WHITE PAPER

EXOGENOUS PROTEASE: KEY LEARNINGS FROM A DECADE OF DEVELOPMENT

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EXECUTIVE SUMMARY

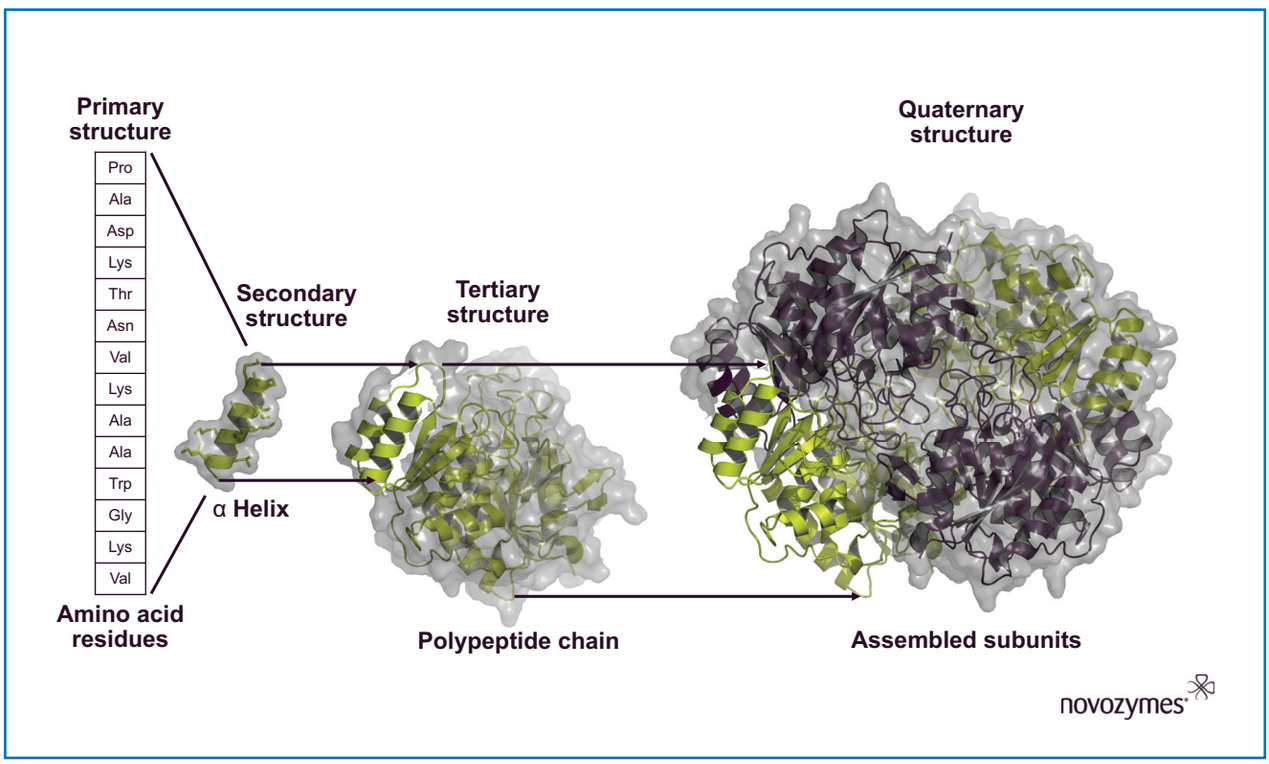
Mono-component exogenous proteases are a relative newcomer to the feed enzyme domain and in some sense application research and development is still catching up with the considerable growth in this feed enzyme segment. Initial motivation for the use of exogenous proteases was simply to reduce dietary crude protein concentration with a commensurate beneficial effect on feed cost and on environmental nitrogen burden. However, as the market segment has matured application optimization is improving as granularity increases for specific effects on individual feed ingredients (both for standardized ileal amino acid digestibility values and energy responses) and adjacent effects of proteases on intestinal resilience, mucin barrier function and nutrient transport. Furthermore, there has been a sustained focus on how exogenous proteases complement the activity of alternative feed enzymes such as phytases and carbohydrases and how they should be combined as part of a wider enzyme admixture with appropriate matrix values in least cost formulation.

There has also been important development in understanding the considerable diversity in the protease super-family which shows clearly that specific characteristics of the protease molecule (such as pH profile, substrate specificity and stability – thermal and gastric) are central to efficacy in the animal. Selection of the 'right' protease for a given application is enormously difficult as the vast majority of the proteases that are available are likely to be unsuitable for e.g. animal feed application. The DSM/Novozymes Alliance has a proven track-record in bringing effective, high quality and scientifically credible solutions to the livestock production industry. The purpose of this short technical review is to highlight some of our key learnings from a decade of development in the application of exogenous protease, to improve the sustainability of animal production.

INTRODUCTION

The exogenous feed enzyme market today is worth around USD$1bn per annum and saves the global feed industry in the region of USD$5bn per annum in reduced nutrient input costs. Additionally, the use of feed enzymes has a substantial impact on environmental, social and economic sustainability of the animal production industry. Initial commercialization of feed enzymes focused on carbohydrases (beta-glucanases and arabino-xylanases) to address the antinutritional effects of high molecular weight soluble pentosans in wheat, barley, rye and triticale and was oriented in Northern Europe, Australia and Canada. Concurrent to the growing carbohydrase segment, phytases emerged (in the early 1990s) to displace finite inorganic phosphates in the diets of non-ruminants and to reduce the antinutritional effects of phytic acid. Subsequently the carbohydrase market has expanded with additional activities such as alpha-amylase, alpha-galactosidase and beta-mannanase and extension to diets based on corn and sorghum and additional focus on the vegetable protein meals e.g. soybean and canola. More recently the feed enzyme marketplace has evolved with the adaption of elevated phytase inclusion concentrations to more quickly and completely degrade dietary phytic acid and the emergence of a new mono-component protease segment.

This series of evolutionary steps has led to unparalleled value creation for the end user of feed enzymes and a fantastic array of solutions for a wide number of nutritional challenges. However, with each evolutionary step the complexity has also increased, both in terms of the number of competing products per se and also appropriate evaluation of the combined value of feed enzymes for feed cost saving and animal performance. Recently, questions have arisen regarding the usefulness of mono-component proteases in diets that contain incumbent carbohydrase and phytase (the implication being that some diets may require only one or two enzyme products with substantive cannibalization of value with each additional enzyme addition). It is therefore the purpose of this short article to summarize the current state of the art in exogenous protease and to suggest how this enzyme class should be most appropriately used in feed formulation to maximise value creation with a focus on the presence of carbohydrases and phytase in the diet.



CHARACTERISTICS OF EXOGENOUS PROTEASES: NOT ALL PROTEASES ARE THE SAME

A common misperception is that any protease is as good as the other. Proteases however, constitute a huge enzyme class which differ significantly in both sequence, structure and functionality. The diversity of proteases is enormous with currently more than 600,000 different protease sequences publicly available (https://www.uniprot.org/), and an impressive diversity in terms of specificity, stability, temperature and pH profiles is found within the protease space. Proteases are categorized into different protease families and sub-families, based on sequence identity in the active site. The 6 main protease families are serine, metallo, cysteine, aspartic, glutamic and threonine proteases (Barrett *et al.* 2012). Furthermore, proteases are grouped into exo- and endo-peptidases. Exo-peptidases including di- and tri-peptidylpeptidases cleave from the end of protein chains releasing either amino acids, dipeptides or tripeptides. Endo-peptidases cleave within the protein chains releasing and solubilizing larger protein fragments. The site of cleavage further depends on the specificity of the protease. Very specific proteases such as the endogenous digestive protease trypsin preferably cleaves next to Lys and Arg, limiting the number of cuts in a protein chain, whereas proteases with a broad specificity have substantially more opportunity to catalyze hydrolytic events.

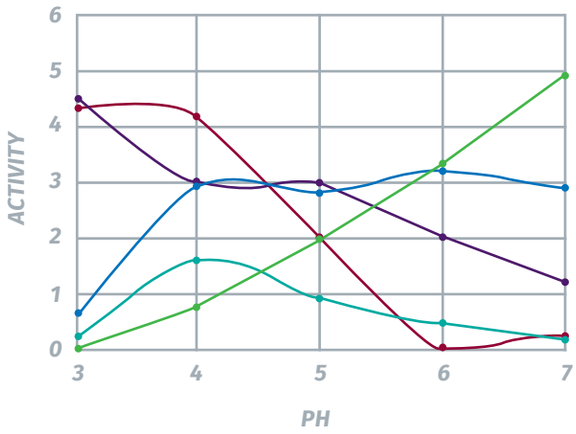
Given the huge diversity within the protease enzyme class, identifying and developing the right feed protease into an effective and valuable commercial product encompasses a large research effort and comprehensive evaluation of the enzyme under application conditions.

For application of proteases to animal feed, whilst the cleaving pattern and specificity is important, the activity profile of the protease as a function of pH and temperature should also be considered, as well as the stability of the protease. Additionally, the compatibility of the supplemental protease with dietary and endogenous proteins must be optimized. Proteases with activity at pH 2-3 could potentially have an effect during gastric digestion, whereas proteases with activity around pH 5-7 may operate more effectively in the small intestine. In Fig. 1, a range of pH profiles for proteases are shown, highlighting the vast difference between different proteases.

Considering stability, particularly pelleting stability and gastric stability are important parameters. The majority of feed for poultry is pelleted and most enzymes are supplied as granulates or otherwise dry products to be mixed with the feed and go through the pelleting process.

FIG. 1:

Examples of pH activity profiles for different proteases, emphasizing the significant difference in pH profiles. Even within a protease family, pH profiles can vary significantly. The activity at different pHs was measured on a 30:70 mixture of SBM-corn by monitoring the increase of amino ends in the solution following a 3h hydrolysis. Only physiologically relevant pH values were considered.



Hence, it is important that the protease can retain its activity during the high temperature and humidity conditions encountered during pelleting. This is often a challenge for enzymes, as survival at high temperatures is not essential in many biological environments. Pelleting stability can in some instances be improved by specific formulation/coating of the granulates, which in turn also offers protection from dust and thereby safe use of enzyme products. In addition to pelleting stability, proteases with a neutral to alkaline activity profile should also be able to survive the quite extreme conditions in during gastric digestion with very low pH, pepsin and mechanical shear, in order to be able to exert activity in the small intestine. Note that even if the pH activity profile of a given protease is moderately high and this enzyme is not expected to have significant activity at gastric pH, the enzyme must tolerate low pH (and aggression from HCl, pepsin and mechanical shear) such that when digesta pH is elevated in the small intestine the protease retains its activity.

Even when a suitable protease is found and appears to satisfy the criteria mentioned above, it is essential that the protease is included in the final product in a sufficient amount to be able to have an effect *in vivo*.

FIG. 2:

Protease activity of different commercial products claiming protease activity. The level of protease activity varies significantly among the products, with some products failing to show any protease activity at any pH. The products were tested at dose levels corresponding to the commercially recommended dose given by the supplier. Activity was measured on casein substrate at pH 3 (acidic), pH 7.5 (neutral) and pH 10.5 (alkaline). A = ProAct, B = Neutral protease, C = Acid protease, D = Keratinase, E = Serine protease, F = Neutral protease, G = Compound enzyme with protease, H = Combination of 3 “proteases”.

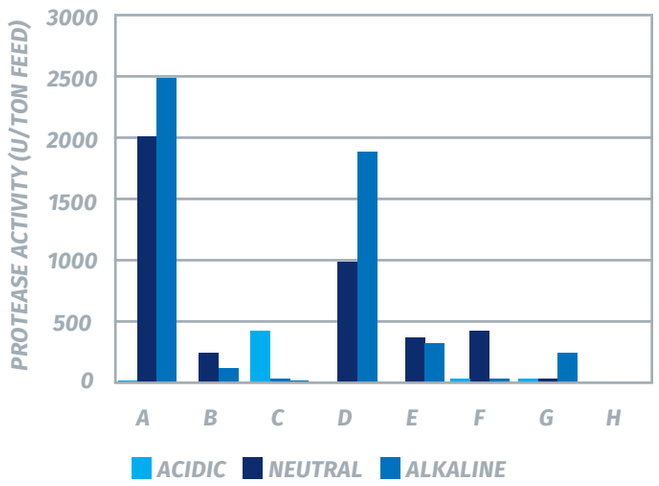
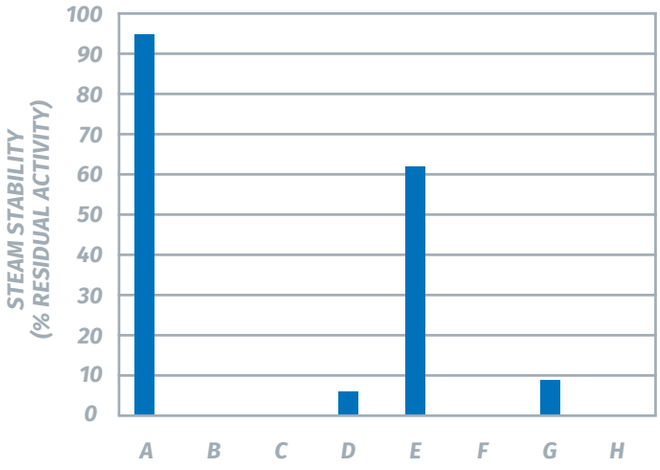


FIG. 3:

Steam stability (proxy for pelleting stability) for different commercial products claiming protease activity. Only a few commercial products can retain the protease activity after being exposed to conditions simulating the pelleting process in terms of high temperature and humidity. The granulated products were steamed at 95 °C for 90 seconds, and the residual activity was calculated by comparison to an untreated sample. A = ProAct, B = Neutral protease, C = Acid protease, D = Keratinase, E = Serine protease, F = Neutral protease, G = Compound enzyme with protease, H = Combination of 3 “proteases”.



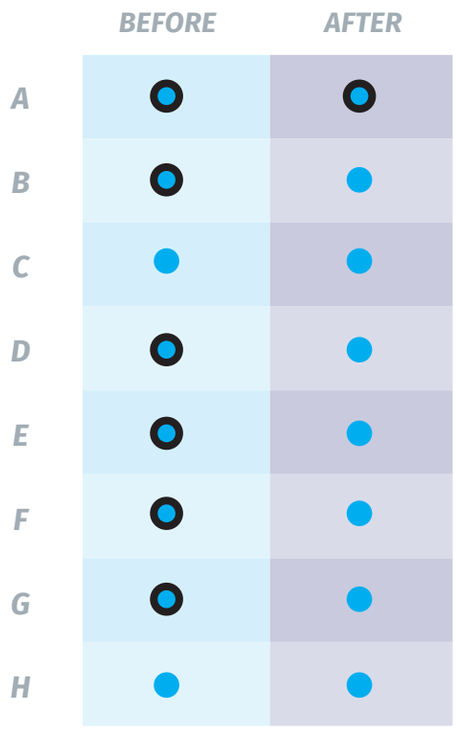
The amount of protease activity per recommended dose differs significantly among commercial proteases and multi-component products claiming proteases activity, as shown in Fig. 2, where the activity is measured on casein at 3 different pHs. Depending on the conditions chosen for activity measurement (pH, temperature, substrate), the ranking of products based on protease activity can vary, so for a useful comparison, physiologically relevant conditions are preferred. It can be argued that casein may not be an adequate substrate for specific proteases but casein is a highly digestible protein source and it is unlikely to find a protease that does not work on casein but works on the much more complex protein structures found in corn or soybean meal.

Steam stability (proxy for pelleting stability) and gastric stability of a range of commercial product claiming protease activity is shown in Fig. 3 and 4. The majority of products either do not offer a very high protease activity at recommended dose and/or contains proteases that are not gastric or steam stable. Thus, it can be concluded that the properties of commercial feed products on the market varies significantly, further adding to the complexity when evaluating the value of feed proteases in general.

Novozymes and DSM have continuously screened proteases for efficacy. In recent years, more than 600 novel experimental proteases have been screened *in vitro* and of those around 70 have been tested *in vivo*.

FIG. 4:

Activity of a range of commercial proteases measured as clearing zones on casein agar plates (pH 7) before and after gastric challenge (pH 3, 15 min). Only one product has retained the protease activity after gastric challenge, meaning that the protease activity would survive the gastric phase in the animal, to be able to be active in the small intestine. Product C was not expected to show any clearing zone before gastric challenge, as clearing zones are measured at pH 7 and this product only has protease activity at low pH (See fig. 2). Product H does not show any clearing zone in accordance with the measurements of protease activity (fig. 2), where no protease activity was detected for this product. A = ProAct, B = Neutral protease, C = Acid protease, D = Keratinase, E = Serine protease, F = Neutral protease, G = Compound enzyme with protease, H = Combination of 3 "proteases".



More than 80% of the proteases tested in vivo had no effect or even a negative effect on protein digestibility in livestock, proving that finding a protease which works efficiently in animals is not a simple task, and of all the possible proteases that potentially could be selected, only a few will be well suited and have a high efficacy as a feed enzyme.

Importantly, several protease suppliers have conducted only few animal trials with their products and in these trials they in general use double (or even higher) of the recommended dose. Barnard *et al.* (2016) is good example of this, where in the digestibility trial they use 10,000U of subtilisin protease/kg feed while the recommended dose is only 4,000U/kg feed. Product consistency starts with how the product is tested and how it is applied in field. In 2008, RONOZYME® ProAct was launched on the market as the first mono-component feed protease. The product contains a serine protease from the serine protease subfamily S1, and displays high pelleting and gastric stability. This endo-protease has a broad specificity and can cleave everywhere in a protein chain with only a slight preference for cleaving close to hydrophobic amino acids. In vitro testing shows the ability of RONOZYME® ProAct to increase protein hydrolysis of several important feed ingredients on top of endogenous digestive proteases, see Fig. 5.

Solubilizing and chopping down protein into smaller fragments to prepare the feed protein for further digestion by endogenous proteases and subsequent absorption is the main benefit of exogenous feed proteases. However, the ability to degrade anti-nutritional factors (ANFs) from e.g. soybean meal (SBM) is also an important property. SBM contains different ANFs such as trypsin inhibitors and lectin, the level of which mainly depend on the processing conditions. Many studies have shown that levels of ANFs differs in different SBM batches and that increasing levels of ANFs has negative consequences on the performance of animals (McNaughton et al. 1981; Palacious *et al.* 2004; Pacheco *et al.*, 2014). RONOZYME® ProAct has the potential to degrade trypsin inhibitors and lectin from SBM as shown in Fig. 6, and thereby help reduce the negative effect of such ANFs on animal performance.

FIG. 5:
Solubilization of protein by RONOZYME® ProAct on different feed-relevant raw materials. RONOZYME® ProAct performed well in all tested raw materials by increasing the solubilization of protein by 6-16 % relative to a control sample (containing pepsin and pancreatic proteases only). These data support RONOZYME® ProActs ability to work on top of the endogenous proteases, already present in the animal.

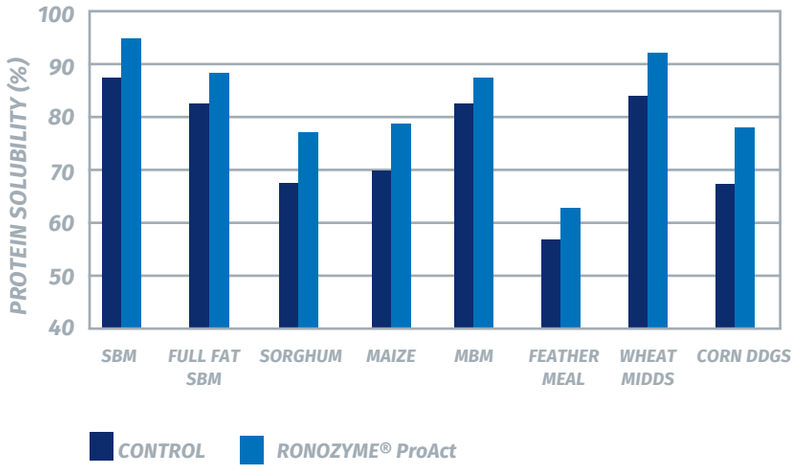
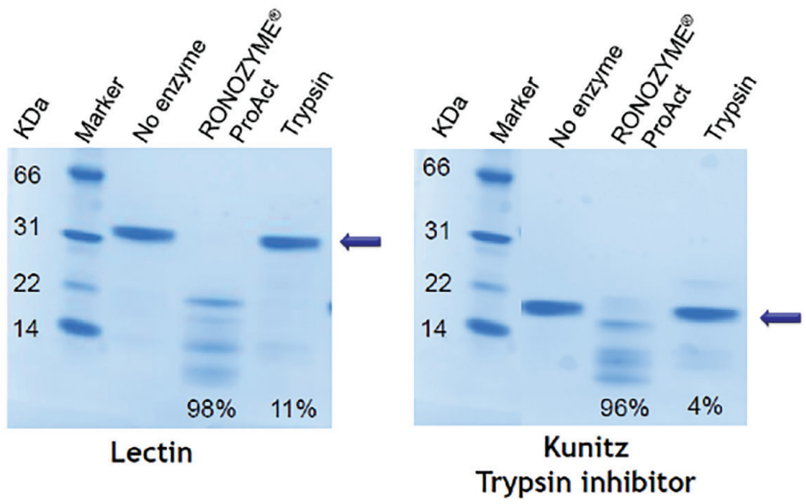


FIG. 6:
RONOZYME® ProActs ability to degrade Lectin and Kunitz trypsin inhibitor from SBM visualized by SDS-PAGE. The percentage numbers below give the degradation efficiency calculated from intensity of bands. 1 mg/ml purified ANF was mixed with 0.1 mg/ml RONOZYME® ProAct or trypsin at pH 7, 40 °C for 2 hours.

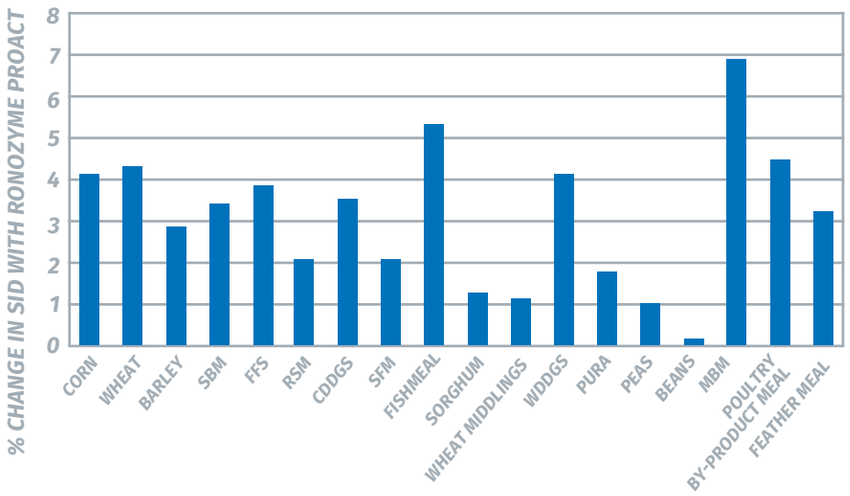


EXOGENOUS PROTEASE AND THE SPECTRUM OF EFFECT IN ANIMAL NUTRITION

Direct Effects

As the name implies, the direct effect of exogenous protease is hydrolysis of high molecular weight polypeptides into lower molecular weight oligopeptides (Glitsø *et al.*, 2012). This effect depends on the characteristics of the protease in question i.e. substrate specificity, pH profile and whether it is endo- or exo-acting etc. as described above. The substrate for exogenous proteases are recalcitrant dietary proteins, proteinaceous antinutrients such as trypsin inhibitors and lectins as well as dietary protein per se (where digestion rate may be increased relative to reliance on endogenous proteases alone). Hydrolysis of these proteins results in a significant increase in dietary protein digestibility (circa +4%; Cowieson & Roos, 2014), the extent of which depends on the amino acid, the raw material in question and the relative inherent quality of the protein therein. DSM Animal Nutrition has conducted more than 120 standardized ileal amino acid digestibility studies with RONOZYME ProAct since 2008 (and in all cases the dose used was the recommended commercial inclusion concentration of 200 g/kg of finished feed). The mean response for the most nutritionally-relevant amino acids (Met, Cys, Lys, Thr, Trp, Arg, Val, Ile and Leu) was +3.1% and this ranged from +1.6% for Leu to +4.8% for Cys. Various feed ingredients have been examined including cereals, vegetable protein meals, by/co-products and animal protein meals. Mean responses to exogenous protease ranged from +0.2% for field beans to +6.8% for meat and bone meal.

FIG. 7:
Effect of RONOZYME ProAct on the standardized ileal amino acid digestibility (SID; average of 9 amino acids) in common feed ingredients. SBM = soybean meal; FFS = full-fat soybean meal; RSM = rapeseed meal; cDDGS = corn distillers grains with solubles; SFM = sunflower meal; wDDGS = wheat distillers grains with solubles; MBM = meat and bone meal



Protease improved the SID of AA in corn by +3.9%, wheat by +4.4% and soybean meal by +3.5%. A summary of these responses is presented in Fig. 7. Importantly, there are substantial differences in the effect of protease by amino acid and also by raw material which necessitates care in the formulation of diets to accommodate protease effects i.e. flat amino acid or protein matrix values are likely to result in variable responses in performance.

It is important to note that not all exogenous proteases have beneficial functionality in the diets of non-ruminants. Cowieson & Roos (2016) summarise the results of a series of experiments with exogenous proteases (of diverse origin) that appear in the scientific literature and many have been observed to have little beneficial effect in the target species. One specific example of this was work by Simbaya *et al.* (1996) who examined the efficacy of a series of five distinct exogenous proteases for broiler chickens. These authors found that only one of these proteases had any beneficial effect on chick performance and even then the extent of this beneficial effect was dependent on the protein content and source in the diet to which it was added. Similar failure to demonstrate the efficacy of novel experimental proteases was recently published by Walk *et al.* (2018) and this is presumably associated with access to limited diversity of suitable protease candidates, overly simplistic diet formulation (no allowance for differential effects across amino acids and raw materials) and no acknowledgement of possible differences in effect with animal species and age.

Indirect Effects

Whilst the direct effect of exogenous protease is hydrolysis of the substrate (proteinaceous antinutrients and dietary protein that would otherwise escape digestion in the intestine), there are a range of significant 'extra-proteinaceous' effects (not unlike the extra-phosphoric effects of phytase). These effects are associated with benefits of protease on gut health (Wang *et al.*, 2008; Peek et al., 2009; Kalmendahl & Tauson, 2012; Cowieson *et al.*, 2015) and the improvement in the digestibility of alternative non-protein nutrients such as energy (mediated through increases in the digestibility of fat and starch). In fact a recent meta-analysis of published work with RONOZYME® ProAct on apparent metabolizable energy (AME) or ileal digestible energy (DE) revealed a mean increase of 81 kcal/kg (Table 1). These effects have been recently confirmed in a net energy (NE) study with broilers (Cowieson *et al.*, 2018a) where an increase in AME of 73 kcal/kg and of 107 kcal/kg in NE was observed in diets that contained both xylanase and a high inclusion concentration of microbial phytase.

TABLE 1:

Summary of effect of exogenous protease on energy digestibility and metabolisability in broilers.

Publication	Diet Type	Broiler Age	Control Diet AME, kcal/kg	ProAct AME, kcal/kg	Delta, kcal *Delta*, %
Fru-Nji et al. (2011)[1]	Corn/SBM	D36	3035	3247	212 7.2
Fru-Nji et al. (2011)[2]	Corn/SBM	D36	3202	3253	51 1.6
Freitas et al. (2011)[3]	Corn/SBM/MBM Low CP/Low ME	D42	3465	3345	-120 -3.5
Freitas et al. (2011)[4]	Corn/SBM/MBM High CP/Low ME	D42	3577	3512	-65 -1.8
Freitas et al. (2011)[5]	Corn/SBM/MBM Low CP/High ME	D42	3523	3553	30 0.8
Freitas et al. (2011)[6]	Corn/SBM/MBM High CP/High ME	D42	3441	3635	194 5.6
Kalmendal & Tauson (2012)[7]	Wheat/SBM	D34	3271	3385	114 3.5
Olukosi et al. (2015)[8]	Corn/DDGS/SBM/Canola	D20	2415	2592	177 7.3
Cowieson et al. (2016)[9]	Corn/SBM	D21	3130	3261	131 4.2
Cowieson et al. (2016)[10]	Corn/canola/DDGS	D21	3095	3123	28 0.1
Sorbara & Cowieson (2018)[11]	SBM	D24	2596	2706	110 4.2
Sorbara & Cowieson (2018)[12]	FFSBM	D24	3279	3409	130 3.9
Cowieson et al. (2018)[13]	Corn/Wheat/SBM/Canola Regular Diet	D24	3178	3238	60 1.9
Cowieson et al. (2018)[14]	Corn/Wheat/SBM/Canola Low protein diet	D24	3164	3250	86 2.7
Summary			3169	3251	81 2.6

[1] also reported an increase in ileal digestibility of fat from 88.6 to 91.1% (P < 0.05). Value reported here is ileal DE, not AME. Protease used was from DSM.

[2] effect reported was ileal DE (P < 0.05) but no significant effect on fat was observed. Protease used was from DSM.

[3] a non-significant increase in fat digestibility was observed (76.5 to 79.5%). AME change was non-significant. Protease used was from DSM.

[4] a non-significant increase in fat digestibility was observed (80.5 to 84.9%). AME change was non-significant. Protease used was from DSM.

[5] a non-significant increase in fat digestibility was observed (83.6 to 86.1%). AME change was non-significant. Protease used was from DSM.

[6] fat digestibility was increased from 80.6 to 85.3% (P < 0.05) and AME change was significant (P < 0.05). Protease used was from DSM.

[7] also reported an increase in total tract digestibility of fat from 89.3 to 91.2% (P < 0.001) and starch from 93.1 to 95.8% (P < 0.001) and a trend (P = 0.09) to increase total tract protein digestibility from 59.7 to 64.3%. Absolute value reported is AMEn on a DM basis. Protease used was from DSM.

[8] also reported an increase in ileal fat digestibility from 83.5 to 84.1% (NS). AME response was significant (P < 0.01). Protease used was from DuPont.

[9] also reported an increase in ileal DE from 3077 to 3154 kcal/kg (P < 0.05) and ileal N digestibility coefficient from 0.775 to 0.788 (P < 0.05). AME value reported on an as-fed basis. Protease used was from DSM.

[10] increase was not statistically significant. Protease used was from DSM.

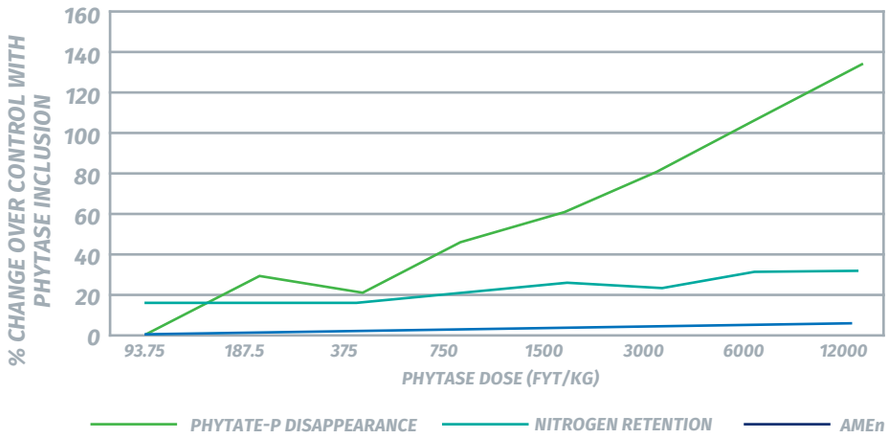
[11] included a total of nine different batches of SBM. Protease used was from DSM.

[12] included a total of nine different batches of FFSBM. Protease used was from DSM.

[13] AME effect was significant. Also reported a significant increase in net energy (2376 vs. 2468 kcal/kg) and retainable energy. A non-significant increase in ileal starch digestibility was reported (94.8 to 95.1%). Protease used was from DSM.

[14] AME effect was significant. Also reported a significant increase in net energy (2398 vs. 2519 kcal/kg) and retainable energy. A significant increase in ileal starch digestibility was reported (93.6 to 97.3%). Protease used was from DSM.

FIG. 8:
Effect of increasing phytase dose on phytate-P disappearance, nitrogen retention and nitrogen-corrected apparent metabolizable energy (AMEn) in broilers fed a corn/soy-based diet (Shirley & Edwards, 2003).

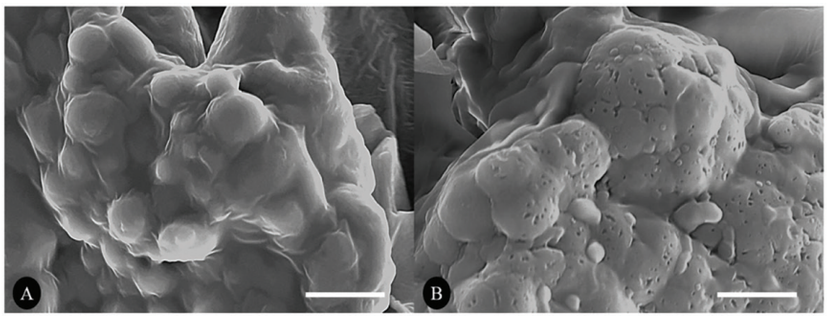


INTERACTIONS BETWEEN PROTEASE AND OTHER ENZYMES ON AMINO ACID DIGESTIBILITY

A common concern with the use of multiple enzymes in the same diet is cannibalization of value. There is no doubt that whilst different enzyme classes (phytases, carbohydrases and proteases) do not share common substrates, the hydrolysis of these divergent substrates results in a range of similar effects in digestibility that have a cumulative (but likely not fully additive) effect on animal performance. For example, carbohydrases (largely xylanases and glucanases) (Cowieson, 2010), phytase (Cowieson *et al*., 2018b,c) and protease (Cowieson & Roos, 2014) have all been shown to increase ileal amino acid digestibility in non-ruminants. In the case of amino acid digestibility improvement with exogenous enzymes these come both from a reduction in endogenous amino acid flow (as is strongly the case for phytase) to more genuine improvements in the digestibility of dietary protein (as is the case with proteases and carbohydrases). The lack of additivity across these enzyme classes is largely associated with the overlap in effect on endogenous loss (as is well appreciated for evaluation of amino acid digestibility in single feed ingredients and led directly to the development of the SID assay). In the case of phytase the effects on amino acid digestibility can be directly related to reduced loss of endogenous proteins (largely from mucin but also from pepsin). These effects are linked to the effect of phytate on protein solubility in the intestine and the increase that this has on endogenous mucin and pepsin production (summarized in Cowieson *et al*., 2009). Indeed, Cowieson *et al*. (2008) noted direct correlation between the effect of phytate and phytase on the flow of endogenous amino acids in broiler chickens and the amino acid profile of mucin and pepsin. Furthermore, the stimulation of excess endogenous amino acid loss by the ingestion of phytic acid can be readily resolved by rather low inclusion concentrations of microbial phytase, with no further benefit evident when inclusion is increased above around 500-750 FYT/kg (Cowieson *et al*., 2018b,c), results that have been observed previously (Shirley & Edwards, 2003; Fig. 8). This fact suggests that any cannibalization of value of carbohydrases or proteases by phytase on amino acid digestibility will be evident with low inclusion concentrations of phytase and this will not change as phytase dose is elevated. In the case of carbohydrases the effects on amino acid digestibility appear largely unrelated to endogenous amino acid flow (based on the fact that a large meta-analysis showed that no single amino acid is benefitted more than any other by xylanase or glucanase addition, a fact that would not be the case if endogenous protein flow was disproportionately influenced relative to dietary protein digestibility; Cowieson & Bedford, 2009). This suggests that the effect of carbohydrases on ileal amino acid digestibility may be largely additive with that of phytase given that the former modifies dietary digestibility (putatively through generic mechanisms involving gastric residency of feed, hind gut fermentation, reduced viscosity of the lumen contents and de-caging of cell wall contents) whereas the latter modifies endogenous protein flow.

Finally, proteases deliver improvements in amino acid digestibility partially by reducing endogenous mucin loss (approx. 30% of their effect) and partially by increasing dietary amino acid retention (Cowieson & Roos, 2016). Thus, some erosion of phytase effect on amino acid digestibility by the addition of protease may be anticipated, especially for the amino acids commonly found in endogenous protein e.g. Thr, Cys, Gly, Ser and Pro.

FIG. 9:
Sections of fixed soybean cotyledon tissue untreated/ treated with RONOZYME® ProAct and visualized by scanning electron microscopy. Numerous holes are observed in the protein storage vacuoles of the enzyme treated tissue. RONOZYME® ProAct concentrations in mg enzyme protein (EP)/mL: (A) 0 mg EP/ mL, control, (B) 1.4 mg EP/mL. Scalebar 10 µm.



PROTEASE AND PHYTATE ACCESSIBILITY

Soybeans store proteins in protein storage vacuoles (PSVs), which are globular structures consisting mainly of protein, phytic acid and minerals (Jiang *et al.* 2001). The phytic acid is concentrated in electron-dense parts of the PSVs called phytate globoids. Because phytate in globoids may be imbedded in proteins, it is not expected to be readily accessible to dephosphorylation by a phytase. Bohn *et al*. (2007) showed that phytase degrades free and soluble phytate significantly faster than phytate located in phytate globoids. The proteins in the globoid may retard the action of phytase by blocking access to phytic acid. RONOZYME ® ProAct has been shown to degrade the proteins in PSVs, which leads to increased solubilization of phytic acid, that can then readily be dephosphorylated by phytase.

The effect of RONOZYME® ProAct on PSVs from soy is shown in Fig. 9. Sections of soybean cotyledon tissue were fixed, dehydrated and incubated with RONOZYME® ProAct and the effect of the protease was visualized by scanning electron microscopy. Numerous holes are observed in the protein storage vacuoles of the enzyme treated tissue, showing the degradation of protein in the PSVs. The effect of protein degradation on solubility of phytic acid was investigated by incubating full fat soybean meal with different dosages of RONOZYME® ProAct and measuring the release of phytic acid after enzyme treatment. An increase in phytic acid release was observed when the protease dosage increased (Fig. 10). The treatment with the highest dosage of protease lead to 9.7% more soluble phytic acid, showing that the protease has an indirect effect on phytic acid release. The results on phytate solubilisation indicates that application of a protease in feed may increase not only protein digestibility but also increase availability of phytic acid, which can then be hydrolyzed by feed phytases. These effects have also been confirmed *in vivo* (Vieira, 2015) where the addition of RONOZYME® ProAct to a corn/soy-based diet that already contained 2000 FYT/kg of RONOZYME® HiPhos resulted in an increase in tibia phosphorus concentration in broiler chickens (from 4.6% to 5.0%).

FIG. 10:
Increase in phytic acid release compared to control (%) plotted against protease dosage (mg enzyme protein/mL). An exponential model was fitted to the values (R^2= 0.997).

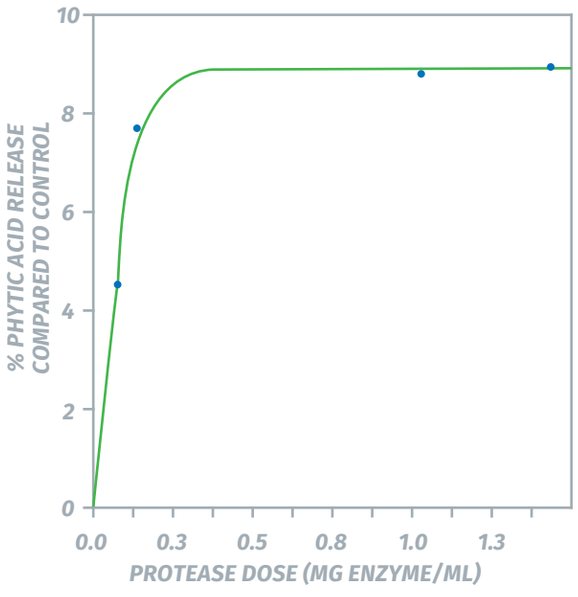
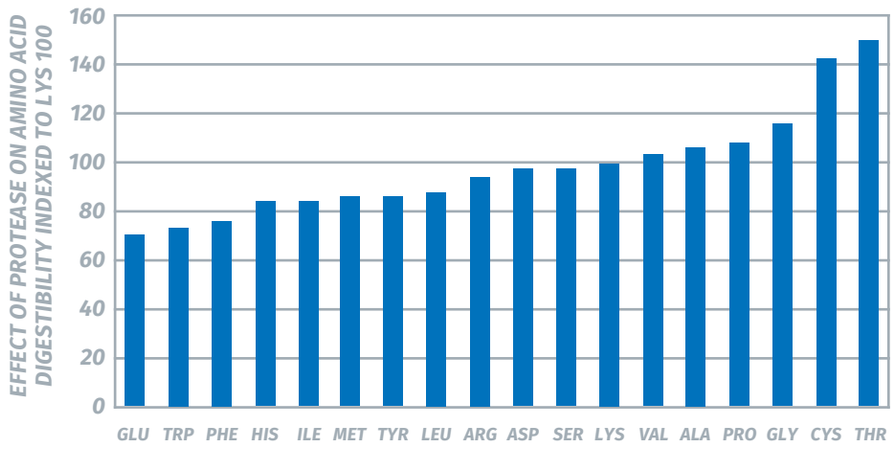


FIG. 11:
Effect of RONOZYME® ProAct on ileal amino acid digestibility in non-ruminants expressed relative to Lys (Cowieson & Roos, 2014).



OPTIMISING THE VALUE CREATION WITH EXOGENOUS PROTEASE

Appreciation of raw material differences

There is no doubt that exogenous proteases do not influence all protein sources to the same extent (Fig. 7) and this is likely associated with the specificity of a given protease for different protein types e.g. hydrophobic or polar proteins, variable charges, amino acid sequences and so on, as well as the inherent digestibility of amino acids from different raw materials. This is also the case for the endogenous protease array where some dietary proteins have been shown to largely escape digestion. For example, Cowieson *et al.* (2017) used proteomics to determine the origin of peptides in the ileum of broilers that had been fed a corn/soy-based diet and noted that the majority of peptide fragments were from undigested storage proteins from soybean meal. Thus, it is not surprising that specific exogenous proteases also 'favour' some protein sources more than others and so have varying compatibility with different dietary raw materials. RONOZYME® ProAct has a particularly strong affinity for protein from animal protein meals and also protein from corn, wheat, barley and soybean meal. Optimization of protease effect *in vivo* can be done by careful consideration of the diet mix (source and proportionality of the dietary protein) to generate a suitable response matrix for that specific diet.

Appreciation for divergence across amino acids

The formulation of diets for non-ruminants relies heavily on maintenance of appropriate amino acid density and also ratios to lysine to ensure balanced provision of amino acids to support growth. As exogenous proteases do not release amino acids in equal proportion (Fig. 8-10; Cowieson & Roos, 2014) it is imperative, if the beneficial effects on digestibility are to be transferred to measurable performance gains, that formulation with proteases are done in acknowledgement of divergent amino acid responses. These responses are specific for individual feed ingredients (see above; Fig. 7) but also for specific amino acids. Generally speaking, relative to Lys, the improvements associated with protease are more substantial for Thr, Cys, Gly, Pro, Ala and Val and less substantial for Glu, Trp, Phe, His, Ile, Met, Tyr, Leu, Arg, Asp and Ser (Fig. 11). This means that the use of protease may change the ideal amino acid ratios in a given diet (and will do so if a flat matrix is applied to the protease that does not acknowledge the different response magnitudes by amino acid). It is therefore critical that formulation of diets to accommodate proteases are not done naïvely with no regard for the influence of a particular raw material mix and amino acid density and balance.

Adjacent factors

In addition to the effect of exogenous protease on specific raw materials and amino acids there are a range of additional considerations that may influence the magnitude and consistency of effect (for a detailed review please see Cowieson & Roos, 2016). One such factor is the quality of the protein in a given raw material e.g. a batch of SBM with an inherently low digestibility will give elevated response to protease compared with a batch of higher quality. It has been conclusively demonstrated that protease effects are substantially greater and more consistent when ileal digestibility of amino acids in a given diet/raw material is below 90% compared to above (Cowieson & Roos, 2014) and this gives considerable latitude for strategic intervention. For example, factors that may promote or demote protein digestibility may be conceptually linked to exogenous protease use e.g. animal species, age, feed processing, particle size and so on. Importantly here it is relevant that creation of a 'negative control' diet to examine exogenous protease effect by stripping protein from the feed is highly likely to be disadvantageous as (a) substrate is being removed (b) amino acid balance is lost (c) the animal reacts to protein insufficiency by upregulation of peptide transporters, so increasing the digestibility of the remaining protein in the diet. Furthermore, ensuring an adequate supply of limiting nutrients e.g. energy and phosphorus is critical to ensuring that the beneficial effect of protease is not lost. Finally, specific to the characteristics of RONOZYME® ProAct, this particular protease is advantaged by maintenance of higher concentrations of added fat, lower limestone use and higher dietary AME (Cowieson & Roos, 2018). The reason for these nuances are not fully clear but it may be related to the need for satisfactory gastric digestion to prepare the incoming protein for the pancreatic proteases (as RONOZYME® ProAct is somewhat similar to chymotrypsin in terms of specificity and so is more compatible with substrate that has undergone some gastric preparation).

CONCLUSIONS

Development of exogenous proteases that are functional in pigs and poultry is extremely difficult. Many such enzymes do not work well as feed enzymes and lack compatibility with endogenous enzymes or the type of substrates presented by common feed ingredients. Furthermore, once a suitable protease has been identified it cannot be simply added to a diet and helpful performance responses observed. Rather, a significant body of work is required to map the effect of the protease across multiple raw materials and amino acids in order to ensure that diet formulation is done strategically. It is also relevant to note that the beneficial effect of exogenous proteases is not restricted to improvements in amino acid digestibility but also extend to energy partitioning/efficiency, environmental sustainability, gut health and other valuable production metrics. Finally, while some moderate erosion of amino acid benefit may be expected when multiple enzymes are added to the same diet these effects are likely to be small and oriented specifically to those amino acids found in endogenous protein e.g. Thr. It can be concluded that exogenous proteases are effective in improving the sustainability of non-ruminant animal production systems. However, they cannot be applied naively without first establishing the foundational knowledge needed to deploy them effectively. Once this foundation is in place exogenous proteases offer significant potential to enhance the profitability of poultry and swine production globally and to assist in maintaining health and welfare of animals under the increasing challenges associated with feeding a growing global population.

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