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Research Article
Effect of a Serine-protease on Performance Parameters and Protein Digestibility of Cultured *Oreochromis niloticus* Fed Diets with Different Protein Levels

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Abstract
Objective: This study was conducted to investigate the effect of a serine-protease supplementation on growth performance and protein digestibility of cultured *Oreochromis niloticus* fed diets with different crude protein (CP) levels. **Materials and Methods:** The diets T1and T2 were formulated to supply 28 and 26% crude protein, respectively. A serine-protease enzyme (Ronozyme ProAct) product with an activity of 75000 Protease (PROT)/g was added to the diets at the level of 0, 200 and 400 mg kg^{-1} diet. In this study 384 fish with an initial body weight of average 36.34±3.68 g were divided into 6 groups with 4 replicates of 16 fish/replicate. The experimental period extended for 56 days. **Results:** The weight gain (WG), specific growth ratio (SGR) and protein efficiency ratio (PER), apparent protein digestibility (APD) were significantly increased when T1 and T2 diets were supplemented with protease enzyme while feed conversion ratio (FCR) was significantly decreased. Fish fed T2 diet supplemented with 400 mg kg^{-1} protease showed a non-significant difference in final weight, SGR, PER and APD comparing with fish fed T1 diet only. **Conclusion:** The results indicated that the protease enzyme supplementation can significantly improve growth performance and protein digestibility in *O. niloticus*. Protease enzyme could be used to reduce the protein content of the diet with maintaining the fish performance.

Key words: Fish, protease enzyme, protein level, performance, protein digestibility

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aquaculture is one of the fastest growing agribusiness sectors, thus it becomes an important component of global food supply[1]. Tilapia production comes in the second rank after carp, which contributes significantly to global aquaculture supply[2,3]. The cost of commercial fish feeds is the major constraint affecting the profitability of this industry especially the cost of animal protein source (fish meal), which constitute 30-50% of the fish feed[4]. To support the higher production of cultured fish in the light of high cost and shortage of animal protein, thus increase the need to find smart solutions to utilize the protein sources more effectively and extend protein resources using plant based ingredients. However, there are limitations on the inclusion levels of plant based ingredients for most fish species due to the presence of various anti-nutritional factors, which impair nutrient utilization and feed efficiency leading to reduce fish growth rate[5,6]. Exogenous enzyme supplementation could be used in aquaculture feed to improve nutrient utilization from plant ingredients based on findings of enzyme applications in swine and poultry diets[7].

Proteases constitute a class of enzymes that break down the peptide bonds in complex proteins to obtain usable amino acids and peptides. Although proteases produced naturally in the digestive system of tilapia, the addition of a specific exogenous protease could improve the protein digestibility of plant ingredients used instead of fish meal in aquafeed[8]. Proteases have been added to poultry and swine diets for many years as part of enzyme admixtures containing xylanases, pectinases, glucanases and amylases[9-11]. Protease supplementation to broiler diet resulted in a significant improvement in crude protein digestability[12,13]. However, studies approved the usage of exogenous protease in aquafeed are limited, so aquaculture nutritionists need to implement more investigations to establish the benefits of dietary protease supplementation to fish feed.

Therefore, the present study was designed to investigate the effect of a serine protease on performance parameters and protein digestibility of cultured *O. niloticus* fed diets with different protein levels.

MATERIALS AND METHODS

Experimental unit: The present study was conducted in the Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Cairo University. The experimental fish were stocked in 24 glass aquaria (80×30×40 cm) supplied with de-chlorinated tap water. The water was aerated continuously by using an air compressor (BOYU S 2000 Air pump, Malaysia). The photoperiod was 12 h light/12 h dark. The water temperature was maintained at 24±1°C using immersion heater (250 W) with a thermostat. The water temperature and the dissolved oxygen level were recorded daily (by Metteler Toledo, model 128, s/No 1242) and the average range of dissolved oxygen was greater than 5.8 mg L^{-1}. Other water quality parameters, including pH and ammonia level were measured every 2 days with a pH meter (Orion model 720A, s/No 13062) and ammonia meter (Hanna ammonia meter), the average range of the total ammonia was 0.12–0.23 mg L^{-1} and the pH was in the range of 7.2±0.5 during the experiment.

Experimental diet: The diet T1 was formulated to supply 28% CP according to NRC[14] and T2 diet was formulated to supply 26% crude protein (low protein diet). Diet composition and chemical analysis are shown Table 1. Ronozyme ProAct a serine-protease enzyme and heat stable formulated product (DSM Nutritional Products SP.z.oo. Poland) with an activity of 75000 PROT g^{-1} was added to the diets (T1 and T2) at the level of 0, 200 and 400 mg kg^{-1} diet. The diets were prepared by individually weighing of each component and thoroughly mixing the minerals, vitamins (premix) and additives with corn. The protease enzyme powder was mixed thoroughly in the stated quantities with a small amount of feed. Water was added until the mixture became suitable for making pellets. The wet mixture was passed through a pellet machine with a 2 mm diameter. The pelleted diets were air dried by electric fan at room temperature for 24 h, then packed in plastic bags and refrigerated at 4°C until use[15]. Calculations and chemical analysis of different diets were performed according to AOAC[16].

Experimental fish: A total of 384 apparently healthy *O. niloticus* were obtained from a private fish farm. Fish acclimated to the laboratory conditions for 2 weeks before being randomly divided into 6 groups with 4 replicates of 16 fish per replicate, representing six nutritional groups. The control groups, control 1 and control 2 were fed on T1 (28% CP) and T2 (26% CP), respectively. Group 3 (G3) were fed T1 diet (28% CP) supplemented with protease enzyme (200 mg kg^{-1} diet), group 4 (G4) received T1 diet (28% CP) supplemented with protease enzyme (400 mg kg^{-1} diet), group 5 (G5) received T2 diet (26% CP) supplemented with protease enzyme (200 mg kg^{-1} diet) and group 6 (G6) received T2 diet (26% CP) supplemented with protease enzyme (400 mg kg^{-1} diet). The experimental fish with average individual initial weight of 36.34±3.68 g were fed the diets at

Table 1: Diet composition and chemical analysis (as fed basis)

	Diet composition along the experimental period	
Items	T1	T2
Feed ingredient (%)		
Fish meal (67%)	8.00	7.00
Soy bean meal (48%)	36.11	35.05
Yellow corn	39.46	42.41
Corn gluten meal (60%)	3.00	1.50
Corn starch	7.24	7.46
Soy and fish oil	3.95	4.25
Mono calcium phosphate (23.7%)	0.20	0.26
Calcium carbonate	1.50	1.52
Premix*	0.30	0.30
Vitamin C	0.01	0.01
D-methionine	0.23	0.24
Total	100.00	100.00
Calculated analysis (%)		
DM	90.81	90.81
Crude protein	28.02	26.17
Ash	6.08	5.91
Ether extract	6.52	6.79
Crude fiber	3.66	3.61
NFE	46.49	48.33
Gross energy# (kcal/100 g)	410.76	410.20
Calcium	0.86	0.85
Total phosphorus (P)	0.63	0.62
Sodium	0.06	0.06
Lysine	1.62	1.53
Methionine	0.73	0.69
Threonine	1.11	1.04
Chemical analysis (%)		
CP	28.05	26.20
EE	6.70	6.74
Ca	0.90	0.90
Total P	0.65	0.66

*Each killogram vitamin and mineral mixture premix contained vitamin A, 4.8 million IU, D_3: 0.8 million IU, E: 4 g, K: 0.8 g, B_1: 0.4 g, Riboflavin: 1.6 g, B_6: 0.6 g: B_{12}: 4 mg, Pantothenic acid: 4 g, Nicotinic acid: 8 g, Folic acid: 0.4 g, Biotin: 20 mg, Mn: 22 g, Zn: 22 g, Fe: 12 g, Cu: 4 g, I: 0.4 g, Selenium: 0.4 g and Co: 4.8 mg, NFE: Nitrogen free extract, #Gross energy: Based on 5.65 kcal g^{-1} protein, 9.45 kcal g^{-1} fat and 4.1 carbohydrate kcal g^{-1}, DM: Dry matter, CP: Crude protein, EE: Ether extract, Ca: Calcium and P: Phosphorus

rate of 3% of a total biomass supplied in 3 daily meals (8 am, 12 pm and 5 pm) for 56 days. Every 2 week all the fish were removed and individually weighed in order to calculate the average weight and to adjust the feeding rate according to NRC[14]. All institutional and national guidelines for the care and use of fisheries were followed.

Growth performance and feed utilization: During 56 days of experiment, the following parameters were determined: Feed Intake (FI) (g), initial weight (g), Final Weight (FW) (g), Weight Gain (WG) (g), Feed Conversion Ratio (FCR), Specific Growth Rate (SGR) and Protein Efficiency Ratio (PER) according to Abu-Elala *et al.*[17].

Apparent protein digestibility: During the last week of the experimental period, the fish were fed the experimental diets mixed with an indicator (chromic oxide 5 g kg^{-1} diet). The fish were fed 3 meals daily between 09:00 and 16:00 h and the feed was offered only as long as the fish were actively feeding, to avoid wastage. After 3 days of accommodation to the indicator, the daily fecal samples from each aquarium were pooled over the 3 successive days according to Bureau *et al.*[18] and Zhou *et al.*[19], 1 h after the last meal, the uneaten feed particles and feces were removed from the system. One-third of the water in the tanks was drained to ensure that the cleaning procedure was complete. The feces were then allowed to settle overnight. Fecal samples were collected each morning at 08:00 h. The feces were immediately collected on filter paper, dried in an oven at 60°C and kept in airtight containers at -20°C for subsequent analysis. The crude protein of feed and dried excreta was analyzed according to AOAC [16].

The Apparent Protein Digestibility (APD) was calculated as follows[20]:

$$APD=1-(F/D\times Di/Fi)$$

where, D is crude protein (%) of diet, F is crude protein (%) of feces, Di is digestion indicator (%) of diet and Fi is digestion indicator (%) of feces.

Biochemical serum analysis: At the end of the experiment 5 blood samples/replicate were collected using clean syringes from the caudal vessels of fish. Non-haemolyzed sera were separated by centrifugation at 1,500× g for 15 min at 4°C, stored in deep freezer at -20°C until analysis to determine serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), urea, creatinine and Blood Urea Nitrogen (BUN) using commercial kits.

Statistical analyses: The obtained data were calculated and statistically analyzed according to Daniel and Cross[21] using SPSS software version 14 for windows. The differences between groups were determined with variance analysis (one-way ANOVA) using the probability level of 0.05 for the rejection of the null hypothesis. Significant differences among means were determined by Duncan's multiple range tests. Data were expressed as means±SEM.

RESULTS

Growth performance and feed utilization: The effects of dietary supplementation of protease enzyme on growth

Table 2: Growth performance of *Oreochromis niloticus* at the end of feeding trial

Items	Initial BW (g)	Final BW (g)	Total weight gain (g)	Total feed intake/fish/56 days (g)	*SGR	**PER	***FCR	****APD
C1	36.28±3.71^{a}	83.75±8.38^{b}	47.48±4.77^{c}	106.96±10.70^{e}	1.12±0.11^{c}	1.59±0.16^{c}	2.25±0.23^{d}	84.73±8.51^{c}
C2	36.35±3.70^{a}	73.53±7.36^{c}	37.18±3.75^{f}	102.96±10.30^{f}	0.94±0.10^{e}	1.39±0.14^{e}	2.78±0.28^{a}	82.23±8.25^{e}
G3	36.38±3.65^{a}	92.81±9.31^{a}	56.44±5.81^{b}	113.97±11.41^{c}	1.25±0.14^{b}	1.77±0.18^{b}	2.02±0.22^{e}	88.12±8.86^{b}
G4	36.25±3.70^{a}	98.13±9.83^{a}	61.88±6.22^{a}	115.80±11.62^{a}	1.33±0.14^{a}	1.91±0.20^{a}	1.87±0.20^{f}	90.68±9.13^{a}
G5	36.11±3.61^{a}	78.50±7.91^{bc}	42.39±4.26^{e}	113.85±11.39^{d}	1.04±0.10^{d}	1.43±0.15^{d}	2.69±0.27^{b}	83.76±8.41^{d}
G6	36.65±3.72^{a}	81.76±8.21^{b}	46.41±4.68^{d}	114.15±11.43^{b}	1.09±0.11^{c}	1.56±0.17^{c}	2.46±0.25^{c}	84.88±8.53^{c}
p-value	1.0000	0.0001	0.0001	0.0003	0.0001	0.0002	0.0002	0.0001

Data represented as Means±SE (n = 16/group/replicate). $^{a-b}$Values with different superscripts significantly different at $p \leq 0.05$, *SGR: Specific growth rate = (Ln Final body weight-Ln Initial body weight)×100/experimental period (days), **PER: Protein efficiency ratio = Weight gain (g) / protein intake (g), ***FCR: Feed conversion ratio = Feed intake (g)/body weight gain (g), ****APD: Apparent protein digestibility, C1: Group (control 1) fed T1 diet (28% CP) without protease enzyme (Ronozyme ProAct), C2: Group (control 2) fed T2 diet (26% CP) without protease enzyme (Ronozyme ProAct), G3: Group 3 fish fed T1 diet (28% CP) plus 200 mg kg^{-1} protease enzyme (Ronozyme ProAct), G4: Group 4 fed T1 diet (28% CP) plus 400 mg kg^{-1} protease enzyme (Ronozyme ProAct), G5: Group 5 fed T2 diet (26% CP) plus 200 mg kg^{-1} protease enzyme (Ronozyme ProAct), G6: Group 6 fed T2 diet (26% CP) plus 400 mg kg^{-1} protease enzyme (Ronozyme ProAct)

Table 3: Serum biochemical parameters of *Oreochromis niloticus* at the end of feeding trial

Items	ALT (U L^{-1})	AST (U L^{-1})	Urea (mg dL^{-1})	Creatinine (mg dL^{-1})	BUN (mg dL^{-1})
C1	21.26±2.30	85.24±8.56	3.23±0.34	0.705±0.071	2.71±0.273
C2	21.13±2.24	84.52±8.52	3.16±0.33	0.683±0.069	2.64±0.266
G3	20.68±2.15	82.68±8.31	3.20±0.32	0.662±0.068	2.50±0.252
G4	20.84±2.92	84.68±8.49	3.22±0.34	0.702±0.072	2.67±0.271
G5	21.08±2.14	83.85±8.41	3.18±0.32	0.658±0.070	2.54±0.257
G6	20.83±2.90	84.05±8.42	3.16±0.32	0.663±0.069	2.62±0.265
p-value	0.070	0.070	0.065	0.070	0.060

Data represented as Means±SE (n = 16/group/replicate), C1: Group (control 1) fed T1 diet (28% CP) without protease enzyme (Ronozyme ProAct), C2: Group (control 2) fed T2 diet (26% CP) without protease enzyme (Ronozyme ProAct), G3: Group 3 fish fed T1 diet (28% CP) plus 200 mg kg^{-1} protease enzyme (Ronozyme ProAct), G4: Group 4 fed T1 diet (28% CP) plus 400 mg kg^{-1} protease enzyme (Ronozyme ProAct), G5: Group 5 fed T2 diet (26% CP) plus 200 mg kg^{-1} protease enzyme (Ronozyme ProAct), G6: Group 6 fed T2 diet (26% CP) plus 400 mg kg^{-1} protease enzyme (Ronozyme ProAct), ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, BUN: Blood urea nitrogen

performance and feed utilization of *O. niloticus* were summarized in Table 2, results revealed that the growth performance parameters of *O. niloticus* were significantly ($p<0.05$) improved when CP 26% diet and CP 28% diet were supplemented with protease enzyme at 200 and 400 mg kg^{-1} in comparing with the non supplemented groups. Fish in group 4 were significantly ($p<0.05$) surpassing all groups regarding weight gain, SGR and PER compared with control groups 1 and 2. Similarly, feed conversion ratio was significantly ($p<0.05$) better in group 4 compared with control groups 1 and 2. Furthermore, fish in group 6 recorded a non significant difference regarding final weight, SGR and PER in comparing with those of control group 1.

Apparent protein digestibility: The effects of dietary supplementation of protease enzyme on crude protein digestibility of *O. niloticus* were illustrated in Table 2, results indicated that the crude protein digestibility of *O. niloticus* was significantly ($p<0.05$) improved when CP 26% diet and CP 28% diet were supplemented with protease enzyme at 200 and 400 mg kg^{-1} in comparing with the non supplemented groups. The highest percentage of crude protein digestibility was recorded in fish group 4 in comparing with other fish groups including control groups. Moreover, the data showed a non-significant difference in CP digestibility in fish group 6 compared with control group 1.

Biochemical serum analysis: Data of serum biochemical parameters at the end of the experiment for the different experimental groups were clarified in Table 3, results showed that the serine-protease (Ronozyme ProAct) supplemented groups had a non-significant difference in ALT and AST, urea, creatinine and BUN in comparing with control groups.

DISCUSSION

The improved growth performance, FCR and feed utilization of *O. niloticus* by serine-protease enzyme (Ronozyme ProAct) supplementation to diet T1 (28% CP) and diet T2 (26% CP) could be attributed to increase protein digestibility and amino acid availability by protease enzyme[8]. Amino acids are essential mainly for growth and they may also be used for metabolic functions[22]. De Silva *et al.*[23] stated that growth rate or net weight gain of red tilapia was correlated to dietary protein consumption irrespective of dietary lipid content. Thus, it is clear from the present study that protease increased the availability of proteins by its proteolysis activity leading to improve growth performance of *O. nilticus*.

Our results of protease enzyme (Ronozyme ProAct) on WG, SGR, PER, FI and FCR are compatible with that of Ayodeji *et al.*[24], who recorded a significant ($p<0.05$) improvement in growth performance parameters including

SGR, PER and FCR in *O. niloticus* fed plant based diet (40.8±0.2% CP) supplemented with Ronozyme ProAct at 0.2 g kg^{-1} in comparison with the control group. Also, Dias *et al.*[8] found that the growth performance parameters of juvenile Nile tilapia were significantly ($p<0.05$) improved when CP 26% diet and CP 28% diet were supplemented with 200, 400 and 600 mg kg^{-1} Ronozyme ProAct. Similarly, Soares *et al.*[25] stated that the inclusion of exogenous protease at 0.0, 0.05, 0.10 and 0.15% gave rise to improve FCR, WG and SGR of tucunare paca (*Cichla temensis)* juvenile and the best results were recorded at 0.1%. Moreover, Drew *et al.*[26] reported a significant improvement in feed efficiency of rainbow trout (*Oncorhynchus mykiss*) by the addition of protease enzyme at 0.25% to diet of canola: Pea mixture. Contrary to this study Adeoye *et al.*[27], who found a non-significant difference between the exogenous protease supplemented *O. niloticus* at 200 mg kg^{-1} and the control group regarding FW, SGR, PER and FCR.

The increase in APD by serine-protease enzyme (Ronozyme ProAct) supplementation to diet T1 (28% CP) and diet T2 (26% CP) may be due to increase amino acid availability by protease enzyme, which break down complex proteins into usable peptides and amino acids[8]. Moyle and Cech[22] and De Silva and Anderson[28] stated that alkaline proteases (which mainly active in an alkaline environment) takes over the role of pepsin in herbivorous and omnivorous fish like Nile tilapia, which not have stomach and lacks pepsin. This was confirmed by the findings of Hara *et al.*[29], who studied the morphology of *Siganus rivulatus* digestive tract as a typical herbivorous fish and found that the stomach in juveniles and adults (called intestine bulb) is a relatively straight tube, not sac like and intestine is extremely long and convoluted.

This study agree with that of Dias *et al.*[8], who recorded 2-8% improvement in APD by the dietary supplementation of Ronozyme ProAct to the CP 26, CP 28 and CP 31 diets of *O. niloticus*. Likewise, Chen *et al.*[30] found a significant improvement in APD in black carp (*Mylopharyngodon piceus*) fingerling by dietary supplementation of neutral protease at level of 0.5, 1, 2 and 3%. Moreover, Drew *et al.*[26] stated that the addition of protease at 0.25g kg^{-1} to canola: Pea mixtures resulted in a significant increase in APD in rainbow trout (*O. mykiss*).

Biochemical serum analysis: The results of serum biochemical parameters indicates that exogenous protease (Ronozyme ProAct) supplementation have the ability to enhance growth performance without apparent impairment of the fish health status. The same were recorded by Ayodeji *et al.*[24], who found that the hematological parameters of *O. nitolicus* were not affected by protease supplementation at 0.2 g kg^{-1} diet.

The present study clarified that the addition of protease enzyme (Ronozyme ProAct) to cultured *O. niloticus* feed had a significant effect on increasing crude protein digestibility and PER which was reflected on final weight gain, SGR and feed conversion ratio. Also, this study revealed that enzyme concentration plays an important role in fish diets. This study is in agreement with latest study with broilers and fish which indicated that not only the enzyme type is important for an animal response (positive or negative) to supplementation but also its concentration to the size and the direction of its application[31,32].

This study will help the researcher to uncover the critical areas which focusing on decreasing the fish feed cost by using exogenous enzymes. Thus, a new theory on reduction of crude protein content of *O. niloticus* diet by using protease enzyme may be arrived.

CONCLUSION

It could be concluded that the exogenous dietary supplementation of protease enzyme (Ronozyme ProAct) can be used safely and economically to improve protein digestibility and reduce protein content of *O. niloticus* diet with maintaining the growth performance parameters.

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