Extraction and characterization of pepsin enzyme from the skipjack vicera (*Katsuwonus pelamis*)

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Abstract. Pepsin is one of the digestive enzymes often used in various fields, especially for industry. Pepsin can be extracted from fish viceras. The aim of this research is to extract pepsin from the stomach of skipjack tuna, purify it, and to characterize several biochemical properties of the pepsin produced. The samples used were skipjack tuna stomachs extracted by adding tris-HCl buffer pH 7.5, centrifuged, and activated. The pepsin crude extract was then characterized by temperature, pH and optimum pepsin substrate. The crude pepsin extract of skipjack tuna showed the best activity in the range temperature 50 °C with a pH of 3. Pepsin purification is carried out using ammonium sulphate with a range of 30-80%. The band produced in each pepsin purification pellet sample is estimated to be between 31-32 kDa, while in the supernatant a thin band remains which indicates that all the proteins in the supernatant have been degraded by ammonium sulphate.

1 Introduction

The average fisheries processing industry according to produces 35% waste from total yield. The amount of waste produced will be very disturbing if no further processing is done. Bone, innards, and fish scales are waste from aquatic organisms produced in tuna products. Waste produced when processing fish can reach 20-60% of raw materials. Tuna fisheries waste consists of 17% head, 8% skin, 5% innards, 4% bone and 2% fins. Innards such as the stomach can be used as a potential source of various protease enzymes [1]. Enzymes are widely used in various industries to hydrolyse protein for example in making collagen [2], cheese [3], and tanning skin [4].

Pepsin, an aspartyl protease that have aspartic acid residues on their active side. Pepsin catalyze the hydrolysis of protein into amino acids and smaller peptides in acidic conditions [5]. Pepsin is released in the form of zimogen as pepsinogen, stable in a neutral environment and a weak alkaline atmosphere at pH 7 to 11 [6]. Pepsin can be found in various animal

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stomachs such as chickens [7], pigs [8], also can be isolated from microorganisms such as *Aspergillus*, *Penicillium*, *Rhizopus*, *and Neurospora* [9].

Pepsin can be extracted by separating gastric juice from the pellet using centrifugation. Previous research used three methods to isolate pepsin from the pig's stomach. The first method of isolation by separating enzymes using water (1.597 U/mL), the second method with coagulation (4,908 U/mL), and the third method is carried out by lipofilisization (3.458 U/mL) shows different enzyme activity [8]. 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. Enzyme from Albacore tuna has activity of 1.235 U.

The difference can be caused by the type of fish used, but can also occur because of the method used [10]. Yellowfin as a raw material for pepsin extraction produces an activity value of 0.127 U/mL [11]. In other studies, the gastric sample of yellowfins (*Thunnus albacares*), hooktooth shark (*Hemigaleus balfouri*), and mackerel tuna (*Euthynnus affinis*) as raw material for making pepsin enzyme amounting to 44.582,07 U/mg and 10.879,99 U/mg [12]. Research on skipjack pepsin is still not much found, therefore, this research was conducted to determine the characteristic of pepsin that produce skipjack tuna, and precipitation with ammonium sulfate to obtain pepsin with high activity

2 Materials and methods

2.1 Materials and tools

The raw material used in this study was skipjack viscera, obtained from Bekasi, West Jawa. Other ingredients used include HCL (Merck), Tris-base (Merck), TCA 10% (Merck), liquid nitrogen, Bovine serum albumin (BSA), distilled water, Haemoglobin (SIGMA), Bradford, Ammonium sulphate (Merck), Acetic acid (Merck). The tools used include tools used in the study were glassware (Iwaki), Erlenmeyer (Iwaki), pH Meter (Hanna Hi 2210), Analytical Scales (Ohaus), Centrifuge (VWR® Mega Star 600R), Microplate UV-VIS Spectro (BMG Labtech), Electrophoresis, Water bath (Yamato).

2.2 Pepsinogen extraction

Samples are obtained in a frozen state. The next sample was disbursed and cleaned from the remaining stomach and blood in the stomach using running water. Next, tuna stomach is cut into a small size1x1 cm using a knife. The tuna stomach is then given liquid nitrogen to solid like ice, then mashed using a blender. The extraction process is carried out with a tris-HCl pH 7.5 buffer. The mixture that has been obtained is then centrifuged for 15 min at 10.000 g at 4 °C. The supernatant obtained is called pepsinogen [13].

2.3 Pepsin activation

Pepsin activation occurs by reducing the pH of pepsinogen to 2 by adding HCl 3 N and left for 10 min, then added $NaHCO_3$ 2 N to increase the pH to 2.75. Samples was precipitated for 6 h, filtered to produce supernatant of pepsin solution. Pepsin obtained was carried out by the pepsin activity test and protein concentration to determine the specific activity of pepsin produced [8].

2.4 Analyses

2.4.1 Evaluation of proteolytic activity

Analysis of pepsin activity carried out using cow haemoglobin as a substrate. The substrate is dissolved with Tris-HCl 10 mm pH 2 with a concentration of 2%. The substrate was taken as much as 0.625 mL into a micro tube and added as much as 0.125 mL of pepsin after that was homogenized and incubated in a period of 15 min at 50 °C. The addition of 10% TCA is then carried out as much as 0.4 mL to stop the reaction. The solution sample is centrifuged at a speed of 10.000 g in 10 min. Making blanks is done by mixing haemoglobin and distilled water. Supernatants are determined with a wavelength of 280 m [8].

To study the pH profile, coarse pepsin proteolytic activity is measured at 50 °C using haemoglobin as a substrate at different pH (pH 2.0; 3.0; 4.0; 4.0 5.0; 6.0; 6,0; and 7.0). For temperature profiles, testing is carried out at various temperatures (30, 40, 50, 60, 70 and 80 °C) at pH 2 [14]. Pepsin activity units are defined as an increase in absorbance of 0.001 per min, so that pepsin activity can be stated as follows:

$$U = \frac{A280 - A0}{0.001 \text{ x } t \text{ x } VE} \text{ x } 100\%$$

U : Activity Unit (AU/mL)
A_{280} : absorbance at a wavelength of 280
A0 : Absorbance of Blank
VE : enzyme volume
t : Incubation time

2.4.2 Evaluation of protein concentration

The analysis was carried out by adding 5 mL of Bradford reagent into a test tube containing 0.1 mL of sample solution. Incubation was carried out for 5 minutes and measurements were taken using a spectrophotometer with a wavelength of 595 nm. The Bradford reagent was made by dissolving 10 mg of coomasie brilliant blue in 5 mL of 95% ethanol, then adding 10 mL of 85% phosphoric acid (w/v) and 250 mL of distilled water until the solution was mixed. The solution was filtered using Whatman filter paper number 1 before use. The standard solution was made by dissolving bovine serum albumin (BSA) with a concentration of 2 mg/mL which was used as a stock solution. The standard stock solution was then given a standard range at a concentration ranging from 0.1-1.0 mg/mL. The standard solution was measured for its absorbance value with a spectrophotometer at a wavelength of 595 nm [15].

2.4.3 Precipitation with ammonium sulphate

Crude extract is precipitated with ammonium sulphate. Purification with ammonium sulphate is done with a variety of saturation of 0-30% to 0-80%. Ammonium sulphate is added little by little into 50 mL of crude enzyme solution while stirring with a magnetic stirrer at medium speed prevent foam formation. The precipitation process takes ± 45 min and is done at room temperature. The solution after adding ammonium sulphate is allowed to stand for 24 h at chilling temperatures (4 ℃). Furthermore, enzyme solution is centrifuged at speed of 7.000 rpm for 10 min at 4 °C. Solution was separated from pellet, to be used at the stage of characterization of enzymes in each fraction [16].

2.4.4 pH and temperature profile

To study pH profile, proteolytic activity of purified pepsins was measured at 50 °C using haemoglobin as a substrate at different pHs (2.0, 3.0, 4.0, 5.0, 6.0, and 7.0,). For temperature profile, the assay was performed at various temperatures (30, 40, 45, 50, 55, 60, and 70 °C) at pH 2.0 [14].

2.4.5 Kinetics study

The activity was assayed with different final concentrations of haemoglobin ranging from 0.5% to 3%. The final pepsin concentration for the assay was 2%. The determinations were repeated twice, and the respective kinetic parameters including Vmax and Km were evaluated by plotting the data on a Lineweaver–Burk double-reciprocal graph [14].

2.4.6 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS-PAGE has the principle of separating proteins based on their migration ability with the help of electric current. The test method consists of pretreatment, gel making, running sample, and fixation. The samples tested were supernatant samples and pellet samples after enzyme purification with ammonium sulphate. Pretreatment was carried out by mixing 10 μL of sample with 10 μL of sample buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercapto ethanol (βME)). The pellet sample was first diluted using 4 mL of tri-HCL pH 4 buffer, then the sample was stored at 0˚C. The stacking gel was made with a concentration of 3% and a separating gel of 15% and left to solidify. A sample of 10 μL was taken and inserted into the well. Running the sample with a current of 13 mA/gel and a voltage of 100 V for 3 h or until the band on the marker reached the red line of the lower limit of the gel. The gel that has been electrophoresed is stained using 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 7% acetic acid for 1 hour, after the staining process the gel is rinsed with 7% acetic acid until the bands are visible [17].

3 Results and discussion

3.1 Characteristics of skipjack pepsin

One of the factors affecting pepsin activity is temperature. The optimum temperature is a condition where pepsin activity reaches the highest value and will decrease when it deviates from that temperature. Optimal temperature measurement is done using an incubation temperature variation. Pepsin is an active protease enzyme in acidic conditions. Pepsin activity is not only influenced by temperature, but is also influenced by the value of the degree of acidity or pH. Measurement of optimum pH characteristic values in the clipped stomach pepsin is measured in the pH range of 2-8. The characteristic temperature and pH of crude enzyme is presented in Figure 1 dan 2.

Figure 1 and 2 shows that crude extract pepsin has the best activity in the temperature 50 °C and pH 3 with activity value 4.392,16 U/mg. Pepsin enzyme activity has increased along with an increase in temperature to reach the optimum temperature limit. This is due to the increasing temperature, it will increase the speed of the reaction, so that the enzyme activity will increase. Just like temperature, enzymes are also affected by the pH of their substrate. Pepsin is an enzyme that works at acidic pH, so with increasing pH value of the substrate, the resulting activity is lower.

The optimum temperature of crude and purified skipjack pepsin (*Katsuwonus pelamis*) has a temperature with the best activity in the range of 40-50 °C, when the temperature is raised pepsin activity has decreased [14]. At the same family, optimum temperature of pepsin from yellowfins (*Thunnus albacares*) stable in a temperature range of 40-60 °C and decreases at 70 °C [11, 12]. Characteristics of pH show pepsin activity will decrease if the pH value deviates from the optimum pH. Pepsin works at low pH because it is included in one of the proteases that work hydrolysing protein in acidic conditions. Decreased value of pepsin activity occurs at a temperature of 60-80°C due to the occurrence of denaturation due to high temperature [14]. Optimum temperature of pepsin is generally in the temperature range of 30-55 °C [6].

The optimum temperature in each pepsin enzyme is not always the same. Optimum temperature difference in pepsin is related to the conformation of enzymes that are influenced by habitat and the environment. Fish derived from cold waters will have high activity at low temperatures, so it is quite vulnerable to high temperatures [18]. The active side of the pepsin under the optimum pH conditions according to the substrate used, so that it will produce a maximum substrate enzyme complex [19]. Pepsin activity generally decreases at higher pH, because the higher pH will produce a basic state. This can be caused by changes in enzyme conformation so that it can reduce activity [20].

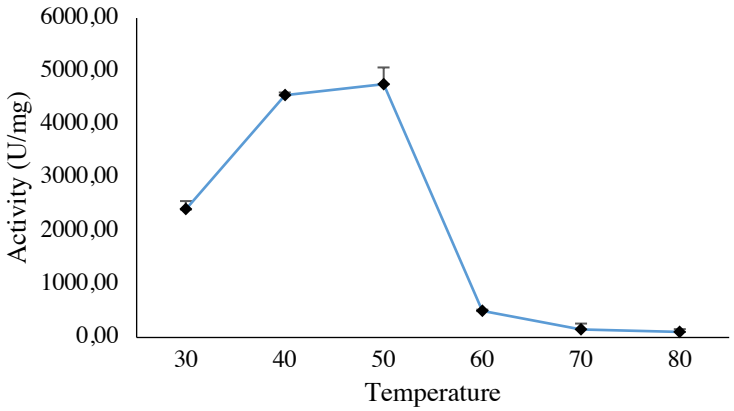

Fig.1. Temperature profile of skipjack pepsin

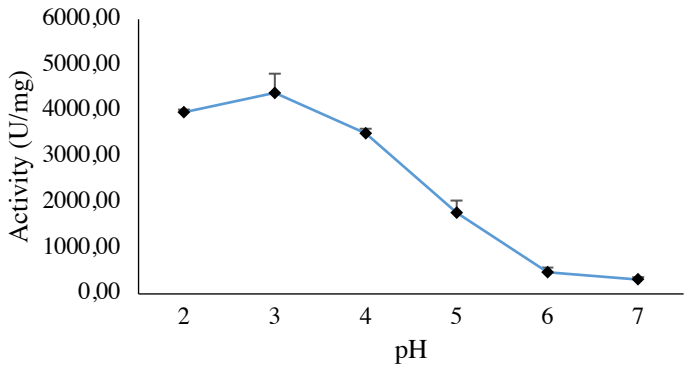

Fig.2. pH profile of skipjack pepsin

3.2 Kinetics assay

Figure 3 shows the relationship between substrate concentration and the activity of crude pepsin extract. These results indicate that pepsin activity is influenced by the concentration of the substrate used. Enzyme activity continues to increase at substrate concentrations of 0 to 2%, but decreases when passing a concentration of 2%. The highest crude pepsin extract activity is at a substrate concentration of 2%, which is 4.967,32 U/mL. Low substrate concentrations will also result in low reaction rates, but will increase as substrate concentrations increase until they reach a limit point or are referred to as maximum rates (V_{max}).

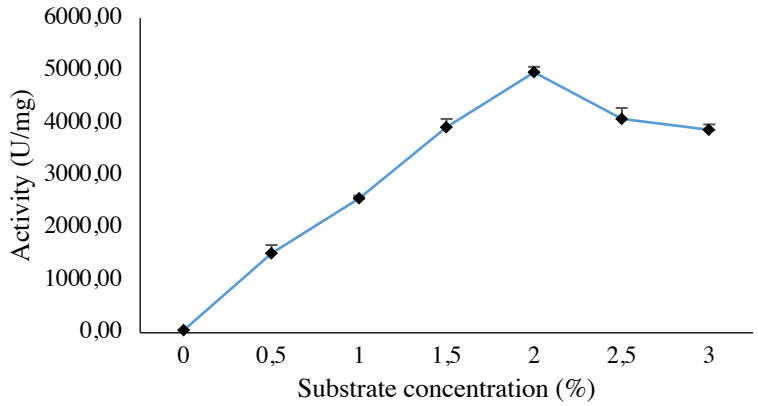

Fig.3. Relationship between substrate and activity

The rate of enzyme reaction can be determined by determining the V_{max} and K_m values using variations in substrate concentration. The activity of the pepsin enzyme can be affected by the concentration of the substrate used. Enzyme activity is related to the rate of enzyme reaction, so the higher the concentration of the substrate used, the enzyme activity and reaction rate will also increase [21]. The maximum rate is achieved because all active sites of the enzyme have bound to the substrate [22]. The Lineweaver-Burk equation of the enzyme is presented in Figure 4

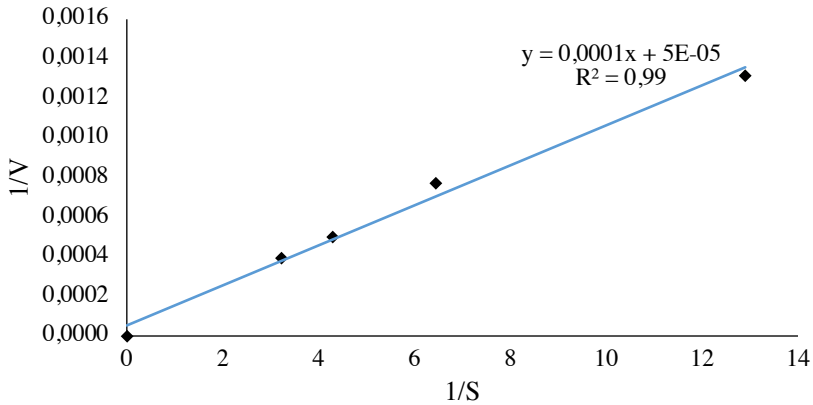

Fig.4. Lineweaver-Burk equation

Figure 4 shows the Lineweaver-Burk equation used to determine the maximum reaction rate (V_{max}) and the Michaelis-Menten Constant (K_m). The crude pepsin extract produced a Vmax value of 20.000 mmol/s and a Km value of 2 mM.

V_{max} is the maximum reaction rate that can be achieved by the pepsin enzyme, while the K_m value is the substrate concentration required by the enzyme to reach half its maximum rate. The K_m values obtained in largemouth bass (*Micropterus salmoides*) 0.039 mM [23] and 54 mM obtained in skipjack pepsin (*Katsuwonus pelamis*) [14].

3.3 Precipitation with ammonium sulphate

Pepsin crude extract that obtained is concentrated with precipitation techniques based on salting out theory, using neutral salt ammonium sulphate $(NH_4)_2SO_4$ with a concentration of saturation of 0-30% to 0-80%. The purpose of concentration with ammonium sulphate is to eliminate some of the impurities contained in the crude extract so that pepsin will have a higher percentage. The protein content of supernatant and pellets of the enzyme pepsin is presented in Figure 5.

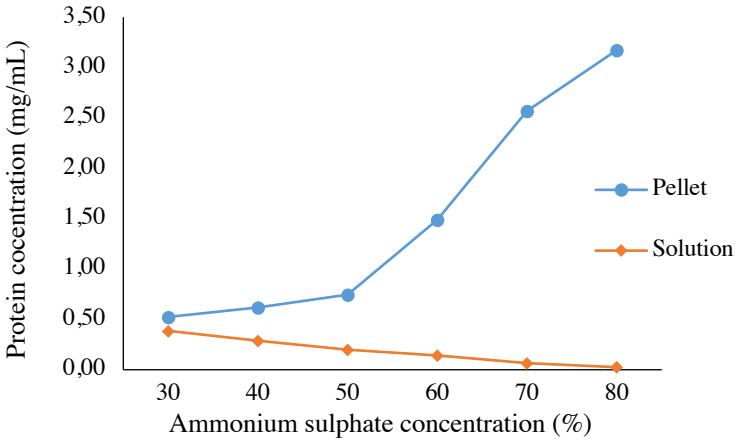

Fig.5. Protein concentration profile after precipitation

Based on Figure 5, we obtained that protein content in pellet samples increases with increased concentration of ammonium sulphate (0-80% saturation). The addition of salt with high concentrations will cause protein to be deposited. This salting out effect can occur due to competition between protein and salt in its interaction with water molecules. Water interacts stronger with salt compared to protein in high salt concentrations, causing protein to have low solubility so that protein experiences deposition [24]. This process also decreases the soluble force that is around the surface of the protein molecule. This is in line with the results obtained in Figure 4 which shows that the concentration of protein in the supernatant section of the results of purification is reduced in line with the increasing concentration of ammonium sulphate used.

Protein that settles mostly is obtained in the precipitate or coarse extract of the enzyme after going through the precipitation or purification process. Another research showed similar results where the concentration levels of enzyme solution protein and pellets produced from the precipitation process using ammonium sulphate were inversely proportional, the results showed that the purifier level of 20% was the best fraction because it produced the highest

specific activity [12]. The use of ammonium sulphate with a high saturation level causes an increase in the electric charge which will attract water molecules from the protein colloid so that hydrophobic interactions between protein molecules will reduce protein solubility so that salting out occurs which causes the protein to precipitate and settle [25]. Furthermore, the solution resulting from the precipitation with ammonium sulphate was then tested for its activity using the optimum pH and temperature presented in Figure 6.

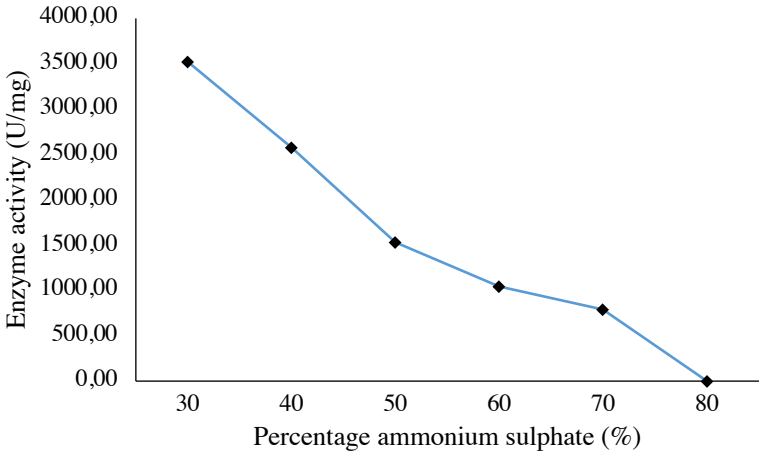

Fig.6. Enzyme activity after precipitation

Based on Figure 6 the value of enzyme activity in the supernatant showed a decreasing number in line with the decreasing levels of dissolved protein after ammonium sulphate precipitation. The decreasing enzyme protein in the supernatant due to the use of ammonium sulphate with a higher saturation level. The decrease in enzyme protein in the supernatant was caused by the use of ammonium sulfate with a higher saturation level. The use of high ammonium sulfate causes enzyme protein to precipitate with non-enzyme protein.

Some research showed similar results where the spesific activity of supernatant was decreased. The decreasing protein levels in the supernatant are also in line with the decreasing specific activity of crude enzyme [12]. In addition, it was also found the percentage of cathepsin inhibition by the pellet from ammonium sulphates precipitation is increased, consistent with an increase in protein levels [19]

3.4 Molecular weight of crude and purified enzyme

Samples used were crude extract, supernatant, and pellets from the purification of Skipjack pepsin. Purification of crude extract used ammonium sulphate with concentrations of 0-30%, 0-40%, 0-50%, 0-60%, 0-70%, and 0-80%. The solution sample was diluted to 10 mL using tris-HCL buffer pH 3. The molecular weight test data are presented in Figure 7 and Figure 8.

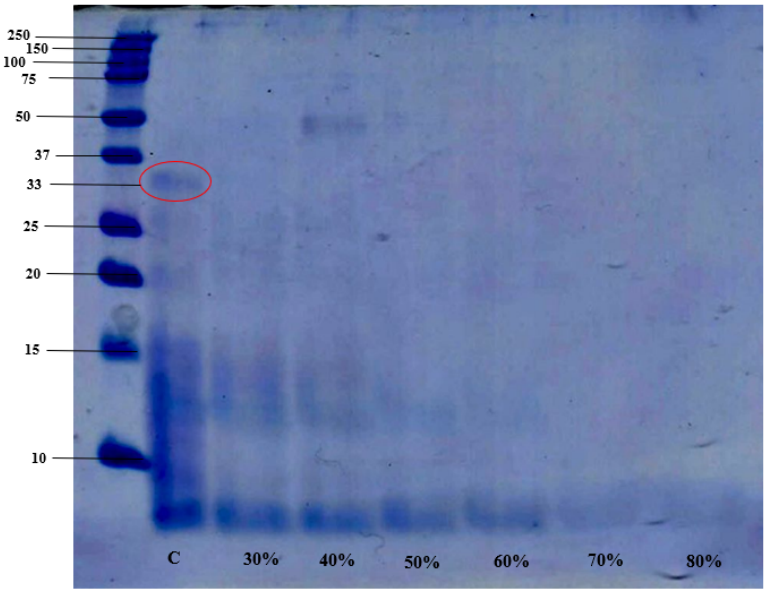

Fig.7. Molecular weight of crude and purified enzyme

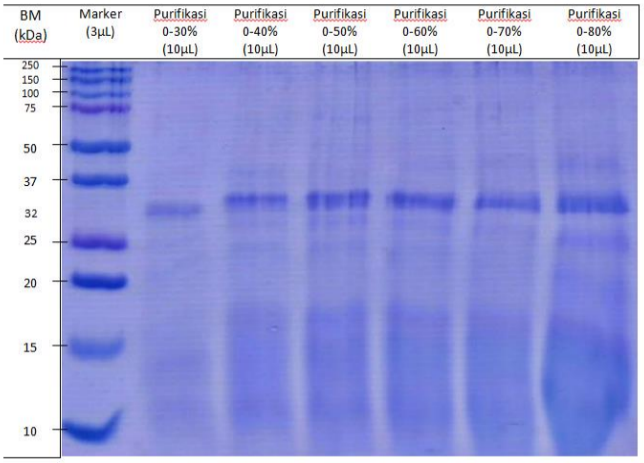

Fig.8. Molecular weight of precipitation pellet

Based on figure 7 the target enzyme pepsin of skipjack from SDS-PAGE showed a thick band. The molecular weight of the enzyme in previous studies was recorded in the range of 31-33 kDa. Meanwhile, the results of the analysis of the molecular weight of crude extract showed a band at 33 kDa, which was estimated to be the target protein, namely the pepsin enzyme. In the supernatant sample with an ammonium sulphate concentration of 0-30% to 0-80%, no spesific band was found indicating the target protein, which was suspected that after the purification process the protein dissolved and precipitated on the pellet, the results of the thick band on the SDS-PAGE supernatant from the purification results were seen below 15kDa, which was not the target protein. This is correlated with a decrease in the protein concentration value of the supernatant (Figure 5). Small concentration of protein resulting the band being thin to invisible. However, on the Figure 8, tested pellet samples showed a thick band at a specific molecular weight of 31-32 kDa.

The resulting band became thicker as the precipitation treatment with ammonium sulfate increased. Samples from several fish species showed differences in molecular weight in pepsin. Molecular weight of skipjack tuna pepsin is similar to that of albacore tuna [10] and Mustelus mustelus [13] ranging from 29 to 32 kDa. Pepsins isolated from Rainbow trout, *Oncorhynchus mykiss* [26] and largemouth bass, *Micropterus salmoides* [23] have values ranging from 32 to 36 kDa.

4 Conclusion

The pepsin enzyme was successfully extracted from the stomach of skipjack tuna using Tris-HCl buffer. Ammonium sulfate precipitation with higher saturation levels resulting yielding greater protein content in the enzyme pellet. Characterization of the crude pepsin extract revealed an optimum temperature of 50 °C and a pH of 3. Molecular weight of approximately 32-33 kDa for the enzyme, with a distinct pepsin band appearing in the pellet fractions.

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