In the case of enzymes such as xylanase, β-glucanase and other NSP-degrading enzymes, a high-molecular-weight (MW) polysaccharide substrate is converted to reaction products consisting of lower-MW oligosaccharides, which are then usually measured by reducing sugar methods such as the DNS (dinitrosalicylic acid) method (Miller, 1959) or the Nelson–Somogyi method (see Bedford and Partridge, 2001, Chapter 4 for a detailed description of the Nelson–Somogyi method). Although arguably the Nelson–Somogyi method is the better (Jeffries *et al.*, 1998), the DNS method has become more popular due to the greater toxicity of the reagents in the Nelson–Somogyi method.

In the case of phytase and protease assays, it is usually the breakdown products – free phosphate in the case of phytase and low-MW peptides in the case of protease – that are measured. While in many cases the reasons for measuring low-MW products are historical, they are also technical; usually, the product of the reaction is a simpler molecule and therefore easier to measure than the disappearance of the more complex substrate. Also, enzyme assays are designed with relatively high substrate concentrations in order to maintain linearity between enzyme concentration and the measured response. Therefore, at the end of an enzyme assay there will always be some intact or partially intact substrate that would make it difficult to measure the relative change in substrate concentration. In some cases the change in substrate concentration is almost negligible, so measuring the products is much more robust than measuring the substrate (Greiner and Egli, 2003).

Viscosity-type assays are also used to measure polysaccharide-degrading enzyme activities. These assays measure the change (decrease) in viscosity due to enzyme activity, and so can be considered closer to the former type of assay in that they essentially measure the disappearance of substrate rather than formation of product. Viscosity-based assays, while they more closely resemble the functionality *in vivo*, can be difficult to quantify, are time consuming (usually only one sample can be tested at a time) and are usually more expensive to perform due to the greater consumption of expensive substrates relative to the colorimetric-type assays (see Bedford and Partridge, 2001, Chapter 4, for more detailed description of a viscometric method).

Once the principle of the detection of enzyme activity has been decided upon, assay methods for the analysis of enzyme activity in feed additives and feeds fall into one of two possible approaches. The first, and certainly the most popular from the quality control approach, is the single time-point method. In this method an enzyme is incubated with substrate for a certain length of time, then the assay is quenched with a reagent that inactivates the enzyme activity (usually irreversibly by a strong acid or alkali) and the amount of product formed by the enzyme is then measured, usually by a colorimetric method (e.g. Engelen *et al.*, 1994; Cosson *et al.*, 1999). This type of method assumes that, between time zero and the time that the reaction is quenched, the enzyme activity (velocity) remains constant. In practice, great care has to be taken with these assays in the choice of dilution of enzyme. Too great a dilution will put the detection (usually a colour reaction) too low on the standard curve, where there

is a high potential for error in activity in the calculation of enzyme activity due to the low signal:noise ratio. If the sample is not diluted sufficiently, the reaction may be in an area that is above the colorimetric standard curve or even below this but in an area where the substrate concentration has become limiting, and so the colour reaction is not responding in a linear way to the enzyme activity.

An alternative method for measuring enzyme activity is by linearity or kinetic analysis. In this type of analysis, continuous or multiple time-point analysis of the enzyme activity is employed, and the activity is measured as the slope of the line. This gives greater detail in understanding enzyme activity and confidence in the results, although is dependent on having a method of detection that can be continuously monitored. For assay of enzymes used in the animal feed industry using other than viscometric methods, this generally entails setting up multiple replicate reactions and then quenching these at different time-points, rather than being able continuously to monitor a single reaction. Alternatively, it could involve setting up a large reaction volume and then taking aliquots from this to test at regular intervals. This makes the process laborious and tends to be utilized in more detailed *in vitro* work (Shen *et al.*, 2005), rather than in routine quality control analysis of feeds. With the correct equipment it may also be possible to measure viscosity continuously, but again it requires relatively expensive rheometers, usually only one sample can be processed at a time and again it involves larger consumption of possibly expensive substrates. There may also be implications for continuous viscometric monitoring because of the effect of the viscometer/rheometer on the rheological properties of the sample itself.

For the measurement of polysaccharide-degrading enzymes such as α-amylase, xylanase and β-glucanase in feed, probably the most significant development in recent years in relation to analysis of these enzyme activities in feed samples has been the development of dyed substrates, available in liquid or tablet form (e.g. Cosson *et al.*, 1999). Normal methods of analysis for xylanase and β-glucanase are not suitable for feed analysis. In addition to sugars that are already present as background noise, there is also the danger that other substrates and the corresponding enzyme activity may be co-extracted from the feed sample into the assay procedure (e.g. starch and α-amylase being extracted into a xylanase assay).

Without dismissing the idea of using a reducing sugar assay to measure enzyme activity in feed, these types of substrate (e.g. from Megazyme International (Republic of Ireland) or Magle (Sweden)) generally allow for better specificity and enhanced sensitivity. The method is based on the determination of water-soluble dyed fragments that are released when a xylanase is added to a xylazyme tablet and allowed to react for a certain time at 50°C. The solubilized fragments have a blue colour that specifically absorbs at 590 nm. Activity is calculated by comparing absorbance values for the sample via reference to an enzyme standard curve prepared from a dilution series of a known amount of enzyme activity. One disadvantage of this method is that it is difficult to express in normal biochemical language (e.g. μmol min^{-1}), and so requires standardization by preparing a standard curve on the dyed substrate from samples of known activity by the parent (e.g. DNS method) in order to express the results of the colour

reaction in terms of quantity of enzyme activity. In theory the standard curve should be prepared with the same enzyme that is present in the feed, because different enzymes may result in standard curves with slightly different slopes. As a result, for feed samples where the added enzyme is of unknown origin, there can be problems with calculating the final activity. These assay methods are described in greater detail in Bedford and Partridge (2001, Chapter 4).

Pre-assay Steps to Avoid Analytical Reproducibility and Recovery Problems in Feed Analysis

Different types of added enzymes all potentially suffer from the same problem of heterogeneity of the sample, regardless of the enzyme activity to be tested, when it comes to analysis in feed. Typically, a sample from a feed mill will be 100–500 g of material. Assuming that all practical steps have been taken at the sampling site to obtain a representative sample for the laboratory, it generally is not practical, for reasons of scale and cost, to run the preparatory steps on, e.g. 500 g of feed material and, therefore, further homogenization and sub-sampling occurs at the laboratory stage prior to the sample entering the actual analysis. The aim of this homogenization is to be able to sub-sample 1–10 g of feed material (e.g. Slominski *et al.*, 2007), which can then be used for the first main laboratory stage of analysis, namely the extraction stage. The principal means by which the relatively large sample obtained from the feed mill is homogenized in the laboratory is by further grinding/milling of the sample in a laboratory mill. An example in the case of phytase analysis is a recommendation to grind 100–150 g of sample using an ultracentrifugal mill with a 1 mm sieve (Engelen *et al.*, 2001). From the larger ground sample the recommendation is to take 2 × 5 g sub-samples for the analysis procedure.

The recently introduced harmonized method for phytase (ISO 30024: 2009) has somewhat abandoned the need for milling, in that this new method recommends the use of 2 × 50 g of unmilled material in the extraction stage in the laboratory, although it does qualify this by also recommending, in the case of inhomegeneity in the sample, to homogenize at least 150 g of sample in an ultracentrifugal mill. Besides the use of an ultracentrifugal mill, laboratory mills come in other suitable designs. Although the principal reason for milling is because the sample has to be ground more finely to have a more homogenized material from which to sub-sample, the milling step may also help to break down enzyme particles (granules). Some enzyme products, due to granulation and possibly coating, have a relatively large particle size (e.g. >99% of the particles are >297 μm for Phyzyme™ XP (Anon., 2008); average of 250 μm for Ronozyme™ NP (Anon., 2009); or up to 800 μm in the case of Natuphos™ 10000G), compared with simple, spray-dried powders, but are nevertheless dosed at relatively low levels in the feed. During the grinding of the sample at the pre-analytical stage in the laboratory mill, coated enzyme products or products of a relatively large particle size and relatively low inclusion rate may actually undergo some particle size reduction, which therefore results in a more even distribution of the enzyme particle throughout the ground sample. Phytase

in-feed inter-laboratory studies (Engelen *et al.*, 2001; Gizzi *et al.*, 2008) have shown that RSD (ratio of standard deviation) values of <20% – and usually <10% – can be routinely achieved with either grinding and small (2 g) sub-sampling or no grinding with a relatively large (50 g) sub-sample.

Similarly, in the case of xylanase, β-glucanase and cellulase, it has been reported that the results of in-feed analysis can achieve similar repeatability/reproducibility to phytases (Cosson *et al.*, 1999; Konig *et al.*, 2002). These sorts of results represent values similar to those achieved for pure enzyme assays (Bailey *et al.*, 1992). Therefore, despite the potential problems associated with distribution of the enzyme within the feed matrix and possible reproducibility problems, there is nevertheless evidence to show that, by applying some effort to homogenize samples pre-analysis, the various restraints can be overcome to give satisfactory results for the major feed enzyme activities.

While the grinding of samples can improve the reliability of results in feed analysis, it is the subsequent stages of analysis that will play a part in effective *recovery* of enzyme activity from the feed. Whether a sample is ground in a laboratory mill or not, it is not possible to analyse enzyme activity in a dry sample, so the first stage of analysis of an enzyme in a feed matrix is to make an aqueous extraction of the sub-sampled, homogenized material. Typically, this involves adding a particular buffer mixture to at least duplicate sub-samples of the dry feed and then agitating (stirring/shaking) this mixture for a period of time in order to solubilize the enzyme protein into the buffer. This extract is then diluted further as necessary for actual assay. Although there is potential for loss of enzyme in subsequent stages of the analysis, an 'inefficient' extraction that actually results in the loss of activity or is not optimized for the solubilization of the enzyme will clearly result in problems of low measured activity – in other words, low recovery.

The analyst will typically use extractions that are based on criteria such as agitation (how forcefully an extraction is stirred or shaken), time, extraction buffer composition and pH, and possibly temperature (Selle and Ravindran, 2007). Most feed extractions are centrifuged or filtered to clarify the solutions, and only the filtrate/supernatant makes it through to the subsequent stages of analysis. This is for simple practical reasons: a solution with large particles may block the small apertures on pipette tips or may cause problems at the detection stage, with 'spikes' of colour, or affect, for example, the correct operation of viscometers.

Analytical Considerations with Vitamin–Mineral Premixes

Sometimes in the case of feed analysis, when low recoveries of enzyme activity in feeds are discovered, the problem may not be due to the feed manufacturing process or interactions with feed ingredients, but may have occurred upstream in the feed manufacturing chain. An intermediate premix is often involved, where the enzyme product is blended with other ingredients. The concept of a 'single premix' that contains all the various trace minerals, vitamins, enzymes, bacteria and other ingredients is a very attractive one, but of course there is

potential for interaction between the ingredients and the enzyme in the dry product during shelf storage. To confirm that there are no adverse problems it is normal to perform shelf-life studies (e.g. Brugger *et al.*, 2009). Almost certainly, granulation and coating of enzyme products will help with stability of the enzyme in these premixes, as these processes will prevent interaction between premix ingredients during the shelf life of the premix.

Ensuring quality control of enzyme activity in these premixes also presents challenges in the analysis. As with feed analysis, so it is in the case of feed premix analysis – the aim is generally to process the sample from dry premix to an extract containing enzyme activity to be further diluted and assayed. Some examples of problems that can be caused by premix ingredients during sample processing for enzyme analysis include the following:

1. High or low pH of the extract. This can usually be simply counteracted by using a buffer for extraction rather than, for example, distilled water. A slightly stronger buffer than that used for normal enzyme extraction and dilution may be required.
2. Adsorption of enzyme to insoluble ingredients. Another problem that can present with premixes is non-specific adsorption of the enzyme to premix components. This can occur especially when adsorbent materials such as silicates are added to premixes, either for use as cheap carriers or to assist in the pelleting process as pellet binders. In these cases it may be useful to add a non-specific 'blocking agent', as is used in other areas of biochemistry, e.g. ELISA. The enzyme protein is present at very low levels and is often the only protein present in a premix, so very easily binds to insoluble adsorbent materials during the extraction stage. To counteract this, typically a relatively high concentration of a non-specific blocking protein is used in the extraction buffer, usually bovine serum albumin (BSA), and in theory this blocks potential adsorption sites on premix ingredients and ensures that the enzyme protein is dissolved into the extract solution. The exact concentration of blocking agent is often only determined by trial and error. BSA is a relatively expensive ingredient and sometimes a cheaper ingredient, e.g. casein, can be used instead.
3. Influence of high concentrations of ions such as $Cu^{2+,}$ Ca^{2+} and Mg^{2+}. High concentrations of mineral ions in the premix extraction can be counteracted by the addition of EDTA (ethylenediaminetetraacetic acid) or another similar chelating agent. As the name suggests, these chelating agents bind or sequester mineral ions and prevent interaction with the enzyme in solution. Care has to be taken with the concentration of EDTA so that carry-over of EDTA into the final assay is not too high.

Analysis of Plant Enzyme Activities

In the case of analysis of plant enzymes, where it is often difficult to achieve a complete extraction, alternative methods may be required. In one reported piece of work (Okot-Kotber *et al.*, 2003), the addition of relatively high levels

of polysacharride-degrading enzyme activities during extraction resulted in higher recovery of plant phytase enzyme activity. In addition, a 'direct incubation' approach may be used (Zimmermann *et al.*, 2002) instead of the extraction approach. In the direct incubation approach no separation of insoluble and soluble components of the feed is required prior to the addition of the substrate. Using this method, over twice the phytase activity was measured in cereals such as rye, wheat and barley (Greiner and Egli, 2003) as compared with the conventional extraction-based technique. These techniques have yet to find popularity in the measurement of exogenous enzymes in feed samples, although they might be useful in the case of low recovery by conventional extraction techniques, where they eliminate the possibility of enzyme adsorption to insoluble feed components as being one reason for the problem observed.

Developments in Phytase In-feed Measurements

Phytase enzymes now vary in their microbial sources and, even in the case of phytases from similar sources, they can vary in their biochemical characteristics and so may behave differently during feed processing and subsequent laboratory analysis. For example, one study (Dalsgaard *et al.*, 2007) observed that when a different buffer strength or composition was used, this altered the measured activity of a phytase. This study used different molarities of acetate and citrate buffers, both of which have been used in published assay methods for phytase, to measure a bacterial phytase. Changing the buffer from a 0.25 M acetate buffer to a 0.20 M citrate buffer reduced the assayed activity by approximately 50%. Use of a 0.10 M citrate buffer increased the activity measured compared with the 0.20 M citrate buffer, but the activity was still 15–20% less than with the acetate buffer. If the same comparisons had been done with a fungal enzyme, there would have been negligible differences.

An inherent thermostability in the protein will obviously be an advantage for survival (and recovery during analysis) of enzyme activity, but does not necessarily mean that the enzyme is therefore easy to recover from the feed sample. It remains possible that low recoveries of phytase activity in feed may be due to a lack of consideration in the analytical method for the interaction of enzyme and feed ingredients during pelleting and conditioning. An example of using a special extraction procedure designed for feed analysis, not used in the analysis of the same enzyme when it is in the pure enzyme formulation, can be seen for example in the development of one of the most commonly used phytase assays (Engelen *et al.*, 1994, 2001). Consideration in the earlier article was given initially to the analysis of readily soluble enzyme samples, and so enzyme samples were extracted in an acetate buffer containing Tween and a low level of calcium. However, although attention was paid to the use of a particular buffer system, the method did not specify any other preparatory criteria, probably because the enzyme was so 'easy' to dissolve and on account of the lack of any major interferences in the sample matrix. Later, when the method was updated for feed analysis (Engelen *et al.*, 2001), the method

subsequently described a milling step required to homogenize the feed sample, a specific 'feed buffer' containing a higher level of calcium chloride (68 mM) and instructions to stir the feed sample for 60 min with subsequent filtration of the extract. The inclusion of a high level of calcium chloride in the extraction buffer helps to precipitate some of the background phosphate (Park *et al.*, 2009) that is removed by filtration/centrifugation prior to the assay procedure. The removal of this background phosphate improves the signal:noise ratio in the final colorimetric step in the assay. Although this method was initially developed for the analysis of Natuphos™ (*Aspergillus*) in-feed enzyme, this method has subsequently found widespread use for the analysis of other enzyme activities in feed: for example, Veum *et al.* (2006), where the two enzymes tested were from *Escherichia coli* and *Peniophora*; Woyengo *et al.* (2008), where the enzyme was Phyzyme™ XP, an *E. coli.* phytase; or Radcliffe *et al*. (2008), where the enzymes tested were Phyzyme™ XP and Natuphos™.

Despite the use of the Engelen method finding widespread use, until recently there was no analytical method that had officially been approved as a standard or harmonized method for phytases of different microbial origin and/or by different enzyme manufacturers. Arising from a collaborative process of several years under the umbrella of FEFANA and a European Commission laboratory, a new method has been published (Gizzi *et al.*, 2008) and recognized as a harmonized phytase method by ISO (International Standards Organization). This method (ISO 30024: 2009) could in many ways be considered to be a derived version of the Engelen method, as the assay determines phytase activity under very similar *in vitro* conditions: it is at the same pH and temperature, uses essentially the same reagents, buffers, substrate preparation, the same detection mechanism, etc. Although there are other procedural differences (the time of the assay is reduced from 60 to 30 min and the new assay is standardized with a phosphate standard curve instead of an enzyme standard curve, for example), one of the main differences compared with the Engelen method is in the extraction procedure. Whereas the original method employed a 68 mM calcium chloride concentration in the extraction buffer, the ISO method uses essentially distilled water as the extraction medium. This method is approved by the relevant manufacturers of Natuphos™, Ronozyme™ P, Phyzyme™ XP and Allzyme™ (FEFANA, 2008). This change of extraction technique could be considered in some ways to be a disadvantage over the old method due to the poorer signal:noise ratio, but the very high calcium levels in the Engelen extraction buffer caused problems with analysis of one of the other products (Cowieson and Adeola, 2005). The direct impact of calcium on phytase activity may be dependent on the particular enzyme, perhaps relating to partial substrate precipitation in the assay rather to than any direct inhibition (Selle *et al.*, 2009). When extracts from the Engelen method are mixed with the substrate to initiate the assay reaction, a distinct precipitate is observed, which indicates significant precipitation of phytic acid. This may lower the available phytate to a suboptimal level in the assay. It should be noted that this ISO method is used only for the analysis of animal feeding stuffs and does not include scope for analysis of enzyme products. If one were to use the newer method for the analysis of an enzyme product it is

likely that the results would be lower than the original (Engelen *et al.*, 1994) method due to the absence of calcium in the assay system of the ISO method. The presence of a low concentration of calcium (1 mM) is stimulatory to many phytases (Ullah *et al.*, 2008), to the effect of adding 10–20% to the observed activity *in vitro*.

As previously mentioned, phytase analysis in feed samples can suffer from a low signal:noise ratio due principally to high P background in the sample. It may be possible therefore to improve phytase analysis by applying further filtration and the use of spin columns (Kim and Lei, 2005) to remove excess P from the feed extract supernatant. Sometimes when aliquots of an extraction are centrifuged, a white layer that is fatty or oily in appearance forms on the top of the supernatant. This makes it impossible to collect all of the supernatant, and even sampling involves passing a pipette through this layer, which could result in fluctuating absorbance values in subsequent stages of the analysis. Filtration with a 0.45 μm membrane removed this layer. Additionally, when spin columns with a MW cut-off of 30,0000 were used, they resulted in improved reproducibility in the analytical method. One European enzyme manufacturer, AB Enzymes, already uses PD10 columns in the analysis of feeds containing phytase (Finase®). These PD10 columns are similar in principle to spin columns, although with a lower MW cut-off of ~5000. They also have the additional advantage of being able to buffer-exchange the sample, useful especially if the extraction buffer is at a different pH to the assay pH. Although this type of step essentially purifies the enzyme activity from background interferences, it can sometimes result in a loss of yield of the enzyme activity. It also adds expense to the method of analysis and makes the analysis more time consuming. Whether the spin columns as described by Kim and Lei or PD10 columns will become more commonplace in the analysis of phytase remains to be seen.

Although the new ISO/FEFANA method has wider regulatory approval than any other previous methods, it still remains that other phytases or new phytases coming on to market may require evaluation to see whether the analytical method will also work consistently with these other products. For example, at least one *E. coli* phytase product, Quantum Phytase™, has shown problems with detection by these methods. Generally, phytase is easily recovered from mash diets by the Engelen or ISO methods but after pelleting, problems have arisen. A specialized and unusual extraction technique at pH 10.0 is therefore required in order to fully solubilize the enzyme into the extraction buffer (Basu *et al.*, 2007). As new phytases come to market, further extraction and assay modifications may be necessary, so that a truly universal method may not be possible.

Overcoming the Problem of Xylanase Inhibitors in Feed Analysis

The analysis of xylanase enzyme activity in feeds that contain wheat has been more problematic than that of other carbohydrases. In the late 1990s the presence of specific proteinaceous xylanase inhibitors was identified that are

probably the reason for this (Debyser *et al.*, 1999). Subsequent research to date has shown that these inhibitors are divided into two types, namely the xylanase inhibitor proteins (XIPs) and the *Triticum aestivum* L. endoxylanase inhibitors (TAXIs) (Sansen *et al.*, 2004; Gusakov and Ustinov, 2009). The TAXI inhibitors are also subdivided into TAXI-I and TAXI-II. Xylanases are normally classified as either 'GH family 10' or 'GH family 11'. The family classification is not dependent on microbial origin and so may be either bacterial or fungal. These inhibitors have different xylanase specificities depending on the family of xylanases. Generally, the TAXI inhibitors affect GH 11 xylanases (which may be bacterial or fungal) although the TAXI-II variant does not affect enzymes with low pH optima. The XIP proteins appear to affect xylanases in both families of xylanases but not if they are bacterial. The levels of inhibitors may also vary according to the source and variety of wheat in the feed (McLauchlan *et al.*, 1999).

In order to overcome these problems during the analysis of xylanase in feeds, several strategies have been suggested. First, it has been suggested that, according to the type of enzyme present in the feed, two separate extraction strategies should be employed. For feeds containing *Trichoderma* spp. xylanases, an extraction buffer of 100 mM acetic acid or 100 mM sodium acetate buffer (pH 4.7) at room temperature should be used. Optimal extraction of *Humicola* spp. xylanases was achieved with a buffer containing 100 mM MES buffer (pH 6.0) and 1% w/v SDS (Megazyme, http://www.megazyme.com).

A second strategy is where enzyme activity is added to a blank feed at different levels to produce a standard curve that takes into account the presence of inhibitors (Cosson *et al.*, 1999). Unfortunately, a blank feed that mirrors the test sample is not always available to the laboratory. In this situation a second strategy is to add or 'spike' more xylanase of known activity to the actual sample extraction. The xylanase reaction is assayed in both spiked and unspiked sample extractions and, on this basis, the activity can be calculated in the original test sample (Megazyme product information, 1999). This method can be effective, but only usually if the enzyme product that has been used in the original feed sample is known, as the spiking needs to be done with the same enzyme preparation as in the original sample. If a sample that is presented to the laboratory is truly 'blind', then this method is not possible.

Another more novel approach, although not one that is likely to see widespread usage, is described in a recent patent (Bauer and De Fontes, 2007). The inventors have produced a xylanase that has virtually none of its original xylanase activity, essentially a catalytically inactive xylanase, but that still retains the ability to interact with xylanase inhibitors. This is pre-dissolved in the extraction buffer at a concentration sufficiently high to swamp the level of inhibitor present in the feed sample, so the xylanase to be determined that is present in the feed is unaffected by the presence of inhibitors. The inactive xylanase molecule binds to xylanase inhibitors in the feed, thereby allowing accurate measurement of xylanase activity of the enzyme contained in the feed.

It is also possible to produce xylanases with altered sensitivity to xylanase inhibitors, or alternatively there are enzymes that are not affected by these

inhibitors, and so with time we may see xylanase preparations coming on to market that are unaffected by this problem.

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12 Holo-analysis of the Efficacy of Exogenous Enzyme Performance in Farm Animal Nutrition

G.D. ROSEN

Introduction

In their preface to the first edition, Bedford and Partridge (2001) concluded that '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'. They also noted that 'Scientific studies describing the use of exogenous enzymes in animal nutrition dates back to the mid-1920s and they now number in excess of 1,300 papers for broilers alone' (Rosen, 2000, personal communication). These themes were variously expounded in their chapters by several other authors. In referring to future research requirements, Thorpe and Beal (2001) concluded that 'There is little published data on the optimum inclusion levels of exogenous enzymes in animal diets'. They pointed out that extrapolation between species considering differences in digestive tract anatomy and physiology may be incorrect. They also raised the pertinent and interesting question of the possible use of exogenous enzymes to alleviate the implications of the European antibiotic growth promoter ban. Choct (2001) stated that 'The concept of predicting the effect of enzymes in a particular feed is attractive, because the producer could then adjust the enzyme amount and/or the nutrient specifications in diet formulations'.

Partridge's chapter (Bedford and Partridge, 2001) on the role and efficacy of carbohydrase enzymes in pig nutrition highlighted the need to answer the question 'When do proven enzymes for pig application give their most effective response?', while referring to the need to understand more clearly the interactions between carbohydrases, phytases and proteases, together with the pre-processing of raw materials and dry versus liquid feed technology. Similarly, Dänicke (2001) invoked the need to consider the types of plant and animal dietary fat and the various dietary grain components used in broiler feeds, while Kornegay (2001) pinpointed the need for a clearer understanding of the non-linear dose–response curves for phytases in both poultry and pig diets. For

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ruminant beef growth and milk production, McAllister *et al*. (2001) underlined the high probability that xylanase and β-glucanase enzyme responses are multifactorial, stressing the role of plant cell wall degradation and the need to consider the potential importance of cutinase, ferulic acid and acetylxylan esterases and arabinofuranosidase.

Regarding the influence of process stability and feed enzyme detection in complete diets, Bedford *et al.* (2001) illustrated the uncertainty of the relationship between *in vitro* enzyme content assays and *in vivo* performances in any approaches to maximizing enzyme benefits. In surveying future horizons, Marquardt and Bedford (2001) regarded enzyme use as yet to be infantile, short of full potential, notwithstanding highly beneficial applications to date. Problems to be solved include: (i) how to select an enzyme from the large multitude on offer; (ii) the need for meaningful assays or measurements to determine not only the amount present in a feed but also its biological relevance; (iii) relative evaluation of the expression of enzymes in plant raw materials compared with their exogenous application; and (iv) a need for better predictions than those from simple linear models. Four areas for future research and development included: (i) more biologically meaningful assays; (ii) fitting enzyme properties to animal species, substrates and environments; (iii) better understanding of animal responses in cereal-based diets; and (iv) the use of models to predict enzyme responses and thereby optimize economy and efficacy of usage.

In this chapter the advent and application of the process of holo-analysis will be presented, with illustrative results and a discussion of its potential contribution to further progress. In this context, it is encouraging to note that the number of potentially relevant papers in broilers, for example, has risen from 1300+ in 2000 to 2800+ in 2008.

Holo-analysis

The term holo-analysis was first introduced by the author in 2004 (Rosen, 2004) and recognized in an invited review (Rosen, 2006a). It was defined as 'the integration of all available data on a specific subject quantifying a dependent nutritional response in terms of all available genetic, chronological, environmental, geographical, managemental, dietary ingredient and nutrient content independent variables'. Holo-analysis was introduced as a self-explanatory descriptor, in the sense that the prefix holo- is very precisely defined in the Oxford English Dictionary as 'whole' or 'entire'. It is therefore self-explanatory per se and free from the uncertainties and imprecision of its progenitor, meta-analysis. More than a century after Airy (1861) used the statistical techniques of Gauss and Laplace to pool collections of star position results and more than 70 years after Pearson (1904) pioneered the use of 11 selected studies on military personnel in assessment of the efficacy of an anti-typhoid vaccine, Glass (1976), an education researcher, introduced the term meta-analysis as 'a rigorous alternative to the casual narrative discussions of research studies', describing meta-analysis as 'the analysis of analyses and the statistical analysis of a large collection of results from individual studies for the purpose of integrating the findings'. How large remained, and still remains, an open question.

Unfortunately, the use of meta- as a prefix is highly imprecise, as may be seen in its 13 disparate dictionary definitions, viz. 'put together', sharing', 'next to', 'connected', 'between', 'behind', 'part of', 'later', 'more developed', 'more comprehensive', 'beyond', 'transformed' and 'transcending'. Thus, it is evident that the scope of meta-analyses can literally range widely from a Glass-like concept of undefined scope down to the provision of an average of two test results.

Relatively speaking, such meta-analytical techniques have been used in nutrition much less frequently than in other topics, as was illustrated in an Internet search coupling the term meta-analysis with different subjects, i.e. hits were medicine (4,180,000 (including human 2,500,000 and veterinary 90,600)), education (3,770,000), environment (2,490,000), statistics (2,260,000) and social sciences (2,110,000). The species proliferation of meta-analyses in nutrition hits can be seen in lower values for human (681,000), animal (319,000), fish (212,000), pets (62,500), chicken (47,000), turkey (46,900), pig (38,500), calf (22,400), dairy cattle (19,300), hen (17,400) and beef cattle (13,100).

The term meta-analysis is not well known, as demonstrated in a pilot study by the author who asked 50 animal nutrition interests 'What is a meta-analysis?' Fewer than half (23/50) knew the term, including 8/10 feed industry suppliers, 6/10 scientific and trade press personnel, 4/10 independent consultants in nutrition, 4/10 academics and 1/10 feed manufacturers and farmers. The other incognisant 27 were asked a second question 'What is a holo-analysis?'. Without prior knowledge they all quite quickly responded with their interpretation of its meaning as comprehensive, complete or holistic analysis. Note that the conduct of holo-analysis can be prolonged, complicated and expensive in the sense that the potential number of variables to be investigated is legion.

The process of holo-analysis comprises ten progressive steps, as follows:

1. Collection of all available, published, negatively controlled feeding test reports.
2. Computer filing of numeric and non-continuous indicator (dummy) independent variables.
3. Calculation or collection from authors of missing variables.
4. Elimination of repeats and errors.
5. Second-phase data per se are inadmissable, lacking a valid negative control.
6. Primary elaboration of comprehensive stepwise multiple regressions relating start-to-finish dependent variable nutritional effects to statistically significant ($P \leq 0.05$ in/$P \geq 0.10$ out) independent variables and possible interactions. (In preliminary holo-analyses of smaller databanks of fewer than 100 tests, exploratory models can also be elaborated using a less-stringent criterion of $P \leq 0.25$ in/$P \geq 0.34$ out.)
7. Determination of best-fit models for effects on dependent variables with maximum correlation coefficient squares (R^2) and minimum root mean square errors (RMSE), excluding aberrant outliers (normally ≥ 3 × standard deviation (SD)).
8. Derivation of auxiliary models for any significantly different subclasses, e.g. test country or individual product.
9. Prepare software, when required, for the calculation of dependent variable responses with confidence limits.

10. Economic integration in financial terms of feed consumption, liveweight gain, mortality, carcass and de-pollution effects to provide an overall nutritive value.

The concept of holo-analysis emerged from a long series of evaluations of the efficacy of 15 different antibiotics in animal nutrition, encompassing the evaluation of a total of 12,153 negatively controlled tests from 1949 to 1991 in 4301 reports, using data from 55 countries (Rosen, 1995). Bans on the veterinary prescription-free use of antibiotics in feed in the European Union in 1999 stimulated an urgent need to set and meet standards for the efficient replacement of pro-nutrient antibiotics in animal nutrition, and foreshadowed the enormous task of thoroughly investigating the efficacy of potential alternatives, such as enzymes, microorganisms, acids, botanicals (including herbs, spices and essential oils) yeasts and derivatives, oligosaccharides, aromatics, metal chelates, etc. The forerunning antibiotic evaluations were in fact only 'meta-analytical' in the sense that not all available independent variables were evaluated, with main attention restricted to the influence of dosage, level of negative control performance, duration, year of test, the influence of anticoccidials and the impact of diagnosed diseases.

Pro-nutrient enzymes are, to date, the most extensively investigated antibiotic replacements, with illustrative holo-analytical results surveyed species by species hereunder.

Broilers

Prototypes of holo-analyses were marked in 2002 (Rosen, 2002a,b) by the publication of Brozyme broiler nutritional response models, based on a total collection of 1322 publications (1925–1999), though a large majority of 1173 (88.7%) was of much more recent vintage (1980–1999). Of these, performance data sets were mined from 575 publications (43.5%). The remainder comprised: general review/mode of action/metabolism with no performance data 450 (34.0%), repeats 98 (7.4%), percentage response 43 (3.3%), no feed/gain/duration/enzyme dosage data 41 (3.1%), no negative control 40 (3.0%), analysis/stability 38 (2.9%) and pullets 37 (2.8%). Exclusion of intermediate-phase data left a total of 1869 start-to-finish tests versus negative controls, utilizing a total of more than 480,000 broilers. Mortalities were reported in only 439 (23.5%) of the 1869 start-to-finish tests. The Brozyme data came from 58 countries, of which the 12 largest contributors, furnishing 84.5% of the total, were the USA (21.8%), Germany (11.3%), Canada (11.0%), the UK (9.5%), Sweden (5.4%), Switzerland (5.3%), Australia (4.3%), the Netherlands (3.9%), Poland (3.7%), Spain (2.8%), Czech/Slovak Republics (2.8%) and Denmark (2.7%).

The extent of variation in nutritional responses in the start-to-finish tests in broilers is illustrated in Table 12.1, which also compares the values for the full collection of results and those tests for which dosages were known. Coefficients of variation between 100 and 200% for LWGeff and FCReff accord with expectation for pronutrients, but the values for FDIeff and MOReff are more than double (444 and 377%, respectively). Although the dosages were known

in only 1869/2573 (73.6%) of the tests, it is interesting to note that the standard deviations and coefficients of variation in both sets are very similar.

Holo-analytical models have been elaborated to assess the magnitudes of start-to-finish nutritional responses to exogenous enzymes in broiler diets in terms of a total of 154 independent variables (Table 12.2). The models in

Table 12.1. Variation in the feed intake (FDIeff), liveweight gain (LWGeff), feed conversion ratio (FCReff) and mortality (MOReff) effects of exogenous enzymes in broiler nutrition.

Variable (n)	Effect (%)[a]	Standard deviation	Coefficient of variation (%)
All			
FDIeff (2573)	+31.3 g (1.5)	139	444
LWGeff (2573)	+53.4 g (5.1)	76.4	143
FCReff (2573)	–0.105 (5.3)	0.195	186
MOReff (439)	–1.71% (26.2)	6.44	377
Enzyme dosage known			
FDIeff (1869)	+31.9 g (1.4)	146	458
LWGeff (1869)	+57.0 g (5.0)	79.0	139
FCReff (1869)	–0.0999 (5.1)	0.194	194
MOReff (365)	–1.80% (26.3)	6.93	385

[a]As percentage of negative control.

Table 12.2. Brozyme independent variables tested (n = 154).

Negative control performance	4	Main cereal	8
Duration	1	Barley percentage	1
Year of test (1900)	1	Hulled barley	1
Age at start	1	Maize percentage	1
Sex (e.g. male, MAL)	4	Oat	1
Cage housing	1	Rye	1
Stocking density	1	Sorghum percentage	1
Not day-old birds	1	Triticale	1
Selected weight birds	1	Wheat percentage	1
Diagnosed disease	1	Animal protein types	11
Mode of action/metabolism test	1	Feed animal protein percentage	1
Factorial data	1	Main vegetable protein	10
Trade press	1	Added oil/fat percentage	11
Phytase dosage	10	Added oil/fat percentage	1
Natuphos	1	Poultry fat percentage	1
Novo Phytase	1	Vegetable oil percentage	1
Finase	1	Rapeseed oil percentage	1
Reducing enzyme dosage	1	Crude protein percentage	1
Broilerase	1	Fat percentage	1
Major countries	12	Fibre percentage	1
Major suppliers	8	Digestible energy	1
Major brands	10	Metabolizable energy	1
Mash feed	1	Net energy	1
Processed feed	1	Lysine	1
Part-purified diet	1	Methionine + cystine	1
Antibiotic type	8	Phosphorus	1
Antibiotic dosage	1	Available phosphorus	1
Anticoccidial feed	12	Calcium	1
Anticoccidial dosage	1	Ration composition known	1

Table 12.3. Broilerase (432 enzymes) models on feed intake, liveweight gain, feed conversion ratio and mortality effects ($P \leq 0.05$ in/$P \geq 0.10$ out).

FDIeff =		−379	−0.0835FDIC	−42.8log(BRO+1)	+10.3DUR	+3.17EXD	+35.6NDO	+25.9MMT	−22.4PRO	+40.9BAR	+76.7OAT
R^2 0.172	SE	72.2	0.007	7.11	0.865	0.697	9.15	7.54	7.55	8.69	19.0
RMSE 133	*P*	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.003	0.000	0.000
n 1869											
			+68.2RYE	−22.2WHE							
			14.4	8.15							
			0.000	0.007							

LWGeff =		−339	−0.0685LWGC	−14.2log(BRO+1)	+4.38DUR	+3.76EXD		+11.8MMT		+26.3BAR	+56.3OAT
R^2 0.171	SE	35.9	0.006	3.83	0.296	0.371		4.08		5.25	6.60
RMSE 72.1	*P*	0.000	0.000	0.000	0.000	0.000		0.004		0.000	0.000
n 1869											
			+46.8RYE	−11.3WHE		+36.0HUB	−14.9MZE				
			8.20	4.96		11.5	5.24				
			0.000	0.023		0.002	0.005				

FCReff =		0.956	−0.623FCRC	−0.0262log(BRO+1)	+0.00863DUR	−0.00638EXD	+0.0987NDO		−0.0334PRO		−0.105OAT
R^2 0.865	Se	0.061	0.010	0.007	0.000	0.001	0.008		0.007		0.019

RMSE 0.128	*P*	0.000	0.000	0.000	0.000	0.000	0.000		0.000		0.000
n 1869											
			–0.115RYE			–0.104HUB		+0.0173CAG	+0.0735PPD	–0.0216MAL	–0.127SEL
			0.014			0.020		0.008	0.014	0.007	0.020
			0.000			0.000		0.027	0.000	0.001	0.000
								+0.0734TRI			
								0.025			
								0.003			
MOReff =		2.56	–0.676MORC				+2.08NDO				
R^2 0.810	SE	0.267	0.026				0.597				
RMSE 3.04	*P*	0.000	0.000				0.001				
n 365											
									–5.22PPD		
									0.946		
									0.000		
				+1.08WHP							
				0.355							
				0.003							

BAR, barley (1 or 0); BRO, broilerase (u g^{-1}); CAG, cage housing (1 or 0); DUR, duration (days); EXD, year of test: 1900; FCRC, control feed conversion ratio (FDIC/LWGC); FCReff, feed conversion ratio effect; FDIC, control feed intake (g); FDIeff, feed intake effect; HUB, hulled barley (1 or 0); LWGC, control liveweight gain (g); LWGeff, liveweight gain effect (g); MAL, male (1 or 0); MMT, mode/metabolic action test (1 or 0); MORC, control mortality (%); MOReff, mortality effect (%); MZE, maize (1 or 0); *n*, number of tests; NDO, not day-old (1 or 0); OAT, oats (1 or 0); *P*, probability; PPD, part-purified diet (1 or 0); PRO, processed feed (1 or 0); R^2, multiple correlation coefficient square; RMSE, root mean square error; RYE, rye (1 or 0); SE, standard error; SEL, selected-weight birds (1 or 0); TRI, triticale (1 or 0); WHE, wheat (1 or 0); WHP, wheat (%).

Table 12.4. Broiler models for the effects of first-generation phytases (14 enzymes) on feed intake, liveweight gain and mortality ($P \leq 0.05$ in/$P \geq 0.10$ out).

FDIeff =		232	–0.136FDIC	+226log(PHY+1)	–514log(PHO+1)	+7.02Ca	–150NAT	–222NPH	–207FIN	+20.0DUR	–78.9CAG
R^2 0.641	Se	56.0	0.014	46.3	84.2	2.38	14.5	23.7	21.3	1.83	13.2
RMSE 62.1	*P*	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000	0.000
n 298											
			+93.0NDO	+65.2COC	–0.573MZP	+71.0AOF	–9.13AFP	–14.7PFP	–11.6VOP	+12.1ROP	
			18.2	12.1	0.183	19.9	2.69	3.11	0.352	2.64	
			0.000	0.000	0.002	0.000	0.000	0.000	0.001	0.000	
LWGeff =		118	–0.231LWGC	+168log(PHY+1)	–339log(PHO+1)		–86.3NAT	–142NPH	–122FIN	+16.4DUR	–49.6CAG
R^2 0.717	SE	33.3	0.017	26.0	29.0		7.71	13.1	12.5	1.08	7.67
RMSE 35.4	*P*	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
n 298											
			+54.2NDO	+48.1COC	–0.176MZP	+105AOF		–0.741PFP		+5.58ROP	–1.97BAP
			8.85	6.72	0.115	13.0		1.74		1.56	0.545
			0.000	0.000	0.000	0.000		0.000		0.000	0.000
			–0.662SOP								
			0.213								
			0.002								
FCReff =		–0.0115	–0.0572FCRC		+0.144(PHO+1)						–0.0334CAG
R^2 0.166	Se	0.036	0.010		0.037						0.008
RMSE 0.0514	*P*	0.746	0.000		0.000						0.000
n 298											
								–0.00787PFP			
								0.002			
								0.000			
MOReff =		3.14	–0.896MORC								
R^2 0.942	SE	1.04	0.040								
RMSE 4.01	*P*	0.050	0.000								
n 31											

AFP, added oil/fat (%); AOF, added oil/fat (1 or 0); BAP, barley (%); Ca, calcium (g kg^{-1}); CAG, cage housing (1 or 0); COC, anticoccidial feed (1 or 0); DUR, duration (days); FCRC, control feed conversion ratio (FDIC/LWGC); FCReff, feed conversion ratio effect; FDIC, control feed intake (g); FDIeff, feed intake effect (g); FIN, Finase (1 or 0); LWGC, control liveweight gain (g); LWGeff, liveweight gain effect (g); MORC, control mortality (%); MOReff, mortality effect (%); MZP, maize (%); *n*, number of tests; NAT, Natuphos (1 or 0); NDO, not day-old (1 or 0); NPH, Novo Phytase (1 or 0); *P*, probability; PFP, poultry fat (%); PHO, phosphorus (g kg^{-1}); PHY, phytase (u g^{-1}); R^2, multiple correlation coefficient square; RMSE, root mean square error; ROP, rapeseed oil (%); SE, standard error; SOP, sorghum (%); VOP, vegetable oil (%).

Table 12.3 are for a notional broilerase, representing all 432 single- and multi-component exogenous enzymes researched in broiler feeds. The models in Table 12.4 are for 14 first-generation phytase products. These broilerase and phytase models each contain a total of 22 statistically significant independent variable terms.

Broilerase effects on feed and liveweight gain are decreased logarithmically with dosage, implying a progressively reduced utilization of the limiting nutrient in the feed. In quadratic dosage models, albeit with lower R^2 values, there are suggestions of maximal effects in the region of 500 units g^{-1} feed. From an economic point of view, as seen in the FCReff model, it appears that the logarithmic pattern of improved conversion is beneficially affected in inferior converters, temporally over the years, in processed feeds, male birds, selected bird tests and in diets containing mainly hulled barley, oats and rye, while it is adversely influenced with bird age, not day-old stock, fed part-purified diets to birds in cages and in feeds with main cereal triticale.

The use of holo-analytical models to assess responses to single types of enzyme products, such as amylases, β-glucanases, lipases, xylanases and phytases, etc., is exemplified herein by the models for 14 first-generation phytase products in Table 12.4 (Rosen, 2003). The 14 phytase products with a total of 311 tests versus negative controls and their test proportions were Alltech phytase (0.3%), EP431 phytase P/L (1.6%), experimental phytase (2.9%), Finase FP 500 (6.0%), IMASPK phytase CZ (2.6%), microbial phytase 1 (1.0%), microbial phytase 2 (1.0%), Natuphos 600, 1000 and 5000 (72.4%), phytase A.f. (1.9%), phytase A.f.N.R.R.L. (2.6%), phytase Novo (5.4%) and phytase SC (2.3%). Note that the model for FCReff has no significant phytase dosage term, but predicted effects with confidence limits on conversion ratio in praxis can of course be determined using the feed intake and liveweight gain models.

Calculations of the effects of phytases on FCReff when used, as in pollution abatement, at dosages of 500–750 u kg^{-1} feed manifest the following indications: (i) praxis versus research conditions +0.044; (ii) in-feed with anticoccidial –0.040; (iii) phytase at 2500 versus 625 u kg^{-1} –0.011; (iv) use of wheat versus maize (62.5%) –0.027; (v) rapeseed oil (5%) versus poultry fat (5%) +0.001; (vi) animal fat (5%) versus vegetable oil (5%) +0.001; and (vii) pairs of NAT, NOV and FIN ±0.003–0.013. Calculations based on these phytase models can also be used to quantify the dosage increase needed to offset the improvement in broiler performance over the years. These models also contradict the observation of Yan (2001) that P equivalency (matrix) values determined in more than marginally phosphorus-deficient diets can provide incorrect overestimates of as much as 100%. This was already apparent in former phytase models in broilers (Rosen, 2002c), which showed that reductions in phosphorus levels (g kg^{-1}) of 8 to 7, 7 to 6, 6 to 5, 5 to 4, 4 to 3 and 3 to 2 required phytase dosages (u kg^{-1}) of +78, +88, +103, +123, +152 and +200 for parity in liveweight gain, i.e. a matrix value measured between 4 and 3 g P kg^{-1} is almost half that between 7 and 6 g P kg^{-1}. This means that the industry is greatly underestimating the equivalency of phytase for P when used commercially.

In future research, it would be of considerable interest to compare the efficacies of second-generation 6-phytases with the first-generation 3-phytases referred to herein. It will also be necessary to correct or to determine all the nutrient equivalency (matrix) values claimed for phytases by calculations for each limiting individual nutrient, including phosphorus, at dosages close to the nutrient requirement.

Interactions of enzymes such as phytases and xylanases is also a potentially fruitful area for future research. On a broader front, there is also considerable scope for detailed modelling of responses to other feed enzymes such as the xylanases, proteases and β-glucanases and to compare their efficacies with those having mixed enzyme contents of two to 11 components.

Layers

A Layzyme holo-analysis (Rosen, 2006b) of 76 enzyme products was a collection of 491 publications dated 1979–2004, containing 136 (27.7%) with performance data, together with 109 (22.2%) with no performance data, repeats 52 (10.6%), no enzyme units 42 (8.6%), mode of action/metabolism tests 39 (7.9%), no negative control 32 (6.5%), not enzyme 27 (5.5%), not controlled 19 (3.9%), no feed data 13 (2.6%), general reviews 12 (2.4%) and no duration 10 (2.0%). Apart from papers on an amylase, a lipase, a mannanase and a polygalacturonase, there were 40 on non-starch polysaccharidases (containing β-glucanase and/or cellulose and/or xylanase), 12 on phytases (including two with declared side-activities) and 20 other polyases (diases, triases, tetrases and pentases), describing the results of 454 tests of duration 21–420 days (mean 140). Responses measured include feed intake, liveweight gain, hen-day production, feed conversion ratio and mortality for hen and egg weight, egg mass day^{-1}, egg specific gravity, cracked eggs, dirty eggs, shell percentage, shell thickness, shell breaking strength, yolk colour score and Haugh units for the egg. For some of these the amount of data was restricted, so the examples of hen and egg responses to a notional layerase (all enzymes tested) given in Table 12.5 are for those with 374 to 377 start-to-finish responses for the 21–420 (mean 120)-day durations involved.

These 12 phytase products comprised experimental phytase (0.5%), fungal phytase (1.0%), microbial phytase (1.4%), Natuphos 600, 1000 and 5000 (72.6%), phytase 1 (2.8%), phytase 2 (6.6%), phytase 3 (1.9%) and phytase CZ (2.8%); and, with declared side-activities, Alltech phytase (8.5%) and Finase FP (1.9%). In each case the main contributor to variation in response was control performance, ranging from 60 to 81%. It is noteworthy that responses in the USA are lower. Phytases as a whole enhance feed intake significantly more than other enzymes, as occurs also in both broilers and pigs, so separate models have, therefore, been elaborated for first-generation phytases (Table 12.6). Table 12.6 summarizes the efficacy of this group of phytase products, based on data mined from 56 publications (1991–2004) with 210 tests in 16 countries on 27,660 layers (86 per treatment mean), 18–108 weeks old for durations of 21–364 days.

Table 12.5. Models of hen and egg production responses to layerase (86 enzymes) in laying hens ($P \leq 0.05$ in/$P \geq 0.10$ out).

FDIeff =		23.1	–0.158FDIC	+2.04PHY	–0.320EXD	+2.24MEN	–2.82USA			
R^2 0.327	SE	9.90	0.022	0.565	0.063	0.659	0.669			
RMSE 4.40	*P*	0.008	0.000	0.000	0.000	0.001	0.000			
n 374										
HDPeff =		108	–0.526HDPC		–0.463EXD		–2.15USA	–0.0750AGS	–5.77PSA	–0.774PRP
R^2 0.636	SE	13.6	0.034		0.123		0.865	0.020	1.54	0.296
RMSE 4.50	*P*	0.000	0.000		0.000		0.014	0.000	0.000	0.001
n 377										
EWTeff =		3.70	–0.222EWTC			+0.810MEN	–1.07USA	+0.0270AGS		
R^2 0.393	SE	4.10	0.028			0.278	0.197	0.006		
RMSE 1.11	*P*	0.369	0.000			0.004	0.000	0.000		
n 377										
EMDeff =		28.1	–0.490EMDC				–3.42USA		–3.85PSA	
R^2 0.552	SE	1.86	0.035				0.579		1.05	
RMSE 3.12	*P*	0.000	0.000				0.000		0.000	
n 377										
ECReff =		1.33	–0.606ECRC						+0.213PSA	–0.300BCP
R^2 0.763	SE	0.066	0.028						0.068	0.010
RMSE 0.206	*P*	0.000	0.000						0.002	0.003
n 374										
			–0.900MMT							
			0.033							
			0.007							

AGS, age at start (weeks); BCP, birds per cage or pen; ECRC, control egg conversion ratio (FDIC/EMDC); ECReff, egg conversion ratio effect; EMDC, control egg mass day^{-1} (g); EMDeff, egg mass day^{-1} effect (g); EWTC, control egg weight (g); EWTeff, egg weight effect; EXD, year of test: 1900; FDIC, control feed intake (g); FDIeff, feed intake effect (g); HDPC, control hen-day production (%); HDPeff, hen-day production effect (%); MEN, metabolizable energy (MJ kg^{-1}); MMT, mode of action/metabolism test (1 or 0); *n*, number of tests; *P*, probability; PHY, phytase (u g^{-1}); PRP, protein (%); PSA, phytase with side-activities (1 or 0); R^2, multiple correlation coefficient square; RMSE, root mean square error; SE, standard error; USA, USA test (1 or 0).

Table 12.6. Models for first-generation phytases (12 enzymes) in laying hens and egg production ($P \leq 0.05$ in/$P \geq 0.10$ out).

FDIeff =		52.8	–0.279FDIC	+7.99log(PHY+1)	–9.14PHO	+0.851PHO^2		+2.81PSA	+5.70WLH	–4.46HYW	+2.87ISB
R^2 0.674	SE	6.05	0.29	2.95	2.27	0.226		1.18	1.01	0.721	1.04
RMSE 3.17	*P*	0.00	0.00	0.007	0.000	0.000		0.000	0.00	0.00	0.000
n 161											
TP 5.4											
HDPeff =		59.2	–0.474HDPC	+6.75log(PHY+1)	–8.30PHO	+0.705PHO^2	+0.185Ca	–2.92PSA	+4.60WLH		
R^2 0.775	SE	6.72	0.026	2.77	2.32	0.232	0.057	1.13	1.01		
RMSE 3.05	*P*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
n 161											
TP 5.9			+0.0110DUR	–0.202SMP							
			0.003	0.054							
			0.000	0.000							
EWTeff =		6.98	–0.0870EWTC	+2.38log(PHY+1)	–0.198PHO				+1.38WLH		
R^2 0.569	SE	1.40	0.021	0.772	0.075				0.287		
RMSE 0.902	*P*	0.000	0.000	0.002	0.009				0.000		
n 161											
				–0.0510SMP	–0.117APP	+1.10MOU					
				0.018	0.026	0.312					
				0.005	0.000	0.001					
EMDeff =		35.4	–0.357EMDC	+6.44log(PHY+1)	–6.35PHO	+0.530PHO^2			+2.07WLH	–1.85HYW	

R^2 0.696	SE	4.14	0.028	2.06	1.67	0.166			0.724	0.447
RMSE 2.33	*P*	0.000	0.000	0.002	0.000	0.002			0.005	0.000
n 161										
TP 6.0			+0.00600DUR							
			0.002							
			0.007							
ECReff =		1.15	–0.544ECRC		+0.0390PHO		–0.007Ca	+0.213PSA		
R^2 0.744	SE	0.107	0.028		0.011		0.002	0.044		
RMSE 0.126	*P*	0.000	0.000		0.000		0.002	0.000		
n 161										
					–0.008APP					
					0.004					
					0.031					
			+0.0960BSE							
			0.025							
			0.000							

APP, animal protein (%); BSE, brown-shelled egg (1 or 0); Ca, calcium (g kg^{-1}); DUR, duration (days); ECRC, control egg conversion ratio; ECReff, egg conversion ratio effect; EMDC, control egg mass day^{-1} (g); EMDeff, egg mass day^{-1} effect (g); EWTC, control egg weight (g); EWTeff, egg weight effect (g); FDIC, control feed intake (g); FDIeff, feed intake effect (g); HDPC, control hen-day production (%); HDPeff, hen-day production effect (%); HYW, Hyline white (1 or 0); ISB, Isa brown (1 or 0); MOU, moulted (1 or 0); *n*, number of tests; *P*, probability; PHO, phosphorus (g kg^{-1}); PHY, phytase (u g^{-1}); PSA, phytase side-activities (1 or 0); R^2, multiple correlation coefficient square; RMSE, root mean square error; SE, standard error; SMP, soyabean meal (%); TP, turning point; WLH, White Leghorn (1 or 0).

The Table 12.6 phytase models were based on 161 tests for which all the required data were available. The contribution to variation of level of control performance ranged quite widely, from 5 to 59%, and responses in egg production and egg weight, but not in feed conversion efficiency, were significantly better in White Leghorns. Whereas the logarithmic pattern of response to phytase dosage had very little contribution to variation (1–6%), the counteractive effects of phytase and phosphorus dosages were somewhat more but not greatly influential (8–15%). Those phytases with declared side-activities enhanced feed intake, adversely affecting feed conversion efficiency effects.

Pigs

The Pigzyme collection of 1316 publications (1951–2001) contained 255 (19.4%) with performance data (Rosen, 2005). The others comprised 343 no performance data/reviews (26.1%), 220 mode of action/metabolism studies (16.7%), 113 repeats (8.6%), 98 non-exogenous studies (7.4%), 94 no enzyme units (7.1%), 90 no negative control/percentage response (6.8%), 52 no feed or gain or duration data (4.0%) and 51 analytical/stability (3.9%). The tests were conducted on 82 enzyme products having one (46), three (12), four (11), five (6), six (6) and seven (1) quantified enzyme components from 36 countries, primarily from the USA (22.4%), Germany (13.9%), Canada (7.0%), Australia (6.4%), the UK (5.9%), Spain (4.3%), France (3.5%), China (3.4%), Poland (3.4%) and the Netherlands (3.0%). The 82 enzyme products contained a total of 221 generic enzyme components.

Tests in the reports mainly concerned either first-generation phytases or non-starch polysaccharidases. The phytases comprised preparations with and without declared side-activities. The polysaccharidases all contained β-glucanase, cellulase and xylanase (becexyase), in some of which there were other declared activities. The phytases comprised 39.3% and becexyases 51.9% of the total resource. In preliminary models utilizing all 82 enzymes (pigases), it was evident that the phytases differed significantly from the remainder, so separate models were developed for the becexyases and phytases.

Tables 12.7 and 12.8 give details of the models for the effects on feed intake, liveweight gain and feed conversion ratio for the becexyase and phytase models, respectively, for conventional ($P \leq 0.05$ in/$P \geq 0.10$ out) and also for less stringent ($P \leq 0.25$ in/$P \geq 0.34$ out) probabilities.

The models for becexyases as yet account for less than half (R^2 = 0.19–0.49) of the variations in nutritional responses, but they suggest the likelihood of a maximum economic effect in the region of 3.0–3.6 u g^{-1} in the feed conversion models. Further dose–response studies in praxis conditions are needed in this connection. Becexyase also seems to stimulate feed intake significantly more in rations containing higher contents of soyabean meal.

The latest phytase models account for 43–57% of the variations in feed, gain and conversion effects. Key features are: (i) a counteractive pattern for phytase and phosphorus dosages, as yet logarithmic, in feed, gain and conversion models, accounting for 14–40% of response variations; and

(ii) stimulating feed and gain effects in maize-based feeds proportionate to maize content. It would be of interest to test phytases at even higher dosages above 33.1 u g^{-1} feed in any attempt to determine a maximum.

Ruminants

The underlying Rumzyme research (Rosen, 2007) yielded a total of 327 publications (1958–2005) of tests conducted in 26 countries comprising the USA (42.5%), Canada (26.6%), Mexico (6.7%), the UK (6.1%), Russia (3.4%), Finland (1.8%), Spain (1.5%), Czechia (Czech Republic) (1.2%), Hungary (0.9%), Iran (0.9%), South Korea (0.9%), the Netherlands (0.9%), Australia (0.6%), Brazil (0.6%), India (0.6%), France (0.6%), Jordan (0.6%), Poland (0.6%), South Africa (0.6%), Croatia (0.3%), Germany (0.3%), Mongolia (0.3%), Norway (0.3%), Slovakia (0.3%), Turkey (0.3%) and Yugoslavia (0.3%). These contained 38 (11.6%) having performance data, of which 27 (8.3%) were Drumzyme (dairy cattle) and 11 (3.3%) were Brumzyme (beef cattle). The relative sparsity of publications with performance data in this field was due to 21 (6.4%) lacking enzyme dosage values, 58 (17.7%) *in vitro* tests, 41 (12.6%) metabolic/mode of action studies, 28 (8.6%) no performance data, 28 (8.6%) sheep, 23 (7.0%) reviews, 20 (6.1%) forage research, 19 (5.8%) repeats, 12 (3.7%) no feed data, 11 (3.4%) non-exogenous enzyme studies, five (1.5%) no negative control, four (1.2%) analytical, four (1.2%) calf milk replacer, four (1.2%) grazing stock, four (1.2%) percentage data, three (0.9%) no duration, three (0.9%) not controlled and one (0.3%) goat.

Beef cattle

The 48 start-to-finish in a total of 65 negatively controlled tests were conducted from 1964 to 2001, of which the bulk (42) were of more recent vintage (1993–2001). The tests were conducted in Canada (73%), the USA (36%) and Iran (9%). The Brumzyme research is based on a total of 966 head, with means of 40 per test and 14.4 per treatment. There were too few tests on criteria such as dressing percentage, back fat, muscle score, rib eye area, marbling and cutability, so the dependent variable effects were for dry matter intake, liveweight gain and dry matter conversion ratio, assessed in terms of 19 tested independent variables, defined and valued in Table 12.9.

The reports concerned 13 enzyme products (Avamorin PK, β-glucanase T.1., cellulase/xylanase FIMNU, Diazyme, Fibrozyme, Finnfeeds fungal extract preparations A+B, Grasszyme, Promote, Spezyme/xylanase B (3/2), Spezyme/xylanase B (9/1), Takamine HT 550F, xylanase T.l. and Xymo-Pabst), mainly comprising six with amylases, eight with cellulases and nine with xylanases together with various minor side-activities, amyloglucosidase, β-glucanase, cellobiase, glucose oxidase, 'gumase', hydroxyethylcellulase, polygalacturonase and protease. The resultant conventional and less stringent models are detailed in Table 12.10.

Table 12.7. Feed intake, liveweight gain and feed conversion ratio models for non-starch polysaccharidases (83 becexyases) in pig nutrition ($P \leq 0.05$ in/$P \geq 0.10$ out[a] and $P \leq 0.25$ in/$P \geq 0.34$ out[b]).

FDIeff[a] =		8.38			+1.60B^2			+66.0MAL	–10.4FIP	+2.28SOP	
R^2 0.193	SE	23.9			0.303			24.5	4.68	0.720	
RMSE 82.3	*P*	0.726			0.000			0.021	0.027	0.002	
n 244											
FDIeff[b] =		166	–0.024FDIC		+1.05B^2			+97.2MAL	–9.28FIP	+1.85SOP	–7.41PRP
R^2 0.250	SE	73.2	0.010		0.369			30.1	5.61	0.811	3.26
RMSE 80.6	*P*	0.024	0.021		0.005			0.001	0.100	0.024	0.024
n 244											
			–28.6MMT	+0.401MZP	+36.8CAN	+35.3GER	+30.0USA				
			14.2	0.246	18.5	15.7	0.113				
			0.045	0.105	0.048	0.026	0.192				
LWGeff[a] =		31.2		–5.53B	+1.55B^2			+20.8MAL			
R^2 0.356	SE	2.85		2.71	0.314			10.1			
RMSE 29.5	*P*	0.000		0.042	0.000			0.041			
n 244											
TP 1.79			–13.8MMT	+ 0.254MZP						–12.0ABF	
			4.74	0.079						4.57	
			0.004	0.002						0.009	
LWGeff[b] =		34.8		–7.71B	+1.68B^2		+0.445C^2	+25.5MAL	–2.86FIP		
R^2 0.393	SE	9.03		2.85	0.318		0.227	10.2	1.93		
RMSE 29.0	*P*	0.000		0.007	0.000		0.051	0.013	0.139		

n 244											
TP 2.29			−17.5MMT	+0.0268MZP	+15.2CAN	+12.2GER	+13.3USA	+5.97APF		−15.6ABF	
			5.08	0.081	6.44	6.34	8.01	4.54		4.97	
			0.001	0.000	0.019	0.056	0.098	0.190		0.002	
FCReff[a] =		0.605	−0.184FCRC	−0.0180B	−0.00300B^2						−0.0180PRP
R^2 0.434	SE	0.095	0.017	0.009	0.001						0.004
RMSE 0.0918	*P*	0.000	0.000	0.035	0.000						0.000
n 244											
TP 3.00								−0.0310APF	−0.00800FAP	+0.0490ABF	+0.00100LWIC
								0.014	0.004	0.015	0.000
								0.027	0.031	0.001	0.000
FCReff[b] =		0.743	−0.207FCRC	+0.0290B	−0.00400B^2	−0.0150C				+0.00200SOP	−0.0180PRP
R^2 0.493	SE	0.188	0.020	0.009	0.001	0.006				0.001	0.000
RMSE 0.0884	*P*	0.000	0.000	0.002	0.000	0.015				0.008	0.000
n 244											
TP 3.63								−0.0260APF	−0.00900FAP	+0.0340ABF	+0.00300LWIC
								0.014	0.004	0.015	0.001
								0.064	0.022	0.023	0.003
			+0.00100LWFC	−0.00200EXD				−0.0440UK	−0.00100BAP	+0490PPD	−0.0250PRO
			0.000	0.002				0.021	0.000	0.037	0.014
			0.000	0.243				0.038	0.074	0.189	0.086

ABF, antibiotic feed (1 or 0); APF, animal protein feed (1 or 0); B, β-glucanase (u g^{-1}); BAP, barley (%); C, cellulase (u g^{-1}); CAN, Canada test (1 or 0); EXD, year of test: 1900; FAP, fat (%); FCRC, control feed conversion ratio (FDIC/LWGC); FCReff, feed conversion ratio effect; FDIC, control feed intake (kg day^{-1}); FDIeff, feed intake effect (kg day^{-1}); FIP, fibre (%); GER, Germany test (1 or 0); LWFC, control final liveweight (kg); LWGeff, feed conversion ratio effect; LWIC, control initial liveweight (kg); MAL, male/castrated pigs (1 or 0); MMT, mode of action/metabolism test (1 or 0); MZP, maize (%); *n*, number of tests; *P*, probability; PPD, part-purified diet (1 or 0); PRO, processed (not mash) feed (1 or 0); PRP, protein (%); R^2, multiple correlation coefficient square; RMSE, root mean square error; SE, standard error; SOP, sorghum (%); TP, turning point (u g^{-1}); UK, UK test; USA, USA test (1 or 0).

Table 12.8. Feed intake, liveweight gain and feed conversion models for first-generation phytases (12 enzymes) in pig nutrition ($P \leq 0.05$ in/$P \geq 0.10$ out[a] and $P \leq 0.25$ in/$P \geq 0.34$ out[b]).

FDIeff[a] =		401		+60.5logPHY	–261logPHO		+38.5FIN				
R^2 0.431	SE	127		21.8	51.4		15.7				
RMSE 70.4	*P*	0.002		0.006	0.000		0.015				
n 150											
				+95.2MAL				–43.4NEN			
				0.306				12.8			
				0.000				0.001			
			+0.880MZP	+0.923SOP		+7.17RBP					
			0.194	0.504		1.72					
			0.000	0.069		0.000					
FDIeff[b] =		–455	–0.0260FDIC	+47.9logPHY	–190logPHO		+91.8FIN	+1.10DUR	+6.52EXD		
R^2 0.568	SE	0.279	0.012	22.8	63.3		18.2	0.300	2.82		
RMSE 64.1	*P*	0.105	0.028	0.037	0.003		0.000	0.000	0.022		
n 150											
			+22.8ABF	+118MAL	+49.5PEL	+126CRU	–48.7RFD	–64.8NEN	+14.7PRP	+1.51BAP	+2.30WHP
			14.1	24.2	20.9	33.6	18.6	15.6	4.23	0.567	0.568
			0.107	0.000	0.020	0.000	0.010	0.000	0.000	0.009	0.000
			+2.81MZP	+3.4850P	+5.80STP	+8.44RBP					
			0.451	0.691	1.36	172					
			0.000	0.000	0.000	0.000					
LWGeff[a] =		–152		+59.5logPHY	–152logPHO						
R^2 0.424	SE	39.9		11.5	26.6						
RMSE 37.6	*P*	0.000		0.000	0.000						
n 150											
						+29.0CRU			+6.14PRP		
						13.7			1.43		
						0.036			0.000		
			+0.755MZP	+0.748SOP			–27.1APF				
			0.108	0.262			22.5				
			0.000	0.005			0.231				
LWGeff[b] =		–335		+49.4logPHY	–178logPHO	+5.39Ca	+25.9FIN		+4.23EXD		

R^2 0.528	SE	127		11.8	32.6	2.15	9.56		1.43		
RMSE 35.1	*P*	0.009		0.000	0.000	0.014	0.008		0.004		
n 150											
				+37.4MAL	+17.8PEL	+48.7CRU	–20.3RFD	–25.3NEN	+6.59PRP		
				12.3	9.70	14.5	9.53	7.43	1.42		
				0.003	0.009	0.000	0.035	0.001	0.000		
			+0.864MZP	+0.820SOP		+2.67RBP		+11.0AOF			
			0.112	2.65		1.05		7.46			
			0.000	0.002		0.012		0.143			
FCReff[a] =		1.40	–0.374FCRC	–0.143logPHY			+0.0620FIN				+0.00500LWFC
R^2 0.557	SE	0.185	0.034	0.038			0.027				0.000
RMSE 0.115	*P*	0.000	0.000	0.000			0.026				0.000
n 150											
			+0.0910ABF	+0.0800MAL					+0.0280PRP		
			0.024	0.036					0.006		
			0.000	0.027					0.000		
					+0.00500STP	+0.00800RBP		–0.101AOF			
					0.001	0.003		0.025			
					0.000	0.003		0.000			
FCReff[b] =		1.40	–0.367FCRC	–0.142logPHY			+0.0650FIN			+0.00200LWIC	+0.00400LWFC
R^2 0.568	SE	0.185	0.036	0.038			0.027			0.002	0.001
RMSE 0.714	*P*	0.000	0.000	0.000			0.020			0.147	0.000
n 150											
			+0.0950ABF	+0.00660MAL					–0.0280PRP		
			0.024	0.037					0.006		
			0.000	0.073					0.000		
					+0.0500STP	+0.00700RBP		–0.105AOF	–0.0370USA		
					0.001	0.003		0.025	0.026		
					0.000	0.013		0.000	0.147		

ABF, antibiotic feed (1 or 0); AOF, added oil/fat feed (1 or 0); APF, animal protein feed (1 or 0); BAP, barley (%); Ca, calcium (g kg^{-1} feed); CRU, crumbed feed (1 or 0); DUR, duration (days); EXD, year of test: 1900; FCRC, control feed conversion ratio (FDIC/LWGC); FCReff, egg conversion ratio effect; FDIC, control feed intake (kg day^{-1}); FDIeff, feed intake effect (kg day^{-1}); FIN, Finase (1 or 0); LWFC, control final liveweight (kg); LWGeff, feed conversion ratio effect; LWIC, control initial liveweight (kg); MAL, male/castrated pigs (1 or 0); MZP, maize (%); *n*, number of tests; NEN, net energy (MJ kg^{-1}); *P*, probability; PEL, pelleted feed (1 or 0); PHO, phosphorus (log u kg^{-1}); PHY, phytase (u g^{-1}); PRP, protein (%); R^2, multiple correlation coefficient square; RBP, rice bran (%); RFD, restricted feed (1 or 0); RMSE, root mean square error; SE, standard error; SOP, sorghum (%); STP, starch (%); USA, USA test (1 or 0); WHP, wheat (%).

Table 12.9. Variables, units, codes, means, standard deviations and ranges of dependent and independent start-to-finish negatively controlled test variables for a notional beefase in beef cattle nutrition (48 tests).

Variable (units)	Code	Mean (%)	Standard deviation (%)	Range
Beefase dosage (u g^{-1})	BUG	869	14.0	0.045–50.8
Duration (days)	DUR	103	56.1	18–205
Year of test (1900)	EXD	91.9	10.8	64–101
Protein (%)	PRP	13.5	2.19	114.0–19.5
Urea (%)	URP	0.102	0.297	0–1.10
Housing individual/ tie stall	HIT	0.354	0.483	1 or 0
Crossbreds	CRB	0.500	0.505	1 or 0
Mode of action/ metabolism test	MMT	0.458	0.504	1 or 0
Feed/implant antibiotic	FIA	0.125	0.334	1 or 0
Vaccinated stock	VAC	0.563	0.501	1 or 0
Canada test	CAN	0.729	0.449	1 or 0
Mixed feed pellet	MFP	0.255	0.441	1 or 0
Mixed feed mash	MFM	0.532	0.504	1 or 0
Barley/rolled barley grain	BRB	0.375	0.489	1 or 0
Barley silage	BSI	0.438	0.501	1 or 0
Liquid enzyme in mash feed	LMF	0.313	0.468	1 or 0
Control dry matter intake (kg day^{-1})	DMIC	9.23	2.05	3.06–12.40
DMI effect (kg day^{-1})	DMIeff	0.00958 (0.104[a])	0.441 (4603[b])	–1.30 to +1.50
Control liveweight gain (kg day^{-1})	LWGC	1.27	0.262	0.705 to +1.700
LWG effect (kg day^{-1})	LWGeff	0.0381 (3.00[a])	0.116 (304[b])	–0.140 to +0.430
Control dry matter conversion ratio	DMCC	7.17	2.51	4.34–13.5
DMC effect	DMCeff	–0.264 (–3.68[a])	0.590 (223[b])	–1.70 to +0.95

[a] Percentage of control; [b] coefficient of variation.

Table 12.10. Models for the effects of beefase (13 enzymes) on dry matter intake, liveweight gain and dry matter conversion in beef cattle ($P \leq 0.05$ in/$P \geq 0.10$ out[a] and $P \leq 0.25$ in/$P \geq 0.34$ out[b]).

DMIeff[a] =		–1.00	+0.0731PRP				
R^2 0.147	SE	0.394	0.028				
RMSE 0.420	*P*	0.015	0.012				
n 42							
DMIeff[b] =		–1.00	+0.0731PRP				
R^2 0.147	SE	0.394	0.028				
RMSE 0.420	*P*	0.015	0.012				
n 42							
LWGeff[a] =		–0.389	+0.0305PRP				
R^2 0.412	SE	0.090	0.006				
RMSE 0.0940	*P*	0.000	0.000				
n 36							
LWGeff[b] =		–0.424	+0.0340PRP	+0.0299VAC			
R^2 0.454	SE	0.091	0.007	0.019			
RMSE 0.0923	*P*	0.000	0.000	0.122			
n 36							
DMCeff[a] =		1.34	–0.117PRP		–0.167BSI		
R^2 0.477	SE	0.525	0.036		0.092		
RMSE 0.451	*P*	0.016	0.003		0.079		
n 36							
DMCeff[b] =		1.51	–0.131PRP		–0.284BSI	+0.572URP	–0.177MMT
R^2 0.605	SE	0.721	0.049		0.104	0.316	0.102
RMSE 0.413	*P*	0.045	0.012		0.010	0.081	0.094
n 36							

BSI, barley silage (1 or 0); DMCeff, dry matter conversion ratio effect; DMIeff, dry matter intake effect (kg day^{-1}); LWGeff, liveweight gain effect (kg day^{-1}); MMT, mode of action/metabolism test (1 or 0); *n*, number of tests; *P*, probability; PRP, protein (%); R^2, multiple correlation coefficient square; RMSE, root mean square error; SE, standard error; URP, urea (%); VAC, vaccinated stock (1 or 0).

The conventional models had only two significant independent variables accounting for 15–48% of response variations. There are, as yet, no significant dosage terms available for beefase (all enzymes tested). Feed intake and gain are enhanced at higher feed protein contents with better conversion rates in barley silage rations. The less stringent models for effects on gain and conversion suggest also that vaccination improves liveweight gain response and enhances feed conversion at higher protein levels in barley silage-containing rations and in metabolic/mode of action tests, but conversion response is inferior in rations containing urea, proportionate to urea content.

There is, therefore, clearly a need for further efficacy studies on the use of exogenous enzymes in beef cattle.

Dairy cattle

The 27 publications on supplementary enzymes in dairy cattle provided 75 start-to-finish negatively controlled tests in a total of 98 results, including intermediate values conducted on 29 different enzyme products. The 29 enzyme products tested were primarily based on β-glucanase, cellulase and xylanase, with minor side-activities of amylase, glucosidase, cellobiase, ferulic acid esterase, glucose oxidase, 'gumase', hemicellulase, hydroxyethylcellulase, limit dextrinase, polygalacturonase, phytase and protease. These tests were effected between 1990 and 2003, mostly in the USA (40%), Canada (29%) and the UK (25%). The research utilized a total of 1348 dairy cows with an average of 12.7 per treatment group. Dry matter intake and milk yield were enhanced by dairyase (all enzymes tested) in 64% and 63% of the tests, respectively, and feed conversion ratio in 52%. Higher protein, fat and lactose milk contents were recorded in 58%, 55% and 51% of the tests, respectively. *In toto*, 30 independent variables have been assessed for dairy cattle, i.e. seven control performances and 23 others in Table 12.11, including dairyase dosage as logarithmic or quadratic. The resultant holo-analytical models are detailed in Table 12.12.

Apart from the constants, there are seven statistically significant independent variables in the conventional models, accounting for 7–36% of the variations in responses for DMIeff, GFMeff, MPPeff, MFPeff and MLAeff. No significant variables have yet emerged for the MPDeff or MCReff models or for dairyase dosage. The less stringent models contain 17 different statistically significant independent variable terms (apart from constants), including quadratic dosage terms for MPPeff (R^2 = 0.875) with a turning point at 42.8 u g^{-1} feed.

The likelihood of significant effects due to the 21 independent variables is elucidated in the 47 significant terms appearing in Table 12.12. Variation accountancy in this set ranges widely, from 9 to 88%. These models are thus indicative of potentially interesting targets in future research, including milk protein and fat content effects, duration, mash feeding, temporal development, role of data from metabolic/mode of action tests, whole cottonseed usage, urea content and UK local factors.

Table 12.11. Variables, units, codes, standard deviations and ranges of dependent and independent start-to-finish negatively controlled test variables for exogenous enzymes (dairyases) in dairy cattle nutrition (75 tests).

Variable (units)	Code	Mean (%)	Standard deviation (%)	Range
Dairyase dosage (units g^{-1} feed)	DUG	12.6	19.2	0.004–72.5
Duration (days)	DUR	68.8	38.3	14–126
Year of test (1900)	EXD	98.1	3.4	90–103
Holstein	HOL	0.560	0.500	1 or 0
Mode of action/metabolism test	MMT	0.440	0.500	1 or 0
USA test	USA	0.400	0.493	1 or 0
Canada test	CAN	0.293	0.458	1 or 0
UK test	UK	0.253	0.438	1 or 0
Mixed feed pellet	MFP	0.373	0.487	1 or 0
Mixed feed mash	MFM	0.533	0.502	1 or 0
Whole cottonseed	WCS	0.307	0.464	1 or 0
Fish meal feed	FMF	0.080	0.273	1 or 0
Blood meal feed	BMF	0.187	0.392	1 or 0
Main vegetable protein soy	VPS	0.333	0.475	1 or 0
Main vegetable protein corn gluten	VPC	0.213	0.412	1 or 0
Grain (%)	GRP	28.1	11.6	0–46.7
Hay (%)	HAP	13.4	11.3	0–43.2
Silage (%)	SIP	38.1	17.2	0–72.2
Urea (%)	URP	0.0875	0.238	0–1.18
Whole cottonseed (%)	WCP	1.77	2.85	0–9.90
Total vegetable protein (%)	TVP	9.41	5.49	0–19.4
Crude protein (%)	CPP	17.9	2.26	9.60–22.20
Neutral detergent fibre (%)	NFP	32.8	4.69	25.3–50.6
Control dry matter intake (kg day^{-1})	DMIC	21.4	3.67	13.4–29.0
DMI effect (kg day^{-1})	DMIeff	0.377 (1.76[a])	0.920 (244[b])	–2.60 to +2.70
Control milk yield (kg day^{-1})	MKDC	30.9	7.24	14.4–48.1
MKD effect (kg day^{-1})	MKDeff	0.797 (2.58)	1.56 (196)	–2.70 to +6.30
Control milk conversion ratio	MCRC	1.44	0.204	0.971–1.920
MCR effect	MCReff	0.0109 (0.76)	0.0795 (729)	–0.210 to +0.210
Control gravimetric 4% fat-corrected milk yield (kg day^{-1})	GFMC	28.3	5.23	18.7–39.5
GFM effect (kg day^{-1})	GFMeff	0.423 (1.49)	1.91 (452)	–3.10 to +6.40
Control milk protein content (%)	MPPC	3.24	0.173	2.87–3.61
MPP effect (%)	MPPeff	0.00914 (0.28)	0.0953 (1043)	–0.250 to +0.280
Control milk fat content (%)	MFPC	3.69	0.444	2.33–5.20
MFP effect (%)	MFPeff	–0.0284 (–0.77)	0.186 (655)	–0.500 to +0.460
Control milk lactose content (%)	MLPC	4.70	12.5	4.51–0.4.91
MLP effect (%)	MLPeff	0.00348 (0.0740)	0.0491 (1411)	–0.110 to 0.080

[a]Percentage of control.
[b]Coefficient of variation.

Table 12.12. Models for the effects of dairyase (29 enzymes) in dairy cattle ($P \leq 0.05$ in/$P \geq 0.10$ out[a] and $P \leq 0.25$ in/$P \geq 0.34$ out[b]).

DMIeff [a] =		8.40		−0.0819EXD		
R^2 0.0934	SE	2.95		0.030		
RMSE 0.878	*P*	0.006		0.008		
n 74						
DMIeff [b] =		8.40		−0.0819EXD		
R^2 0.0934	SE	2.95		0.030		
RMSE 0.878	*P*	0.006		0.008		
n 74						
MPDeff[a] no significant independent variable						
MPDeff [b] =		8.10	+0.0212DUR	−0.0876EXD		−0.526MMT
R^2 0.174	SE	6.15	0.008	0.061		0.308
RMSE 1.40	*P*	0.193	0.013	0.154		0.092
n 65						
			−0.912UK			
			0.451			
			0.048			
MCReff [a] no significant independent variable						
MCReff [b] =		0.124	+0.000597DUR		+0.0277MFM	
R^2 0.190	SE	0.080	0.000		0.012	
RMSE	*P*	0.129	0.052		0.031	
0.0730						
n 54						
			+0.0877UK	−0.000987SIP		−0.00435NFP
			0.042	0.001		0.002
			0.043	0.203		0.057
GFMeff [a] =		0.683			+0.472MFM	−0.623WCS
R^2 0.153	SE	0.232			0.216	0.231
RMSE 1.79	*P*	0.004			0.032	0.009
n 69						

GFMeff[b] =		0.683						+0.472MFM	−0.623WCS		
R^2 0.153	SE	0.232						0.261	0.231		
RMSE 1.79	*P*	0.004						0.032	0.009		
n 69											
MPPeff[a] =		0.249									
R^2 0.118	SE	0.136									
RMSE	*P*	0.072									
0.0956											
n 57											
			−0.0126PRP	−0.136URP							
			0.007	0.054							
			0.093	0.015							
MPPeff[b] =		2.71	−0.721MPPC	−0.00681DUG	+0.0000796DUG^2	−0.00664DUR		+0.106MFM	−0.0958WCS	+0.0274WCP	+0.0825MMT
R^2 0.875	SE	0.356	0.093	0.002	0.000	0.001		0.017	0.029	0.009	0.019
RMSE	*P*	0.000	0.000	0.001	0.020	0.000		0.000	0.003	0.004	0.000
0.0496											
n 43											
TP 42.8			−0.376CAN			+0.395UK	+0.00196SIP	−0.00309HAP	+0.0110NFP	−0.218BMF	−0.0845HOLS
			0.078			0.088	0.002	0.002	0.004	0.037	0.051
			0.000			0.000	0.211	0.122	0.005	0.000	0.102
				−0.00469TVP							
				0.004							
				0.234							
MFPeff[a] =		−0.00921				−0.00225DUR				+0.0267WCP	
R^2 0.357	SE	0.047				0.001				0.007	
RMSE 0.152	*P*	0.844				0.000				0.000	
n 63											
					+0.106FMF						
					0.034						
					0.003						

Continued

Table 12.12. *Continued*

MFPeff[b] =		0.692	–0.0988MFPC		–0.00355DUR	+0.0705MFM	–0.0757WCS	+0.0912MMT
R^2 0.568	SE	0.211	0.058		0.001	0.022	0.026	0.037
RMSE 0.133	*P*	0.002	0.093		0.001	0.003	0.006	0.018
n 51								
				–0.517URP		–0.00577HAP		
				0.144		0.002		
				0.001		0.014		
MLAeff[a] =		0.0165			–0.000322DUR			
R^2 0.0695	SE	0.014			0.000			
RMSE 0.0493	*P*	0.260			0.084			
n 44								
MLAeff[b] =		–0.127			–0.000757DUR			
R^2 0.286	SE	0.102			0.000			
RMSE 0.0466	P	0.221			0.003			
n 36								
							+ 0.00537NFP	+ 0.0226BMF
							0.003	0.009
							0.116	0.022

BMF, blood meal feed (1 or 0); CAN, Canada test (1 or 0); DMIeff, dry matter intake effect; DUG, dairyase dosage (u g^{-1}); DUG^2, dairyase dosage square (u $g^{-1})^2$; DUR, duration (days); EXD, year of test (1900); FMF, fish meal feed (1 or 0); GFMeff, gravimetric 4% fat-corrected milk yield effect (kg day^{-1}); HAP, hay (%); HOLS, Holstein (1 or 0); MCReff, milk conversion ratio effect; MFM, mixed feed mash (1 or 0); MFPC, control milk fat content (%); MFPeff, milk fat effect (%); MLAeff, milk lactose effect (%); MMT, mode of action/metabolism test (1 or 0); MPDeff, milk/day effect (kg); MPPC, control milk protein content (%); MPPeff, milk protein effect (%); *n*, number of tests; NFP, neutral detergent fibre (%); *P*, probability; PRP, feed crude protein (%); R^2, multiple correlation coefficient square; RMSE, root mean square error; SE, standard error; SIP, silage (%); TP, turning point (u g^{-1}); TVP, total vegetable protein (%); UK, UK test (1 or 0); URP, urea (%); WCP, whole cottonseed (%); WCS, whole cottonseed (1 or 0).

Overview

Holo-analyses to date have revealed statistical significance for 87 independent variables in monogastric and 22 in ruminant farm animals, as determinants of the nutritional values of exogenous enzymes. It is also interesting to note that the only significant variable in common in the five species is mode of action/metabolism test. The spread of independent variables as the primary contributor to response variation in Table 12.13 (for those models containing two or more statistically significant variables) manifests: (i) level of control performance (37.5%); (ii) ingredient or ingredient percentage inclusion (27.5%); (iii) enzyme and phosphorus dosage terms (17.5%); (iv) duration and breed (5%); and (v) year of test (1900), cage housing and dairy mixed feed mash (2.5%).

Thus, it may be concluded to date that level of control performance, dietary composition and enzyme or phosphorus dosage terms are the most important contributors to the magnitude of response to exogenous enzymes. Specific dietary ingredients involved therein are maize, whole cottonseed, urea, silage and crude protein content.

Uses and Applications of Holo-analyses

Software based on holo-analytical models can be prepared in order to: (i) predict responses at optimum dosage unique in time and place to maximize the efficient use of a pro-nutrient exogenous enzyme product, together with confidence limits for the estimate thereof; and (ii) compare the efficacy of products, in order to select the best product from competitive offers. In this connection a seven-question test, below, can usefully be deployed.

1. How many properly controlled feeding tests do you have on the efficacy of product x?
2. How many of these have no negative controls?
3. Can you supply a bibliography for (1)?
4. How many times out of ten does product x improve liveweight gain and feed conversion?
5. What are the coefficients of variation in gain and conversion responses you claim for your product?
6. What dosage of product x will maximize the return on my investment?
7. Can you supply me with a holo-analytical model to predict responses with confidence limits to product x under my specific conditions today?

Appropriate answers to the questions, respectively, comprise (1) minimum 20, but preferably ≥ 50; (2) 0; (3) yes; (4) 7/10 is the norm for pro-nutrient feed additives; (5) 100–200% is satisfactory; (6) x ppm because …; and (7) yes.

Until now, holo-analysis has mainly been used in broiler, turkey, layer, pig and beef and dairy cattle studies on the efficacies of acids, enzymes and

Table 12.13. Comparison of main independent variable contributions to response variations R^2 (%) to eight exogenous enzymes in five farm animal species ($P \leq 0.05$ in/$P \geq 0.10$ out[a] and $P \leq 0.25$ in/$P \geq 0.34$ out[b]).

Species	Enzyme	Model	Independent variable	R^2 contribution (%)
Broiler	Broilerase	FDIeff[a]	Duration	29.7
		LWGeff[a]	Control liveweight gain	39.8
		FCReff[a]	Control feed conversion ratio	92.9
		MOReff[a]	Mortality	96.4
Broiler	Phytase	FDIeff[a]	Log phosphorus dosage	28.1
		LWGeff[a]	Log phosphorus dosage	26.4
		FCReff[a]	Cage housing	29.5
		MOReff[a]	Control mortality	100.0
Layer	Layerase	FDIeff[a]	Control feed intake	59.9
		HDPeff[a]	Control hen-day production (%)	77.4
		EWTeff[a]	Control egg weight	33.9
		EMDeff[a]	Control egg mass day^{-1}	73.3
		ECReff[a]	Control egg feed conversion ratio	80.8
Layer	Phytase	FDIeff[a]	White Leghorn	50.1
		HDPeff[a]	Control hen-day production (%)	56.0
		EWTeff[a]	White Leghorn	58.5
		EMDeff[a]	Control egg mass day^{-1}	53.6
		ECReff[a]	Control egg feed conversion ratio	84.3
Pig	Becexyase	FDIeff[a]	Becexyase dosage square B^2	57.5
		FDIeff[b]	Becexyase dosage square B^2	44.4
		LWGeff[a]	Becexyase dosage square B/B^2	72.8
		LWGeff[b]	Becexyase dosage square B/B^2	65.9
		FCReff[a]	Control feed conversion ratio	34.1
		FCReff[b]	Control feed conversion ratio	30.0
Pig	Phytase	FDIeff[a]	Maize (%)	29.1
		FDIeff[b]	Maize (%)	22.8
		LWGeff[a]	Maize (%)	34.0
		LWGeff[b]	Log phosphorus dosage	32.9
		FCReff[a]	Control final liveweight	27.5
		FCReff[b]	Control final liveweight	25.6
Beef cattle	Beefase	DMIeff[a]	Protein (%)	100.0
		DMIeff[b]	Protein (%)	100.0
		LWGeff[a]	Protein (%)	100.0
		LWGeff[b]	Protein (%)	100.0
		DMCeff[a]	Protein (%)	87.2
		DMCeff[a]	Protein (%)	76.9
Dairy cattle	Dairyase	DMIeff[a]	Year of test (1900)	100.0
		DMIeff[b]	Year of test (1900)	100.0
		MPDeff[a]	–	–
		MPDeff[b]	Year of test (1900)	69.5

Continued

Table 12.13. *Continued*

Species	Enzyme	Model	Independent variable	R^2 contribution (%)
		MCReff[a]	–	–
		MCReff[a]	Mixed feed mash	29.7
		GMFeff[a]	Whole cottonseed in feed	51.7
		GMFeff[b]	Whole cottonseed in feed	83.5
		MPPeff[a]	Urea (%)	56.0
		MPPeff[b]	Silage (%)	46.6
		MFPeff[a]	Whole cottonseed in feed	67.2
		MFPeff[b]	Urea (%)	29.1
		MLPeff[a]	Duration	100.0
		MLPeff[b]	Duration	38.7

DMCeff, dry matter intake effect; DMIeff, dry matter intake effect; ECReff, egg conversion ratio effect; EMDeff, egg mass day^{-1} effect; EWTeff, egg weight effect; FCReff, feed conversion ratio effect; FDIeff, feed intake effect; GFMeff, gravimetric 4% fat-corrected milk yield effect; HDPeff, hen-day production effect; LWGeff, liveweight gain effect; MCReff, milk conversion ratio effect; MFPeff, milk fat content effect; ; MLPeff, milk lactose content effect; MOReff, mortality effect; MPPeff, milk protein content effect.

oligosaccharides. Others in view include anticoccidials, antihistomonials, antimicrobials, antioxidants, aromatics, botanicals, metal chelates and microbials.

An example of product comparisons using the models in Table 12.4 quoted paired comparisons of the three phytase brands studied, which showed only small differences between pairs of feed conversion differences of 0.3–1.3 points that were, in fact, statistically insignificant.

A further application of holo-analytical models concerns the validation of so-called matrix values and their use in feed formulation and/or nutrient economy. For example, as above, the use of a low-level linear segment of the dose–response curve to determine phytase/phosphorus equivalencies revealed in broiler models (Rosen, 2002c) to enhance the dosage of phytase required to offset weight gain loss by 73% if measured for 3–4 P kg^{-1} feed compared with 6–7 P kg^{-1} feed. It is therefore essential to reassess and validate matrix values at points close to the nutrient requirement.

Future Research

In addition to the topics for future research already mentioned in the broiler, pig and beef and dairy cattle sections, it will be of interest to elaborate models for individual enzyme products having sufficient tests or, at least as a start, to assess their comparative value using a dummy product variable added to the models for broilers (Table 12.3), layers (Table 12.5), pigs (Tables 12.7 and 12.8), beef cattle (Table 12.10) and dairy cattle (Table 12.12).

Finally, there is a question of how often a holo-analytical model should be updated to take account of ongoing research on the efficacy of exogenous enzymes. In this context it will be relevant, as a start, to compare the models based on the collection of 1322 papers used to provide the models in Table 12.3 with new models constructed from: (i) a collection of 1512 subsequent publications; and (ii) the total of 2834. This updating will also enhance progress in the longer-term replacement or minimization of temporal or geographical variables by biological terms.

Conclusion

The review above illustrates the extent to which the advent of the holo-analysis concept meets the eight future requirements referred to in the Introduction. Holo-analysis has been shown to be capable of predicting nutritional responses to exogenous enzymes with associated confidence limits in chickens, laying hens, pigs and beef and dairy cattle, with promising features for future extensions to include the use of enzymes in turkey, fish, rabbit, horse and pet feeds and foods. Holo-analysis will also be invaluable in the investigation of the comparative values of enzymes versus, or in combination with, other pro-nutrients, such as acids, aromatics, botanicals (including essential oils, herbs and spices), chelates, microbials and saccharides.

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13 Feed Enzymes, the Future: Bright Hope or Regulatory Minefield?

M.R. Bedford and G.G. Partridge

Introduction

From the initial commercial use of feed enzymes to the present day spans only 20 years, highlighting the youth of this industry. Both the 'carbohydrase' enzymes (i.e. those targeted principally against non-starch polysaccharides) and then, subsequently, 'phytases' were slow in their initial uptake, in a market that was estimated to be worth around US$100 million in the mid-1990s. However, since that time, the industry has rapidly developed into a market today worth approximately US$550–600 million, with an estimated annual growth rate around 10%. Clearly, the recent past has been generous to the feed enzyme industry, but what does the future hold?

Our understanding of what is required of a feed enzyme, from a functional viewpoint, has advanced considerably since the early 2000s. Concurrent with this have been considerable advances in our ability to search nature and evolve new products better to suit the role for which they are intended. Consequently, there seems to be a plethora of potential enzyme candidates for use in the animal feed industry, but this is sharply counterbalanced by the spiralling regulatory costs of bringing such products to market. These constraints in many cases are fully warranted in that they are a safety check on the feed industry that has suffered several food/feed safety concerns since the early 1990s (e.g. BSE, dioxins, melamine, *Salmonella*, *E. coli*, etc.). As a result, the regulatory authorities in the major markets are rightly in no mind to relax their vigilance through reductions in their safety requirements. Nevertheless, given that the major markets around the world still differ significantly in their regulatory demands, the costs of delivering a new product just to the major swine and poultry categories (e.g. sows, weaner pigs, grower-finisher pigs, broilers, broiler-breeders, layers, turkeys and breeding stock) in all parts of the world are probably in excess of US$2 million. The potential for delivery of new

products to the market is therefore huge, but the entry costs are also substantial. As a result, the number of enzyme candidates making it through the process will probably be quite limited. This chapter focuses on how we have arrived at this point and speculates where the future might lead.

Early Markets

Products

Early in the 1980s there was a small market for β-glucanases in the Scandinavian markets, particularly Finland, where it was discovered that such enzymes enabled the inclusion of significant amounts of barley in poultry rations and, to a lesser extent, pig rations. This was achieved without any loss in bird performance or initiation of wet litter problems, and resulted in considerable savings in feed costs. This application spread to the UK and Northern Europe in the early 1990s, with xylanases entering the market in 1990–1991, predominantly for wheat-based rations. The bulk of these products at the time were opportunistic, many having been developed for alternative applications such as the pulp and paper industry, or for brewing. The products were often relatively crude, in that they were not mono-component enzymes or genetically modified to optimize production of the desired enzyme activities. There would often be more than three or four enzyme activities present in the product in appreciable quantities, but often only one or two of these were assayed for routine quality control in any one batch. Since the efficacy of a feed enzyme product can either be enhanced or, equally, compromised by the presence of certain ancillary enzyme activities, this meant that the early products were prone to considerably more variation in response than modern-day, mono-component products.

Regulatory environment

At this time there were several small-scale feed enzyme producers, some being simply small divisions of very large enzyme companies. However, the market itself was small and, as a result, interest from both the regulatory authorities in Europe and the feed industry itself was limited. Enzyme products were treated as being fermentation products, much in the same way as brewer's yeast by-products, and as a result were considered more as feed ingredients rather than feed additives. This changed rapidly in the early 1990s with the increasing recognition that enzymes should be considered as feed additives, putting them into a class of compounds requiring considerable safety testing. All products developed for sale in the EU from this point in time onwards had to undergo a minimum of a 90-day chronic toxicity test using a rat model, and were subject to several other exposure and efficacy tests as well.

Mid-life

Products

In the mid- to late 1990s the feed enzyme market progressively developed. The enzyme phytase started to be used in the Netherlands as a result of evolving legislation penalizing excessive phosphorus application on arable land, which ultimately forced feed manufacturers to look for solutions to reduce the phosphorus content of manure. This market was, and remained, small in comparison with the carbohydrase enzyme market and did not spread geographically for several years, as a result of the lack of similar environmental legislation outside the Netherlands and parts of Germany. Whereas carbohydrase enzymes were cost effective in most wheat- and barley-based poultry feeds, often saving multiples of the cost of investment, the use of phytase was only cost effective when environmental penalties were factored into the purchase decision. The return on investment did not exist for phytase at that time in most markets, and as a result the enzyme market was predominantly carbohydrases.

The imposition of the meat-and-bone meal ban in monogastric diets in the EU in the mid-1990s had an immediate effect on the value of phosphorus, and consequently on the value of phytase. Meat-and-bone meal is an animal protein source that is also rich in phosphorus and calcium and which, consequently, could supply a large part of the animal's needs for these minerals. Its routine use kept inclusion rates of expensive inorganic phosphate sources to a minimum. However, with the ban on this protein source the reliance on inorganic phosphates increased and as a result the 'shadow price', or value, of phosphate in monogastric diets increased significantly. In a very short period of time phytase moved from being far too expensive to consider to becoming economically feasible in feed formulation, and its sales began to grow rapidly outside the Netherlands.

Activity in the carbohydrase sector was also increased, the focus being on a better understanding of the substrate with the introduction of much more targeted products. At the same time reductions in feed enzyme production costs allowed improvements in return on investment from using the products, and the market continued to expand. Mannanases, pectinases, amylases, α-galactosidases and proteases appeared, sometimes as stand-alone products, but more usually as part of a combination of enzyme activities designed to attack several components in the diet simultaneously. The first carbohydrase products targeting maize (= corn)-based diets were introduced during this period, although success was relatively limited at first since the responses to such products were usually smaller compared with traditional wheat and barley applications for carbohydrase enzymes. Their effects were consequently more difficult to prove statistically and more subtle at farm level. Equally, the feed industry in those parts of the world feeding maize-based diets was less convinced at that time that maize was anywhere near as variable as wheat or barley as a feed ingredient. This perceived 'gold standard' status for maize has since been seen to be misplaced, following more detailed research in recent years.

Regulatory environment

During this phase, the implementation of more rigorous regulatory rules, particularly in the EU, and an intensification of competition resulted in a number of smaller producers and enzyme blenders exiting the market. The market came to be dominated by three or four companies, between them commanding more than 75% of the market share, and product development took on a more systematic rather than opportunistic approach. In some respects the regulatory entry hurdle into the EU acted as an incentive to the larger companies to invest, as new products would be exposed to less competition in such a controlled environment. The process to get a product registered in the EU involved each member state (12 at the time) scrutinizing and questioning the dossier where they felt it appropriate, and since this process was not coordinated between member states it could take as long as 3 years and cost around €2 million. This clearly was beyond the means of the smallest enzyme producers or blenders, many of whom dropped out of the EU market. Such a regulatory hurdle was not apparent in most other markets around the world and, as a result, there were several 'new' products that were introduced into the EU many years after their use had become commonplace elsewhere in the world. Several changes were being made, however, that would simplify this process in the EU so that the timescale was more predictable, but nevertheless the costs of achieving registration were, and still remain, high.

2000 to the Present

Products

The products on the market today are an evolution of those present 5–10 years ago. To the authors' knowledge there is no new class of enzyme currently on the market that was not present 10 years ago. While there are some that have remained unchanged, the majority have been improved through various methods better to meet the challenges that they face. Thermostability has been an issue for both carbohydrase and phytase enzymes, and developments have been made in terms of better formulations (e.g. thermostable coatings for enzymes that would otherwise succumb to thermal degradation in the pelleting process), better post-pellet liquid application systems and genetic evolution of the parent enzyme into a more thermostable variant.

Further improvements have also been made in productivity through improved nutrition and genetics of the production systems employed (e.g. fungal, yeast and bacterial). This has resulted in the average price of enzymes falling considerably in recent years. For example, the end-user cost for enzyme treatment of 1 tonne of barley-based poultry feed has fallen approximately tenfold in the 20 years since it was first introduced. Such reductions in cost have resulted in routine use of feed enzymes in diets where previously it was not cost effective. Coupled with this have been some significant changes in the feed ingredients market. The cost of key raw materials (e.g. cereals, soybean

meal, fat and inorganic phosphates) had, until the end of 2007, been relatively stable. Since then prices have been extremely volatile, in some cases doubling in a few months. Such events, coupled with reduced enzyme prices, resulted in unprecedented increases in both phytase and carbohydrase use to deal with the rising costs of both inorganic phosphates and fat, in particular. Species and segments of the market where phytases did not previously fit, from an economic viewpoint, suddenly became much more cost effective. It is probable that both markets increased by more than 30% between 2007 and 2008.

The dramatic increase in inorganic phosphate prices also resulted in more novel use of phytase, namely via variable dosing. Up until 2007, phytases had been routinely used at a nominal 500 FTU kg^{-1} feed for many applications. It was well known that the relationship between dose and response was log-linear, so that further benefits were accrued at higher dosages of phytase, but the scale of these additional benefits above 500 FTU kg^{-1} feed were insufficient to justify the cost of the extra enzyme. However, when inorganic phosphate prices started to exceed US$1000 t^{-1}, the economic optimum inclusion rate of most phytases was well in excess of 1000 FTU kg^{-1} feed, at which point almost 50% more savings could be realized. At this time many feed manufacturers understandably increased their dosages, thereby immediately increasing the global market size. Similarly, increases in the price of fat drove the shadow price of energy to almost double that of its historic value, and the opportunities for savings in ration costs in many cases overcame the traditional reticence of some feed compounders to test carbohydrase enzymes in maize-based diets.

The use of multifactorial models to describe the animal's responses to both phytases and carbohydrases is also emerging as a useful tool to maximize profitability from the use of feed enzymes. Such tools, through holo-analysis of all data available for a given product, identify variables that can positively or negatively influence the response observed. As a result, it is now understood that the response to phytase, for example, is moderated by various ration and husbandry factors that previously were not considered as part of the recommendations for use of the product.

Regulatory environment

The major developments since the early 2000s have been the introduction of a new feed additive regulation in the EU and a request for efficacy data for registration in the USA. The regulation in the EU changed the process such that only one scientific body, EFSA (the European Food Safety Authority), was responsible for the evaluation of the data presented in the dossier and, once satisfied, the dossier was then passed to the Commission for ratification at parliamentary level. This replaced the tortuous member state scientific appraisal process and brought a great deal more clarity and openness to the procedure. The data requested were still related to safety of use of the product for the animal, the consumer and the worker handling the product, as well as for efficacy in designated target animal species. Enzymes were classified as either digestibility enhancers, gut flora stabilizers or substances favourably affecting

the environment. The efficacy data collected for each species had to fit into one of these three categories for the product to be registered.

In the USA the main development was related to a need to provide efficacy data for poultry and swine, in the form of two positive trials for each. This was limited to one representative category for poultry, usually broilers, and one for swine, usually piglets. Unfortunately, the end point or variable deemed acceptable to prove efficacy did not overlap with that for the EU efficacy studies and, as a result, additional animal trials are needed.

Future

Products

As our understanding of target substrates and the conditions under which they exist *in vivo* increases, our ability to find and evolve enzymes better to suit the application will improve correspondingly. For example, it is becoming clear that an 'ideal' phytase for feed application needs to be highly thermostable, function at a low pH within a proteolytic gastric environment and should excel equally at both phytate (IP_6) destruction and phosphate release. It should have a high specific activity and be produced at very high expression rates so that the dosage of enzyme can be markedly increased compared with the dosages employed today, with little if any increase in cost. The product should also be simple to quality control with a rapid and quantitative assay. With such information to hand it is possible to target the search and evolutionary strategy for far more specific, feed-relevant phytases. Search strategies have improved and increased in number such that it is possible to screen nature far more efficiently for potential candidates. Once a suitable candidate is found, powerful evolutionary techniques are available to further hone that candidate for the intended task. If successful, the fruits of this strategy should present an enzyme that is better at surviving the pelleting and digestive processes, better at phytate hydrolysis and equally cheaper to produce than all previous feed phytases – in essence, a clear market leader. The key to this process is marrying our understanding of the operating environment of the enzyme with the molecular techniques required to find and evolve such a product. This requires a multidisciplinary approach, involving the melding of many disparate sciences, in a significant and costly coordinated effort. However, the potential gains are large.

Regulatory environment

If such a strategy, as described above, were to succeed then the enzyme currently would still have to go through the regulatory process before it could be marketed commercially. One potential issue is that should the candidate be the product of an environmental DNA screening strategy, it is possible that the identity of the 'donor' organism is not known. While most regulatory processes

would cater for this within the existing 90-day chronic toxicity test, and it is recognized that it is the production organism and not the donor that is the most likely source of any toxin, such knowledge may still unnerve a regulatory authority that is prone to invoke a 'precautionary principle' approach.

With authorities increasingly moving their interests into the efficacy arena, and the definition of a category of animal expanding from species to, for example, broilers, layers, breeders, turkeys, ducks, etc., the costs for providing such efficacy data have and will probably continue to increase. This is particularly so when the stringencies applied to documentation, trial design and statistical power demand almost good laboratory practice-like conditions. Currently, the costs for registering a product for broilers, layers, turkeys, piglets and grower-finisher pigs in the EU is estimated in excess of €1.0–1.5 million. Approximately 25% of this is spent on all the safety studies, with efficacy studies demanding the remaining 75%. Even though the above-mentioned categories cover perhaps 90% of the usage of a given monogastric feed enzyme, such a registration may well limit the vendor to less than 60% of the market, by the simple fact that the feed compounder often produces feed for many categories of animals from the same mill. Feed for piglets, grower-finisher pigs and sows is often produced at the same feed mill and, as a result, will require an enzyme with registration in all of these categories. Similarly, a poultry feed mill may produce duck, breeder and pullet feed, and for the same reason would require a product registered in all categories. The successful candidate therefore needs to prove efficacy in all categories of any significance, which raises the efficacy costs probably by another €350,000–450,000. A typical product coming on to the market today will probably take between 4 and 7 years to get from the discovery process to the market, the efficacy requirements being responsible for 1–2 years of this process. Such a large upfront investment has two immediate consequences:

1. Smaller companies are likely to be excluded.
2. Smaller market segments are and will be ignored.

Perhaps it is the second consequence that is most unnerving for the future. With a clear need to improve food security within Europe, attention has focused on the use of alternative protein sources to reduce our traditional dependence on imported soybean meal. Rapeseed meal, lupins, linseed and several other 'home-grown' sources are candidates, but traditionally have been avoided as they are not as well digested or utilized as soybean meal. The potential for enzymatic upgrading of these vegetable protein meals in feed has been demonstrated in the literature, but the solutions to date have not been cost effective or consistent enough to attract widespread commercial interest. The problem stems from the fact that such a targeted enzyme (or enzyme combination) would be capable of creating sales only in the limited markets where such meals would be used. Some markets are simply too small to warrant the upfront investment required to bring such a product to market, and as a result the potential will remain untapped.

While the safety of the animal and the consumer is of paramount importance, it is becoming increasingly clear that the costs of proving efficacy

are now disproportionately onerous in some markets compared with others. The potential for new, better targeted products is great, but there may well be a distinct geographical divergence in their availability to the end user, which ironically could well be to the detriment of food security in these heavily regulated areas.

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