Optimizing Performance and Profit for Better Sustainability: A Review on Protease Application in Aqua Feed

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Abstract

Optimizing utilization of nutrients in aqua feeds by supplementing various dietary enzymes have recently been considered by nutritionists and formulators worldwide to combat fluctuating price, availability and quality of commonly used plant and animal proteins. Biological factors such as growing stage, age, species types, environmental factors such as temperature, pH and dissolved oxygen, and feed composition appeared to significantly affect endogenous enzyme production. A better understanding of these effects on digestive proteases will allow optimizing the use of supplementary enzymes, and in turn, will assist in better utilization of dietary nutrients.

The main objective of using dietary protease has been to compensate digestive enzymes to promote growth and efficiency of nutrient utilization and reduce nutrient excretion. Little focus has been paid to assessing their effects on improving digestibility of raw materials or feed quality or gut health and specific immune response. This review mainly digestive proteases, their interactions with various ingredients or diet composition in different fed species, use of dietary proteases and its implications on the industry in optimizing performance and profit.

Keywords: digestive enzyme; dietary protease; aquafeed; profit optimization;

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Introduction

The historical use of dietary enzymes started with the application of amylases and proteases to the diets of various farm animals for better productivity in 1950s. Since then, the use of enzymes has been expanded to other carbohydrases, phytates and recently, to lipase in terrestrial animals (1). The feed enzyme market is dominated by phytases and carbohydrases. However, as with the increasing cost of proteins, application of protease is on the rise specifically in poultry feed and expected to exceed those of phytase in the coming decade.

Application of protease in aqua feed is not new and the research endeavor can be traced back to 1977, when graded level of a commercially available bovine trypsin was used in common carp diets (2). Until recently, studies on application of dietary protease in aqua feed have been sporadic (3). Dietary protease can compensate the deficiency of endogenous enzymes especially for young animal, and assist in the breakdown of macromolecular proteins, which are difficult to digest. Until now, studies regarding exogenous protease supplementation in aquafeeds have been reported in rainbow trout (*Oncorhynchus mykiss*) (4, 5, 6, 7, 8,), tilapia (*Oreochromis aureus* × *O. niloticus*) (9, 10), common carp (*Cyprinus carpio*) (11), Atlantic salmon (*Salmo salar L.*) (12), and Pacific white shrimp (*Litopenaeus vannamei*) (9, 13, 14).

Digestion, a complex physiological process, depends on molecular activation, recognition and hydrolysis of food in specific conditions. During the process, the digestive enzymes break down polymeric macromolecules into smaller building blocks facilitating and enhancing nutrient absorption. Presence of proteases for protein, amylases for carbohydrate, and lipases for lipid digestion in various fish (15, 16) and shrimp (17, 18, 19) species has already been reported. Detail knowledge on digestive physiology of aquatic animals is a prerequisite when formulating feeds. There are great differences in specific activities of alkaline protease for various fish and crustacean species. And within a species, activity of specific enzymes showed great deal of variations during larval developments,

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food and feed composition and understanding these variations is of great importance for the development of artificial feeds (20).

This paper highlights dietary influence on digestive protease activity and effects of dietary inclusion of alkaline proteases on performance in various fed aquaculture species.

Digestive protease

Dietary protein level, protein sources, age or life-stage, all can affect digestive enzyme activity in aquatic animals. For example, significant changes in digestive enzymes of rainbow trout (*Oncorhynchus mykiss*) not in sea bream (*Sparus aurata*) were reported when dietary fish meal were gradually replaced by plan proteins (0% - PP0, 50% - PP50, 75% - PP75, and 100% - PP100) in their diets (21). Alkaline protease activity in trout fed the PP0 diet peaked at 3 h post-feeding (7.17 U protease/mg protein/min), showing a 3/36 h TPA (total protease activity) ratio of 2.07. Trout fed diets PP50 and PP75 showed only a slight post-feeding increase in this activity, with 3/36 TPA ratios of 1.43 and 1.40, respectively. PP100 fed trout did not register post-prandial peak, showing a 3/36 TPA ratio of 0.73. Similar to rainbow trout, a decreasing trend in total protease activity in intestine and hepatopancreas of hybrid tilapia (*Oreocromis niloticus* x *O.* aureaus) was reported when fed diets with increasing level of soybean meal, where 0%, 25%, 50%, 75% and 100% replacement of dietary fish meal (20%) were replaced in a recent study (27). The authors reported a significantly higher rate of decrease in activity in hepatopancreas (from 123 U/mg protein in those fed 0% SBM to 12 U/mg protein in 100% SBM fed fish) than in intestine (from 415 U/mg protein in those fed 0% SBM to 216 U/mg protein in 100% SBM fed fish). Dietary protein level might also influence enzyme secretion e.g., in tilapia fed 30% and 48% CP diets (23). However, under starving conditions, the differences are notable among various carp species reflecting their natural feeding habit and conditions (24, 25). But when fed a same diet, the differences in proteolytic activity among the same group of species could be minimal as reported in a study with silver carp and common carp (26).

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Among the various types of protease enzymes, trypsin, carboxy-peptidase A, carboxy-peptidase B, amino-peptidase, chymotrypsin, elastase like, collagenase, and esterase have been reported in major farmed crustacean species such as *Litopeneaus vannamei*, *Peneaus monodon*, and *Macrobrachium rosenbergii* (27). For all three species, trypsin activity tend to decrease significantly from early metamorphosis stages i.e., during zoea and mysis stages to the post larval stages and onward.

In crustaceans, molting stages also affect digestive protease activity. In a study with red shrimp *Pleoticus muelleri*, authors reported significantly higher trypsin and chymotrypsin activity during the post-molt stage (2.4±0.19 and 3.7±0.29 abs/min/ mg, respectively) than those during the inter-molt (0.9±0.0.6 and 1.2±0.10 abs/min/mg, respectively) or pre-molt (1.4±0.07 and 2.1±0.07 abs/min/mg, respectively) stages (28). In Pacific white shrimp, trypsin activity was also higher during post-molt stages. The higher trypsin and chymotrypsin activity during the post-molt stages observed in this study corresponds well with the higher nutritional requirement for growth during this period.

Dietary protease

In most cases, supplementation of dietary protease has been proved to improve production performance of various farmed species (4, 7, 8, 10). For example, supplementation of an exogenous protease (175 mg/kg) in pelleted diet containing 30 g/kg fish meal significantly increased weight gain and decreased FCR of tilapia (10). In another study, a mixture of proteolytic enzymes and carbohydrases in diet containing 339 g/kg soybean meal significantly improved the growth and feed efficiency of Atlantic salmon (12). In contrast, no effect on growth performance was observed in a study with rainbow trout (6) fed dehulled lupin-based diets supplemented with protease and carbohydrases alone or in combination.

In a recent study on tilapia, low fish meal diet (30 g/kg) supplemented with a protease complex (175 g/kg) showed significant improvement in ADC of DM and CP while

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no effects were observed in those fed the high fish meal (90 g/kg) diets (10). The more pronounced improvement in digestible nutrient concentration of a poorly digestible diet supplemented with a protease than the highly digestible diets are also evident in other species such as poultry (29). In another study (4), authors reported better feed efficiency of rainbow trout by the addition of protease in diet containing canola seed and pea, and was improved numerically in diets containing flax seed and pea. The authors also reported significant improvement in the ADC of CP, lipid, energy and dry matter for diets containing coextruded canola:pea when supplemented with protease but not for the coextruded flax:pea diets. The lack of positive effects on ADC of CP, crude lipid and gross energy was also reported in Nile tilapia fed diets supplemented with 1.5 g/kg enzyme complex containing neutral protease, β-glucanase and xylanase despite a higher ADC of DM in 1.0 g/kg group compared to those 0 g/kg group (9). These results implicate the variable affects of dietary protease based on their types, composition of various enzymes, as well as substrate types and their composition.

A challenge remains for the feed formulators whether to chose a single protease, a protease complex or multi-enzyme complex containing protease, phytase and carbohydrases. There are very few studies comparing the effects of various types of protease or protease complex or multi-enzyme complex on animals. In a recent digestibility study with rainbow trout comparing effects of a single protease and multi-enzyme complex on soybean meal, rapeseed meal and sunflower meal, authors reported significant improvement in ADC of CP in soybean meal when supplemented with the single protease (7). Interestingly, authors could not find any effect of single protease or multi-enzyme complex on the digestibility of the other two raw materials.

In shrimps, effects of dietary protease have been investigated on various species such as black tiger shrimp, *Peneaus monodon* (30) and Pacific white shrimp, *Peneaus vannamei* (13). In *P. monodon* fed graded levels of canola meal (20%, 54% and 64%), performance (weight gain and FCR) was improved only in those fed 20% canola meal diets supplemented with 0.25% protease compared to the corresponding non-supplemented diets.

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In *P. vannameii*, effects of graded level (0, 0.2 or 0.4%) of a commercial feed grade protease (ENZECO® Bromelain FG, EDC, NY) on ADC DM and ADC CP were tested. The ADC CP was signifcantly higher (74.3%) in shrimps fed 0.4% protease diets than those fed the control diets (65.3%). In the subsequent growth trial, authors reported a decrease in growth performance (weight gain, feed efficiency, and protein efficiency ratio) in shrimps fed the diets with protease. This negative response was more pronouced in high protease (0.4%) diets than those with a lower concentration (0.2%) of the enzyme.

In a recent study, growth performances of *P. vannamei* fed high fish meal diet (HFD) and low fish meal diet supplemented with a protease complex (LFD+P) were significantly better compared to those fed the low fish meal diets (10). Compared with the LFD group, the addition of protease (LFD+P) improved weight gain by 11.0% ($P<0.05$) and decreased FCR by 0.13 ($P<0.05$). In the same study, shrimps fed the HFD diet, or LFD+P diet had a higher activity of hepatopancreatic protease than those fed LFD diet ($P<0.05$). However, no significant difference was detected in intestinal protease activity among the groups ($P>0.05$).

Application of Protease

There are about 14 different types of alkaline proteases identified in animals that include but not limited to trypsin like, several chymotrypsin and elastase like, and carboxypeptidases. Each of these proteases has specific temperature and pH optima, and the ability to breakdown protein molecules vary from substrates to substrates. A list of preferred substrates for activity analysis of some of these enzymes are listed in table 1.

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Table 1. A list of preferred substrates for the activity assays of various alkaline proteases

Enzyme types	**Preferred substrate**
Chymotrypsin-like	Benzoyl-L-tyrosine ethyl ester (BTEE)
Carboxypeptidase A	Hippuryl-L-phenylalanine (HPA)
Carboxypeptidase B	Hippuryl-L-arginine (HA)
Leucino aminopeptidase like	L-leucine-p-nitroanilide (LAPNA)
Total protease	Azocasein
Serine protease	Benzoyl-DL-arginine *p*-nitroanilide (BAPNA)
Chymotrypsin	Succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAPNA) OR N-succinyl-L-phenylalanine-*p-nitroanilide* (Suc-Phe-p-Nan)
Amino peptidase	Aminoacyl β-naphthylamide (AA-NA) with Arg, Leu, Phe, Val, Lys as substrates.

The substrate specificity of each enzyme may well explain why a single protease was able to improve digestible nutrient content in soybean meal but not in sunflower meal or rapeseed meal (7). However, in a subsequent study (8), authors showed ability of the multi-enzyme to breakdown some non-starch polysaccharides. These types of information are very useful to improve our understanding on protease enzymes and variations in their effects on different substrates. For example, a single protease working very well on soy-based diets may well not be able to exert similar effects on diets predominantly containing other protein sources. On the other hand, a multi-enzyme complex containing a protease may not be able to exert the same effects as a single protease or a protease complex.

Conclusion

This review highlights the need for better understandings of digestive enzyme types and their activity as ingredients, diets, age, size, molting stage, culture conditions, and pH,

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temperature and dissolved oxygen of the culture environment can significantly influence their specific activity. The range of digestive enzymes specifically proteases is diverse. Composition of these enzymes and their interactions with environment and diets influence digestive capacity and immune response of the animals. Further studies are recommended to enhance our knowledge on digestive enzymes and their relationships with modern diets, culture systems and environment. Total protease activity or activity of various protease enzymes may also vary significantly based on analytical methods chosen for example, substrates or inhibitors used and incubation parameters such as pH, temperature and duration.

Dietary enzymes are being considered one of the available solutions to improve quality of feeds. Supplementation of enzyme can improve gut health, compensate digestive enzymes when needed, and may also improve immune responses. However, feed manufacturing industry remains skeptical because of concerns on heat-stability of the enzymes, interactions with other enzymes, safety of recommended dosages, and lack of knowledge of suitable substrates for each type of enzyme.

It is important to understand that each specific enzyme usually attack specific active sites in a complex molecule. With the omnipresence crisis for quality raw materials, an industry wide understanding of dietary enzyme application in aqua feed has become essential. Further research is needed to understand the effects of different types of dietary proteases on improving the quality of proteins in feed, their interactions with various ingredients, gut health, and nutrient utilization. Appropriate application of dietary proteases available in the market has also been an issue because of the concerns on heat stability and fear of their destruction during manufacturing. Some better heat stable enzymes are available in dry powder form, can be readily added to the mixer, and can keep majority of their activity intact. The rests are available in liquid form to be applied after pelleting requiring specific mechanical tools. We need to better understand advantages and disadvantages of the various application methods when selecting an enzyme. Ability to apply directly to the mixer may have several advantages. In addition to the ease of

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handling, the protease may also alter the quality of feed proteins during cooking or conditioning resulting in better quality feed. We are still at infancy in our understanding of the complexity of the protease world. A renewed and targeted focus in our research agenda is recommended to improve our understandings.

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