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Toward optimal value creation through the application of exogenous mono-component protease in the diets of non-ruminants



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ABSTRACT

Exogenous protease has formed part of several commercial enzyme admixtures for the past 15–20 years but has become available as a mono-component enzyme only in the last 5–10 years (Cowieson and Adeola, 2005; Fru-Nji et al., 2011). As part of an enzyme admixture (often also containing various carbohydrases and/or phytase) it is difficult to separate the contributions of the component parts and the efficacy of each has implicit rather than explicit value. However, the effectiveness of mono-component enzymes can be readily assessed under different production and nutritional circumstances and their complementarity with alternative enzymes is readily rendered transparent. The motivation for the use of exogenous protease in non-ruminant nutrition is typically the reduction of feed cost at constant animal performance. This objective is usually achieved via displacement of expensive protein/amino acid sources in the diet through the assignment of a nutrient matrix on the enzyme in least cost formulation. Recently attention has moved from this classical approach to protease application to so-called ‘extra-proteinaceous’ effects including environmental benefits, litter management, enteric resilience, uniformity and microbial stability. Whilst these ‘secondary’ effects may not be the principal motivator for the use of proteases in animal production they are increasingly visible and require explanation. Furthermore, these responses, if understood, allow positive translation to production metrics such as reduced FCR and improved weight gain. It is the purpose of this review to briefly summarise the current state of the art in mono-component exogenous protease use in non-ruminants and to suggest mechanisms whereby the ‘extra-proteinaceous’ effects may be explained. Finally, key diet/animal factors that may promote beneficial effects of exogenous protease on live performance will be discussed.

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1. Introduction

Feed enzymes are an exciting success story. From humble beginnings in Scandinavian Europe with beta-glucanases, to growth in arabino-xylanases across Western Europe, Canada and Australia to the emergence of phytases, amylases and protease on a global basis (Bedford and Partridge, 2010). The global feed enzyme market today is very close to US\$1bn per annum and growing rapidly. Proteases, as stand-alone enzymes, are a newcomer to the feed enzyme market having only

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been the focus of specific marketing and research attention in the last 5–10 years (Cowieson and Adeola, 2005; Fru-Nji et al., 2011). However, protease activity has been declared in a range of admixture products for almost two decades though the beneficial effects were implicit rather than explicitly communicated. The research 'life cycle' of feed enzymes is somewhat predictable with initial focus on the target nutrient e.g. carbohydrases and energy, phytase and phosphorus, lipase and lipid and protease and protein but with extension to alternative nutrients following rapidly. In the case of phytase the early focus was on phosphorus and to a certain extent also on calcium, with amino acids and energy following thereafter (Selle and Ravindran, 2007). Today many nuances have emerged in phytase and phytate that consider effects on enteric health, protein accretion, involvement of *myo*-inositol, ion balance, phytate source and variation in characteristics etc (Cowieson et al., 2009). Given the foundational knowledge generated by at least 2–3 decades of sustained research in commercial application of feed enzymes (spearheaded by phytase) the technical knowledge cycle of protease is moving at an accelerated pace. Initial research in the usefulness of exogenous protease in animal production considered effects on live performance (predominately piglets and broiler chicks), with digestibility of nitrogen following thereafter (a more complete description is found below). Ileal digestibility of amino acids and alternative non-proteinaceous nutrients such as fat were explored and the interaction between protease and various dietary ingredients. Under contemporary terms of reference protease research is actively pursuing evidence that proteases beneficially alter gut health through changes to mucosal integrity, amino acid transport, environmental sustainability and gut tensile strength. It is the purpose of the present review article to describe the literature in the area of exogenous proteases in non-ruminant animal production and to suggest mechanisms which may be responsible for the effects of protease beyond amino acid and protein digestion. Finally, various factors that influence the magnitude and consistency of the efficacy of proteases are suggested and implications discussed.

2. Effect of exogenous protease on animal performance

Supplementation of production animal diets with exogenous protease was pioneered by Lewis et al. (1955) and Baker et al. (1956). Lewis et al. (1955) published a series of three piglet studies (one of which was conducted and reported in three separate parts resulting in a total of six experiments) and observed that pepsin, pancreatin and papain addition (1% inclusion) resulted in significantly reduced FCR. Furthermore the beneficial effects of pepsin and pancreatin on FCR appeared independent and when results from three studies were combined the combination of pepsin and pancreatin resulted in an increase in weight gain of around 27% and a reduction in FCR of around 43 points (2.49 vs. 2.06). When the various enzymes were fed independently, both pepsin (2.88 vs. 2.20) and papain (2.82 vs. 2.36) reduced FCR demonstrating that proteolytic activity was an important contributing factor rather than alternative enzymes in the pancreatin mixture. The focus of the work of Lewis et al. (1955) and Baker et al. (1956) was on weaning of pigs and the potential of soybean meal to replace skimmed milk powder.

Following the pioneering work of Lewis, Baker and colleagues in the 1950s with crude proteolytic extracts contemporary work has moved largely to explore the effect of proteases obtained from microbial fermentation rather than those sourced directly from e.g. pancreatic extraction. The first contemporary published report on the efficacy of a specifically produced mono-component exogenous protease in animals may be Castanon and Marquardt (1989) who assessed the potential of various enzymes (including a protease from *Bacillus licheniformis*) to improve the nutritional value of field beans for Leghorn chicks. The addition of 0.25% (but not 0.50%) protease to a diet consisting of >900 g/kg field beans resulted in a significant increase in weight gain from day 14–21 (61.0 g vs. 67.9 g) and a significant decrease in FCR from day 7–21 (2.63 vs. 2.49). Similar responses were not observed when the field beans were autoclaved prior to enzyme addition suggesting that some of the beneficial effects of the enzyme were associated with degradation of heat labile antinutrients.

Following the early work of Castanon and Marquardt (1989), Huo et al. (1993) noted that fungal and bacterial proteases could reduce the activity of trypsin inhibitors and lectins in raw soybean meal, albeit in vitro. Subsequently, Guenter et al. (1995) showed that protease improved weight gain (3%) and FCR (2%) of chicks fed a semi-purified canola-based diet. Following data presented by Guenter et al. (1995), Hessing et al. (1996) assessed the potential for an acid- or an alkaline-protease to reduce the antinutritional effects of lectin, trypsin and antigenic proteins in soybean meal. It was concluded that an acid- but not an alkaline-protease had the capacity to degrade glycinin and beta-conglycinin as well as Kunitz soybean trypsin inhibitor in vitro though there was no effect of either protease on lectin. Whilst these responses were reflected in performance outcomes (significant increase in feed intake and weight gain from day 7–27 and in coefficients of apparent ileal N digestibility; 0.76 vs. 0.85) the authors conclude that care must be taken when ascribing such effects purely to changes in the antinutritional effects of various proteins in soy. More recently Rooke et al. (1998) demonstrated that an acid-, but not alkaline-protease improved live weight gain of newly weaned piglets and reduced the antigenicity of soybean protein in vitro. For further reading the papers published throughout the 1990s on the effect of proteases on reduction in the effect of antinutritional factors in (mostly) soybean are well summarised by Thorpe and Beal (2001).

The interest in protease use specifically to degrade proteinaceous antinutrients appeared to decline in the late 1990s and into the new millennium and the focus moved to more classical nutrition and production metrics. Marsman et al. (1997) used a commercial neutral protease to explore the potential for improvement in the nutritional value of a semi-synthetic soy-based diet for chicks but observed no effect on weight gain or FCR despite a significant improvement in coefficients of apparent ileal N digestibility (0.837 vs. 0.852) and NSP disappearance (0.145 vs. 0.206). O'doherty and Forde (1999) observed improvements in the weight gain (0.862 vs. 0.887 kg/day) and FCR (2.62 vs. 2.50) of growing and finishing pigs fed diets containing 400 g/kg peas when supplemented with exogenous neutral protease. Ghazi et al. (2002) assessed the potential

of various proteases to improve the nutritional value of soybean meal in juvenile and adult broilers. Two proteases were considered, an alkaline protease from *Bacillus* and an acidic protease from *Aspergillus*, and it was noted that whilst both proteases reduced chick serum anti-soya antibodies on day 27 only the protease isolated from *Aspergillus* increased weight gain (36 g/day vs. 41 g/day) and apparent ileal N digestibility (0.78 vs. 0.83). Interestingly, using a precision feeding method, the acid protease from *Aspergillus* promoted an increase in true metabolisable energy from 11.4 MJ/kg to 13.1 MJ/kg in one experiment and from 11.0 MJ/kg to 12.6 MJ/kg in a second whereas the protease from *Bacillus* had no effect.

Differences in efficacy among various exogenous proteases has been demonstrated on several occasions and is not easy to explain given the limited detail on the proteases used. For example, [Simbaya et al. \(1996\)](#) observed significant differences in efficacy between 5 alternative proteases in improving broiler chick performance when fed on semi-synthetic diets based on canola meal. Unhelpfully the 5 proteases examined in this work are labelled G, D, M, N and O and no further information is shared on the source organisms, characteristics of the protein etc. Nonetheless, protease 'G' reduced FCR of chicks from 1.52 to 1.42 whereas, for example, protease 'D' had no effect (1.52 vs. 1.53). Interestingly, in a second experiment the effect of protease 'G' was confirmed in a diet containing 220 g/kg crude protein but not in a diet containing 170 g/kg crude protein suggesting that part of the reason for variable effects of protease on chick performance may be associated with crude protein concentration in the control diet. Finally, [Simbaya et al. \(1996\)](#) note that protease 'G' (the protease which generally gave the most promising responses across several experiments) successfully reduced FCR in a practical diet based on wheat and soybean meal (1.41 vs. 1.35) but had no effect in a wheat/canola-based diet (1.42 vs. 1.43).

[Odetallah et al. \(2003\)](#) noted improved performance of broiler starters when a corn/soy-based diet was supplemented with a keratinase from *Bacillus licheniformis* but the response was inconsistent across the three separate experiments that were reported. Furthermore, the beneficial effect of keratinase addition appeared skewed toward juvenile birds and the beneficial effects did not persist to market weight, an observation that was confirmed subsequently ([Odetallah et al., 2005](#)). [Angel et al. \(2010\)](#) assessed the dose sensitivity of a mono-component protease isolated from *Nocardioptis prasina* in young broiler chicks fed a corn/soy-based diet that had been reduced in crude protein and digestible amino acids by around 10% (22.5% in the positive control and 20.5% in the negative control with amino acid reductions proportional to these changes in crude protein). Weight gain from day 7–22 in the positive control was 711 g and in the negative control 661 g. Addition of the mono-component protease increased weight gain from 661 g to 708 g in a dose-dependent manner. Optimum weight gain and FCR responses were recorded at around 200 g/t of the product. Similar improvements in performance were observed in a study reported by [Freitas et al. \(2011\)](#) with the same exogenous protease. These authors fed broiler chicks from day 1–40 either a nutritionally adequate corn/soy-based diet or the same diet with slightly diluted crude protein and amino acids. The mono-component protease had no effect on weight gain of the birds but reduced FCR from 1.75 in the diet with reduced protein and digestible amino acids to around 1.72, depending on the dose applied (a quadratic response was observed).

[Wang et al. \(2008\)](#) explored the interactive effects of dietary protein source (SBM or cottonseed meal) and exogenous protease on performance of broiler chickens. Effects of protease were generally positive though higher in the starter phase than in the finisher phase, especially for weight gain. [Barekatin et al. \(2013\)](#) observed increased weight gain in broiler chickens fed a diet based on corn and soybean meal with graded concentrations of sorghum distillers dried grains with solubles when the diets were supplemented with exogenous protease. Furthermore, the effects were more pronounced, especially for FCR, when higher concentrations of sorghum distillers grains were included. [O'shea et al. \(2014\)](#) assessed the interaction between xylanase and protease in grower-finisher pigs fed diets based on rapeseed meal and wheat distillers grains with solubles and observed limited effect on performance but a significant increase in the apparent ileal digestibility of energy with the protease. Recently, [Zuo et al. \(2015\)](#) found that the addition of protease to the diets of weaned piglets increased average daily gain from 258 g to 293 g and reduced FCR from 1.21 to 1.17.

3. Effects on amino acid digestibility

It is curious that though logically an exogenous protease would be added to the diets of non-ruminant animals in order to increase the digestibility of protein and amino acids this was not the principal focus of much of the foundational research in this field. Nevertheless, the effect of exogenous protease on ileal amino acid digestibility is significant and moderately consistent and has been recently reviewed by [Cowieson and Roos \(2014\)](#). A total of 25 independent experiments were considered in the meta-analysis resulting in a total of 804 datapoints. The mean response in apparent ileal amino acid digestibility was +3.7% and this ranged from +5.6% for Thr to +2.7% for Glu ([Figs. 1 and 2](#)). The effects of protease on apparent ileal amino acid digestibility were independent of geographical influences, species (layers, broilers, pigs and turkeys) and diet composition (semi-synthetic, practical, wheat, corn, soy, meat meal etc). However, a substantial (47%) portion of the variability in the effect of exogenous protease on ileal amino acid digestibility was explained by the inherent digestibility in the control diet. When the inherent digestibility of amino acids in the control diet was less than 70% protease addition improved amino acid digestibility in 90% of cases with a mean improvement of around 10%. When the inherent digestibility of amino acids in the control diet was more than 90% there was a protease-mediated improvement in digestibility of around 2% ([Cowieson and Roos, 2014](#)). Similar responses have been previously observed for both phytase and xylanase ([Cowieson and Bedford, 2009; Cowieson, 2010](#)) and highlight the importance of routine raw material quality surveillance in order to maximise the consistency and value of exogenous enzymes. Furthermore, these observations bring into relevancy factors that may alter amino acid digestibility (and so axiomatically the efficacy of exogenous protease) either directly e.g. hydrothermal

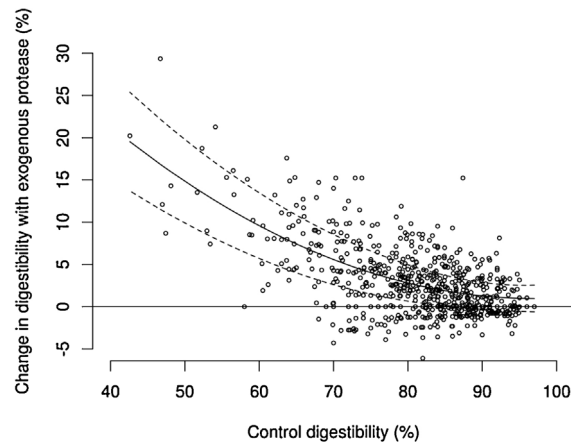


Fig. 1. Correlation between inherent amino acid digestibility in the control diet (%) and the effect of exogenous protease (% change relative to control diet). Solid quadratic line indicates the best fit model. Dotted lines are 95% confidence intervals. Solid horizontal line indicates zero effect of protease (from Ref. [Cowieson and Roos, 2014](#)).

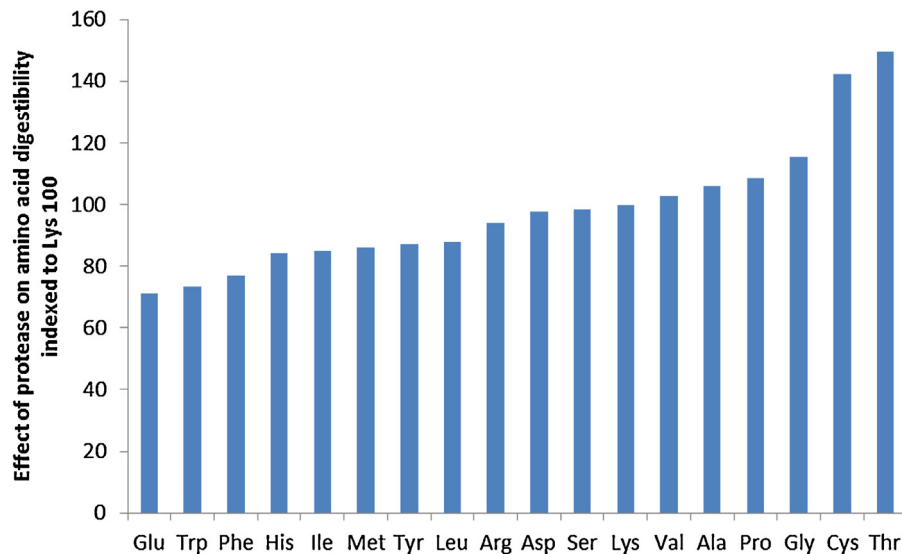


Fig. 2. Effect of protease on ileal amino acid digestibility in pigs and poultry index Lys 100.

conditioning, particle size, alternative zootechnical additives or indirectly e.g. species, animal age, lighting regime, stocking density, health status.

4. Extra-proteinaceous effects of exogenous protease

The effect of exogenous protease on ileal amino acid digestibility is clear and responses are summarised above. However, there is increasing interest in the past few years in ‘extra-proteinaceous’ effects of protease such as influences on enteric resilience, litter quality, interactions with non-protein nutrient digestibility e.g. fat or starch, carcass yields and so on.

The origin of the promotional effect of protease on gut health is not clear but may be a combination of several interacting factors. Such factors may include a reduction in putrefaction in the distal digestive tract ([Windey et al., 2012](#)), hydrolysis of proteinaceous antinutrients and antigenic proteins ([Rooke et al., 1998](#); [Ghazi et al., 2002](#); [Cowieson et al., 2015](#)), a shift in the site of macro-nutrient digestion to more proximal segments of the intestine ([Liu et al., 2013](#)) or enhanced gut physiology e.g. increased Glu for enterocyte energy metabolism ([Wu et al., 2014](#)), reduced viscosity of lumen contents ([Odetallah et al., 2003](#); [Barekatian et al., 2013](#)), altered flow of NSP in the intestine ([Olukosi et al., 2015](#)), improved retention of calcium and phosphorus ([Olukosi et al., 2015](#)), enhanced availability of amino acids for mucin synthesis ([Cowieson and Roos, 2014](#)), and enterocyte turnover or tight junction integrity ([Cowieson et al., 2015](#)). Putrefaction of protein in the distal intestinal tract has a range a potentially harmful consequences that depend on the extent of the putrefaction and the amino acid composition of the fermented protein. [Windey et al. \(2012\)](#) submits that while bacterial fermentation of carbohydrate generates mostly useful

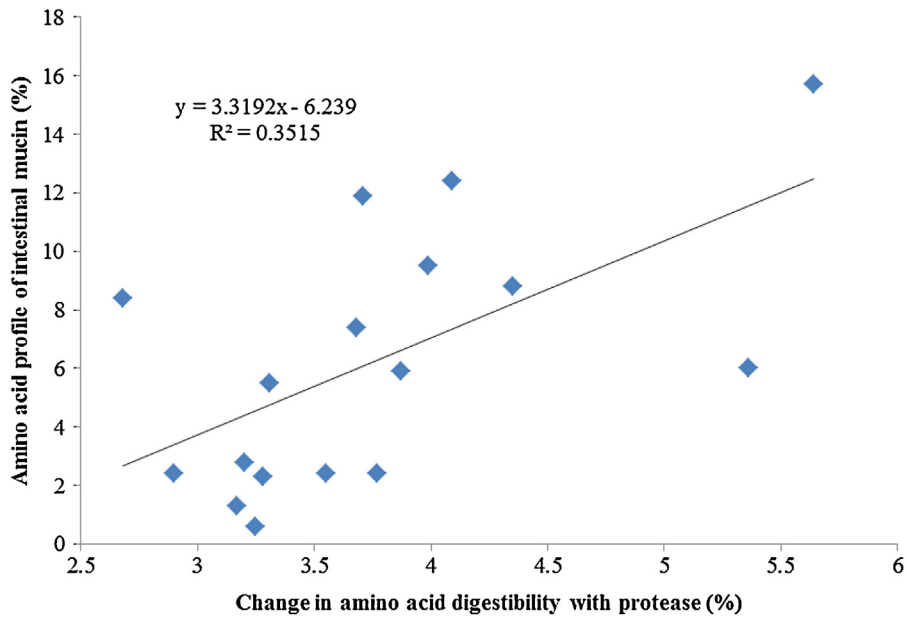


Fig. 3. Correlation between the amino acid profile of intestinal mucin and the effect of protease on ileal amino acid digestibility ($P=0.012$).

short-chain fatty acids such as butyrate the fermentation of protein generates a variety of harmful metabolites. Fermentation of branched chain amino acids (Val, Ile, Leu) generates branched chain fatty acids such as isobutyrate, isovalerate and 2-methylbutyrate. Aromatic amino acid (Phe, Tyr, Trp) metabolism by bacteria in the intestine produces phenolic and indolic compounds such as skatole. Fermentation of sulphur-containing mucoproteins and amino acids (Met, Cys and taurine) results in the production of hydrogen sulphide (Windey et al., 2012). Thus, the concentration, amino acid profile, digestibility and rate of digestion (both of which are influenced by exogenous proteases) of dietary protein and the magnitude of endogenous amino acid losses into the caudal segment of the gut will contribute to the extent of putrefaction and gut health in general.

Interestingly, such responses in enteric health are by no means restricted to recent literature and there have been several papers published in the past few decades that touch on this area. For example, Mynott et al. (1991) demonstrated that oral delivery of 'an enterically-protected protease preparation' Detach; Enzacor Technology Pty. Ltd., Melbourne, Australia was able to substantially reduce enterotoxigenic *Escherichia coli* (ETEC) attachment to the intestinal mucosa of rabbits. Protease supplementation also resulted in a significant reduction in colony-forming units per centimetre of intestine and a significant reduction in diarrhoea and diarrhoea-related death. Attachment of ETEC to the intestinal mucosa is facilitated by pilus adhesions known as colonization factor antigens and ingestion of certain exogenous proteases may modify the intestinal mucosa sufficiently to disrupt attachment (Sellwood, 1980). The observations made by Mynott et al. (1991) were confirmed subsequently in pigs (Mynott et al., 1996) and the area is well reviewed by Jin and Zhao (2000). Finally, Zuo et al. (2015) noted that protease addition to a corn/soy-based diet for newly weaned piglets resulted in a significant reduction in diarrhoea index (3.37% in the control, 1.84% with 200 mg protease/kg diet), a result that is in agreement with the early observations by Mynott and colleagues.

Involvement of mucin in the effects of exogenous protease was implied by Cowieson and Roos (2014). A statistically significant correlation between the effect of protease on amino acid digestibility and the amino acid profile of intestinal mucin was observed (Fig. 3) that was not apparent for alternative sources of endogenous protein. From this relationship it is plausible that a portion of the beneficial effect of protease on amino acid digestibility is conferred via a reduction in the loss of mucoprotein from the intestine, with self-evident implications for gut health. Importantly, Peek et al. (2009) noted that protease addition to a corn/wheat/soy-based diet alleviated the negative effects of coccidial (*E. acervulina*, *E. maxima*, *E. tenella*) infection in broilers, significantly increasing weight gain equivalent to uninfected birds. Furthermore, birds that received the diet supplemented with exogenous protease had a significantly thicker adherent mucus layer in the duodenum, jejunum and caecum compared with birds that received the unsupplemented control diet (Fig. 4). This result is in agreement with Cowieson and Roos (2014) as mentioned above and it may be that exogenous protease reduces the metabolic demand for mucoprotein by reducing the erosion of this layer by various antinutritional factors in the incoming feed matrix or through beneficial changes in the microbiome.

Cowieson et al. (2015) considered the effect of a mono-component exogenous protease on immune competence and jejunal health of broilers fed either a SBM-free diet (based on corn, canola meal and DDGS) or a SBM-based diet (standard corn/soy). Bird performance was more obviously improved in the diet based on SBM compared with the diet based on canola meal though ileal N digestibility was improved equivalently in both diets. Furthermore, protease application resulted in upregulation of Claudin1 and in various amino acid transporter proteins in the jejunum of broilers on day 21, an effect

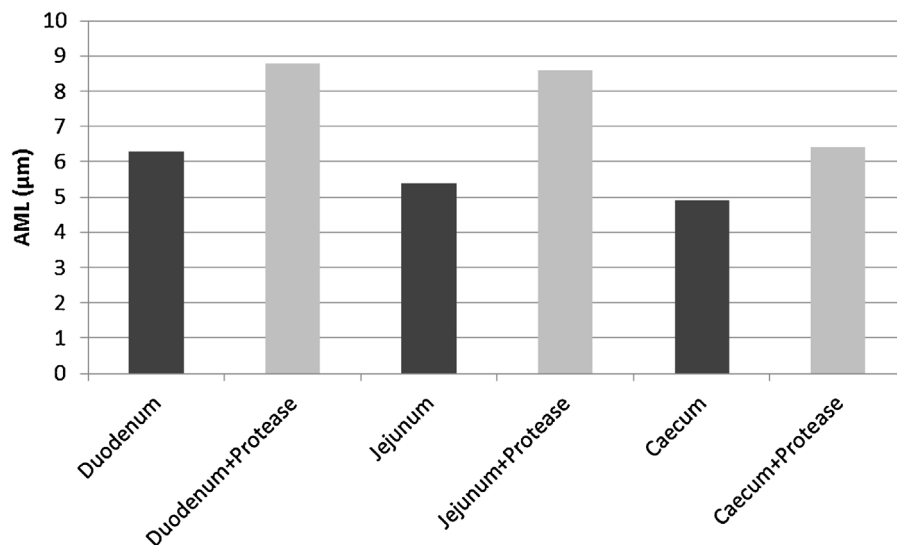


Fig. 4. Effect of exogenous protease on the thickness of the adherent mucus layer (AML; μm) in three intestinal segments in broilers. The effect of protease was statistically significant in each segment (adapted from Peek et al., 2009).

suggestive of increases in the integrity of tight junctions in the intestine and absorptive capacity *per se*. Tight junctions in the intestine control passage of nutrients and other luminal contents through the paracellular space between epithelial cells (Turner, 2009). These tight junctions are complex and involve tight junctions *per se* as well as subjacent adherens junctions, collectively known as the apical junctional complex (Turner, 2009). Failure of the apical junctional complex reduces the capacity of the gut to prevent influx of undesirable compounds and bacteria into the blood and so well-functioning tight junctions are desirable. The mechanism for increases in tight junction integrity with exogenous protease is not clear but may be related to improved availability of Lys and Pro by protease effect and the bioconversion of these to hydroxylated forms for collagen synthesis. This contention is partially supported by Cowieson et al. (2016) who observed complementary effects of added ascorbic acid (responsible for hydroxylation of Lys and Pro for collagen synthesis) and exogenous protease in broilers fed practical diets. For example, the tensile strength of the gut was increased from 3.8 N to 4.26 N by the addition of ascorbic acid and protease even though this was associated with a decrease in epithelial thickness suggesting substantially improved gut integrity. Further supporting evidence of the role of exogenous protease in gut morphology is presented by Wang et al. (2008) and Zuo et al. (2015) who observed significant increases in villus height and decreases in crypt depth when an exogenous protease was added to corn/soy-based diets for newly weaned piglets (Zuo et al., 2015) and broilers (Wang et al., 2008). It is also notable that Zuo et al. (2015) observed a significant reduction in diamine oxidase in the plasma of weaned piglets fed a diet based on corn and soybean meal. Diamine oxidase can be used as a biomarker for mucosal maturation and intestinal integrity suggesting beneficial effects of protease on such metrics.

Nutrients in most feed ingredients are present in a complex matrix involving starch and non-starch carbohydrate, protein, lipid and various minerals and vitamins. It is not surprising therefore that feed enzymes have a wide influence beyond their target/focal nutrients. For example, xylanases promote amino acid digestibility (Cowieson et al., 2009; Cowieson and Bedford, 2009) despite being considered principally as 'energy enzymes' and phytases have been shown to increase metabolisable energy (Selle and Ravindran, 2007). Similarly, proteases often increase the digestibility of non-protein nutrients and this may be associated with gross changes to the macrostructure of the nutrient matrix in the feed following proteolysis or perhaps via nuances involving endogenous secretion, gut health, active transport etc. Cowieson et al. (2015) noted that protease addition to a diet based on corn and soybean meal increased ileal digestible energy from 3077 kcal/kg to 3154 kcal/kg and apparent metabolisable energy from 3130 kcal/kg to 3261 kcal/kg. Kalmendal and Tauson (2012) reported that supplementation of a wheat-based diet for broilers with exogenous protease significantly increased the ileal digestibility of starch from 93 to 96%, of fat from 89 to 91% and AME from 13.68 to 14.16 MJ/kg. Increased energy digestibility in broilers associated with a mono-component protease (70.6 vs. 77.8%) was also observed by Fru-Nji et al. (2011) and improved fat digestibility (80.3 vs. 84.0%) by Freitas et al. (2011). Olukosi et al. (2015) reported an increase in AME of a corn/soy-based diet for broiler chickens from 2415 to 2453 and 2592 kcal/kg respectively for 0, 5000 or 10,000 protease units/kg of diet respectively. Further, Wang et al. (2008) noted that protease increased the total tract retention of starch significantly in young (day 18–21) and older (day 39–42) broilers fed either a diet based on soybean meal or cottonseed meal (increases from around 98% to 99.3%). These effects are too substantial to be explained by increases in protein digestibility alone and are likely to involve increases in the digestibility of starch and/or fat. Indeed, Cowieson et al. (2016) noted that protease addition to a wheat/soy-based diet resulted in a significant reduction in the concentration of taurine in the jejunal digesta (1097 vs. 870 mg/kg digesta) which is indicative of a reduction in bile secretion. Yuan and Wang (2010) noted that elevated taurine concentrations in the jejunum of broilers inhibited intestinal development by increasing total bile acid concentrations, an observation that was recently

confirmed (Huang et al., 2014). It is therefore possible that exogenous protease enhances fat digestibility by disrupting the feed nutrient matrix and reduces bile acid synthesis, secretion and intestinal taurine concentration which in turn promotes intestinal development.

5. Factors that may promote protease efficacy

The effect of exogenous protease on ileal amino acid digestibility, the major factor that promotes the magnitude and consistency of the effect is the inherent digestibility of amino acids in the diet or feedstuff in question (see above). Other factors which may promote the effect of exogenous protease on ileal amino acid digestibility per se are not clear but are likely to be related to the tertiary structure of the incoming protein and its characteristics e.g. globular, fibrous, hydrophobic or polar etc and also the presence of antinutrients that may stimulate an increase in endogenous protein flow. These two factors (the compatibility of the substrate with the exogenous protease and the presence of proteinaceous antinutrients) are likely to be centrally involved in scaling the response magnitude and in the consistency of the response per se. However, when it comes to the effect of exogenous protease on factors not directly related to ileal amino acid digestibility the picture is considerably more complex. For example, as mentioned above, application of protease often results in performance responses that are beyond what could be explained by the sum of effect on amino acid digestibility. The effect of exogenous protease on performance is likely to be promoted under certain dietary regimes and demoted by others.

Following a substantial meta-analysis of more than 50 independent broiler trials including more than 300 'control' diets, each fed with and without a mono-component exogenous protease (Cowieson et al., unpublished), several statistically significant factors emerged that are particularly associated with performance benefits. These factors include (+/- indicates whether this factor promotes or demotes a favourable effect of protease on weight gain and FCR): added fat/oil (+), amino acid balance (+/- depending on the individual amino acids and their ratios), presence of full-fat soybean meal (+), the dose of enzyme used (the higher the dose, the higher the response), limestone inclusion (-) and the balance of amino acids relative to energy (+/- depending on the individual amino acid). The relevance of some of these key factors above will be briefly reviewed.

Additional added fat promoted the beneficial effect of exogenous protease on weight gain and FCR in broilers. The influence of added fat on enzyme effect has been discussed before. For example, Cowieson et al. (2010) observed that reducing added fat in a broiler starter diet by 20 g/kg resulted in a significant (3–4%) reduction in ileal amino acid digestibility measured on day 21 and so blunted the efficacy of carbohydrases. Gehring et al. (2011) noted elevated efficacy of an enzyme blend when added to a corn/soy diet with increasing addition of mixer-added fat (1, 2.5 and 4%). These observations support previous reports 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 emptying which is driven in part by dietary fat concentrations (Gentilcore et al., 2006) i.e. low fat diets may reduce residency time of feed in the proventriculus/gizzard, or even residency of food in the intestinal tract per se (Mateos and Sell, 1980). It is interesting that the amino acids most detrimentally influenced by the removal of added fat are those amino acids which have been shown to be released last from the sequence of endogenous proteolytic mechanisms (Low, 1980). Importantly these observations have been made in both mash and pelleted diets suggesting mechanisms that extend beyond the effect of fat on thermo-mechanical damage to protein (including enzyme) in the pellet press, pellet quality and so on. The relevance of this for exogenous protease is not fully clear. However, most commercially-available proteases available currently are chymotrypsin-like semi-alkaline endo-peptidases (Glitsko et al., 2012) and function most readily on substrate that has had prior exposure to pepsin and low pH. It is conceivable therefore that additional added fat in the ration will delay gastric emptying and therefore increase the exposure time of dietary protein to pepsin and HCl, increasing the completeness of conversion of high molecular weight proteins to lower molecular weight soluble polypeptides, which in turn increases substrate availability for exogenous (as well as endogenous) peptidases.

That higher dietary concentrations of full-fat soybean meal leads to above average beneficial effects of protease on live performance is unsurprising and this has been well covered above. It is likely that full-fat soybean meal will draw heat labile antinutrients into the diet such as trypsin inhibitors and lectins as well as antigenic proteins such as glycinin and beta-conglycinin that may lead to inflammatory responses in the GI tract. Cowieson et al. (2015) show clearly that the ingestion of even fully processed SBM leads to sub-clinical inflammation in the intestine which exogenous proteases can partially mitigate. It is plausible that full-fat soybean meal leads to slight depression in bird performance depending on the thoroughness of thermal processing and that exogenous protease is able to address, even partially, some of these nutritional obstacles.

Dose response studies with exogenous protease are not commonly reported in the literature compared with similar titrations of phytase. However, studies by Angel et al. (2010) and Freitas et al. (2011) suggest that the beneficial effects of protease are increased by elevated inclusion concentrations in a linear (Angel et al., 2010) or quadratic (Freitas et al., 2011) manner. The dose response curve of any enzyme will be inextricably linked to both substrate availability and the outcome metric in focus and it is likely that dose responses will differ as diet composition, bird age etc change. However, most feed enzymes follow a distinct log-linear dose response curve and it is likely that this will also be true for exogenous protease. Optimising dose based on diet characteristics, bird age and which outcome is being specifically targeted e.g. meat yield, litter quality, feed cost reduction, is an area for future exploration.

Table 1

Current (as of 2016) commercially-available exogenous proteases. This table includes only products that are marketed as proteases and not products which include protease as a side-activity or as part of a wider admixture.

Product	Supplier	Declared activities by supplier
RONOZYME ProAct	DSM/Novozymes	Protease from <i>Nocardiopsis prasina</i> expressed in <i>Bacillus licheniformis</i>
CIBENZA DP100	Novus International	Protease from <i>Bacillus licheniformis</i>
AVIZYME 1512	DuPont/Danisco	Protease from <i>Bacillus subtilis</i>
POULTRYGROW250	Jefo	Protease from yeast (<i>Streptomyces</i> spp.)

Diets with lower limestone inclusion concentrations are more responsive to exogenous protease than those with higher limestone levels. This observation was unexpected and the reasons for this are not clear. One possible explanation for this observation is that lower limestone inclusion would typically be associated with (a) diets containing meat and bone meal or similar animal protein sources (b) phytase use (c) older animals i.e. grower/finisher stages. It is therefore possible that limestone is not directly involved in promoting protease effect but is a surrogate term that is multifactorial and is captured simply by the empirical nature of the exploratory model used. Limestone is however a buffering agent in the intestine and can interfere with protein solubility so it is possible that high limestone inclusion concentrations directly influence substrate availability via reduction in the effectiveness of gastric digestion (perhaps especially in young animals). These mechanisms require further specific exploration.

Finally, protease responses are linked to a variety of amino acid concentrations and ratios to each other and to energy. These interrelationships will be explicitly presented in a future publication and are too complex for full explanation here. However, amino acids that appear central to the translation of exogenous protease effect to beneficial performance outcomes are digestible Lys, Thr, SAA, Leu and Ile. Alternative amino acids were non-significant throughout the model development. The effect of protease on amino acid digestibility (Fig. 2) relative to Lys is relevant and may change the ideal protein balance in the diet. The interaction between dietary energy and specific amino acids, especially Leu and Ile is an area that warrants future study.

6. For the future

It is clear from the published literature that currently available exogenous proteases are effective tools to increase amino acid digestibility in non-ruminants. Furthermore, substantial beneficial effects on animal performance have been recorded that cannot be fully explained by the sum of the effect on amino acid digestibility, responses that point to 'extra proteinaceous' effects of protease. Presently there are rather few commercially-available mono-component proteases available for use in non-ruminant animal production, at least with reasonably wide regulatory coverage (Table 1) and all are alkaline serine endopeptidases (albeit with different kinetic characteristics and substrate specificity) which most closely resemble chymotrypsin (EC 3.4.21.x) in the endogenous protease array. However, considerable opportunity exists to develop novel proteases which are more functional at low pH e.g. pepsin-like or which specifically target proteinaceous antinutrients such as lectins, trypsin inhibitors, antigenic proteins or certain species of bacteria e.g. anti-adhesion proteases. Pepsin (aspartic endopeptidase EC 3.4.23.x) has shown some promise in early studies (see above) and there are several other proteases in the peptidase EC 3.4.x super-family such as cysteine proteases (EC 3.4.22.x) e.g. papain and bromelain metalloproteases (EC 3.4.17) e.g. carboxypeptidase A and B, that may have value in animal nutrition. Central to success will be the ability of the supplier to provide such proteases at costs sufficient to deliver substantial return on investment and to ensure regulatory coverage is as flexible as possible in dose and species application. Furthermore, the future of protease is highly likely to mirror phytase in that the focus will move from the primary target (protein/amino acids) to extra-proteinaceous effects just as the focus of phytase research today is not limited to phosphorus. As the understanding of the wide range of effects of protease grows the market for this unique enzyme will also expand and will lead to novel application options and multifactorial solutions to ongoing production concerns.

Moving forward, the animal protein industry is likely to come under increasing scrutiny in areas such as animal welfare, environmental sustainability and the interaction with human health and disease. Exogenous enzymes, including protease, play an important role in improving environmental sustainability of pig and poultry operations via reduced N emissions (Leinonen and Williams, 2015). Finally, considerable potential exists to more closely align enzymes such as protease (and phytase, carbohydrases etc) with alternative zootechnical additives such as pre-biotics, pro-biotics and phytochemicals to provide options for the animal protein industry as antibiotic growth promoters are displaced (Dersjant-Li et al., 2015).

7. Conclusions

It can be concluded that exogenous proteases have substantial value in animal production and effects that extend well beyond 'simple' amino acid and protein release values. Exogenous proteases disrupt the feed matrix liberating fat and starch, reduce the antinutritional effects of various dietary antagonists and promote intestinal resilience through enhanced emulsification of fats, reduced endogenous protein flow and improved mucin and intestinal integrity. Exogenous protease research is at an exciting tipping point akin to that of phytase 10–15 years ago when the questions asked are less to do with accepted responses in the focal nutrient and more to do with its central role in a multifactorial nutritional solution. The

benefits of protease use are wide ranging and will form an important part of responses to issues in weaning piglets, rearing animals without the use of prophylactic antibiotics, management of the environment at a local and global level and using protein sources of poorer quality to reduce nutritional competition between humans and animals. In short, when applied properly, exogenous proteases can create value at virtually every point in the food chain and despite some two decades of focussed research their potential remains tantalisingly untapped.

Conflict of interest

The authors declare no conflict of interest.

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