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Characterization and Activity of Digestive Enzymes in Different Sizes of Nile Tilapia (*Oreochromis niloticus* L.)

Rungkan Klahan[1]*, Nontawith Areechon[1], Ruangvit Yoonpundh[1] and Arunee Engkagul[2]

ABSTRACT

The activity of protease, amylase and lipase from the pseudostomach, upper intestine, lower intestine and liver of 5.7, 35.8 and 92.1 g male Nile tilapia (*Oreochromis niloticus* L.) was determined. Protease activity had an optimal pH of 12, 9 and 9 for the 5.7, 35.8 and 92.1 g fish respectively and the highest specific activity was found in the 35.8 g fish. Amylase activity had an optimal pH of 6, 7 and 2, respectively and the highest specific activity was found in the 92.1 g fish. Lipase activity had an optimal pH of 8, 7 and 8, respectively and the highest specific activity was found in the 35.8 g fish. In all sizes of tilapia, amylase activity was significantly higher in the liver than other organs ($P<0.01$). Protease and lipase activity in the lower and upper intestine respectively of the 5.7 and 35.8 g fish were significantly higher than in other organs ($P<0.01$). This study indicated that different sizes of fish had different levels of enzymatic activities. Middle-sized fish appeared to have the highest specific activity of protease and lipase, while the large fish had the highest specific activity of amylase. These results will be used as a basis for suitable feed formulation for different sizes of Nile tilapia so that optimum nutritional values and cost-effectiveness can be achieved.

Key words: digestive enzymes, Nile tilapia, protease, amylase, lipase

INTRODUCTION

Tilapia are fast–growing, resistant to disease and handling, easy to reproduce in captivity and able to tolerate a wide range of environmental conditions. They are commonly cultured in tropical and subtropical regions of the world and constitute the third largest group of farm finfish (El–Sayed, 1999). Tilapia continues to show tremendous growth in output with global production totalling 2,348,656 tons in 2006, half of which was traded internationally. In 2002, tilapia became an aquaculture trade commodity (Merican, 2006) and the species has been cultured under an intensive system at high stocking rates, thus emphasizing the need for more nutritionally-complete diets and a more efficient digestive enzyme system (Shiau, 2002), which includes protease, amylase and lipase. In herbivorous and omnivorous fish such as the Nile tilapia, which has no stomach and lacks pepsin (which is utilized as a low–pH, proteolytic enzyme (Moyle and Cech, 2000), the role of pepsin is taken over by alkaline proteases, which are most active in an alkaline environment (De Silva and Anderson, 1995). There is initially low activity of alkaline protease in early juvenile stages and the general protein digestion is heavily dependent on the

1 Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand.

2 Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

* Corresponding author, e–mail: supatcharin5@hotmail.com

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alkaline tryptic rather than the acidic peptic enzymes. A fall in protease activity was correlated with a decrease in the proportion of fish meal in the diet (De Silva and Anderson, 1995). Lipase activity has been found in extracts of the pancreas, pyloric ceca and upper intestine, but was almost non–existent in the stomach and in the lower intestine. The principal site for lipase activity is the mucosal layer but in some species, like the Siberian sturgeon, there is hydrolysis in the stomach by gastric lipase (Gisbert *et al*., 1999). However, the primary site of lipid hydrolysis for most species appears to be in the pyloric ceca and anterior intestines (Halver and Hardy, 2002), and all fat–digestive enzymes are known to act in alkaline media. Carbohydrase (α-amylase) is produced in the pancreas and, like lipase, has been identified in pancreatic juice, the stomach and intestines but not necessarily in all sites of all investigated species. The products of carbohydrate hydrolysis are polysaccharides and monosaccharides, which are more easily assimilated, and carbohydrase activity responds to the level of dietary carbohydrate. In general, the activity of amylase in particular, differs from species to species and appears to be related to their feeding habits. The aims of this study were to determine the activity of digestive enzymes including protease, amylase and lipase in three sizes of Nile tilapia. The results from this study will be used as a basis to develop feed formulation suitable for different sizes of Nile tilapia, so that optimal nutritional values and cost–effectiveness can be obtained.

MATERIALS AND METHODS

Fish and sample preparation

Three hundred male Nile tilapia (*Oreochromis niloticus* L.) from the Faculty of Fisheries, Kamphaeng Saen campus, Kasetsart University were used in this study. They were divided into 3 groups (100 fish per group) with an average weight of 5.7±0.5, 35.8±2.5 and 92.1±5.9 g, respectively. Fish were acclimated for one week by feeding twice per day with 28, 30 and 35% protein feed for the 92.1, 35.8 and 5.7 g fish, respectively. The upper and lower intestines, pseudostomach and liver were collected from 100 fish in each size group and weighed 16 hours after feeding. Samples were stored at -80°C until used.

Crude enzyme preparations

The upper and lower intestines, pseudostomach and liver were homogenized (1:2 w/v) with 50 mM Tris – HCl buffer (pH 7.5) in an ice water bath, using a tissue homogenizer. The preparation was centrifuged at 10,000 × g for 15 min at 4°C. The floating lipid fraction was removed and the aqueous supernatant was recovered and kept at -20°C until used (Gimenez *et al*., 1999).

Enzymatic assay

Characterization of protease

Protease activity was monitored in triplicate by measuring the increase in cleavage of short chain polypeptides using the method of Bezerra *et al*. (2005) with slight modifications. The total protease activity was determined using 1% (w/v) azocasein, prepared in buffers at various pH levels: 0.1 M glycine–HCl pH 2; 0.1 M citrate buffer pH 3–5; 0.1 M phosphate buffer pH 6–8; 0.05 M carbonate buffer pH 9–10; 0.05 M Na_2HPO_4 buffer pH 11; and 0.1 M KCl - NaOH buffer pH 12–13. The substrate (500 µl) was incubated with crude extract (20 µl) and buffer solution (200 µl) at various pH levels for 60 min at 30°C. Then, 500 µl of 20% (w/v) trichloroacetic acid (TCA) was added to stop the reaction. After 15 min, centrifugation was carried out at 10,000 g for 10 min. The supernatant (1.0 ml) was added to 1 M NaOH (1.5 ml) in a glass cuvette and the absorbance was measured at 440 nm against a blank similarly prepared, but without the crude extract sample. The protease-specific activity was expressed by the change in absorbance per min per mg protein of the enzyme extract (ΔAbs min^{-}

^{1} mg protein^{-1}).

Characterization of amylase

The amylase activity was determined in triplicate by the 3,5–dinitrosalicylic acid (DNS) method (Bernfeld, 1951). Starch substrate (1% w/v) was prepared in buffers at various pH levels: 0.1 M glycine–HCl pH 2; 0.1 M citrate buffer pH 3–5; 0.1 M phosphate buffer pH 6–8; 0.05 M carbonate buffer pH 9–10; 0.05 M Na_2HPO_4 buffer pH 11; and 0.1 M KCl–NaOH buffer pH 12–13. The substrate (500 µl) was incubated with crude extract (50 µl) and buffer solution (400 µl) at various pH levels for 60 min at 30°C. Then, 1.5 ml of 1% dinitrosalicylic acid (DNS) solution was added and boiled for 5 min. After boiling, 1.5 ml of distilled water was added to the mixture and the cooled solution was read at 550 nm. Blanks were similarly prepared, but without the crude enzyme extracts. Maltose (10–100 mM) was used for the preparation of the calibration curve. The amylase-specific activity was defined by the mmol of maltose produced per min per mg protein at the specified condition.

Characterization of lipase

The lipase activity was measured in triplicate by the method modified from Markweg *et al*. (1995) using 0.01 M para–nitrophenylpalmitate (pNNP) dissolved in iso–propanol as the substrate. The substrate (100 µl) was incubated with crude enzyme extract (50 µl) and buffer solution (800 µl) at various pH levels: 0.1 M glycine–HCl pH 2; 0.1 M citrate buffer pH 3–5; 0.1 M phosphate buffer pH 6–8; 0.05 M carbonate buffer pH 9–10; 0.05 M Na_2HPO_4 buffer pH 11; and 0.1 M KCl - NaOH buffer pH 12 – 13; for 60 min at 30°C. Then, 250 µL of 0.1 M Na_2CO_3 was added to stop the reaction and centrifugation was carried out at 10,000 g for 15 min. The absorbance of the supernatant was measured at 410 nm against a blank similarly prepared, but without the crude extract sample. Para–nitrophenol (pNP) at a concentration of 100–1000 µg/ml was used for the preparation of the calibration curve. The lipase-specific activity was defined by the µmol of p–nitrophenol produced per min per mg protein at the specified condition.

Protein concentration

The protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Statistical analysis

This experiment was conducted using a completely randomized design (CRD) and the data obtained from the enzyme activity in various pH buffers, organs and sizes of fish were subjected to a one way analysis of variance (ANOVA) followed by Duncan's new multiple range test at $p = 0.01$.

RESULTS AND DISCUSSION

Characterization of digestive enzyme: Optimal pH

Optimal pH of protease

The activity of protease from the pseudostomach, upper and lower intestines and liver was highest at a pH of 9, 9, 12 and 10 in 5.7 g fish, at pH 8, 10, 9 and 10 in 35.8 g fish and at pH 9, 10, 10 and 9 in 92.1 g fish, respectively (Figures 1, 2 and 3). The results were in line with the work of Kuz'mina and Ushakova (2007), which showed that the protease activity of 620 g turbot decreased considerably at pH 5.0 and increased at pH 8.5. Alarcon *et al*. (1998) also reported that the alkaline protease activity of gilthead seabream and common dentex was maintained over a wide pH range (5–11) and showed a well-defined optimum at pH 10.0. Sheng *et al*. (2006) reported the pH range for maximal activity of protease extracted from the intestines of 55–225 g Nile tilapia at 9.5–10.5 and the alkaline protease activity remained high over a wide range of pH values (6–11). Protease activity was apparently highest in 35.8 g fish (Figure 1). Proteases and other enzymes can also be classified

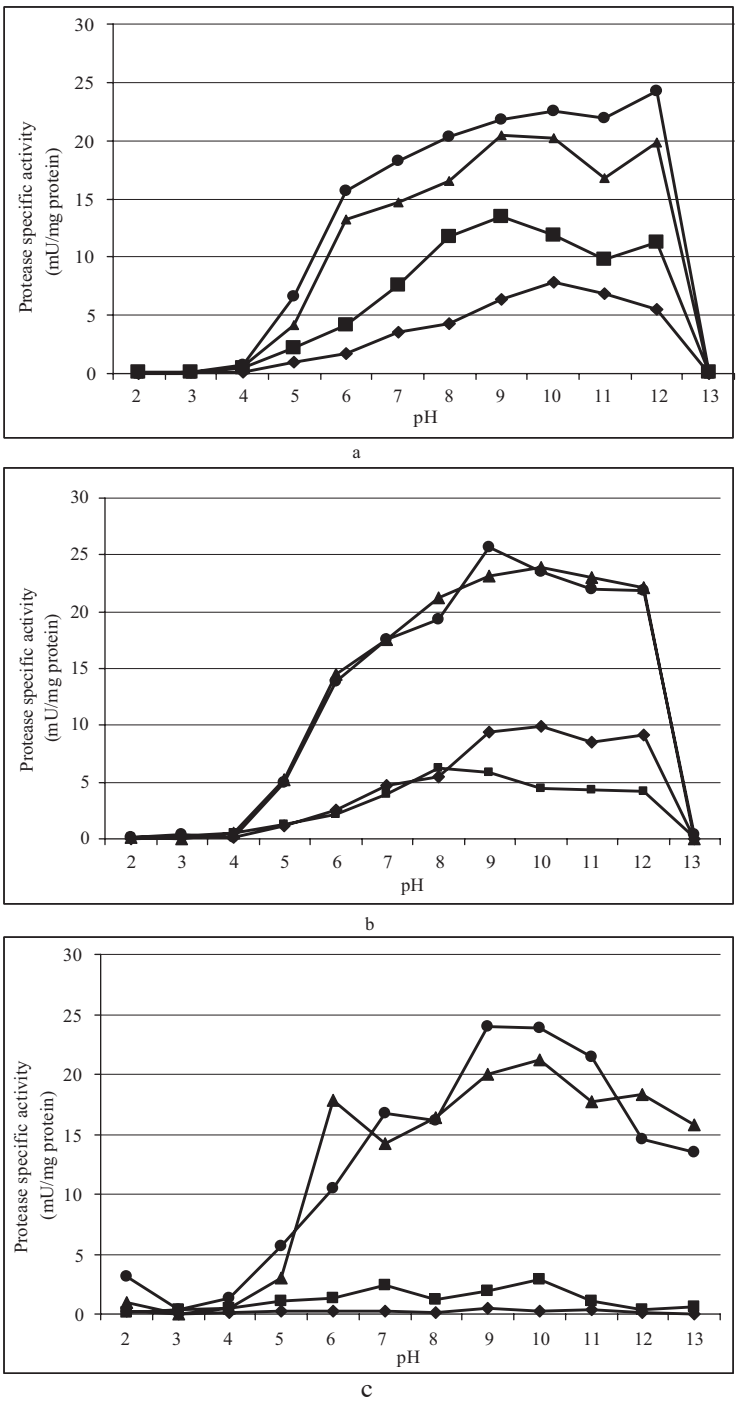

Figure 1 Protease activity (specific activity) in liver (-◆-), pseudostomach (-■-), upper (-▲-) and lower (-●-) intestines at pH 2 – 13 of 5.7 (**a**), 35.8 (**b**) and 92.1 g (**c**) tilapia (*Oreochromis niloticus* L.).

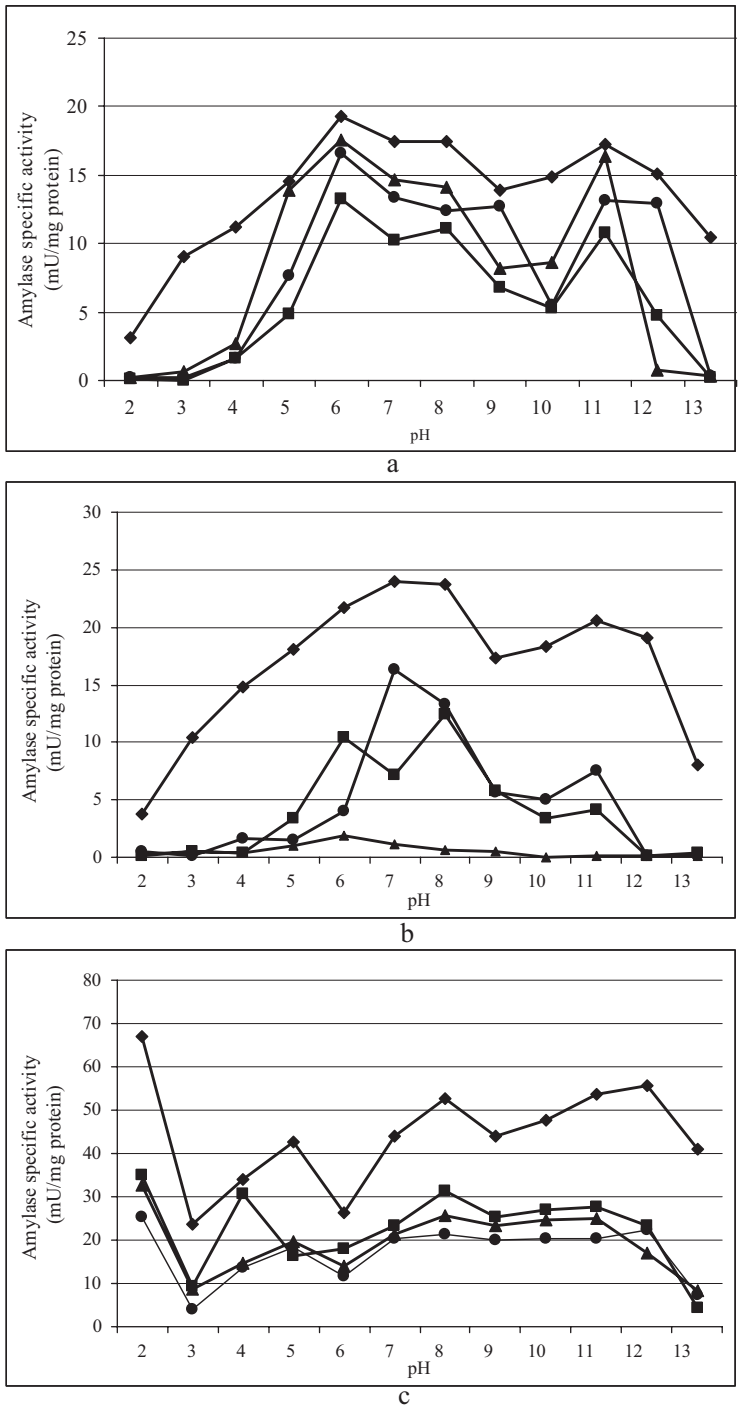

Figure 2 Amylase activity (specific activity) in liver (-◆-), pseudostomach (-■-), upper (-▲-) and lower (-●-) intestines at pH 2 – 13 of 5.7 (**a**), 35.8 (**b**) and 92.1 g (**c**) tilapia (*Oreochromis niloticus* L.).

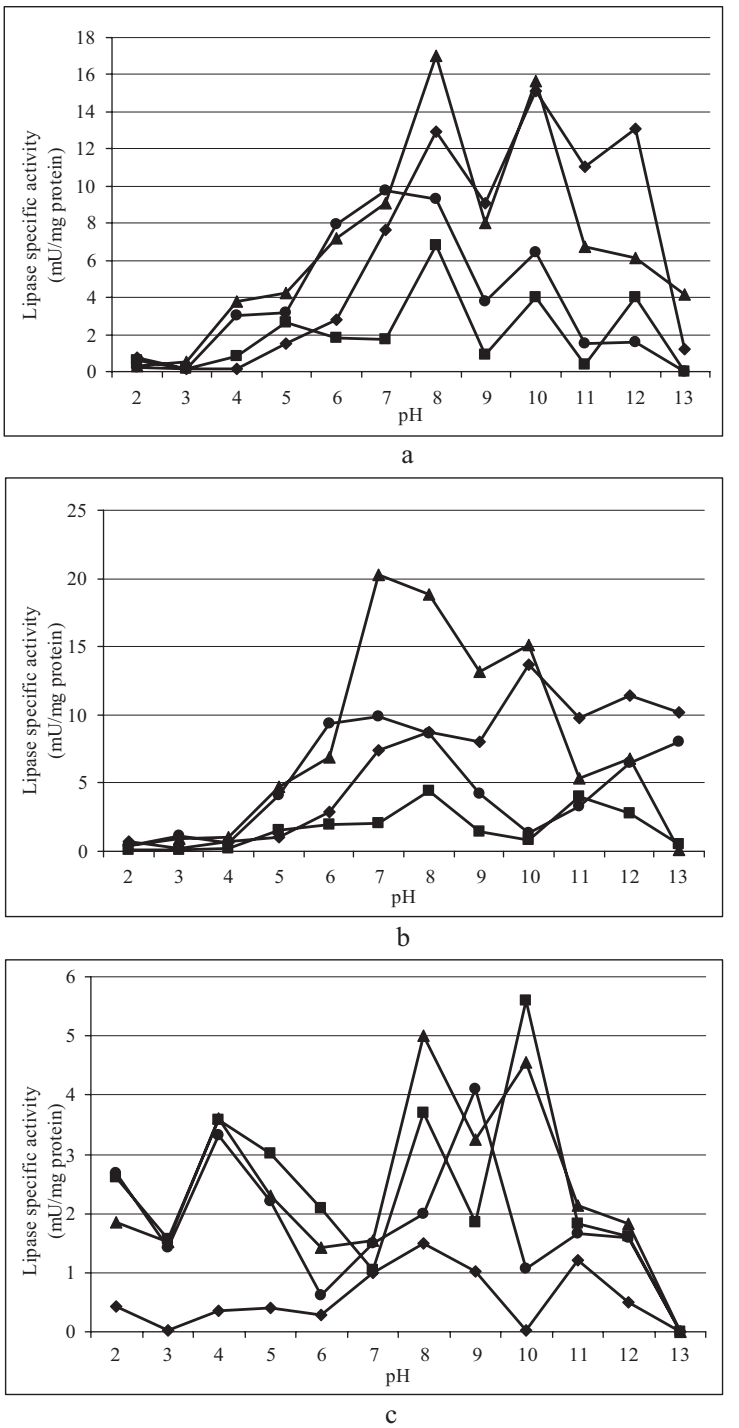

Figure 3 Lipase activity (specific activity) in liver (-●-), pseudostomach (-■-), upper (-▲-) and lower (-●-) intestines at pH 2 – 13 of 5.7 (**a**), 35.8 (**b**) and 92.1 g (**c**) tilapia (*Oreochromis niloticus* L.).

based on their optimal pH (acid or alkaline) and the tissue of production (gastric, pancreatic and mucosal) (Halver and Hardy, 2002). Proteases reported in this study were serine proteases (alkaline protease) because the k_{cat}–pH of serine proteinase–catalyzed reactions showed a bell–shaped curve reflecting a maximum activity close to pH 8 (Wong, 1995). The result was similar to that reported by Amaral *et al*. (2005) who showed that the optimal pH for the soluble enzyme (trypsin) extracted from the intestines of Nile tilapia was 8.0. In stomach-less fish like tilapia, digestion of protein is undertaken by alkaline or serine proteases, which are most active in an alkaline environment. However, the highest activity levels of protease in tilapia at all sizes were not that different and all were found in the lower intestine.

Optimal pH of amylase

The amylase activity level from the liver, pseudostomach, upper and lower intestines of 5.7 g fish was highest at pH 6. For 35.8 g fish, the activity level of amylase from these organs was highest at pH 7, 8, 6 and 7, respectively and at pH 2 for 92.1 g fish (Figure 2). Comparison of the amylase activity in the three sizes of fish showed that the activity was highest in 92.1 g fish. The amylase activity of 5.7 and 35.8 g fish was similar to that from the study of Zeng and Cohen (2000), which showed peaks of amylase activity at pH 6.5 in the salivary glands of *Lygus hesperus* and *L. lineolaris*. Sheng *et al*. (2006) reported a pH level of 7.5 for maximal activity of amylase extracted from the intestines of 55–225 g Nile tilapia. Moreau *et al*. (2001) found that the activity of amylase extracted from the intestines of 50 g fish had a pH of 7.0 as the optimum. Amylase has been identified in the pancreatic juice, stomach, intestines and bile, however, the main producers were the pancreas and the liver (De Silva and Anderson, 1995). The optimal pH for amylase activity varied depending on the source of the enzyme, with a range of pH values reported for amylase in mammals of 6.0–7.0 and 4.8–5.8 for *Aspergillus oryzae*, 5.85–6.0 for *Bacillus subtilis*, 5.5–7.0 for *Bacillus licheniformis* and 6.0–8.0 with a temperature tolerance from 20 to 40°C for porcine pancreatic α–amylase (Wong, 1995). The carbohydrate is thus digested at a non-acidic pH.

Optimal pH of lipase

The activity of lipase from the liver, pseudostomach, upper and lower intestine of 5.7 g fish was highest at pH 10, 8, 8 and 7, at pH 10, 8, 7 and 7 in 35.8 g fish and at pH 8, 10, 8 and 9 in 92.1 g fish, respectively. However, the highest activity of lipase was detected in 35.8 g fish (Figure 3). Gjellesvik *et al*. (1989) reported similar results with cod lipase activity at an optimal pH above 8.25 while Sheng *et al*. (2006) reported the maximal activity of lipase from the intestines of hybrid juvenile tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) at a pH level between 6.0 and 9.0. Triacylglycerol lipase or lipase are commonly found in animals, plants and microorganisms. Animal lipases include pancreatic, gastric and intestinal lipase (Wong, 1995). Pancreatic lipase hydrolyzes only the primary ester bond of the lipid yielding tri–, di– and mono–acylglycerol, respectively. The general digestive process for lipids involves the extracellular hydrolysis of lipid in the stomach, intestines and cecal lumen by a variety of lipases and colipases (Higgs and Dong, 2000). However, the primary sites of lipid hydrolysis for most species appear to be in the pyloric ceca and anterior intestines (Halver and Hardy, 2002). All fat–digesting enzymes act in an alkaline media and the optimal pH varies slightly from species to species. Lipase from the intestinal mucosa has an optimal pH between 7.0 and 7.5, while intestinal esterase is most active between a pH of 8.0 and 9.0 (De Silva and Anderson, 1995), in agreement with the values reported here.

Digestive enzyme activities of various sizes of tilapia

The enzyme activities in the liver, pseudostomach, upper and lower intestine of 5.7, 35.8 and 92.1 g fish are summarized in Tables 1, 2 and 3, respectively. Protease activity appeared to be significantly higher in the lower intestine than in other organs of the 5.7 and 35.8 g fish (*P<0.01*) since the protease in tilapia is a serine protease, which is most active in an alkaline environment in the intestines (De Silva and Anderson, 1995). The amylase activity in the liver of 5.7, 35.8 and 92.1 g fish was significantly higher than other organs (*P<0.01*) due to the pancreas being the main producer of carbohydrases (amylase). The pancreas in fish can be either discretely located in one organ or diffused with pancreatic nodules spreading through the adipose tissue, mesentery and liver (Halver and Hardy, 2002). Amylase may also be produced in the liver (De Silva and Anderson, 1995). The lipase activity was significantly higher in the upper (anterior) intestine of 5.7 and 35.8 g fish (*P<0.01*). The findings were similar to the work of Natalia *et al*. (2004), which showed the highest activity level of lipase was in both the upper and lower intestines and the pancreas of 251.5 g *Scleropages formosus*. The primary site of lipid hydrolysis in most fish appeared to be in the pyloric ceca and upper intestine (Halver and Hardy, 2002).

Table 1 Activity of digestive enzymes extracted from various organs of 5.7 g tilapia (*Oreochromis niloticus* L.).

Organs	Specific activity (mU mg protein^{-1})		
	Protease	Amylase	Lipase
Liver	7.894 ± 0.301^{d}	19.252 ± 0.109^{a}	15.100 ± 0.923^{a}
Pseudostomach	13.515 ± 0.134^{c}	13.284 ± 0.675^{c}	6.831 ± 1.318^{c}
Upper intestine	20.410 ± 0.070^{b}	17.538 ± 0.133^{d}	17.033 ± 0.492^{a}
Lower intestine	24.260 ± 0.183^{a}	16.545 ± 0.023^{b}	9.767 ± 0.938^{b}

^{abcd} Mean values in the same column with different letters are significantly different (*P<0.01*)

Table 2 Activity of digestive enzymes extracted from various organs of 35.8 g tilapia (*Oreochromis niloticus* L.).

Organs	Specific activity (mU mg protein^{-1})		
	Protease	Amylase	Lipase
Liver	9.921 ± 1.308^{c}	23.971 ± 1.058^{a}	13.684 ± 0.111^{b}
Pseudostomach	6.225 ± 0.644^{d}	12.433 ± 0.583^{c}	4.383 ± 0.238^{d}
Upper intestine	22.918 ± 0.745^{b}	1.878 ± 0.012^{d}	20.248 ± 0.421^{a}
Lower intestine	25.694 ± 0.977^{a}	16.363 ± 0.138^{b}	9.872 ± 1.123^{c}

^{abcd} Mean values in the same column with different letters are significantly different (*P<0.01*)

Table 3 Activity of digestive enzymes extracted from various organs of 92.1 g tilapia (*Oreochromis niloticus* L.).

Organs	Specific activity (mU mg protein^{-1})		
	Protease	Amylase	Lipase
Liver	2.509 ± 0.108^{b}	57.153 ± 3.786^{a}	1.501 ± 0.125^{c}
Pseudostomach	2.852 ± 0.270^{b}	35.149 ± 5.106^{b}	5.598 ± 0.066^{a}
Upper intestine	21.752 ± 1.904^{a}	27.655 ± 0.668^{b}	5.010 ± 0.342^{ab}
Lower intestine	23.923 ± 1.406^{a}	25.497 ± 4.242^{b}	4.107 ± 0.962^{b}

^{abc} Mean values in the same column with different letters are significantly different (*P<0.01*)

Variation in the activity of each type of enzyme is shown in Figure 4 for the three sizes of tilapia. The activity of protease and lipase in the smaller fish (5.7 and 35.8 g) was higher than that in the large fish size (92.1 g). Amylase activity, on the other hand, was lower in the smaller fish and increased in the large fish. The results agreed with the report by Rathore *et al*. (2005), which also found that the amylase activity of common carp was high in large fish. The growth of fish is highly variable, being greatly dependent upon a variety of interacting environmental factors, such

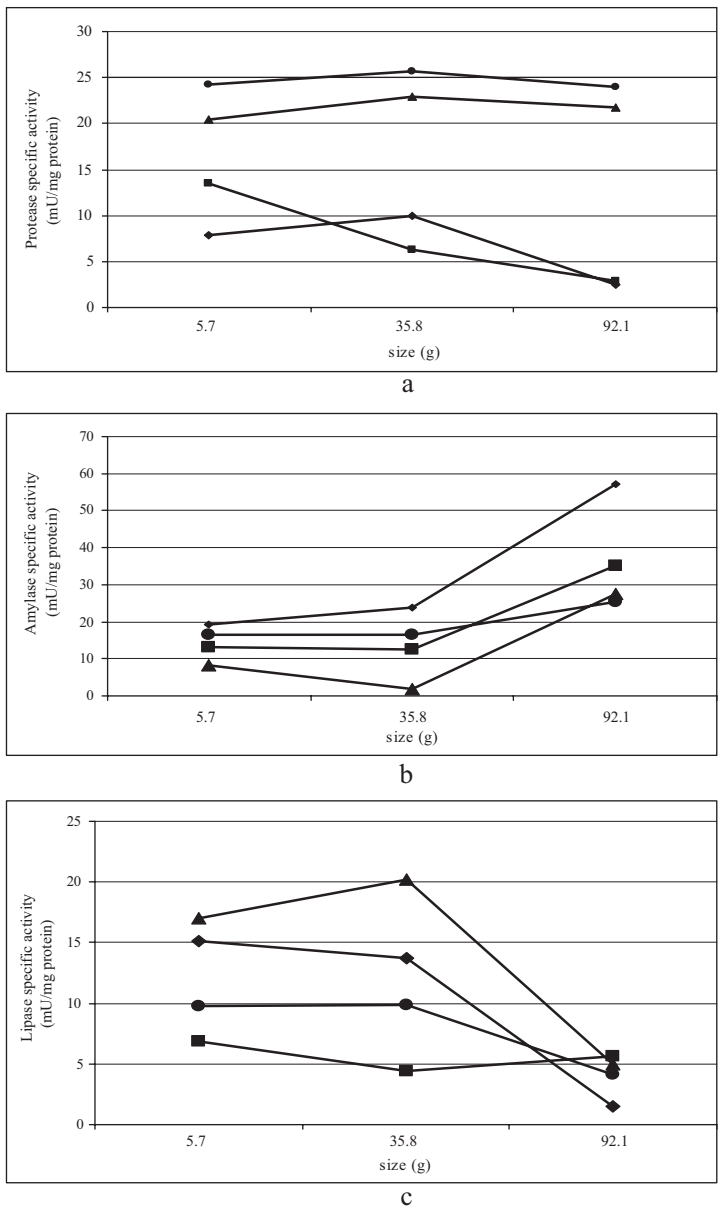

Figure 4 Activity of digestive enzyme (protease (a), amylase (b) and lipase (c)) extracted from liver (-◆-), pseudostomach (-■-), upper (-▲-) and lower (-●-) intestines of 5.7, 35.8 and 92.1 g tilapia (*Oreochromis niloticus* L.)

as water temperature and with other factors, such as: the degree of competition; the amount and quality of food ingested; and the age and state of maturity of the fish (Moyle and Cech, 2000). Food quality is also important, with a complete diet including essential amino acids, fatty acids, minerals and vitamins being required for a high growth rate in fish. The growth rate of fish is also controlled by growth hormone (GH), with the two major metabolic functions of GH being protein accretion and lipid metabolization (Björnsson, 1997). GH could increase the rate of protein synthesis in rainbow trout, leading to an increase in the growth rate. GH also stimulates an increase in triacylglycerol lipase activity in the liver, which breaks down lipids to usable fatty acids and glycerol. Dietary proteins, lipids and carbohydrates are essential for growth (anabolism) and for energy to run the body machinery (catabolism). Proteins, which consist of chains of amino acids, seem to be essential mainly for growth although they may also be used for catabolic functions (Moyle and Cech, 2000). Dietary lipid is the only source of essential fatty acids needed by fish for normal growth and development. Lipids, especially phospholipids, are important for cellular structure and the maintenance of membrane flexibility and permeability (Shiau, 2002). From the results of the current study, the activities of protease and lipase were high in the small fish, with this activity being essential for the utilization of protein and lipid from feed to produce a high growth rate at an early age. Amylase, which is needed for the hydrolysis of carbohydrates, responds to the level of dietary carbohydrate. *Oreochromis mossambicus* developed a higher level of amylase activity when they were changed to a starch–rich diet (De Silva and Anderson, 1995). Thus, carbohydrase (amylase) and protease activity are related to the feeding habits of fish.

CONCLUSION

The study clearly demonstrated that there were variations in the digestive enzyme activity (protease, amylase and lipase) in different sizes of tilapia. The protease and lipase activities were high in small-sized fish while amylase activity was high in the large size. Digestive enzyme activities also depended on the organs studied. The protease and lipase activities appeared to be high in the intestines while amylase was more active in the liver. Different digestive enzyme activities in various sizes of fish can be used to select the suitable feed stuffs for the highest digestibility and the lowest cost in each fish size**.** Thus, the results from this study can be used as a basis for suitable feed formulation that will be effectively utilized by different sizes of Nile tilapia.

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