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by Sri Hastuti

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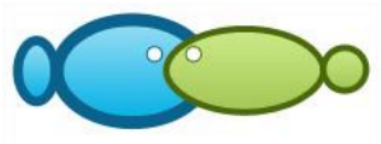

Blood serum biochemistry responses and digestive enzyme activities of tilapia (*Oreochromis niloticus*) according to different dietary protein level consumption

Sri Hastuti, Subandiyono Subandiyono

Department of Aquaculture, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang, Indonesia. Corresponding author: Sri Hastuti, hastuti_hastuti@yahoo.com.

Abstract. The present experiment was carried out for 40 days to determine the effects of dietary protein on haematological parameters and digestive enzyme activities of tilapia (*Oreochromis niloticus*). The four isolipid test diets contained 28, 32, 36, and 40% of protein. The diet was formulated to be tested randomly on 600 tilapia (5.0±0.5 g). The experiment used three replications. The fish was reared in 60x40x50 cm aquaria; each unit was equipped with aeration and thermostat. Results indicated that haematological parameters including triacylglycerol, aspartate aminotransferase, and alkaline phosphatase decreased as the dietary protein levels increased. The lowest pepsin activity was observed in 28% of dietary protein, which indicated the lower availability of protein as a substrate for protease activities. Furthermore, the highest amylase activity was found in the fish fed on 28% of protein, as was indicated and related to higher carbohydrate level of the diet. Based on the polynomial regression analysis of pepsin and amylase activities, the dietary protein requirements for the trial tilapia were estimated at 35.73% and 35.82%, respectively, with the dietary lipid level of 10.05%.

Key Words: amylase, dietary protein requirements, pepsin, protease, tilapia haematological profile.

Introduction. Digestibility of feed ingredients is crucial in learning fish energy needs and assessment from various diets containing different protein levels. Digestive enzymes are produced in the fish body to break down nutrients in the food consumed, so that it can be absorbed easily. The role of digestive enzymes is very important for the digestive process in the body of the fish. The ability of fish to digest food is very dependent on the availability of digestive enzymes (Fitriliyani 2011). Digestive enzyme activity is an indicator to determine digestive capacity. Whenever digestive enzyme activity is high, it can be physiologically indicated by the abilities of the fish to process the diet consumed (Infante & Cahu 2007). Therefore, the activity of digestive enzymes are influenced by dietary nutrients consumed by the fish (Nurhayati et al 2014; Yamin & Palinggi 2007; Suzer et al 2007).

Feed ingredient digestibility in the fish is affected by several factors, including the feed type, the feed nutritional content (protein), and the digestion enzyme content and types in the fish digestive tract (NRC 1993; Tillman et al 1991; Hepher 1990). Fish ability in digesting feeds is very dependent on the availability of digestive enzymes. It seems that the nutrient content of feed (protein) also affects the enzyme activity digestion. Therefore, digestive enzyme activity indicators to measure dietary protein requirements are used.

Biochemistry of blood serum such as total cholesterol, triglycerides, or aminotransferase is usually used as indicators for assessing the fish nutritional and health status (Shi et al 2016). Variations in dietary nutritional profiles can cause changes in the physiological functions of fish (Kamalam et al 2017). The relationship between enzymatic and intermediate activity metabolic steps may be responsible for controlling nutrient uptake as well as specific metabolic pathways for each nutrition (Kamalam et al 2017).

Trends in increasing tilapia (*Oreochromis niloticus*) production from the aquaculture industries on 2030 reached 128 million tons (FAO 2017). As a result, the need for pellet feeds to support tilapia production will also increase. Currently, pellet feeds is commonly applied in tilapia farming. Furthermore, natural food resources in the form of trash fish and earthworms were declining (Tacon & Metian 2008) as were being used for fish feeds. Therefore, the development of pellet diets for tilapia farming at certain growth stages is important to formulate. Protein play an important role in the feed formula and it is required for better growth of *O. niloticus*. Reducing dietary protein level could affect the fish growth performances. On the other hand, the increasing of protein content above the optimal level will increase costs, as well as waste production and implicit ambient water pollution (Xu et al 2011). Yet, despite the fact that the potential production of pellet feeds for tilapia is very high, there is no accurate information available on protein requirements of the tilapia in relation to the blood serum biochemistry responses and digestive enzyme activities. This study was designed to compare the biochemistry of blood serum and digestive enzymes in different levels of dietary protein.

Material and Method

Experimental design. The experiment was arranged in a completely randomized design with total of 4 treatments and performed in triplicates. Four levels of dietary protein, namely 28, 32, 36, and 40%, each for diet 1, 2, 3, and 4, respectively, were tested on tilapia (weight 5.0±0.5 g). The experimental fish were reared in 60×40×50 cm aquaria for 40 days. Each aquarium was equipped with an aerator and thermostat. This research was conducted at the Laboratory of the Fish Seed Center (FSC), Siwarak, Semarang, Central Java, Indonesia.

Experimental diets. Four experimental diets were formulated to contain different levels of crude protein, namely 28, 32, 36, and 40% (Table 1).

Table 1

Formulation and composition of test diets

Feed ingredients (g 100 g^{-1})	*Diets (Crude protein, %)*			
	Diet 1 (28)	*Diet 2 (32)*	*Diet 3 (36)*	*Diet 4 (40)*
Fish meal	40.50	40.50	40.50	40.50
Casein	0.00	4.50	9.15	11.55
Fish oil	7.15	7.15	7.15	7.15
Wheat powder	11.15	11.15	11.15	11.15
Starch	32.70	27.20	21.65	16.05
Vitamin premix*	1.00	1.00	1.00	1.00
Mineral premix**	2.00	2.00	2.00	2.00
Choline chloride	0.50	0.50	0.50	0.50
$Ca(H_2PO_4)_2$	1.00	1.00	1.00	1.00
Cellulose	1.00	2.00	2.90	6.10
Sodium carboxymethyl cellulose	2.00	2.00	2.00	2.00
Phagostimulant	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00
Proximate composition (%)				
Ash	7.96	8.05	7.96	7.78
Lipid	10.05	10.05	10.05	10.05
Crude protein (CP)	27.90	32.03	36.05	40.06
Gross energy(kJg^{-1})	15.58	15.55	15.61	15.65

*Vitamin (mg kg^{-1} of diet): VB_1 50 mg, VB_2 200 mg, VB_6 50 mg, VB_{12} 20 mg, folic acid 15 mg, VC (30%) 325 mg, pantothenate 400 mg, inositol 1,500 mg, *D*-biotin (2%) 5 mg, niacin 750 mg, VA 2.5 mg, VE (50%) 100 mg, VD_3 2 mg, VK 20 mg. **Mineral (mg kg^{-1} of diet): $Ca(H_2PO_4)_2$ 1,800 mg, KH_2PO_4 1,350 mg, NaCl 500 mg, $MgSO_4 \cdot 7H_2O$ 750 mg, $NaH_2PO_4 \cdot 2H_2O$ 650 mg, KI 1.5 mg, $COSO_4 \cdot 6H_2O$ 2.5 mg, $CuSO_4 \cdot 5H_2O$ 15 mg, ZnSO4·7H2O 350 mg, $FeSO_4 \cdot 7H_2O$ 1,250 mg, $MnSO_4 \cdot 4H_2O$ 80 mg, Na_2SeO_3 6.00 mg.

Fish meal and casein was used as the main source of protein, while fish oil was used as the main lipid source. All macro-ingredients were sieved through a 160 µm-mesh size and then mixed using a blender. The micro-ingredients (vitamin premix, mineral premix, choline chloride and calcium carbonate) were mixed in a plastic container before being added to the macro-ingredients. Fish oil was homogenized before added to the mixture. The final mixture was then passed through a meat grinder to form a 3 mm-diameter pellets. Furthermore, the pellet feed was dried in an oven at temperature of 60°C. Dry pellets were packaged in plastic bags and stored at 0°C until usage.

Experimental fish and culture management. Six hundred tilapia with the body weight of 5.0±0.5 g were obtained from the Fish Seed Center (Siwarak, Semarang) and used as experimental subjects. After 2 weeks-acclimatization period, the fish were randomly assigned to twelve aquaria of 60×40×50 cm with a density of 50 fish in each aquarium. Acclimatization of the experimental diet was carried out gradually, where the fish was used to be fed on commercial feed, then the test diet was proportionally added up to 20, 40, 60, 80, and finally 100%. During the 40 days trial, the fish were fed three times a day by applying the ad satiation method. During the experiment, feed intake was recorded and manure was daily removed from the aquarium. The temperature was maintained around 29.92±2.55°C, with dissolved oxygen level of >3 mg L^{-1} and ammonia nitrogen <0.05 mg L^{-1}.

Sample collection and analysis. After fasting for 24 hours, all fish in the aquarium were anesthetized, counted, and weighed at the end of the experiment. The results of these measurements were used to calculate growth and survival variables. The gastrointestinal tract including the stomach and intestines of six fish per treatment (n=24) were weighed and then homogenized as described by Li et al (2012) for determination of digestive enzyme activities. The extract was centrifuged at 1,150 rpm at 4°C for 10 minutes, then the supernatant was collected as an enzyme source. Pepsin, amylase, and lipase activities were analyzed using a commercial kit.

Blood samples of three fish from each treatment (n=12) were collected via the caudal vein. Blood serum was prepared by centrifugation (1,150 rpm, 10 minutes, 4°C) for measurement of haematological parameters, including total cholesterol, triacylglycerol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase using the colorimetric method.

Statistical analysis. Results on the measurement of the enzyme activity, blood serum hematology and growth and SR are presented as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test with SPSS 22.0 software. The significance test was carried out with a probability level of $p<0.05$. Furthermore, the polynomial regression model was calculated to estimate the optimal dietary protein requirements.

Results

Biological parameters. Biological parameters, including body weight, growth performances, and survival of the experimental fish fed on four test diets (diets 1-4) for 40 days are shown in Table 2. Fish survival rates ranged from 91.69 to 95.18%, which was similar amoung groups ($p<0.05$). Furthermore, dietary protein levels significantly affected feed consumption and *O. niloticus* growth ($p>0.05$).

Table 2

Biological parameters of *Oreochromis niloticus* fed on different dietary protein levels

Biological parameters	*Diets (Crude protein, %)*			
	Diet 1 (28)	*Diet 2 (32)*	*Diet 3 (36)*	*Diet 4 (40)*
Initial weight	5.02±0.17	5.25±0.14	5.42±0.62	5.48±0.59
Final weight (g)	18.90±1.37^{c}	23.40±1.71^{b}	25.52±2.16^{a}	26.10±1.57^{a}
Feed consumption (g)	19.29±0.22^{b}	22.69±0.31^{a}	23.12±0.87^{a}	24.74±0.58^{a}
SGR (% day^{-1})	3.31±1.26^{c}	3.74±0.27^{b}	3.87±0.32^{a}	3.90±0.21^{a}
Survival rate (%)	91.69±3.40^{a}	95.18±3.30^{a}	94.07±4.46^{a}	94.96±5.58^{a}

Data are expressed as mean±SD of three replications. Values on the same row with different superscripts show statistically significant differences ($p<0.05$).

Digestive enzyme activities. The activities of digestive enzymes, including lipase, amylase, and pepsin are presented in Table 3. Furthermore, the response of digestive enzyme activities to dietary protein levels is presented in Figure 1. Second order of polynomial regression analysis of the pepsin (Figure 1a) and amylase (Figure 1b) in relation to the dietary protein levels, followed the equations of $Y = -8.2894x^2 + 592.4x - 9889.5$ ($R^2=0.9558$) and $Y = 0.0084x^2 - 0.6018x + 10.972$ ($R^2=0.9994$), respectively. Therefore, the optimal dietary protein level calculated from the equation were 35.73 and 35.82%, with the feed lipidic level of 10.05%.

Table 3

Digestive enzyme activities of *Oreochromis niloticus* fed on different dietary protein levels

Enzymatic parameters	*Diets (Crude protein, %)*			
	Diet 1 (28)	*Diet 2 (32)*	*Diet 3 (36)*	*Diet 4 (40)*
Lipase (U g^{-1} protein)	0.99±0.24^{a}	1.01±0.05^{a}	1.30±0.12^{a}	1.12±0.12^{a}
Amylase (U mg^{-1} protein)	0.74±0.16^{a}	0.35±0.07^{b}	0.25±0.11^{b}	0.40±0.07^{b}
Pepsin (U mg^{-1} protein)	216.47±13.38^{c}	525.61±25.26^{b}	747.99±23.10^{a}	525.61±32.86^{b}

Data are expressed as mean±SD of three replications. Values on the same row with different superscripts show statistically significant differences ($p<0.05$).

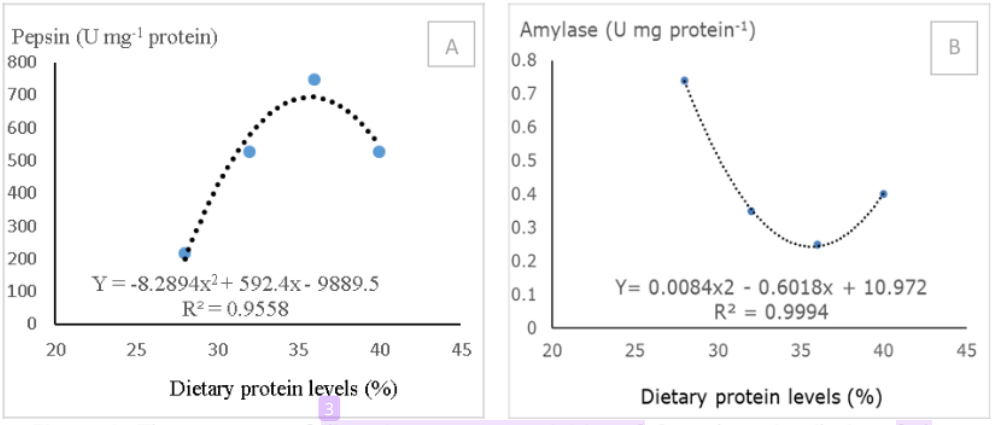

Figure 1. The response of digestive enzyme activities of *Oreochromis niloticus* fed on different dietary protein levels.

Blood serum biochemistry. The haematological parameters of *O. niloticus* are presented in Table 4. The total cholesterol value and triacylglycerol activities decreased as the levels of dietary protein increased, but there was no significant difference for the triacylglycerol content between the 3-4 feed treatments ($p>0.05$). Concerning the alanine aminotransferase (ALT) activities, only in the case of treatment with 28% protein level showed significant higher value, while in the other treatments (i.e. 32, 36, and 40% of protein) the ALT activity was equal. On the other hand, a continuous decreasing trend was observed for the aspartate aminotransferase (AST) activities. Fish that were fed on a diet containing 28% of protein showed the highest alkaline phosphatase level.

Table 4

Haematological parameters of *Oreochromis niloticus* fed on diets containing different protein levels

Haematological parameters	*Diets (Crude protein, %)*			
	Diet 1 (28)	*Diet 2 (32)*	*Diet 3 (36)*	*Diet 4 (40)*
Total cholesterol (mg dL^{-1})	318.60 ± 26.68^{a}	210.73 ± 3.48^{b}	194.10 ± 5.03^{c}	156.98 ± 6.96^{d}
Triacylglycerol (mM)	5.38 ± 1.22^{a}	2.75 ± 0.32^{b}	0.88 ± 0.08^{c}	0.5 ± 0.06^{c}
Alanine aminotransferase (U L^{-1})	3.43 ± 1.46^{a}	2.43 ± 0.32^{b}	2.7 ± 0.88^{b}	2.56 ± 0.25^{b}
Aspartate aminotransferase (UL^{-1})	55.2 ± 19.25^{a}	26.0 ± 6.33^{b}	$12.6\pm0.75b^{c}$	9.76 ± 3.28^{c}
Alkaline phosphatase (UL^{-1})	4.33 ± 1.27^{a}	1.66 ± 0.45^{b}	1.30 ± 0.26^{b}	2.46 ± 0.7^{b}

Data are expressed as mean±SD of three replications. Values in the same row with different superscripts are significantly different ($p<0.05$).

Discussion. The diet that is consumed by tilapia and then enters the digestive tract will undergo to the digestion processes. As long as in the fish intestine, the diet is digested and hydrolyzed by various enzymes into simpler forms or nutrients it can be more easily absorbed by the intestinal walls, and finally entering the circulatory systems (Tu et al 2015; Infante & Cahu 2007). The substrate availability is a factor that regulates enzyme activities in fish (Kuzmina 1996). High protein content in a diet is associated with low starch levels (Table 1), thereby the increasing of pepsin activities (Table 2). Similar results were found in rainbow trout (*Oncorhynchus mykiss*) (Hepher 1990). In the present study, dietary protein levels significantly affected the digestive enzyme activities of *O. niloticus* (Table 1). Furthermore, optimum dietary protein requirements for the maximum enzyme activity are among 35.73 to 35.82% (Figure 1).

Pepsin is one of the key enzymes responsible for proteolysis. In the present study, pepsin activity was relatively low in *O. niloticus* consumed diet 1. This was due to the lower availability of dietary protein as a substrate for pepsin activity; similar phenomenon was reported in hybrid catfish (*Clarias batrachus* × *Clarias gariepinus*) (Giri et al 2003). In addition, lipase activities remained constant among the four diet groups (Table 3), in accordance with the equal lipid concentrations in the related four diets (Table 1); similar results were reported for *Labeo rohita* seeds (Debnath et al 2007) as well as for goldfish (*Cyprinus carpio* var. Jian) juveniles (Liu et al 2009). Amylase activities in the fish group of diet 1 was markedly higher than that found in the other of three test diets. This phenomena is related to the highest carbohydrate and the lowest protein level of diet 1, as well as the previous studies (Giri et al 2003; Liu et al 2009; Mohanta et al 2008).

Digestible enzymes also affect the utilization of dietary protein for fish growth (Tu et al 2015). Results of the present study showed that the growth of *O. niloticus* increased as increaed the dietary protein levels (Table 2) as well as on the pepsin activities (Table 3). Dietary protein is the most important component affecting the growth performances of fish (Lee & Kim 2005). Furthermore, the higher activities of digestive enzymes (i.e. pepsin in diet 3) indicates that *O. niloticus* are physiologically able to utilize the protein consumed, being in accordance with the findings of Infante & Cahu (2007). Therefore, *O. niloticus* that consumed diets containing higher protein level (Table 3) tends to undergo better growth (SGR) (Table 2). Our SGR values obtainedn are higher than that of the study conducted by Kushayadi et al (2020), 3.5±0.02% for *O. niloticus* (6.7±1.2 g) fed on a diet containing 30% of protein.

Blood serum biochemistry of *O. niloticus* including total cholesterol, triacylglycerol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase were influenced by dietary protein levels ($p<0.05$) (Table 4). The increase in dietary protein content tends to decrease the blood serum biochemical values. Otherwise, the decrease in starch content (Table 1) is thought to be a factor that reduces the blood serum biochemical values. *O. niloticus* fed on diet 1 (i.e. containing 32.70% of starch) produced the highest values of total cholesterol, triacylglycerol, ALT, AST, and alkaline phosphatase (Table 4). The increasing of cholesterol content might be caused by the excessive consumption of fat (Sun et al 2015), and carbohydrates (Anand et al 2015). Diets containing high carbohydrates will increase the levels of fructose 2,6 biphosphate, so that the phosphorfructokinase-1 being more active and stimulating glycolysis reaction. The increment on glycolysis will increase the converted glucose to become fat. Furthermore, these free fatty acids are combined with glycerol to form triglycerides (Tsalissavrina et al 2006).

Triglycerides as the result of esterification process between glycerol and three fatty acid also called triacylglycerol. Triacylglycerol levels related to cholesterol levels in the blood. Therefore, the increase in triacylglycerol could cause the total cholesterol level in the blood increase, too (Tsalissavrina et al 2006). The total cholesterol value of *O. niloticus* in diet 1 was 318.60±26.68 mg dL^{-1} (Table 3), which was above the normal values. The normal value of blood cholesterol levels of *O. niloticus* is 64-299 mg dL^{-1} (Hrubec et al 2008).

Quantitatively, hepatic ALT and AST activities were the most important degrading enzymes in amino acid catabolism (Metón et al 1999). Results of the present study showed that the high level of AST in *O. niloticus* fed on diet 1 (Table 4) was as an indicator of malnutrition and responsible for the poor growth performance of this treatment. Additionally, AST was more active than ALT in *O. niloticus* fingerlings, and was consistent with that in tiger puffer (Takifugu rubripes) juveniles (Kim & Lee 2009). The activities of alkaline phosphatase could be used as markers for well-differentiated intestinal brush border membranes (Kvåle et al 2009). Higher alkaline phosphatase activities in diet 1 indicated abnormal intestinal digestive function in fish fed on this diet. Furthermore, triacylglycerol content was gradually decreasing from diet 1 to diet 4 (Table 4), which suggested the deduction of lipid transportation as dietary protein levels increased.

Conclusions. It was concluded that the optimum protein levels required by *O. niloticus* fingerlings were ranged between 35.73 to 35.82%, based on the polynomial regression analysis of pepsin and amylase activities. Dietary protein levels significantly influenced on the growth performances, blood serum biochemistry, and digestive enzyme activities of the experimental fish.

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Authors:
Sri Hastuti, Department of Aquaculture, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang 50275, Central Java, Indonesia, e-mail:hastuti-hastuti@yahoo.com
S.Subandiyono, Department of Aquaculture, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang 50275, Central Java, Indonesia, e-mail:s_subndiyono@yahoo.com
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