Pepsin activity from gastric of milkfish and catfish in Indonesian Waters

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Abstract. Pepsin is the main protease enzyme in the gastric including the gastric of the fish. The enzyme has many benefits for both food and non-food. The purpose of this study was to determined the pepsin activity of the catfish and milkfish. The study was divided into 2 stages, namely pepsin extraction stage and pepsin characterization. The results showed that pepsin from milkfish and catfish can be extracted properly using tris-Cl buffer with the activity of 526.5 U/mL (catfish) and 397 U/mL (milkfish). The pepsin isolated from the gastric of catfish and milkfish can work well at a temperature range of 20-40°C and pH 1-3. Pepsin from the gastric of catfish had $K_m = 0.3333$ mM and $V_{max} = 1666.6667$ mmol/s. Pepsin from milkfish has $K_m = 0.091$ and $V_{max} = 909,091$ mmol/s.

Keywords: activity, catfish, extraction, milkfish, pepsin

1. Introduction

Milkfish and catfish are cultured fish that are widely produced in Indonesia. In 2017 milkfish production reached 701,426 tons (KKP 2018). Milkfish has a chemical composition, namely protein (24.17%), fat (0.8%), carbohydrate (2.7%), water (70.78%), and ash (1.40%). The proportion of milkfish viscera reaches 6-8% of the weight of the fish (Hafiludin 2015). Alhana (2011) explained that the proportion of catfish meat was 38.56%, skin 14.43%, bones and head 43.28% and viscera by 3.37%. During this time, the use of fish viscera, especially the gastric is still limited. Actually, gastric can be used as a source of enzymes, namely pepsin.

Pepsin is an aspartic protease enzyme that can be found in the digestion of vertebrate and invertebrate animals. So far, pepsin used in Indonesia derived from pigs and are imported goods. The value of enzyme imports based on the Ministry of Trade of the Republic of Indonesia in 2017 amounted to 464.8 million dollars and in 2018 increased to 514.1 million dollars (Kementrian Perdagangan Republik Indonesia 2019). Pepsin functions to accelerate the reaction of protein hydrolysis into amino acids and peptides. The zymogen from pepsin is in the form of pepsinogen. Pepsinogen can be classified into five
namely pepsinogen A, B, C, F and prochymosin (Foltman 1981). Pepsinogen is activated into pepsin by decreasing the acidity using HCl (Jurado et al 2012).

Based on the results of the study, pepsin can be isolated from the stomach of goats (Suzuki et al 1999), cattle (Chow and Kassell 1968) and pigs (Jurado et al 2012). Pepsin can also be isolated from freshwater fish or seawater fish. Research on pepsin from seawater fish includes albacore tuna (Nalinanon et al 2010), yellowfin tuna (Pasaribu et al 2018), and smooth hund (Musletus musletus) (Bougatef et al 2008). Pepsin extracted from freshwater fish derived from cork fish (Channa argus) (Chen et al 2009) and European eel (Anguilla anguilla) (Wu et al 2009). Therefore, fish pepsin can be an alternative for industries in Muslim countries like Indonesia.

There are several methods for extracting pepsin from the gastric of fish. Bougatef et al (2008) have successfully isolated pepsin using the Tris-Cl buffer. Jurado et al (2012) have successfully isolated pepsin using distilled water and coagulation. The different methods and raw materials used will affect the activity of pepsin produced. Therefore, the purpose of this study was to determine the pepsin activity of the catfish and milkfish with three extraction methods, namely buffer tris Cl, distilled water, and coagulation.

2. Materials and methods

2.1. Materials
The raw material is gastric of catfish obtained from the IWA-KE OISHI Fish Factory in Ciseeng, Bogor, West Java and gastric of milkfish which obtain from one of domestic industry of milkfish presto, Pemalang, Central of Java. Other materials include distilled water, ice, liquid nitrogen, tris base (Sigma-Aldrich), hydrochloric acid (Merck), NaHCO₃, 2N, trichloroacetic acid (Merck, for analysis), and bovine hemoglobin (Sigma).

The equipment used in this research are ruler, analytical balance (Quattro), pH meter (Hanna instrument), centrifuge (Backman Colter), spectrophotometer (UV-Vis Genesys 10 Thermo), homogenizer (Nissei ÂM-Series), 10 mL micropipette (Pyrex), incubator (IS 900), thermometer, water bath (Memmert W350), and vortex (V1-Biosan).

2.2. Methods
The study was divided into 3 stages, namely preparation of the sample, extraction of pepsinogen, and characterization of pepsinogen. Before analysis, the pepsinogen was activated using HCl 3 N.

2.2.1. Preparation of sample. The gastric of milkfish was separated from other fish viscera and then frozen at a temperature of -18°C±2°C. In frozen conditions, frozen gastric was transported to the laboratory by using cooled bag which added ice in a ratio of 1:2. Viscera of catfish were brought from the company using an icebox which added ice with a ratio of 1:2 to the laboratory and then gastric was separated from other viscera. The gastric were analyzed morphometrically. The gastric then were frozen until they are used.

2.2.2 Extraction of pepsinogen. Pepsinogen extraction was carried out using three different methods. The first extraction method refers to the research of Bougatef et al (2008) using Tris-Cl buffer solvents and other extraction methods refer to studies by Jurado et al (2012) using distilled water and coagulation. The crude extract of pepsin was then tested for its enzyme activity (Jurado et al 2012). The highest activity of enzymes then characterized which includes determining the optimum temperature and pH, as well as the kinetics of the enzyme.
The first method of extracting pepsinogen was based on the research of Bougatef et al. (2008). The frozen gastric of milkfish was melted and then cut into small sizes. The gastric was added with liquid nitrogen until the sample was submerged and then frozen. The gastric was homogenized into flour. Samples were added with Tris-Cl 10 mM buffer pH 7.5 with a ratio of 1:2 (w/v). The process was carried out at low temperatures. The sample was centrifuged at a speed of 10,000 g for 15 minutes at 4°C. The supernatant was a crude extract of pepsinogen 1.

The second pepsinogen extraction method refers to the research of Jurado et al. (2012) by modifying the centrifuge speed to 10,000 g. The second pepsinogen extraction method was done the same as the first pepsinogen extraction method by substituting the Tris-Cl buffer solvent using 1:3 (w/v) distilled water and stirring for 1 hour. The next step was the same as the first pepsinogen extraction method. The pepsinogen obtained called pepsinogen 2.

The third pepsinogen extraction method was carried out the same as the second pepsinogen extraction method and the coagulation stage of the resulting supernatant was continued. The supernatant was then coagulated by reducing the pH of the supernatant using 3N HCl solution to pH 4. Pepsinogen will be coagulated and separated using centrifuge at a speed of 4000 g. The obtained supernatant was then discarded while the pellet is weighed. The pellet was diluted with distilled water 1:10 (w/v). The solution formed is used as pepsinogen 3.

2.2.3. Activation of pepsin. Activation of pepsin was done by adding HCl 3 N to pepsinogen to pH 2, then allowed to stand for 10 minutes. Then the pH was increased to 2.75 with NaHCO3 2 N. Samples were soaked for 6 hours. The solution was pepsin.

2.2.4. Characterization of pepsin. The characterization of pepsin includes determining the optimum temperature, optimum pH, optimum substrate concentration, and the kinetics of enzyme reactions. Determination of the optimum temperature was carried out by the method of Nalinanon et al. (2010) with a hemoglobin 2% substrate. The temperature tested was around 20-60°C. The determination of the optimum pH was carried out by the method of Zhao et al. (2011). The pH of the tested substrate was 1-6. The buffer used for pH 1-4 is 0.25 M sodium acetate-HCl and the buffer used at pH 5 and 6 was 0.25 M sodium acetate-acetic acid. Determination of optimum substrate was done by referring to the method of Zhao et al. 2011. Hemoglobin was used as a substrate with a concentration of 0.5-2.5% and a pH of 2.0. The reaction kinetics were calculated using the Lineweaver Burk equation.

2.2.5. Assay of pepsin activity (Jurado et al. 2012). As a substrate used hemoglobin 2%, pH 2. As much as 1 mL substrate was added 0.2 mL pepsin. The enzyme solution was incubated for 10 minutes at 37°C. The solution was then added with 5% TCA of 2 mL and precipitated for 50 minutes then filtered using filter paper. The obtained filtrate results were analyzed using a spectrophotometer at a wavelength of 280 nm. One unit of activity is expressed as an increase of 0.001 at 280 nm per minute. Enzyme activity can be calculated by the following equation:

\[ A = \frac{(A_{280} - A_0)}{(0.001 \times t \times \text{VE})} \]  

Information :
- \( U / \text{mL} \) = Unit of enzyme activity
- \( A \) = enzyme activity in U per mL
- \( \text{VE} \) = Volume of pepsin solution in the activity test
- \( A_{280} \) = Absorbance at 280 nm
- \( t \) = incubation time (minutes)
- \( A_0 \) = Absorbance of samples prepared with the same treatment without adding pepsin to the substrate
2.2.6. Determination of reaction kinetics. The kinetics of the pepsin reaction can be calculated based on the Lineweaver-Burk equation.

\[
\frac{1}{V_0} = \frac{1}{V_{maks}} + \frac{K_m}{V_{maks}} \cdot \frac{1}{[S]}
\]  

\(V_0\) = initial reaction speed  
\([S]\) = substrate concentration  
\(V_{maks}\) = the maximum speed that can be achieved in an enzymatic reaction  
\(K_m\) = Michaelis-Menten's constant refers to the concentration of the substrate needed to reach ½ maximum reaction speed

2.2.4 Analysis of data. The research data were analyzed using the Completely Randomized Design (CRD) method with one factor (catfish or milkfish) and two repetitions referring to Mattijk and Sumertajaya (2002). The experimental design model is

\[Y_{ij} = \mu + A_i + \varepsilon_{ij}\]  

Information:  
\(Y_{ij}\) = the observation result of the \(i^{th}\) treatment with the \(j^{th}\) test  
\(i\) = different treatment used  
\(j\) = test in each treatment  
\(\mu\) = general midpoint  
\(A_i\) = effect of method treatment \(i^{th}\)  
\(\varepsilon_{ij}\) = effect of error

The hypothesis of the different test methods for extraction of the enzyme activity produced was as follows:  
\(H_0\) = difference in the extraction method has no effect on milkfish or catfish pepsin extract activity  
\(H_1\) = different extraction methods give effect on milkfish or catfish pepsin extract activity

3. Results and discussion

3.1 Description and morphometric of milkfish and catfish gastric  
Visualization of the hull of milkfish and catfish was presented in figure 1. The gastric of milkfish (Chanos chanos) was brownish, round in shape and small in size (figure 1A). Coad (2015) stated that the entire surface of the gastric was covered by mucous cells that contain acidic mucopolysaccharides to protect the stomach wall from the action of hydrochloric acid found in the gastric. The milkfish gastric functions to store food temporarily and mix food with gastric sap. The milkfish gastric also functions to channel food into the intestine and can digest plankton efficiently. Catfish gastric are faded red, curved cone-shaped (figure 1B).  

(A)  
(B)  

Figure 1. Visualization of gastric (A) milkfish dan (B) catfish.
The gastric has a fibrous and elastic texture. The elastic of the gastric functions to accommodate food that enters the body. Catfish is one type of omnivorous fish that tends to be carnivorous (Darmawan and Fokusari 2014). Catfish gastric has a slippery surface and a little slimy. Gildberg (1992) explained that the gastrointestinal organs can secrete gastric juice in the form of hydrochloric acid and pepsinogen. Hydrochloric acid functions in the process of activating pepsinogen into pepsin. Morphometric observations of milkfish and catfish stomach are presented in Table 1.

Table 1. Morphometric of milkfish and catfish gastric.

| No | Parameter | Milkfish* | Catfish** |
|----|-----------|-----------|-----------|
| 1  | Weight (g) | 1.45±0.50 | 3.53±1.29 |
| 2  | Length (cm) | 2.01±0.37 | 4.72±0.90 |
| 3  | Height (cm) | 1.01±0.06 | 2.82±0.59 |

*n=124; **n=51

Table 1 showed that gastric of milkfish have bigger size than gastric of catfish. Coad (2015) stated that milkfish was one type of plankton-eating fish that obtains its food by filtering water from its habitat using gills which long and tight. Djumanto et al (2017) and Triyanto et al (2014) stated that the composition of milkfish is phytoplankton and zooplankton as the main food. Triyanto et al (2014) also explained that milkfish also eat other foods, such as detritus, plant litter, worms, and insects. Purnamaningtyas and Tjahjo (2013) state that milkfish are omnivorous because they utilize zooplankton and phytoplankton as food. Coad (2015) stated that milkfish has a gastric that can digest planktons efficiently.

Catfish has a gastric that functions to accommodate the food that is entered and will be digested. The natural feed of catfish in the wild can be tiny shrimps, small fish, rotifers, small insects, and foliage (Tiengtam et al 2010). Catfish feed in a culture system is usually in the form of pellets and natural food in a pond.

3.2 Activity of crude extract of pepsin from milkfish and catfish

Pepsin extracted by different methods produces different activities. Figure 2 showed the activity of pepsin produced by different methods. Pepsin from milkfish and catfish extracted using Tris-Cl buffer have higher activity compared to coagulation methods, but the same activity of an enzyme which extracted using distilled water.

![Figure 2](image-url)

**Figure 2.** The activity of pepsin from milkfish (A) and catfish (B) which extracted by different methods.
Zhao et al (2011) stated that the use of buffer types in pepsin extraction is very important and the pH of the buffer used is in the range of 7-7.5 which is a good condition to keep the enzyme stable. Generally, the buffer used is the sodium phosphate buffer, Tris-Cl, and distilled water. Pepsin extraction with Tris-Cl buffer (pH 7.5) is also used in smoothhound fish (Bougatef et al 2008) and discus fish (Chong et al 2002) and pepsin extraction with distilled water used in Atlantic cod (Gildberg 2004) and monterey fish sardines (Castillo-Yanez et al 2004).

3.3 Characterization of crude extract pepsin from milkfish and catfish

The characteristics of pepsin from milkfish and catfish were presented in table 2. Table 2 showed that pepsin from fish with different habitats has different characteristics. Catfish that were cultivated in freshwater ponds had a wide optimum temperature (20-40°C). Milkfish that were kept in ponds with brackish water which had an optimum temperature of 30°C. Pepsin extracted from omnivore fish tended to be carnivores which had a higher maximum activity of pepsin compared to the omnivore banding which tended to be herbivorous. The optimum temperature of pepsin from cork fish (Channa argus) was 40°C (Chen et al 2009). Bougatef et al (2008) which stated that the optimum temperature of smoothhound fish pepsin (Mustelus mustelus) was 40°C. The optimum temperature of pepsin in largemouth bass (Micropterus salmoides) was at 40°C to 50°C (Miura et al 2015). Mazumder et al (2018) stated that differences in habitat and fish species can result in different pepsin activities.

Catfish had a narrow optimum pH (pH 2) but milkfish had optimum pH 1-2. Pepsin of largemouth bass (Micropterus salmoides) had an optimum pH of 1.5 to 3.5 (Miura et al 2015), cork fish (Channa argus) at pH 3 (Chen et al 2009), and in African oelacanth fish (Latimeria chaleumnae) at pH 1 to 3 (Tanji et al 2007). The pH has an important role in the binding process of the substrate by the enzyme. pH values that were too high can cause polypeptide chain conformation or denaturation (Nalinanon et al 2010).

Based on these data it can be concluded that pepsin which includes aspartic protease had an optimum pH of 1-3.5.

| No | Parameter               | Species of fish |
|----|-------------------------|-----------------|
| 1  | Optimum temperature (°C)| Catfish 20-40   |
|    |                         | Milkfish 30     |
| 2  | Optimum pH              | Catfish 2       |
|    |                         | Milkfish 1-2    |
| 3  | V_max                   | Catfish 1,666.67 mmol/s |
|    |                         | Milkfish 909.09 mmol/s |
| 4  | K<sub>m</sub>           | Catfish 0.33 mM |
|    |                         | Milkfish 0.09 mM |

K<sub>m</sub> value obtained by Wu et al (2009) in Anguilla anguila of 0.088 mM, El-Beltagy et al (2004) on Oreochomis niloticus at 0.77 mm, and Jatmiko (2018) on Thunnus albacares at 0.25 mM. Enzyme constant value is the amount of substrate concentration needed by the enzyme to be able to reach half the maximum speed. The value of K<sub>m</sub> pepsin in each type of fish shows a different value. The value of K<sub>m</sub> pepsin from cork fish (Channa argus) was 0.14 mM (Chen et al 2009), largemouth bass (Micropterus salmoides) 0.039 mM (Miura et al 2015), rice eel (Monopterus albus) 0.069 mM (Weng et al 2011), and pepsin in yellowfin tuna (Thunnus albacares) 0.25 mM (Jatmiko 2018).

Enzyme activity is related to enzyme kinetics. Zhao et al (2011) stated that enzyme kinetics is related to the speed of enzymatic reactions and the factors that influence it. Reaction speed value (V<sub>max</sub>) is the maximum speed that can be achieved in a reaction. The constant value (K<sub>m</sub>) is the substrate concentration needed to reach half the speed of the maximum speed. Saropah et al (2012) revealed that the smaller the K<sub>m</sub> value indicates the enzyme-substrate complex the better because the enzyme has a high value of enzyme affinity for the substrate. Each enzyme has different V<sub>max</sub> and K<sub>m</sub> values with substrate concentrations that are specific to a certain temperature and pH.
Conclusion

Both pepsin from milkfish and catfish can be extracted properly using tris-Cl buffer with the activity. The pepsin isolated from the gastric of catfish and milkfish can work well at a temperature range of 20-40°C and pH 1-3. Pepsin from the gastric of catfish had $K_m = 0.3333$ mM and $V_{max} = 1666.6667$ mmol/s. Pepsin from milkfish has $K_m = 0.091$ and $V_{max} = 909.091$ mmol/s.

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