Proteases: Potential for use in poultry nutrition  
Roselina Angel  
Department of Animal and Avian Sciences  
University of Maryland  
College Park, MD 20742  
rangel@umd.edu  
and  
Nelson E. Ward  
and  
Roland Brugger  
DSM Nutritional Products  
Parsippany NJ

Introduction

Most microorganisms and plants can biosynthesize all 20 standard amino acids, while animals (including humans), must obtain some of the amino acids from the diet. Key enzymes in the animals’ body amino acid biosynthetic for specific amino acids are not present at all or not present in enough quantity in animals. The amino acids that an organism cannot synthesize, or synthesized at an insufficient quantity, are referred to as essential amino acids. If amino acids are present in the environment, microorganisms can conserve energy by taking up the amino acids from their surroundings and down-regulating their biosynthetic pathways and thus they start requiring outside sources of these amino acids.

In animals, amino acids are obtained through the consumption of foods containing protein. Ingested proteins are broken down through digestion, which typically involves denaturation of the protein through exposure to acid and hydrolysis by enzymes called proteases. Some ingested amino acids are used for protein biosynthesis, while others are converted to glucose through gluconeogenesis, or fed into the citric acid cycle. This use of protein as a fuel is particularly important under starvation conditions since it allows body proteins - particularly those found in muscle - to be used to support life. Amino acids are also important dietary sources of nitrogen.

The global enzyme market has achieved success in the animal feed sector (Figure 1). The availability of a cost-effective phytase reduces feed costs and inorganic phosphate use. High ingredient costs have spurred interest of xylanases, amylases and others for corn/SBM diets during the past 2-3 years. Use of xylanases in wheat based diets has been the norm in commercial diets since 2000. The global industrial enzyme market is expected to be $2.7 billion by 2012 with the greatest growth in animal feeds.
Commercial proteases account for 60% of estimated worldwide sale of enzymes (Rao et al., 1998), largely within the food and dairy sectors. The protease market has experienced notable growth, owing to their ability to serve degradative and synthetic functions. Viable proteases are focused on specific applications, thus the advent of protein engineering (recombinant DNA technology and site-directed mutagenesis) allows for rapid and effective modifications for new enzymes.

![Figure 1. Global Enzyme Market by Applications Sector ($ millions)](image)

**Proteases**

Proteases perform a variety of roles in biology. These enzymes function in important physiological processes, including homeostasis, apoptosis, signal transduction, reproduction and immunity (Hedstrom, 2002). In addition, proteases are involved in blood coagulation and wound healing. To accomplish a wide range of tasks, over 500 proteases exist in humans; a similar number is present in other organisms.

From a nutritionist’s perspective, the hydrolysis of proteins to individual amino acids and peptides in the intestinal tract is a key function for proteases. Several intestinal proteases exist, and comprise a “protease system” in the intestinal tract for the utilization of various protein sources.

Passage of hydrochloric acid, produced in the proventriculus, and peptides through the proventriculus and gizzard into the duodenum stimulates the release of the hormones secretin and pancreozymin from the mucosa of the duodenum (Rothman and Wells, 1967). These hormones promote the secretion of pancreatic juice containing a number of enzymes and bicarbonate ions. The production of an alkaline solution quickly neutralizes the acid entering the duodenum (Barash et al., 1993). Small intestinal enzymes function
best at pHs close to neutral or slight below neutral and thus, insufficient alkaline bile, lowers enzyme activity in the intestine (Zentler-Munro, 1985).

Pancreatic proteases are secreted from the pancreas only in the form of zymogens. All known cellular proteases are synthesized as zymogens, or the inactive precursor, to prevent unwanted protein degradation at the point of origin or thereabouts (Kahn and James, 1998). The conversion of the zymogens to the active protease requires low pH (autocatalysis) or limited proteolysis. Primary pancreatic zymogens are trypsinogen, chymotrypsinogens A and B, proelastase, and procarboxypeptidases A and B. Trypsin is activated after being attacked by enterokinase, found in the brush border membrane. The active trypsin then hydrolyses bonds in the other zymogens, releasing the active enzymes.

**Digestive Proteases**

*Pepsin* is an acidic protease secreted in the stomach of most animals. Released as pepsinogen, it is activated in the presence of hydrochloric acid. It is active at low pH and inactive at pHs above 6 with variance across species (Crevieu-Gabriel et al., 1999). This protease hydrolyzes peptide bonds mainly between two hydrophobic amino acids. It falls within the group of carboxyl proteases.

*Trypsin* is the primary protease in the intestinal tract. Produced as the inactive trypsinogen by the pancreas, its active form hydrolyzes at the carboxyl side of lysine and arginine, except when followed by proline. It has an optimal operating pH of about 8 or less, and temperature of 37°C (Goldberg et al., 1969).

*Chymotrypsin* is also secreted by the pancreas as the zymogen or precursor, and is activated by trypsin. It hydrolyzes peptide bonds in which the carboxyl groups come from one of the three aromatic amino acids (phenylalanine, tyrosine, and tryptophan).

*Elastase* is produced by the pancreas and cleaves at the carboxyl end of the hydrophobic amino acids glycine, valine and alanine. These amino acids are common in connective tissue in muscle. Elastase, like trypsin and chymotrypsin, falls within the category of serine protease.

*Carboxypeptidases A and B* are exopeptidases also secreted by the pancreas. Exposure of carboxy-terminal aromatic or nonpolar amino acids by chymotrypsin and elastase are cleaved by carboxypeptidase A; carboxypeptidase B cleaves carboxy-terminal basic amino acids exposed by trypsin (Krehbiel and Matthews, 2003).

Pancreatic proteases experience a progressive fall in activity as digesta passes through the small intestine (Goldberg et al., 1969). The decline in importance of these primary proteases is paralleled by a gain in brush border and cytosolic peptidase activity. An estimated 70-85% of all luminal amino acids are taken up from the small intestinal digesta as peptides (Krehbiel and Matthews, 2003). Yet, approximately 85% of this
quantity appears in hepatic portal blood as free amino acids because of intracellular hydrolysis (Ganapathy et al., 1994).

Protease Classifications
Proteases can be classified in different ways. By organism (plant, animal, bacterial, fungal), where animal and plant derived proteases are produced by extraction while fungal and bacterial derived proteases are produced by fermentation. Proteases are also classified by their pH activity range (acid, neutral, alkaline, high alkaline) and by their peptide bond specificity (endopeptidases, exopeptidases, or amino acid specific proteases). Another type of classification for proteases is based on their proteolytic mechanism (serine, threonine, cystine, aspartic acid, glutamic acid, or metallo proteases).

Serine peptidases or proteases reflect the presence of serine in the active site of the protease. Often referred to as a “catalytic triad,” the active site usually contains serine, histidine and aspartic acid (Hedstrom, 2002). These proteases effectively hydrolyze a wide range of peptide bonds via the serine hydroxyl group of the protease when it conducts a neutrophilic attack on the peptide bond (by the carbonyl carbon) of the substrate.

Serine proteases include the pancreatic enzymes trypsin, chymotrypsin, elastase. Although these enzymes are similar in structure and have the same catalytic triad, they act on distinctly different peptide bonds.

Proteases classification can also be based on location within the protein molecule at which it cleaves: exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino or carboxyl end or terminus of the protein. These can be further classified as amino- and carboxypetidases. Endopeptidases cleave peptide bonds within the inner regions of the protein away from the C and N end. There are four subgroups based on their catalytic mechanism: serine proteases, aspartic proteases, cysteine proteases and metallo proteases.

Ingredient Protein Digestibility
From a practical nutritionist’s standpoint, the value of ingredients as a protein (amino acid) source generally comes down to the total amino acids it contains, the ratio of amino acids and availability of the amino acids (aside from cost, ingredient accessibility, etc.). Numerous studies and tables on protein and amino acid availability (the digestibility, absorption and utilization by the animal) have been published. The common thread that runs through such summaries is that ingredient improvements can yet be made in digestibility of protein.

In one large scale study (Ravindran et al., 2005), apparent ileal digestibility was determined for various ingredients for broilers. For cereals, the overall amino acid digestibility coefficient for eight samples of corn was 0.81 (range 0.77 to 0.85). For
soybean meal, the digestibility coefficient was 0.82 (range 0.81 to 0.83). In particular, the groups of meat and bone meal (avg 0.62) and meat meal samples (avg digestibility coefficient 0.65) showed low amino acid digestibility values. This was accompanied by marked variation.

Similarly, University of Illinois work has reported a number of studies on amino acid digestibility of ingredients. Low values and high variation for meat and bone meals is typical (Parson et al., 1997; Parsons et al., 1998). More recent collaborative work with The Ohio State University, Purdue University and University of Illinois further verifies such data (Adedokun et al., 2007).

Generally, however, digestibility data indicate that there is room for improving ingredient amino acid digestibility. And this holds true for ingredients that generally are considered to have good digestibility, and certainly for ingredients such as animal by-product meals.

Enzymes and enzyme combinations added to poultry diets
Most of the work published to date with protease is with proteases in combination with other enzymes (Zanella et al., 1999; Zyla et al., 2001; Ghazi et al., 2002; Hong et al., 2002; Cowieson and Adeola, 2005; Cowieson et al., 2006; Yu et al., 2007; Cowieson and Rivandran, 2008a and b). In most cases it is difficult to tease out the impact of the protease alone.

Cowieson and Ravindra (2008) reported that 500g/metric ton of an enzyme cocktail containing endoxylanaze, alpha-amylase, and subtilisin protease in corn-soy diets improved broiler weight gain and feed efficiency, and increased dry matter and AME over the diet without enzymes. Because the enzymes were not used individually, the impact of the protease cannot be determined. In a separate study (Cowieson and Ravindran, 2008b) with the same combination of enzymes in corn soy diets, enzyme addition improved broiler weight gain and feed efficiency. AME and N retention were also increased, but there was no impact on calcium and phosphorus digestibility. Yu et al. (2007) compared the effects of a protease in a commercial enzyme complex that included alpha amylase and endo-1, 4 beta xylanase with a pure protease. In corn soy diets, the pure protease improved in vitro digestibility of soybean meal, whereas the enzyme complex did not. In vivo, again, it was the pure protease that resulted in positive impacts on gain and feed to gain.

Multiple enzyme complexes (xylanase, protease, and amylase) have resulted in performance improvements in corn-soy diets (Zanella et al., 1999; Ghazi et al., 2002). In combination, phytases and carbohydrases have shown greater response on broiler growth than when used individually (Cowieson and Adeola, 2005). Hong et al. (2002) found that the use of an enzyme cocktail (xylanase, amylase and proteases) improved the digestibility of a corn-SBM diet for ducks. Zanella et al. (1999) reported a 3%
improvement in energy and amino acid digestibility of a corn-based diet for broilers when xylanase, amylase and protease were included in the diet.

Ambiguity exists across a number of these studies. Differences in dietary ingredients, proteases, various combination and levels of enzymes, animals and management, etc., all impact the interpretation and effectiveness measured for a particular enzyme or group of enzymes.

**Proteases**

The interest in proteases has increased in the last few years and new commercial proteases have entered the market recently, either in the US or abroad. The protease (ProAct®, DSM) is an alkaline serine protease derived from *Nocardiopsis prasina* and the production strain *Bacillus licheniformis*. Pepsin stability experiments find that 97% of this protease remains intact and active after being exposed to pepsin for 1.5 hrs, pH 3 and at 40°C (Novozymes Internal Reports).

The protease was found to be effective across a wide range of peptide bonds and considered to be a non-specific protease (Fischer et al., 2009. This protease is characterized by an optimal pH range that begins at pH of 5-6, and continues into the area of alkalinity. This range in operating pH complements existing endogenous proteases such as pepsin and others that operate optimally in an acidic pH.

Carvalho et al. (2009 a and b) and Bertechini et al. (2009 a and b) presented ileal digestibility results with this protease, which is summarized in Figure 2. This work confirms the ability of this protease to improve ingredient protein digestibility.
Figure 2. Apparent essential AA digestibility improvement in corn, soybean meal (SBM) full fat SBM and meat and bone meal (MBM) when 200 ppm protease were added over no protease added. From Carvalho et al. (2009ab) and Bertechini et al. (2009ab).

These researchers used the NFE diet or basal diet substitution methods to determine amino acid digestibility in different ingredients. This method is patterned after the procedure developed by Matterson et al. (1965) in which birds were fed a reference diet with significant portions being replaced by test ingredients. Across all ingredients tested, corn, soybean meal, full fat soybean meal and meat and bone meal, the protease improved amino acid digestibility. The improvement over the non-protease control ranged from about 2% to 14%.

Figure 3. Apparent essential amino acid digestibility improvement in different diet with 200 ppm ProAct® protease. Adapted from Favero et al., 2009, Maiorka et al., 2009 and Vila and Broz, 2008.

Other studies from Spain and Brazil were completed (Figure 3) with different diets. The digestibility improvement across essential amino acid was approximately 4% with the use of 200 ppm ProAct® protease.

In a study done at the University of Maryland in 2009 (Angel, 2010) with Ross 708 straight run broilers were fed corn soy diets with graded concentrations of this protease from 7 to 22 d of age in batteries. Six diets were fed. A positive control (PC) that was formulated based on AgriStats (2008) met or exceeded all NRC (1994) nutrient recommendations. The PC diet contained 22.5% crude protein. All other diets were negative control diets with the protease (0, 100, 200, 400, 800 ppm). The negative control diet contained 20.5% crude protein and a 9% reduction in the concentration of...
Lys, Met, TSAA and Thr was forced into the diet. All diets were isocaloric. The content of the distal ileum was removed with water, freeze dried, and ground and analyzed for marker, nitrogen and amino acids. Analysis of the diets confirmed levels close to formulated levels.

**Figure 4** contains amino acid digestibility results. There was no clear dose response for any of the essential amino acids. Overall, the improvement in essential amino acid digestibility due to the protease was 4.8% when 200 ppm was used with the greatest impact seen in threonine. The non essential amino acids were improved by an average of 3.72% with Asp and Ser being the most positively affected.

**Figure 4.** Amino acid digestibility improvements across various levels of ProAct protease (Angel, 2010)

Across several studies, threonine digestibility is improved to the greatest extent with the ProAct® protease. This is true for most individual ingredients, as well as corn/SBM diets fed for ileal amino acid digestibility determinations.
Conclusion
There is little information in the literature as to how proteases work in the intestinal tract, where in the tract they have the most impact and how they interact chemically with other exogenous enzymes as well as endogenous enzymes. It will be important as the role of proteases gains commercial application and importance that we better understand how these enzymes work and how they interact with the animal and other enzymes.

References

Angel, R.A., 2010. Use of innovative enzymes to reduce poultry feed costs: the protease case. Feeding the Genetics of Today, DSM Technical Symposium, Atlanta GA.


Fischer,


