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

M.F. Isaksen, A.J. Cowieson and K.M. Kragh

## 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.

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## 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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