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ORIGINAL RESEARCH

Identification of amylase activity from digestive tract of vannamei shrimp's (*Litopenaeus vannamei*) using size exclusion chromatography method

Abstract. Vannamei shrimp (*Litopenaeus vannamei*), also known as whiteleg shrimp is widely cultivated and consumed by many people. However, most consumers were removed the shrimp head which integrates with its digestive tract. The digestive tract of whiteleg shrimp contains digestive enzymes, including amylase. This study aimed to determine the protein content and amylase activity from vannamei shrimp's digestive tract using size exclusion chromatography method. The protein isolation of size exclusion chromatography was prepared in three steps; centrifugation, precipitation, and dialysis. The protein from dialysis step was purified by gel filtration chromatography. Each fraction that obtained was determined the protein content and amylase activity by spectrophotometer UV-Vis method. The results showed that the fraction of 108th has highest protein content and amylase activity with a value of 0.85 mg/ml and 25.66U/ml respectively.

Keywords: amylase activity, *Litopenaeus vannamei*, protein content, size exclusion chromatography

INTRODUCTION

Vannamei shrimp is one of the fisheries commodities that have high economic value in both domestic and global markets, and its production is also increasing every year. Therefore, there is also a lot of waste produced from shrimp and most of them are head and shell waste. Head of shrimp contain whole digestive tract with digestive enzymes, including amylase, lipase, and protease.

Amylase is classified as saccharidase (an enzyme that cuts polysaccharides). Amylase is a digestive enzyme, mainly carried out by the pancreas and salivary glands (1). Amylase enzymes have many essential roles in food, detergent, textile, paper, and bioethanol industries. In the pharmaceuticals field, amylase is used as a raw material for drug digestion disorders.

The use of amylase as a result of isolation is actually considered ineffective because its enzyme activity is still low, so size exclusion chromatography is a method that can increase the activity of the enzyme. The separation and purification of the enzyme is necessary to increase enzyme activity and obtain optimal and efficient catalysts (2). Enzyme separation was carried out to obtain protein fraction of the digestive tract of Vannamei shrimp (*Litopenaeus vannamei*) which had the highest amylase enzyme activity.

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The process of enzyme separation was carried out through precipitation and gel filtration chromatography method, while precipitation is carried out using ammonium sulfate. Ammonium sulfate is used because it has several benefits. These include having high solubility, does not affect the activity of enzymes, has sufficient deposition power, has a stabilizing effect on most enzymes, can be used at various pH and low prices (3).

Gel filtration chromatography is a method of separating proteins based on the molecular size of a protein by passing it into a column containing expanded gel particles (4). The principle of gel filtration chromatography process is similar to the column chromatography, differing only stationary phase used where gel filtration chromatography uses sephadex G-100 as stationary phase.

The previous study revealed that gel filtration chromatography was able to separate amylase enzyme from digestion of vannamei shrimp was 2.97 ± 0.11 units/mL (5). Other studies showed that amylase activity isolated from shrimp with several probiotic Bioremediation-Bacillus sp was 60.3 ± 3.8 units/ml (6). Anhar (2018) reported that 80% of ammonium sulfate was

able to precipitate amylase enzyme 30.52 U/ml (7). The aims of this study are to isolate and determine amylase from the digestive tract of shrimp Vannamei shrimp (*Litopenaeus vannamei*) by using gel filtration chromatography method.

METHODOLOGY

Materials

Vannamei shrimp, phosphate buffer, 14,000 Da cellophane membrane, ammonium sulfate, $BaCl_2$, HCl, EDTA alkaline, Bovine Serum Albumin (BSA), distilled water, Sephadex G-100, DNS reagent, 1% starch, Coomasie blue R250, ethanol 95 ethanol %, 85% phosphoric acid and Na_2CO_3.

Extraction and Precipitation

As much as 540 g sample of digestive tract from head of the shrimp was mashed with a grinder machine while adding drop by drop of cold phosphate buffer pH 7. After that the mixture was centrifuged (Thermo Scientific) in cold temperature at 10,000 rpm for 15 minutes. The supernatant that obtained then added dropwise an ammonium sulfate solution under constant stirring to precipitate the enzyme. The precipitation was centrifuged again at 10,000 rpm 4°C for 15 minutes in cold temperature. The obtaining residue then used for dialysis.

Dialysis

The cellophane tube bag is filled with a deposit resulted from the deposition process which will then be dialyzed, and the other tip is bound again. The result of precipitation deposited into cellophane with a size of 14 kDa and then dialyzed by immersing cellophane with pH 7 buffer solution. Dialysis was carried out using magnetic stirrer at 125 rpm, at 4°C for 7x24 hours with solvent replacement every 3 hours. Then, mix the phosphate buffer solution pH 7 with $BaCl_2$ and HCl (1: 1), if no dialysis deposits have been formed (8).

Determination of Protein

Determination of protein content begins by creating two parts, namely blank and test. Pipette 0.1 ml of enzyme and add 5 ml of Bradford are used in the test, while for the blank, it contains 0.1 ml of distilled water and 5 ml of Bradford reagen. The test and blank were then incubated with waterbath at 37 °C for 10 minutes. After incubation, the test and blank will be measured at a wavelength of 595 nm (9).

Size Exclusion Chromatography

Sephadex was developed by using phosphate buffer. 1.9 ml of the enzyme fraction with the highest specific activity value was obtained from the deposition of ammonium sulfate, then it put into a column with a sephadex G-100 matrix (column length of 20.5 cm and diameter of 2.4 cm) which was balanced with phosphate buffer and the elution rate is regulated. The sample was eluted with the similar phosphate buffer (10). The volume of each fraction collected was 1.5 ml and each fraction absorbed was measured then the enzyme activity test was carried out.

Amylase Activity

Amylum 1% as much as 0.1 ml was added to the crude extract as much as 0.1 ml, then incubated at 37°C for 5 minutes. Enzyme activity was stopped by adding 2 ml of DNS, incubated at 37°C for 5 minutes. Amylase activity was determined by measuring using a spectrophotometer at a wavelength of 540 nm (11).

Data Analysis

Amylase activity:

$$\frac{M\ Glucose\left(\frac{\mu g}{ml}\right) x\ 1000\ x\ fp}{Mr\ Glucose\ x\ Incubation\ time} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where M Glucose is the glucose produced from starch hydrolysis, fp is the dilution factor, and Mr Glucose is the relative molecules of glucose.

RESULTS AND DISCUSSION

Extraction and Precipitation

The Vannamei shrimp was determined in the Zoology section of the Indonesian Institute of Sciences' Biology Research Center (LIPI) Cibinong. The results of these determinations indicate that the sample is indeed Vannamei shrimp. The determination of the sample aims to find out the truth of the sample used, which is Vannamei shrimp. Determination is conducted so that there is no error in the preparation of the sample that will be used in the research.

Enzymes are one of the materials that can be used for health, food, and industry needs. This study uses a protein obtained from the digestive tract of shrimp. The digestive tract in the head of the Vannamei shrimp can be a source of enzymes (12). The sampling result obtained a digestive tract of 540.0985 g.

Enzyme extraction separates one or more components in a mixture using a solvent with the principle of polarity. The principle of polarity in extraction applies PBS (phosphate buffer saline) pH 7. Isolation is conducted because the enzyme is in the cells of the shrimp digestive tract. Therefore, the extraction process is conducted by destroying the digestive tract of shrimp (13). Vannamei shrimp

gastrointestinal enzyme extraction results can be seen in (Table 1).

Table 1. Results of vannamei shrimp digestion enzyme extraction.

No.	Information	Result
1.	Vannamei Shrimp Samples	7.11 kg
2.	Vannamei Shrimp Digestion	540.0985 g
3.	Enzyme axtract	1080 ml
4.	Supernatan	968 ml
5.	Precipitate	30 ml
6.	% Yield Vannamei	7.5963%
7.	Shrimp Digestion % Yield Sediment	5.5545%

Based on the table above, the shrimp digestive tract obtained is 540.0985 g from 7.11 kg of Vannamei shrimp. The digestive tract yield of Vannamei Shrimp obtained a yield of 7.5963%. These results achieve higher results when compared with Anhar's result (7). Anhar (2018) has a Vannamei shrimp digestive tract of 520.39 g and a digestive tract yield of 4.3365% (7). The factor that caused the difference was the weight of the shrimp used in this study was heavier by 20 g so that the yield and the number of shrimp digestive tract samples obtained were higher.

Enzyme precipitation is carried out with the aim of deposition of the protein and the protein is precipitated to separate it from other molecules besides protein. Enzyme precipitation uses 70% ammonium sulfate. According to Purwanto states that higher levels of ammonium sulfate will precipitate a more hydrophilic protein. Therefore, the protein deposited in this process shows that many crude enzyme extracts have proteins with high hydrophilic properties (3).

The crude enzyme extract obtained as much as 1080 ml and then it was centrifuged. The first centrifugation is done to separate proteins and cell debris. Cells that are not expected are still mixed with proteins, so they must be separated from proteins. The second centrifugation process produces a precipitate that was 30 ml. The 30 ml of pellet contains a lot of protein because it is deposited due to the salting-out process. The withdrawal of water by salt so that the binding of protein with water becomes weak and the bond between protein and protein so that the protein becomes precipitated (3). The deposition of protein causes high salt levels in the sample, so it needs to be separated through a dialysis process.

Dialysis

The purpose of the dialysis process is to remove excess salts from the precipitation, and then the low molecular protein comes out of the sample through the cellophane membrane. Ammonium sulfate salt can be removed by the process of dialysis using cellophane membranes (14). The cellophane membrane has a size of 14 kDa. Therefore, the cellophane membrane can hold molecules larger than 14 kDa.

Size Exclusion Chromatography

Gel filtration chromatography is one of the methods used for enzyme separation. Large molecules will pass through the pore of the sephadex first and smaller particles will go down longer because they are stuck in the pore. Spectrophotometer readings were performed at a wavelength of 280 nm. A wavelength of 280 nm is used to determine whether there is a protein concentration in it. Therefore, it is necessary to test the activity to get more specific results.

The results obtained from filtrate gel filtration chromatography were 139 fractions. Based on Figure 1 of the 139 fractions obtained, 14 fractions with absorbance above 0.15 were separated and carried out enzyme activity tests. The reason is that the 14 fractions are considered to have more amylase enzymes compared to other fractions. The amylase enzyme obtained is expected to have the highest amylolytic activity in breaking down starch into glucose. The results obtained from the dialysis process are in the form of dialysate. The dialysate was obtained 20 ml. The dialysate contains an enzyme extract that does not contain ammonium sulfate and low molecular protein. The final result of dialysis was identified using the sulfate test which was characterized by the absence of white deposits.

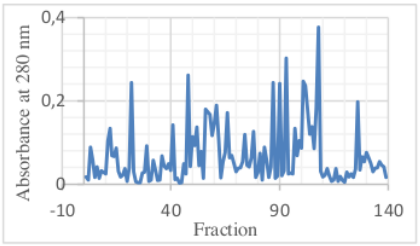

Figure 1. Chromatogram of protein absorption at 280 nm.

Amylase Activity

Amylase enzyme activity values are obtained through calculations integrated into the linear regression formula y = 0.0534 + 0.02165x. The value of y in the regression equation is the

difference between the absorbance of blanks and tests, while the x value is obtained from the glucose level collected. The r-value in this study was 0.9968. r-value is expressed as close to 1, and it can be concluded that the correlation coefficient is feasible, which means the points on the calibration curve are close to the slope.

Total enzyme activity was calculated in U (units) per ml of enzyme extract. One unit of an enzyme (U) is defined as the number of ml of the catalyst needed to produce 1 µmol substrate every minute. This shows that the amylase enzyme in the 108th fraction has the highest amylolytic activity in converting starch to glucose. The highest activity of amylase, which was obtained in the 108th fraction with an activity of 25.66 U/mL (Table 2).

Table 2. Amylase activity of selected fraction.

Fraction	Total Enzyme Activity (U/mL)	Fraction	Total Enzyme Activity (U/mL)
22	15.44	87	14.09
48	17.27	90	12.43
56	3.00	93	19.82
57	3.26	101	2.58
58	1.95	102	15.27
61	7.56	**108**	**25.66**
66	5.29	126	2.43

The results of the protein level test from the vannamei shrimp digestive tract can be seen in Table 3. The protein concentration was determined by Bradford method. Based on Table 3, protein levels were concentrated of 0.1215 mg/ml (before dialysis process), 0.1181 mg/ml (dialysate), and 0.0852 mg/ml (108th chromatography fraction). The results show the highest protein content in the sample before dialysis process. Samples before dialysis contain a lot of protein, caused by the absence of a separation process. Then, there is a decrease in protein content, especially dialysate, because of the protein precipitation with 70% ammonium sulfate salt. That is not all protein separated from the extracted sample so that the protein content after dialysis gets lower yields compared to before dialysis. The reduction of protein level in the 108th fraction is also caused by dilution occurs during the chromatography process.

Table 3. Protein concentration at each stage of separation.

Stage	Protein (mg/mL)	Total Amylase Activity (U/mL)	Specific Activity (U/mg)
Salting-Out	0.1215	17.08	140.51
Dialysis	0.1181	19.96	169.01
108th chromatography fraction	0.0852	25.66	301.17

The data of amylase and protein on the white shrimp digestive tract in the current study indicated an increase in enzyme activity and a decrease in protein levels along with the level of purity. The enzyme activity is concerning to the total protein present (i.e., the specific activity) can be determined and used as a measure of enzyme purity. It is clear that the higher the level of purification, the greater the cost of enzyme activity (15). Anhar (2018) reported the protein concentration and the amylase activity from salting-out precipitation of 12 kg white shrimp, respectively, about 0.117 mg/mL and 30.52 U/mL. Our current research might be explained by different amounts of white shrimp and ammonium sulfate percentage, affecting different protein levels and the activities. In addition, dilution factors and the absence of protease inhibitors, like PMSF, could influence the decrease in enzyme content.

CONCLUSION

In summary, the highest activity from size exclusion chromatography (SEC) was obtained at 108th fraction with an amylase activity of 25.6571 U/mL and specific activity was 30.10 U/mg. This value shows that protein fraction of Vannamei shrimp digestion has amylase activity, even though it is not high enough. Further investigations on SEC conditions, such as pH, temperature, retention factor, and gel matrix type, are considered to find maximum enzyme activity.

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