The Age Dependent Activities of Digestive Enzymes in Rasbora, *Rasbora lateristriata* Blkr., (Pisces: Cyprinidae)

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ABSTRACT

This study was to evaluate the digestive enzyme activity included a protease (trypsin & chymotrypsin), lipase, carbohydrase (amylase & cellulose) and alkaline phosphatase in Rasbora. This research was carried out using three different ages (2, 4 and 6 months) and in each age consisted of six groups (replicates). In this study 150 fish (±2 months age), 120 fish (± 4 months age) and 90 fish (± 6 months age) were used. All digestion enzyme activity was measured by the spectrophotometric method, except the lipase activity was by the titration method. The results showed that the distinctness of age resulted in a significant difference on total protease, trypsin, lipase, cellulase and alkaline phosphatase ($P < 0.05$), but no significant difference in amylase activity ($P > 0.05$). Total protease and trypsin activities were higher in fish of age two months than fish age four and six months, but the activity of lipase, cellulase and alkaline phosphatase were higher in fish age of four months compared to two months age fish. Fish, with distinct age has the different nutrient digestion capacity as expressed by differences in the activity of the enzyme digestion, except amylase. These results contribute to the future development of digestive physiology, especially in Rasbora.

Keywords: alkaline phosphatase, carbohydrase, lipase, protease, Rasbora

INTRODUCTION

The ability of fish to digest the feed is highly dependent on the activity of digestive enzymes, such as protease, lipase, carbohydrase, and alkaline phosphatase. Acid protease or pepsin is particularly active in the fish with stomach, as observed in *Mormyrus Rume* (Odedeyi & Fagbenro, 2010), *Etroplus suratensis* and *Oreochromis mossambicus* (Sankar et al., 2014), but not in fish without stomach (Day, German & Tibbetts, 2011a) and the Mediterranean fish (Caruso, Denaro, & Genovese, 2009).

The activities of alkaline protease such as trypsin and chymotrypsin, have also been studied in a variety of fish (Kamarudin, Otoi, & Saad, 2011; Savona, Tramati, & Mazzola, 2011). These enzymes showed different activities in the fish digestive tract, according to feed quality (Debnath *et al*., 2007; Chaudhuri, Mukherjee, & Homechaudhuri, 2012; Abdel-Warith, Younis, & Abdualla, 2013), feeding guilds (Falcon-Hidalgo, Forrellat-Barrios, Farnes, & Hernandez, 2011; Langeland, Lindberg, & Lund, 2013), phylogeny (German, Horn, & Gawlicka, 2004; Kumar *et al*., 2007), and sex (Thongprajukaew & Kovitvadhi, 2013).

Lipase and carbohydrase activities are varied among fish with different feeding guilds in which omnivorous fish show a lower lipase activity than carnivores (Langeland *et al.*, 2013). Amylase activity is generally higher in herbivorous fish than in omnivores and carnivores (Day *et al*., 2011b). The ability of fish to digest cellulose is highly dependent on the presence of microbial symbionts in their digestive tracts (Maity, Kundu, Pramanik, & Patra, 2011; Ganguly & Prasad, 2012).

Past studies have shown cellulase activity in *Oreochromis niloticus* (omnivore), but not in *Horabagrus brachysoma* (omnivore) and carnivorous *Parachanna obscura* and *Gymnarchus niloticus* (Fagbenro *et al*., 2005; Prasad & Suneesha, 2013). Alkaline phosphatase activity was also affected by changes in the feed quantity and quality (Liu, Zhang & Wang, 2010), but studies on *Labeo rohita* indicated otherwise (Debnath *et al*., 2007). The effect of age or size of the fish on the digestive enzyme activity has also been documented in previous studies (Klahan, Areechon, Yoonpundh, & Engkagul, 2009; Thongprajukaew, Kovitvadhi, Engkagul, & Rungruangsak-Torrissen, 2010; Susilo, Yuwono, Rachmawati, Priyanto, & Hana, 2015). However, the effect of age on enzyme

activity changes when diet and feeding guilds differ (Day *et al*., 2011a; Savona *et al*., 2011; Pena *et al*., 2015). In spite of elaborate study on digestive enzyme on various fish species, no published data are available for Rasbora digestive enzyme activities related to its age.

Feed consumed by fish will be used for growth if it has been digested in the gastrointestinal tract of fish. The digestive process of feed involving digestive enzymes such as proteases, lipases and carbohydrases, requires the compatibility between the feed consumed and the capacity of fish digestion, in order to achieve feed efficiency (Caruso *et al*., 2009). The digestion product by the enzyme is a simple compound and readily absorbed by the fish intestine to support growth. Among the digestion products is a phosphate-bonded compound which is a substrate for alkaline phosphatase, so the hydrolysis product becomes easily absorbed (Ducasse-Cabanot *et al*., 2007; Silva *et al*., 2010). Therefore, a good feed quality supported by its enzyme capacity will be an important factor to support fish growth, otherwise when low feed quality will have an effect on the poor of nutrient intake for fish growth (Thongprajukaew *et al*., 2011; Santigosa *et al*., 2011).

Rasbora (*Rasbora lateristriata* Blkr.) is a wild fish that has the potential to be developed, because it has good taste and high nutritional value. Biological studies of the genus Rasbora have been conducted, but mostly focused on the taxonomy aspects of the fish (Kottelat, Whitten, Kartikasari, & Wirjoatmodjo, 1993), its length to weight relationships (Muchlisin, Musman, & Azizah, 2010), fecundity and spawning frequency (Muchlisin, Musman, Fadli, & Azizah, 2011), natural feed (Sulistiyarto, 2012) and its distribution (Rosadi, Yuli, Setyohadi, & Bintoro, 2014).

A scientific study related to the digestive physiology of fish will be necessary to support the domestication efforts of Rasbora in the future. Therefore, a research focusing on the digestive capacity associated with digestive physiology as expressed by digestive enzyme activity provides new insight , especially for Rasbora. The objective of this study was to evaluate the digestive enzyme activities of total protease, trypsin, chymotrypsin, lipase, amylase, cellulase and alkaline phosphatase with different age in Rasbora.

EXPERIMENTAL SECTION

Materials and Instruments

Nα-*p*-tosyl-L-arginine hydrochoride methyl ester (Sigma-Aldrich, AG), N-benzoyl-L-tyrosine ethyl ester (Sigma-Aldrich, AG), Sodium acetate (Merck, AG), Folin & Ciocalteu's phenol reagent (Sigma-Aldrich), Starch (Bio Basic Canada, High Purity), carboxymethylcellulose (Merck, Technical Grade), Tris (hydroxymethyl) aminomethane (Tris) (Sigma-Aldrich, ACS reagent, >99.8%), hydrochloric acid (Merck, 36.5-38.0%), 3,5-dinitrosalicylic acid (DNS) (Sigma-Aldrich, >98%), *p*-nitrophenyl phosphate (*p*NPP; Sigma-Aldrich, AG), *p*-nitrophenol (Sigma-Aldrich, AG), NaOH (Sigma-Aldrich, AG), single centrifuge (Eppendorf, 5415 R), spectrophotometry (Hitachi, U-2900), channel pipette (Serana), waterbath (JEIO-TECH, WB-20E).

Experimental Design

The fish sample (F1) was obtained from Wet Laboratory of Biology Faculty, Unsoed, Purwokerto. Fish with three different age maturity (age 2, 4 and 6 months) were rearing in three difference concrete tanks (125 x 250x50cm) and feed twice days at 08:00-09:00 pm and 15:00-16:00 pm. Fish were fed with artificial diet containing crude protein 33.71 %, crude lipid 5.67 % and NFE 38.96 % as a previous study on Rasbora (Susilo, Sukardi & Affandi, 2016) during two weeks rearing. In this study 150 fish (age ±2 months, mean weight 0.28±0.04 g), 120 fish (age ±4 months, mean weight 0.44±0.08 g) and 90 fish (aged ±6 months, mean weight 0.74± 0.12g) were used. Fish was fasted for 24 h before the sampling. At the end rearing fish from each concrete tanks divided into six pools sample.

Crude enzyme preparation.

Using a tissue Homogenizer (Heidolph Dixie 900), the digestive organs were isolated and homogenized (1: 8 w/v) in a cold solution of 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaCl. Homogenates were centrifuged at 12.000 RPM (Eppendorf, 5415 R) for 15 minutes at 4 °C and the supernatant was stored in the refrigerator at -80 °C (Thongprajukaew *et al*., 2010) until used for enzyme activity measurements. Soluble protein contents in the supernatant were determined using albumin as a measurement standard.

Determination of Digestive Enzymes Activity

A modification of casein hydrolysis was used to measure the activity of total protease (Thongprajukaew *et al*., 2010). The buffers included glycine-HCl 0.1 M (pH 2.0), citrate 0.1 M (pH 5.0), phosphate 0.1 M (pH 6.9), Tris-HCl 0.1 M (pH 8.1) and Glycine-NaOH 0.1 M (pH 10.0) and KCl-NaOH (pH 12.5) 0.1 M. The reaction mixture consisting of casein 1% (w/v) in buffer (450 µL), buffer (450 µL) and crude enzyme extract (100 µL) were incubated for 60 min at 37 °C. This reaction of the mixture was stopped by the addition of 1000 µL TCA 8% (w/v) reagent.

The same procedure was done to blank tube except that the enzyme extract was added after the TCA 8%(w/v). After storing for 1 hour in 4 ^{o}C, the sample was centrifuged at 6000 rpm for 10 minutes. The absorbance of supernatant was measured at a wavelength of 280 nm. Protease activity was calculated using a standard curve of tyrosine. The amount of enzyme required to catalyze the formation of 1 µg tyrosine min^{-1} was defined as one unit of enzyme activity.

The trypsin activity was measured using Nα-p-tosyl-L-arginine hydrochloride methyl ester (TAME; Sigma-Aldrich, AG) as substrate (Khishimura, 2008). Reagent mixture consisted of 100 µL of enzyme extract and 300 µL of 10 mM TAME in 2600 µL of 46.0 mM Tris-HCl buffer at pH 8.1 with 11.5 mM $CaCl_2$. Reactions were started by adding the enzyme extract into a mixture of TAME and buffer solution. TAME hydrolysis were measured as the change in absorbance at 247 nm for 3 minutes at 30 ^{o}C. Trypsin activity was expressed as the change in absorbance min^{-1} mg^{-1} protein in enzyme extract.

N-benzoyl-L-tyrosine ethyl ester (BTEE;Sigma-Aldrich, AG) was applied as a substrate to measure the activity of chymotrypsin (Lazzari *et al*., 2010). Reagent mixture consisted of 100 µL of enzyme extract and 1400 µL of 2.0 mM BTEE in 1500 µL of 80.0 mM Tris-HCl buffer at pH 7.8 with 0.1 M $CaCl_2$. Reactions were started by addition of enzyme extract to the mixture BTEE and buffer solution. BTEE hydrolysis was measured as change absorbance at 256 nm for 3 minutes at 30 ^{o}C. The activity of chymotrypsin was expressed as the change in absorbance min^{-1} mg^{-1} protein in enzyme extract.

Titration method with olive oil emulsion as substrate was used to measure lipase activity (Borlongan, 1990). Reagent mixture consisted of a substrate (2000 µL), 0.1 M Tris-HCl buffer at pH 8.0 (3000 µL) and extract enzyme (500 mL) was incubated for 2 h at 37 °C, then stopped by the addition of 3000 µL ethyl alcohol 95 %(v/v). The reaction mixture was titrated with 0.01 N NaOH using 0.9 % (w/v) phenopthalien in ethanol as indicator. The same procedure was conducted to blank tube except enzyme extract was added after ethyl alcohol and immediately before titration. Lipase activity expressed as units of activity per mg protein. Lipase activity unit (U) was defined as the volume of 0.01 N NaOH required to neutralize the fatty acid released during the 2 hours of incubation of the substrate and after correction by the blank sample.

A standard protocol of 3,5-dinitro-salicylic acid (Sigma-Aldrich, AG) using starch as substrate was used to measure amylase activity (Klahan *et al*., 2009), whereas phosphate 0.1 M (pH 6.9) and Tris-HCl 0.1 M (pH 8.1) reagents were used as the buffer. A Reagent mixture which consisted of a substrate (750 µL), buffer (700 µL) and extract the enzyme (50 µL) was incubated for 20 min at 37 °C and the reaction was stopped by the addition of 1500 µL DNS 1 %(w/v). The reaction mixture was placed in boiling water for 5 minutes.

The same procedure was conducted to blank tube, except enzyme extract was added after the DNS 1 %(w/v). The absorbance of the reaction mixture was measured at 540 nm, after previously added 3000 µL of doubly distilled water. The amount of maltose removed from substrate was determined from a standard curve of maltose. Amylase activity was calculated as the amount of maltose released (µmol) min^{-1} mg^{-1} protein in enzyme extract.

Measurement cellulose activity used 3,5-Dinitrosalicylic acid (Sigma-Aldrich, AG) method with carboxymethylcellulose (CMC) as substrate (Savona *et al*., 2011). Reagent mixture consisted of a substrate (300 µL), 0.1 M sodium acetate buffer (300 µL) and enzyme extract (60 µL) were incubated for 10 minutes at 40 °C then the reaction was stopped by the addition of 900 µL of DNS 1% (w/v) reagent. The reaction mixture was placed in boiling water for 15 minutes. The same procedure was

done at blank tube, except enzyme extract was added after the DNS 1 % reagent. The absorbance of the reaction mixture was measured at 640 nm, after previously added 3000 µL of doubly distilled water. The amount of glucose released from substrate was determined from the glucose standard curve. Cellulose activity was calculated as the amount of glucose released (µmol) min^{-1} mg^{-1} protein in enzyme extract.

A modified method using *p*-nitrophenyl phosphate (*p*NPP; Sigma-Aldrich, AG) as the substrate was used to measure alkaline phosphatase activity (Debnath *et al*., 2007). The reaction mixture consisted of 1000 µL of substrate buffer (0.1 M Glycine, pH 10.4; 3 mM p-nitrophenyl phosphate in 1 mM $MgCl_2$) and the enzyme extract (50 µL) were incubated for 30 minutes at 30 ^oC, then the reaction was stopped by the addition of 1500 µL of 3.0 N NaOH. The same procedure was conducted to blank tube, except enzyme extract was added after of 3.0 N NaOH. The product of hydrolysis of *p*-nitrophenyl phosphate (pNPP) was measured at 405 nm. Alkaline phosphatase activity was determined from a standard curve of *p*-nitrophenol. Alkaline phosphatase activity was expressed in U (µg *p*-nitrophenol liberated) mg^{-1} protein in enzyme extract.

Statistical Analysis

Enzyme activity values were tested by analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. Statistical analysis was performed using SPSS 18.0 package version of Windows software.

RESULTS AND DISCUSSION

There were significant differences of total protease activity between the age of the fish (p <.05) and the high protease activity was found at pH 6.9 to pH 10, as well as the fish ages of two months have the highest protease activity (**Table 1**).

In this study, the total protease activity was not found at pH 1.9 and it showed a low at pH 5.1. This phenomenon indicated that there was no pepsin-like activity that was active at acidic pH. These results were different from previous studies on *Betta splendens* (Tongprajukaew *et al*., 2010) , *Tilapia rendali* (Hlophe & Moyo, 2013) and *Horabagrus brachysoma* (Prasad & Suneesha 2013) and *Etropus suratensis* and *Oreochromis mossambicus* (Sankar *et al*., 2014).

Tabel 1. The total protease activity of Rasbora at different ages and pH activity

Age of fish (months)	Activity on various pH (U mg^{-1}protein)					
	1.9	5.1	6.9	8.1	10	12.5
2 (n=6)	-	9.15±1.52a	127.56±26.11a	140.48±39.29a	131.81±42.00a	-
4 (n=6)	-	2.94±1.59b	91.92±24.02b	100.33±35.10ab	90.06±26.14ab	-
6 (n=6)	-	3.52±2.98b	80.21±13.86b	75.41±10.53b	69.26±26.02b	-

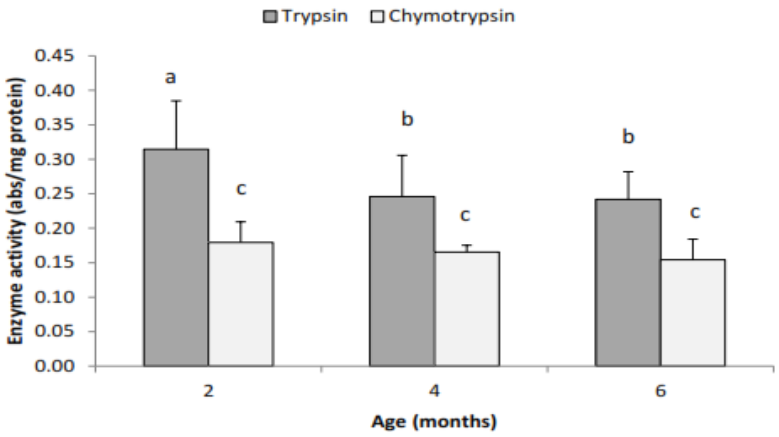

Figure 1. Trypsin and chymotrypsin activities in Rasbora at different age. Means (+sd) with different superscript letter are significantly different (P<.05; n=5).

The existence of the activity of pepsin or pepsin-like seems to be related to the presence of a functional stomach. Rasbora on this research may be included in the category of fish with non functional stomach since there is no pepsin-like activity. This funding has also confirmed with *Zenarchopterus buffonis* (Abidin *et al*., 2016). At all the fish ages the total protease activity measured at pH 6.9 to 10.0 showed a high activity indicating that protease digestion of the fish is active at a neutral to alkaline condition which occurred in the segment of intestine.

The results of this study do not differ from previous research on *Betta spledens* (Thongprajukaew *et al*., 2010), *Odontobutis obscures* (Ye, Chen & Zhu, 2013) and *Lutjanus guttatus* (Pena *et al*., 2015). The total protease activity was also found higher in two months old fish than in four and six months old fish, which indicates that young fish have a higher protein digestive capacity than the older. This is in contrast to results of previous studies on fish omnivore *Oreochromis niloticus* (Klahan *et al*., 2009) and herbivore *Hyporhamphus regularis ardello* (Day *et al*., 2011a) and Eurasian perch, *Perca fluviatilis* (Langeland *et al*., 2013). The higher activity of protease in two months old fish than in four and six months old fish may be associated with the higher protein requirements for growth, as was observed in *Oreochromis niloticus* (Abdel-Tawwab, Ahmad, Kattab & Halaby, 2010).

Trypsin activity was significantly different among the age or weight of the fish (p <.05) with the highest trypsin activity found in fish age of two months (**Figure 1**), but the activity of chymotrypsin was not significantly different among age or weight of the fish (p>.05). The results of this study did not differ from previous research on *Lates niloticus* (Namulawa *et al*., 2013), *Horabagrus brachysoma* (Prasad & Suneesha, 2013) and *Labeo rohita* (Umalatha, Kushwaha & Gangadhar, 2016), but it is contrary to the results on *Limia vittata* & *Gambusia punctate* (Falcon-Hidalgo *et al*., 2011) and *Hyporhamphus regularis ardelio* (Day *et al*., 2011a).

So it seems that the Rasbora changes in protein digestive capacity, as indicated by the increased activity of total protease, dominated by the activity of trypsin and not by the activity of chymotrypsin. Trypsin activity is higher than chymotrypsin, reflecting the absence of growth inhibition in the fish, and the high activity of trypsin can be an indicator of increased growth and efficiency of feed on fish (Chan *et al*., 2008; Rungruangsak-Torrissen *et al*., 2009).

Lipase activity was found significantly different between the age of the fish (P <.05), and the lowest activity was found in the fish with two months of age, but the activity of lipase with four and six months of age did not differ between the two (**Figure 2**). The results of this study were lower than previous studies on *Glyptosternum maculatum* (Xiong, Xie, Zhang & Liu, 2010). The incubation time differences in this study were shorter than previous research indicating one cause of the differences in the results of this research. However, the result is consistent with previous research on *Oreochromis niloticus* (Klahan *et al*., 2009). Results of previous studies had also shown that the activity of lipase was in the range of neutral to alkaline pH, which indicated that the lipase working on the digestive tract of fish was a lipase secreted by the pancreas or hepatopancreas (Zambonino-Infante & Cahu, 2007) with the intestine as the main segment of fat digestion (Wu, Hong & Zhang, 2010).

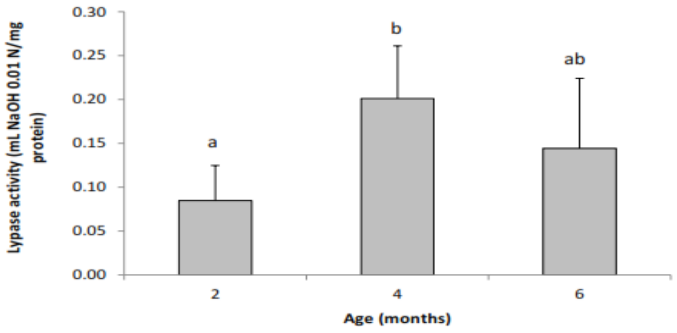

Figure 2. Lypase activity in Rasbora at different age. Means (+sd) with different superscript letter are significantly different (P<.05; n=6)

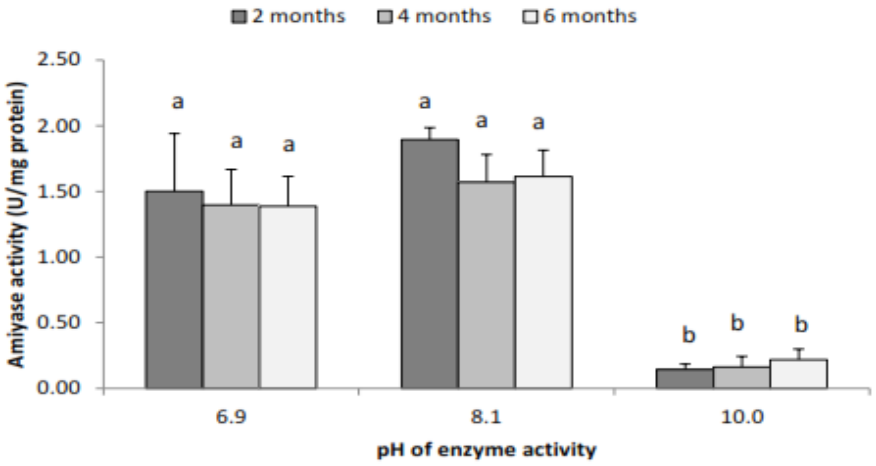

Figure 3. Amylase activity in Rasbora at different age. Means (+sd) with different superscript letter are significantly different (p<.05;n=6).

The higher activity of lipase in a four month old fish than in two months old fish in this study, was in line with the decrease in protease activity. The phenomenon of a decrease in protease and increased lipase activity has been documented in fish that obtained intake of protein and high carbohydrate as occurred in *Dentex dentex* (Perez-Jimenez *et al*., 2009), *Labeo brick* (Mondal, Kaviraj, & Mukhopadhyay, 2012) and *Megalobrama amblycephala* (Habte-Tsion *et al*., 2013).

There was no significant differences amylase activity between age of the fish (P>.05), but amylase activity was significantly influenced by the pH of incubation (P <.05) with the lowest activity at pH 10 (**Figure 3**). In this study, the highest amylase activity was found at pH 6.9 and 8.1, but it decreased at pH 10.0. The results of this study are lower than in previous studies on fish *Betta splendens* (Thongprajukaew *et al*., 2010) and *Diplodus puntazzo* (Savona *et al*., 2011). High amylase activity at neutral to slightly alkaline pH, which reflects that the starch hydrolysis process occurs at a neutral to alkaline condition.

Amylase, that is active in such condition generally secreted by the pancreas or hepatopancreas and works in the area of intestine. Activities of amylase were dominant in the intestine with the alkaline condition has been documented in *Odontobutis obscures* (Ye *et al*., 2013), *Lates niloticus* (Namulawa *et al*., 2013), *Etroplus suratensis* and *Oreochromis mossambicus* (Sankar *et al*., 2014) and *Tilapia rendali* (Hlophe & Moyo 2013). Differences in age of the fish in this study seem to also produce no significant difference of amylase activity, which indicates no change in the capacity of fish to digest complex carbohydrates, especially starch. A similar phenomenon occurred in fish that has no stomach as *Hyporhamphus regularis ardelio* (Day *et al*., 2011a), but different results were seen in *Horabagrus brachysoma* (Prasad & Suneesha, 2013).

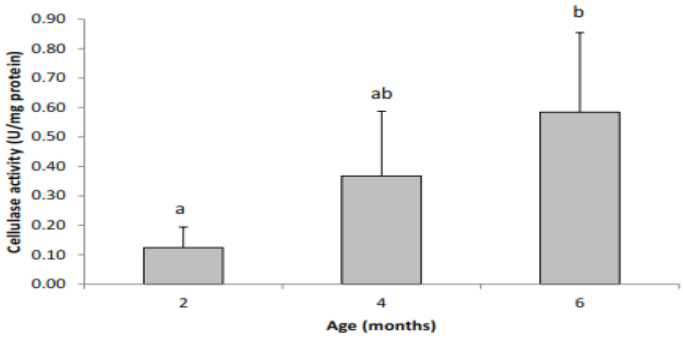

Figure 4. Cellulose activity in Rasbora at different age. Means (+sd) with different superscript letter are significantly different (P<.05;n=6).

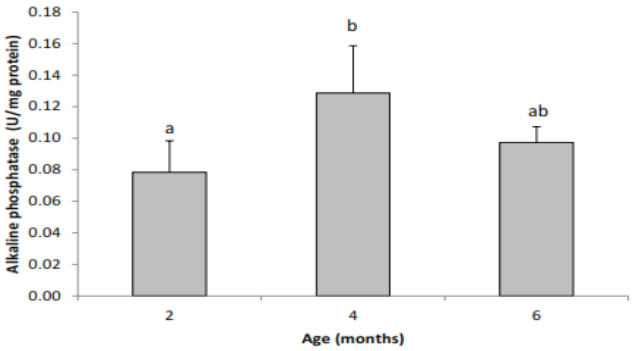

Figure 5. Alkaline phosphatase activity in Rasbora at different age. Means (+sd) with different superscript letter are significantly different (P<.05;n=6)

In this study the cellulose activity was significant differences between the ages of the fish (P <.05) and high activity in fish ages of four and six months (**Figure 4**). Cellulose activity changed with the increase age of the Rasbora. Six months old fish had higher cellulose activity than two months old fish, but did not differ from four months old fish. The results of this study indicated that Rasbora at six months age had higher ability to digest crude fiber than fish of two months age. Increased capacity to digest crude fiber of the fish indicated that in fish intestine, it is suspected that symbiotic microorganisms synthesize cellulose, because cellulose in the fish intestine derives from the secretions of the gut of microbes (Krogdahl, Hemre & Mommsen, 2005; Kar & Ghosh, 2008).

The activities of cellulose in the intestine of fish were also shown in previous studies on fish omnivore (Fagbenro *et al*., 2005; Tongsiri, Mang-Amphan & Peerapornpisal, 2010; Savona *et al*., 2011). However, previous studies showed that cellulose activity was related to the composition of the feed in the digestive tract. Carnivorous fish that consumed macro algae had higher cellulose activity compare to fish that did not consume the macro algae (Chaudhuri, Mukherjee, & Homechaudhuri, 2012).

Alkaline phosphatase activity showed a significant difference between the age of the fish (P <.05), and high alkaline phosphatase activity in fish age of four months (**Figure 5**), but it was not different with six months old fish. Increased activity of alkaline phosphatase in four month old fish in this study reflected the increase of nutrient absorption ability with decreasing the activity of protease and increasing lipase activity in Rasbora. The phenomenon of the increase of alkaline phosphatase activity with the increase in lipase activity, was documented in fingerling *Mesopotamichthys sharpeyi* (Rahimi, Sayed Mohammad & Mohammad, 2015). Alkaline phosphatase is an enzyme involved in the breakdown of phosphate bonds so that it is easily absorbed in the substrate of proteins, lipids and carbohydrates, and the decrease in the intake of nutrients such as lipids can reduce its activity (Ducasse-Cabanot *et al*., 2007), and generally alkaline phosphatase works on the surface of intestine enterocytes microvillus (Minjoyo, Tan-Fermin, & Macaranas 2003), so, the highest activity of alkaline phosphatase in larvae *Rutilusfrisiikutum* occur after microvillus is formed (Hassanatabar, Ouraji, Esmaelli & Babaei, 2013).

CONCLUSIONS

Fish with two months of age had higher protein digestion than the older one, but the ability to digest carbohydrates, especially starch was not influenced by differences in age. The ability of Rasbora to digest fat and fiber increased with the increase age of the fish as well as feed absorption efficiency, improved with the increase of age reflected by the activity of alkaline phosphatase.

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