Characterization of ammonium sulphate fraction of pepsin from fish stomach

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Abstract. Pepsin is one of the digestive enzymes often used in various fields, especially for industry. Pepsin can be isolated from fish viscera. This study was aimed to obtain the source of pepsin having high activity and the characteristics of the pepsin. Fish used in this study including tuna, Hemigaleus balfouri, skipjack, and cob. Pepsin was purified using ammonium sulfate. The results showed that pepsin from tuna and H. balfouri had the highest activity. The activity of the purified pepsin as 101,061 AU/mg. The pepsin worked in a temperature range of 40-60 °C and pH 2 having activity up to 9,000 AU mL, and 9,800 AU/mL, respectively.

Keywords: enzyme activity, pepsin, tuna

1. Introduction

Protease is an enzyme that catalyzes the hydrolysis reaction of protein-peptide bonds (Rani et al 2012). One of the protease enzymes is pepsin. Pepsin is included in the digestive enzymes in animal stomach. Pepsin comes from pepsinogen which is activated by acid in the stomach. Pepsinogen is produced from the chief cells of oxyntic glands located in the stomach wall epithelium (Kageyama 2002). During the process of activation of pepsinogen into pepsin, there is a residue that is cut off so that conformational changes occur, hands the active side of pepsin is open and can carry out activities as an enzyme (Ritcher et al 1998).

Pepsin is an enzyme that is widely used in various fields, one of which is the industry in making detergents. In addition, pepsin is used in making protein hydrolysate, surimi, and hydrolysis of collagen. However, so far the production of pepsin comes from pigs, that is forbidden for many users in Indonesia. One alternative source of pepsin is fish stomach. Nonetheless because the amount of innards from fish processing reaches 20% (Bougatef 2013, Khandagale et al 2017). Isolation of pepsin from fish stomach has been carried out such as from Sparus latus fish (Zhou et al 2007), albacore tuna (Nalinanon et al 2010), skipjack tuna (Nalinanon et al 2011), mandarin fish, that produce four types of pepsin with various temperatures (Zhou et al 2008). Pectoral rattail (Albatrossia pectoralis) obtained two types of
pepsin (Klomkao et al 2007). In addition, pepsin and pepsinogen have also been isolated from \textit{H. balfouri} species (Bougat et al 2008).

In Indonesia, there are various types of carnivorous fish that can produce pepsin, such as yellowfin tuna. Pasaribu et al (2018) showed that pepsin from yellowfin tuna from Gorontalo waters can work at 60°C and acidic pH. In addition to yellowfin tuna, there are still many types of carnivorous fish that can be used, such as \textit{H. balfouri}, cob, and skipjack. However, the three types of fish are not yet known which types of fish have the highest specific activity, including yellowfin tuna. Therefore, this research was conducted to determine the types of fish that produce pepsin with the highest specific activity and characterization of pepsin from the ammonium sulfate fraction derived from the species of pepsin producing fish with the highest activity.

2. Materials and methods

2.1. Materials

Tuna (\textit{Thunnus albacares}), skipjack (\textit{Katsuwonus pelamis}), \textit{H. balfouri}, and cob (\textit{Euthynnu affinis}) stomachs originating from Indian ocean waters, Bovine hemoglobin, tris base, HCl, liquid nitrogen, glycine, Na\textsubscript{2}HPO\textsubscript{4}, KCl, citric acid, dan Trichloroacetic acid (TCA).

2.2. Methods

This research was conducted through 4 stages, namely 1) isolation of pepsin from the stomach of tuna fish, skipjack, \textit{H. balfouri}, and cob, 2) determination of highest specific activity from pepsin-producing fish, 3) purification of pepsinogen and pepsin from pepsin-producing fish with highest specific activity and 4) characterization of pepsin fraction using ammonium sulfate.

2.2.1. Isolation of pepsinogen. Isolation of pepsinogen was carried out using a method based on Bougat et al (2008). The frozen stomach was thawed in the cooler box. The stomach was chopped into a size of 1-1.5 cm and weighed as much as 20 g. The stomach was homogeneous with a 10 mM Tris-HCl buffer pH 7.5 using an Elvehjem mortar. Homogenates were centrifuged at a speed of 10,000 g for 15 minutes at 4°C. The supernatant was taken (crude extract of pepsinogen) and stored at -4°C until ready for use for further testing.

2.2.2. Pepsinogen activation. Pepsinogen activation was carried out by modifying method based on Zhou et al (2007) and Nalinanon et al (2009). A crude extract of pepsinogen was added with 1 M HCl until the pH reaches 2 and homogenized. Then allowed to stand at 4°C for 30 minutes. The suspension was centrifuged at a speed of 10,000 g for 30 minutes at 4°C. The supernatant was taken and tested its specific activity and protein content using the Bradford method.

2.2.3. Determination of pepsin protein activity and levels. Determination of pepsin activity was carried out by modification of the methods of Bougat et al (2008), Nalinanon et al (2010), and Jurado et al (2012). Pepsin activity test was carried out using a substrate in the form of bovine hemoglobin. A total of 0.125 mL of the enzyme was put into a test tube and added with 0.625 mL of 2% hemoglobin in the buffer with the optimum pH of the results of previous experiments. The mixture of solutions was homogenized and incubated at the optimum temperature for 10 minutes. The mixture of the solution was then added with 0.4 mL of 10% TCA and homogenized. The mixture was centrifuged at a speed of 10,000 g for 15 minutes. The supernatant portion was taken and measured at a wavelength of 280 nm. A blank solution was made with a mixture of hemoglobin and distilled water. The determination of protein content was carried out using the method of Bradford (1976). The pepsin activity unit was calculated using the following equation.
U = \frac{A_{280} - A_0}{0.001 \times t \times VE} \tag{1}

U : Activity units (AU/mL)
A_{280} : Absorbance samples at wavelength 280 nm
A_0 : Absorbance blank
VE : Enzyme volume
T : Incubation time

2.2.4. Purification of pepsin with ammonium sulfate. Purification in this study using ammonium sulfate based on Englard and Seifter (1990) methods. A crude extract of pepsinogen (F.Pn) and pepsin (F.P) from fish with the highest specific activity was fractionated using ammonium sulfate. Purification was carried out from saturation of 0-20%, 20-40%, 40-60%, 60-80%. The purification process was carried out at 4°C using thermometer and ammonium sulfate was added gradually during 1 hour of stirring with a magnetic stirrer. The next step was centrifugation at a speed of 10,000 g for 15 minutes. Pellet was resuspended with a 10 mM buffer Tris-HCl pH 7.5 for pepsinogen and a 10 mM buffer Tris-Cl pH 2. Ammonium sulfate fractions were measured for their activity and protein content to determine the best fraction.

2.2.5. Determination of the optimum temperature of pepsin. The determination of optimum temperature was done at different temperatures including 20°C, 30°C, 40°C, 50°C, 60°C, and 70°C using hemoglobin as the substrate.

2.2.6. Determination of the optimum pH of pepsin. The optimum pH determination was carried out using 3 types of buffers namely KCl/Cl 50 mM buffer (pH 1, 1.5, and 2), glycine-Cl 50 mM buffer (pH 2, 2.5, 3, 3.5) and McIlvaine buffer (pH 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6). The mixture was incubated for 15 minutes at the optimum temperature that was obtained.

3. Results and discussion

3.1. Optimization of pepsin sources
The results of the optimization of pepsin sources showed that pepsin from *H. balfouri* produced the highest specific activity (44582.07 U/mg) (figure 1). The fish that produces the second-highest specific activity is tuna (10879.99 U/mg). This shows that *H. balfouri* and tuna are potential sources of pepsin. However, stomach of *H. Balfouri* is difficult to obtain compared to that of tuna because the tuna processing industry produces very high amounts of tuna viscera waste. Therefore, pepsin was derived from tuna.

![Figure 1. Specific activities of various types of fish.](image-url)
3.2. Purification pepsinogen and pepsin

Purification carried out in this study was using ammonium sulfate and based on the salting-out event. The results (as seen in figure 2) showed that purification using enzymes in the form of zymogen (pepsinogen) produces better specific activities than other actively enzymes. This can be caused by the active site of the enzyme is still protected by protein residues that will be hydrolyzed during the enzyme activation. This study was comparable with Nalinanon et al (2009) where the purification of inactive form enzymes resulted higher activity compared to the purification of activated pepsin. The results showed that the purifier level of 20-40% was the best fraction because it produced the highest specific activity. The study of Pasaribu et al (2018) showed that the best fraction was at 30-40% purity, comparable to this study. However, the higher of the purity level, the specific activity produced decreases. The decreasing can be caused by protein denaturation by salt (Klomkao et al 2005).

![Figure 2. Specific activities of crude extract, pepsinogen fraction, and pepsin fractioned by ammonium sulfate.](image)

3.3. Optimum temperature and pH

Each enzyme has the optimum temperature and pH respectively, even the same type of enzyme will have a different optimum temperature and pH. This depends on the habitat and sources (the type of animal, bacterial and plant) of enzymes to be isolated. Tuna pepsin works in a temperature range of 40-60°C (figure 3). This results were concordance to by Pasaribu et al (2018) who stated that yellowfin tuna pepsin works in the temperature range of 40°C-60°C. According to Zhao et al (2011) pepsin works in a temperature range of 30°C to 55°C. In addition to that, the optimum temperature of fish pepsin is very dependent on fish species (cold or warm water species), regarding to Shahidi and Kamil (2001). Fish from cold waters have lower optimum temperatures compared to warm water fishes (Noda and Murakami 1981, Squires et al 1986, Zhou et al 2008).

Enzymes also work at their respective pH. As with temperature, the same enzyme will have a different optimum pH. In general, pepsin works at low pH because it is included in acidic protease. Tuna pepsin showed optimum work at pH 2 using a glycine-Cl buffer (figure 4). However, with this buffer, pepsin activity will decrease as pH increases in addition to the glycine buffer, contrary to the McIlvaine buffer. Pepsin activity in McIlvaine buffer decreases when the buffer pH reaches more than pH 4. Pasaribu et al (2018) also show the optimum pH in the range of pH 2 to 3.5.
Figure 3. The effect of temperature on pepsin activity.

Figure 4. The effect of pH on pepsin activity (KCl/HCl, Glisin-Cl, McIlvaine)

4. Conclusions

Tuna pepsin produces the second-highest specific activity and the best ammonium sulfate fraction at 20-40% precipitation. Tuna pepsin works in the temperature range of 40°C-60°C and at pH 2.

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