Digestive Enzyme Activities in Barred Loach (Nemacheilus fasciatus, Val., 1846.): Effect of pH and Temperature

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Digestive Enzyme Activities in Barred Loach (*Nemacheilus fasciatus*, Val., 1846.): Effect of pH and Temperature

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ABSTRACT. This study aims to determine the total protease, lipase, and amylase activities at different pHs, as well as pepsin and trypsin-like at different temperatures. A total of 240 individuals have been used in this study. Enzyme activity was measured by the spectrophotometer method. The effect of pH was evaluated on protease, lipase, and amylase activity, while the effect of temperature was evaluated on pepsin and trypsin-like activities. The results showed that the total protease activity at pH 7.0-10.0 was significantly higher than pH 1.7-5.0 ($P < 0.05$). Furthermore, the activity of lipase was significantly higher at pH 5.0-7.0 than pH 1.7, 3.4, and 10.0. Also, the activity of amylase at pH 7.0-8.0 was significantly higher ($p < 0.05$) than pH 1.7-5.0 and pH 10.0. Moreover, the pepsin-like activity in the anterior gut was significantly higher ($p < 0.05$) than the posterior gut. Conversely, trypsin-like activity in the posterior gut was significantly higher ($p < .05$) than the anterior gut. Additionally, the pepsin-like activity was significantly higher at 45°C compared to different temperatures ($p < 0.05$), whereas trypsin-like was significantly ($p < 0.05$) higher at 60 °C than other temperatures. Conclusively, the total protease and amylase activity was higher under neutral to slightly alkaline conditions, while lipase was higher under acidic to neutral conditions. Furthermore, the pepsin-like activity was only found in the anterior gut, whereas trypsin-like was higher in the posterior gut. The optimal temperature for pepsin-like and trypsin-like activity was 45 °C and 60 °C, respectively.

Keywords: *Nemacheilus fasciatus*, pepsin-like, pH, temperature, trypsin-like

INTRODUCTION

Fish uses nutrients, such as protein, fat, and starch, contained in feed through the digestive process that takes place in the intestine. The digestion of these nutrients occurs enzymatically by enzymes such as protease, lipase, and amylase. However, several factors, including pH and temperature influence their activities.

Previous studies have shown various digestive enzymatic activities that are related to differences in optimal pH. Thongprajukaew et al. (2010) demonstrated that *Betta splendens* had protease activity in the pH range of 7.0 - 8.0. Ye et al. (2013) also showed that the protease activity in *Odontobutis obscures* increases at pH range of 7.5-8.0, however, those in hybrid sturgeon are between a pH of 8.0-8.5 (Ji et al., 2012). Also, the higher lipase activity in the intestine as compared to the stomach was manifested in the *Glyptosternum maculatum*, and the most were found in the anterior gut at pH 6.0 (Xiong et al., 2011). The result indicates that the intestines are the primary location for digesting fat and the lipase activity in *B. splendens* was found at a pH range of 7-11 (Thongprajukaew et al., 2010). Previous research also showed that *O. obscures* had relatively high amylase activity at pH 7.0 - 8.0 and reaches the highest at pH 7.5 (Ye et al., 2013). In addition, *Rasbora lateristriata* has the highest amylase activity at pH 6.9-8.1, and the lowest in alkaline conditions at pH 10.0 (Susilo et al., 2018).

The activity of the digestive enzyme is greatly influenced by temperature, and there are varieties of tolerance to temperature changes. Furthermore, previous studies have shown the diversity of acid protease activity of pepsin on *Cichlasoma beani* fish which is optimal at 55 °C (Martinez-Cardenas et al., 2017), *G. maculatum* and *Micropterus brachyurus are* 30 °C and 35 °C, respectively (Xiong et al., 2011; Martinez-Cardenas et al., 2020) as well as *Lambeo fimbriatus* at 40 °C (Biswajit, 2020). Meanwhile, the activity of trypsin or alkaline protease shows variation among the examined species. Furthermore, *Cirrinus mrigala* has high trypsin activity at 30-40 °C (Khangembam and Chakrabarti, 2015), *Lutjanus guttatus* and *Paralichthys orbignyanus are* optimal at 50 °C (Pena et al., 2015; Candiotto et al., 2015), while *Sardinella longiceps* and *Acanthopagrus latus* have optimal activities at 60 °C (Khandagale et al., 2017; Namjou et al., 2019).

The barred loach, *N. fasciatus is a* wild fish that inhabit rivers with rocky bottoms and clear waters. It has a maximum standard body length of 7.4 cm, consumes benthic and detritus organisms, and is distributed in Sumatra and Java, Indonesia (Kottelat et al., 1993). However, declining water quality and overfishing have reduced their population in nature (Tjahjo et al., 2017). Therefore, there is a need for the domestication of barred loach to increase their population in nature and also meet the needs of the community. Consequently, adequate biological knowledge is required as the initial basis for supporting the domestication of barred loach. Furthermore, previous studies on barred loach were conducted, these include those related to adaptation and growth (Prakoso et al., 2017a), reproductive biology and growth (Prakoso et al., 2017b), genetic and phenotypic diversity (Ath-thar et al., 2018), oxygen consumption (Prakoso and Kurniawan, 2020) as well as protease, lipase, and amylase activity (Susilo and Rachmawati, 2020). However, there are no research related to the activity of digestive enzyme, especially pH and temperature, this is therefore a novelty, particularly in barred loach. As a result, this study aimed to determine the activity of digestive enzymes at different pH and temperature conditions. The differences in pH were investigated for their effects on protease, lipase, and total amylase activity, and the impacts of temperature on pepsin-like and trypsin-like activities were examined. Furthermore, the pH and temperature tolerance of digestive enzymes in barred loach contribute to the preparation of feed formulas and protease applications in the future.

EXPERIMENTAL SECTION

Materials and Instruments

Casein (Merck), Folin & Ciocalteu's phenol reagent (Sigma-Aldrich), Starch (Bio Basic Canada, High Purity), Tris (hydroxymethyl) aminomethane (Tris) (Sigma-Aldrich, ACS reagent, >99.8%), Tichloroacetic acid (TCA) (Merck), Hydrochloric acid (Merck, 36.5-38.0%), 3,5-dinitrosalicylic acid (DNS) (Sigma-Aldrich, >98%), p-nitrophenyl phosphate (pNPP; Sigma-Aldrich, AG), p-nitrophenol (Sigma-Aldrich, AG), NaOH (Sigma-Aldrich, AG), quartz cuvette (Purshee), single centrifuge (Eppendorf, 5415 R), spectrophotometry (Hitachi, U-2900), channel pipette (Serana), waterbath (JEIO-TECH, WB-20E), pH meter (Eutech Instruments), electric homogenizer (Heidolph Diax 900).

Fish Sample

A total of 220 were used with an average length and weight of 6,09 ± 0,28 cm and 1.34 ± 0.27 g, respectively caught in the Logawa tributary, Karanglewas, Purwokerto at coordinates 07º25'02.95"S. and 109°11'45.41"E. The captured fish were placed in a box filled with ice and then taken to the Animal Physiology Laboratory of the Faculty of Biology, Jenderal Soedirman University, Purwokerto, for further treatment.

Isolation and Homogenization of Digestive Organs

A total of 100 barred loach fish were divided into four pool sample groups, with each pooled sample containing 25 fish. Subsequently, surgical operation was performed to obtain their digestive organs without intestinal partitioning, and the same procedure was carried out on 120 other barred loach divided into six pool sample groups, with each pooled sample from 20 fish. Furthermore, the digestive tract was partitioned into the anterior and posterior gut and the samples were then used to measure protease, lipase, and total amylase activity. Subsequently, anterior and posterior samples were used to measure pepsin-like and trypsin-like activity. The digestive organs, including the entire system and the intestines, which had partitioned were destroyed by electric homogenizers. The digestive organs were homogenized using a cold buffer solution of 0.05 M Tris-HCl (pH 7.5) with a ratio of 1:8 (b: v) and was collected in a 1.5 mL Eppendorf tube and centrifuged at a speed of 12000 rpm (temperature 4 °C) for 15 minutes. Also, the supernatant obtained as a crude extract of the enzyme was collected in a 1.5 mL Eppendorf tube and stored in a freezer at -80 °C, subsequently, it was used to measure enzyme activity. The dissolved protein content in the enzyme extract was measured using Folin-phenol reagent and albumin as the standard (Umalatha, et al., 2016). This content was used to calculate the specific activity of the enzyme.

Measurement of Digestive Enzyme Activities

The casein hydrolysis method was used to measure protease activity (Thongprajukaew et al., 2010; Susilo et al., 2018). The reaction mixture, consisting of casein substrate (350 μL), buffer (350 μL), and the enzyme extract (50 μL) was incubated at 37 °C for 30 minutes, after which 750 ml μL of 8% TCA solution was added to stop the reaction. The mixture was allowed to stand for 60 minutes in the refrigerator and then transferred to 1.5 mL Eppendorf tubes and centrifuged at 6,000 rpm for 10 minutes. The supernatant obtained was then measured for its absorbance on a spectrophotometer with a wavelength of 280 nm. The resulting tyrosine concentration was calculated using a standard tyrosine curve and the protease-specific activity was expressed as U (μg.h^{-1}) .mg $protein^{-1}$

Lipase activity was measured using the p-nitrophenylpalmitate (p-NPP) hydrolysis as a substrate following the method of Susilo et al. (2018). The reaction mixture consisting of buffer (1800 μL), 0.01 M p-NPP substrate (400 μL), and the enzyme extract (100 μL) was incubated at 37 °C for 30 minutes. At the end of the incubation, 700 mL of 0.1 M Na_2CO_3 solution was added to stop the reaction. After it is cooled, the contents of the test tube were transferred to 1.5 mL volume Eppendorf tube and centrifuged at 10,000 rpm for 15 minutes. Subsequently, the obtained supernatant was measured for its absorbance at 410 nm, the p-nitrophenol content was calculated from the standard curve and lipase-specific activity was expressed as U (μmol.h^{-1}) .mg protein^{-1}.

Furthermore, the amylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method with starch as the substrate following the procedure of Susilo et al. (2018). The reaction mixture, which consists of 1% starch substrate (350 μL), buffer (350 μL), and the enzyme extract (50 μL) was incubated for 15 minutes at 37 °C. At the end of the reaction, 750 μL of 1% DNS reagent was added and all the mixture was placed in boiling water for 5 minutes. After all the test tubes cooled, the reaction mixture was diluted by adding 3000 μL distilled water and then measured for absorbance at 540 nm. Furthermore, the amount of maltose was calculated from the standard curve and the specific activity of amylase was expressed as U (μmol.h^{-1}).mg protein^{-1}.

The pepsin-like activity was measured by the Folin-Ciocalteu's method with casein as a substrate (Rungruangsak and Utne, 1981). Additionally, the enzyme extract was activated with 0.01 N HCl before tests. Also, the reaction mixture consisted of 1% casein substrate in a buffer solution of 60 mM HCl (300 μL), and the enzyme extract (100 μL) were incubated for 45 minutes at 37 °C. The reaction was stopped by adding 600 μL of 5% TCA reagent and after 30 minutes at room temperature, the mixture was centrifuged at 6000 rpm for 10 minutes. Afterward, a total of 400 μL of supernatant was placed in 1.5 mL Eppendorf tubes and then, 800 μL of 0.5 M NaOH solution and 240 μL of Folin-Ciocalteu's reagent was added. It was then homogenized and allowed to stand for about 10 minutes, before measuring the absorbance at 720 nm. Subsequently, the amount of tyrosine was calculated from a standard curve and the specific activity of pepsin-like was expressed as U (μmol.h^{-1}).mg protein^{-1}.

The activity of trypsin was measured by Folin-Ciaocalteu's method with casein as a substrate (Rungruangsak and Utne, 1981). Furthermore, the reaction mixture consisted of 1% casein substrate in a buffer solution of 0.1 M Tris-HCl (350 μL), and the enzyme extract (50 μL) incubated for 45 minutes at 37 °C. The reaction was stopped by adding 600 μL of 5% TCA reagent. After 30 minutes at room temperature, it was centrifuged at 6000 rpm for 10 minutes. Afterward, a total of 400 μL of supernatant was placed in a taken 1.5 mL Eppendorf tubes mixed with 800 μL of 0.5 M NaOH solution and 240 μL of Folin-Ciaocalteu's reagent. The mixture, which serves as an instrument of homogeneity was allowed to stand for about 10 minutes before measuring the absorbance at 720 nm. Also, the amount of tyrosine produced were calculated from a standard tyrosine curve, and the specific activity of trypsin-like was expressed as U (μmol.h^{-1}).mg protein^{-1}.

Measurement of pH Effect on Enzyme Activity

Protease, lipase, and amylase activity were measured using six different pH levels, namely 1.7 (0.1 M KCl-HCl Buffer), 3.4 (0.1 M Glycine-HCl buffer), 5.0 (0.1 M buffer M acetate), 7.0 (0.1 M phosphate buffer), 8.1 (0.1 M Tris-HCl buffer), and 10.0 (0.1 M buffer Glycine-NaOH). Furthermore, enzyme activity was measured in duplicate, and each temperature treatment was repeated four times.

Enzyme Activity in Different Gut Segments.

The activities of pepsin and trypsin-like were measured in both the anterior and posterior gut segments and the incubation temperature was 37 °C. The measurement in this stage determines the part of the intestine used to measure pepsin and trypsin-like activities at different temperatures.

Effect of Temperature on Enzyme Activity

The temperatures tested include 30, 45, 60, and 75 °C. Also, pepsin-like and trypsin-like activity was measured in the anterior and the posterior gut, respectively. Furthermore, the data from the measurement of enzyme activity were analyzed using a one-way analysis of variance (ANOVA) and Tukey's test.

RESULTS AND DISCUSSION

Total Protease Activity

The results showed a low and high protease activity at acidic, between pH 1.7-5.0 and 7.0-10.0, respectively (Figure 1.). The results showed that protease was more dominant in neutral to alkaline conditions. The protease involved in the digestion of feed protein was the pancrease since it requires a neutral to the alkaline environment for their activities (Izvekova et al., 2013). The results of this study were not different from *Cichlasoma urophthalmus*, which had optimal protease activity at pH 9.0 (Cuenca-Soria et al., 2014), and the *R. lateristriata*, was also high at pH 7-10 (Susilo et al., 2018). Furthermore, the presence of protease activity in the intestine with alkaline environmental conditions was shown in *Salmo salar* (Krogdahl et al., 2015) and *Scorpaena notata* (Aissaoui et al., 2017). Therefore, it is assumed that the digestion process of protein in barred loach mostly occurs in neutral to alkaline conditions, however, the protease activity is acidic and also present in the barred loach. Additionally,The presence of acid protease activity indicates that barred loach is a fish that has a stomach. This is contrary to *R. lateristriata*, which does not have acid protease activity (Susilo et al., 2018).

Lipase Activity

The results showed a low pancrease activity since it is measured in mUnits (mU), however, there was a significant difference between the pH of the enzyme incubation applied ($p < 0.05$). **Figure 2** shows the presence of lipase activity in acidic conditions (pH 1.7-3.4), however, it was low in alkaline (pH 10.0), and high activity is at pH 5.0 - 8.0. These indicate that lipase activity was found under acidic to slightly alkaline conditions.

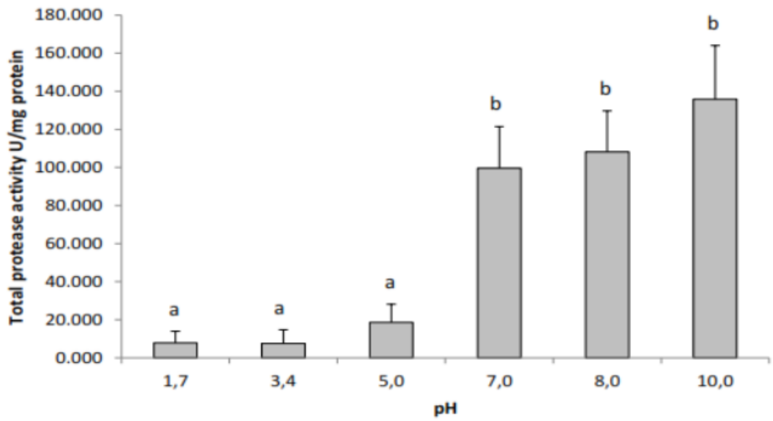

Figure 1. Average (+ sd) total protease activity in barred loach at different pH. Note: Different letters represent significant differences.

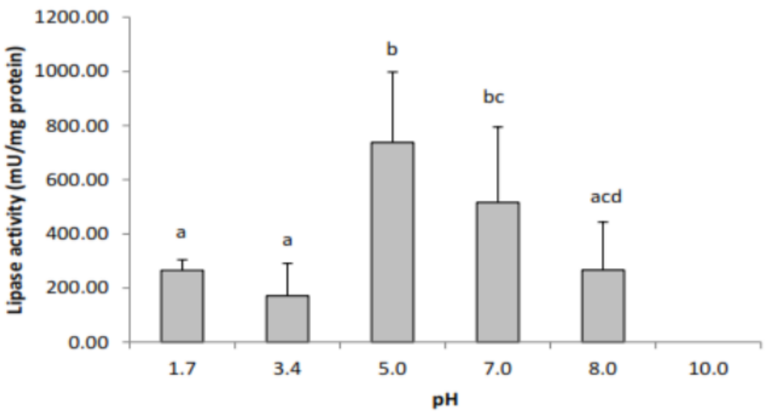

Figure 2. Average (+ sd) lipase activity of barred loach at different pH. Note: Different letters represent significant differences

The presence of lipase activity at acidic pH and high activity at pH 5-8 is in line with previous studies on *Cirrhinus reba*, which has optimal activity at pH 5.5 (Islam et al., 2009), and *Sardinella aurita*, with stable lipase activity in the pH range of 4.0-5.0 (Smichi et al., 2010). However, this was different from previous studies on *Cyprinus carpio*, in which optimal lipase activity was found at pH 8.0 and no lipase at pH 6.0 (Görgün and Akpinar, 2012).

Furthermore, the results of this study are not in line with the bioecological study of barred loach, whose stomach contents mostly contain insects and their larvae or tend to be carnivores (Tjahjo et al., 2017), which have high lipase activity. However, the presence of lower lipase activity in carnivorous fish compared to herbivores was demonstrated in *Xiphister mucosus* (herbivores) and *Xiphister atropurpureus* (carnivores) (German et al., 2004). Additionally, the difference in lipase activity in this study as compared with the previous is related to the fish species and feeding habits of fish. The results of this study were also not different from previous studies on *Sparidentex hasta* (carvivores) which showed low intestinal lipase activity (Jahantigh, 2015). However, this was different from a study on the omnivore fish *Oreochromis niloticus, Gymnocypris przewalkskii* which showed high lipase activity in the intestine (Santos et al., 2016; Tian et al., 2019),

Amylase Activity

The results of amylase activity showed a high value of 15.47±5.13 U/mg protein found at pH 7.0 and 14.88±4.69 U / mg protein at pH 8.0 (**Figure 3**) which are significantly different from other pHs (P <0.05). Furthermore, the high amylase activity in the intestine was neutral and slightly alkaline, whereas the intestinal environment has a neutral to an alkaline state. Previous studies have shown that the gastrointestinal or intestinal tract of *O. mossambicus*, *Tilapia rendalli*, and *Clarias gariepinus* have higher amylase activity than the stomach (Hlophe, et al., 2014). In addition, the phenomenon of high amylase activity in the intestine was found in *Lates niloticus* (Namuwala et al., 2014). Studies on *Carassius auratus gibelio, Leuciscus idus*, C. *carpio*, *Perca fluviatilis*, and *Sander lucioperca* also showed high amylase activity at pH 7.0 and 9.0 (Solovyev et al., 2015). Furthermore, *N. fasciatus*, which had optimal amylase activity at pH 7-8 has no significant difference with the results of previous studies. Moreover, amylase at pH 5.0 and 10.0 is lower, because acidic and alkaline conditions are believed to be unsuitable for their activity. The phenomenon of the lower activity under acidic conditions was also found in *Pangasius* sp. (Thy et al., 2011), and *Anguilla japonica* (Murashita et al., 2013). Also, the decreasing activity at pH 10 was found *O. obscures* (Ye et al., 2013), therefore, it is assumed that both acidic and alkaline conditions are not unfavourable media for amylase activity.

Pepsin and Trypsin-like Activities

Pepsin activity was higher in the anterior gut than in the posterior (**Figure 4**), where there is a stomach. However, the posterior gut does not have pepsin-like, because generally, the posterior gut is a site of an enzyme that requires neutral to alkaline conditions.

Contrary to the pepsin-like activity found only in the anterior gut, the trypsin-like is found in both the anterior and posterior gut, with the highest found in the latter (**Figure 5**). The results of the variance test also showed a significant difference in trypsin-like activity (P. <0.05) between the anterior and posterior gut. Furthermore, the presence of high trypsin-like activity in the posterior gut was thought to be related to the enzymes secreted by the pancreas, which require neutral to alkaline conditions.

The presence of pepsin activity, which is active in acidic conditions was demonstrated in *Horabagrus brachysoma* and *Bostrichthys sinensis* (Renxie et al., 2010; Prasad and Suneesha, 2013), as well as *Archosargus probatocephalus*, which had pepsin with optimal activity at pH 2.0 (Merino-Contreras et al., 2018). Furthermore, a different condition was found in the posterior gut, which indicates the absence of pepsin-like activity. Previous studies suggest that the posterior gut, which was identical to the intestine, is an area with a neutral to base environment, as shown in *Lota Lota* (Izvekova et al., 2013) as well as *C. auratus gibello*, *L. idus, C. carpio, P. fluviatilis,* and *S. luciperca* (Solovyev et al., 2015). It was also suggested that the presence of HCl secretion was believed to make the anterior gut a suitable location for pepsin-like or acid protease activity.

Contrary to pepsin-like, trypsin-like activity in barred loach fish was found in both the anterior and posterior gut, however, the action was higher in the posterior gut. Furthermore, the results of this study were significantly different from those found in *S. hasta, Ctenopharyngodon idella,* and *Hoplias malabaricus*, which showed a higher protease activity in the anterior compared to the posterior intestine (Jahantigh, 2015; Gioda et al., 2017). Nevertheless, this was similar to studies on *G. przewalskii, Mystus nemurus,* and *N. fasciatus*, which showed lower alkaline or trypsin protease activity in the anterior compared to posterior gut (Tian et al., 2019; Rahmah et al., 2020; Susilo and Rachmawati, 2020). Moreover, the variation in optimal protease activity between the anterior and posterior gut in the various species studied is believed to be influenced by differences in feeding habits.

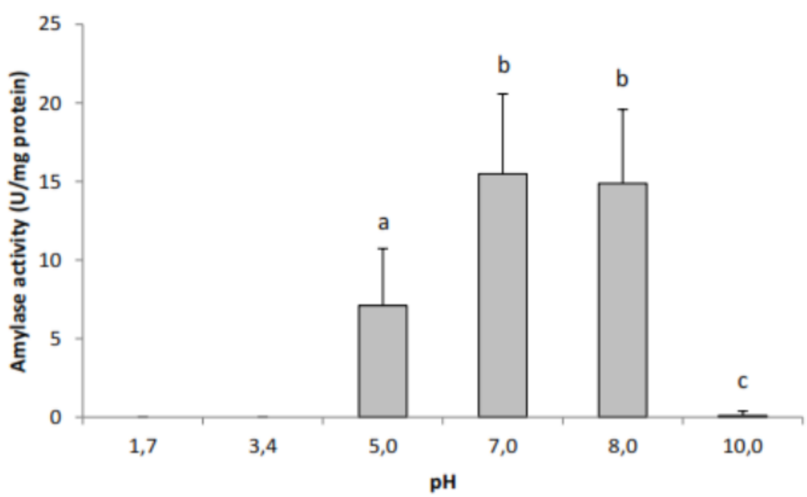

Figure 3. Average (+ sd) amylase activity in barred loach at different pHs. Note: Different letters represent significant differences.

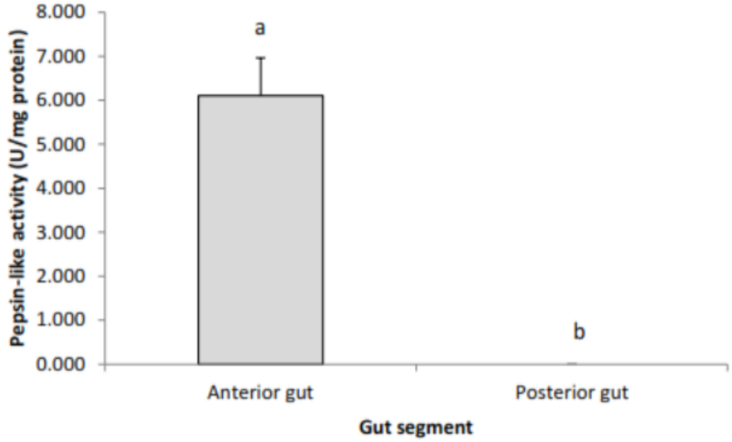

Figure 4. Average (+ sd) pepsin-like activity in barred loach at different gut segments. Note: Different letters represent significant differences.

Effect of Temperature on Pepsin and Trypsin-like Activities

Pepsin-like activity was measured at different incubation temperatures, and the results showed that an increase up to 45 °C result in a higher pepsin-like activity, while 60 °C caused a decrease (**Figure 6**). Furthermore the results are not different from previous studies on *Pangasius gigas* (Vannabun et al., 2014) and *Cichlasoma beani* (Martinez-Cardenas et al., 2017) which had optimal temperatures of 40 °C and 55 °C, but decreased at 60 °C. This condition is different from *Centropomus undecimalis* (Concha-Frias et al., 2016) which has optimal acid proteinase activity at 75 °C. In addition, the difference in enzyme tolerance to temperature exposure is likely due to the variation in habitat and enzyme structure between species.

The results showed an increase in trypsin-like activity up to 60 °C, which was the optimal temperature. However, an increase up to 75 °C resulted in decreased activity (**Figure 7**). These are similarly to previous studies on *Paralichthys olivaceus* (Kim and Jeong, 2013), *Sardenella longiceps* (Khandagale et al., 2017), and *Acanthopagrus latus* (Namjou et al., 2019). However, they are different from *Helicoverpa armigera* (Grover et al., 2018) and *O. niloticus* (Prihanto et al., 2019) which have optimal alkaline protease activity at 50 °C and 35 °C and decreased at 60 °C. Furthermore, it is believed that the variation in the tolerance of alkaline protease or trypsin-like to the temperature is the cause of the different denaturation effects that occur.

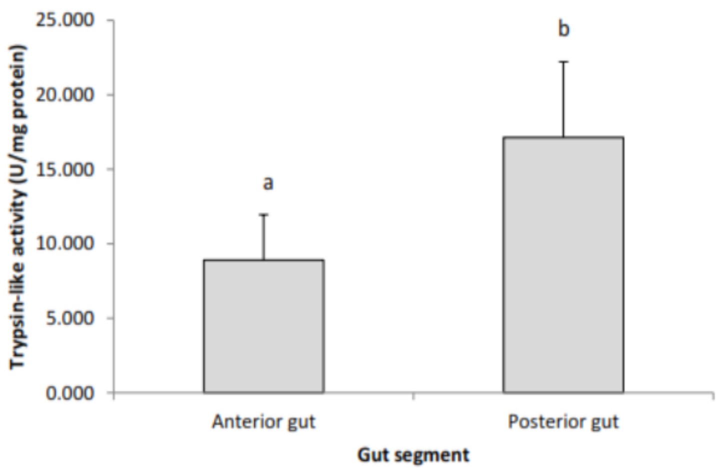

Figure 5. Average (+ sd) trypsin-like activity in barred loach at different gut segments. Note: Different letters represent significant differences

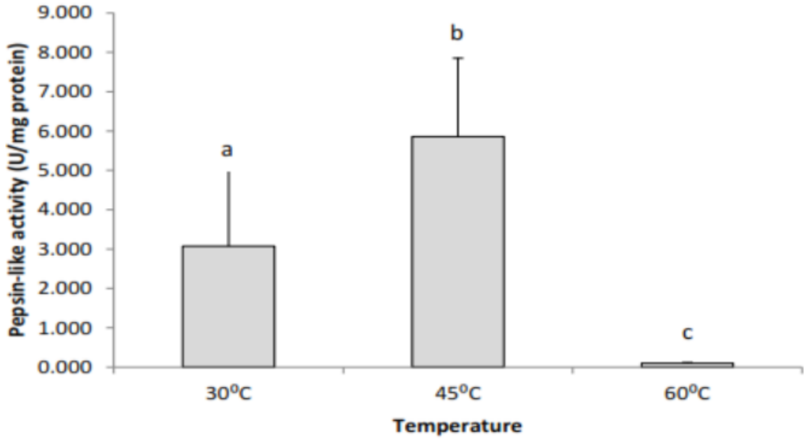

Figure 6. Average (+ sd) pepsin-like activity in barred loach at different temperatures. Note: Different letters represent significant differences.

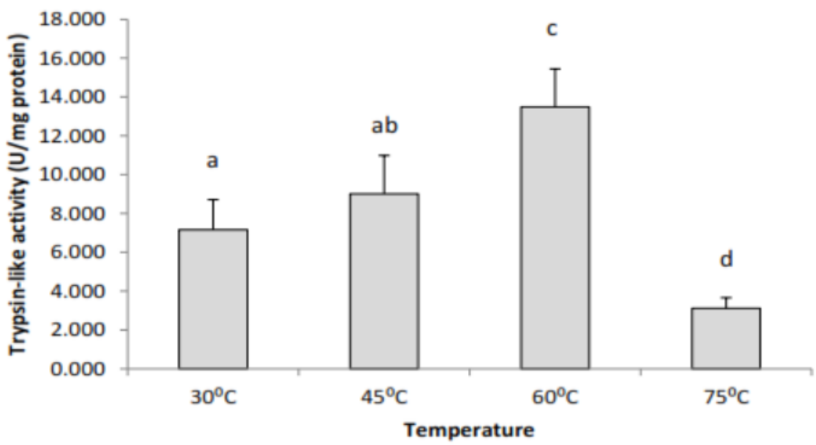

Figure 7. Average (+ sd) trypsin-like activity in barred loach at different temperatures. Note: Different letters represent significant differences

CONCLUSIONS

In conclusion, the protein and starch hydrolysis by total protease and amylase activities were high at neutral up to slight alkaline condition, whereas lipid hydrolysis by lipase was at slight acid up to neutral condition. Furthermore, the pepsin-like activity was found only in the anterior gut, whereas trypsin-like was present in both the anterior and posterior gut. However, the activity in the posterior was higher. The optimal temperature for pepsin-like and trypsin-like activity are 45 °C and 60 °C, respectively. Further studies related to the effect of temperature on lipase and carbohydrase activity, as well as on the digestive capacity of barred loach on feed, need to be carried out to obtain more comprehensive information.

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