Jurnal Natural
Vol. 21, (3) 2021
DOI 10.24815/jn.v21i3.18874
pISSN 1411-8513
eISSN 2541-4062

ORIGINAL RESEARCH

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

HARIYANTI[1], RENA NADIA PERMATA ASRY[1], HANIFAH RAHMI[1]*

[1]Faculty of Pharmacy and Science, Universitas Muhammadiyah Prof. Dr. HAMKA, DKI Jakarta, Indonesia

Abstract. Vannamei shrimps (*Litopenaeus vannamei*), also known as white leg shrimps are widely cultivated and consumed by many people. However, most consumers have removed the shrimp's head which integrates with its digestive tract. The digestive tract of white leg shrimp contains digestive enzymes, including amylase. This study aimed to determine the protein content and amylase activity from Vannamei shrimps' digestive tract using the size exclusion chromatography method. The protein isolation of size exclusion chromatography was prepared in three steps, namely; centrifugation, precipitation, and dialysis. The protein from the dialysis step was purified by using gel filtration chromatography. Each obtained fraction was determined the protein content and amylase activity by using spectrophotometer UV-Vis method. The results showed that the fraction of 108th had the 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 domestic and global markets, and its production is also increasing every year. Therefore, there are a lot of wastes produced from shrimps and most of them are from the head and shell wastes. The head of shrimp contains the whole digestive tract with digestive enzymes, including amylase, lipase, and protease.

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

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

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

The previous study revealed that gel filtration chromatography was able to separate amylase enzyme from the digestion of Vannamei shrimp was 2.97 ± 0.11 units/mL (5). Other studies showed that amylase activity isolated from

*Corresponding Author:
hanifah_rahmi@uhamka.ac.id

Received: December 2020| Revised: September 2021 | Accepted: September 2021

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). This study aimed to isolate and determine amylase from Vannamei shrimps' (*Litopenaeus vannamei*) digestive tract by using gel filtration chromatography method.

METHODOLOGY

Extraction and Precipitation Stages

Researcher used as much as 540 g sample of the digestive tract from the shrimp head then mashed with a grinder machine, while it was adding drop by drop of cold phosphate buffer pH 7. After that, the mixture was centrifuged (Thermo Scientific) at a cold temperature of 10,000 rpm for 15 minutes. The supernatant that was obtained was added drop wise an ammonium sulfate solution under constant stirring to precipitate the enzyme. The precipitation was centrifuged again at 10.000 rpm 4ºC for 15 minutes in cold temperature. The obtaining residue is used for dialysis.

Dialysis

The cellophane tube bag was filled with a deposit resulted from the deposition process which it would be dialyzed, and the other tip was bound again. The result of precipitation was deposited into cellophane with a size of 14 kDa and then it was also dialyzed by immersing cellophane with a pH 7 buffer solutions. Dialysis was carried out by using a magnetic stirrer at 125 rpm, at 4°C for 7x24 hours with solvent replacement every 3 hours. If there were not any dialysis deposits have been formed from this stage (8), the researcher would mix the phosphate buffer solution pH 7 with $BaCl_2$ and HCl (1: 1).

Determination of Protein

Determination of protein content began by creating two parts, namely blank and test. Pipette with 0.1 ml of enzyme and added it with 5 ml of Bradford were used in the test, while for the blank, it contained 0.1 ml of distilled water and 5 ml of Bradford reagent. Then, the test and blank were incubated with a water bath at 37 ºC for 10 minutes. After incubation, the test and blank would be measured at a wavelength of 595 nm (9).

Size Exclusion Chromatography

Sephadex was developed by using phosphate buffer. As much as 1.9 ml of the enzyme fraction with the highest specific activity value was obtained from the deposition of ammonium sulfate 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 was regulated. The sample was eluted with a similar phosphate buffer (10). The volume of each fraction collected was 1.5 ml and each fraction absorbed was measured by the researcher, then the enzyme activity test was carried out.

Amylase Activity

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

Data Analysis

Amylase activity:

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

From the formula above, it can be explained as 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

Vannamei shrimps were determined in the Zoology section of the Indonesian Institute of Sciences' Biology Research Center (LIPI) Cibinong. The results of these determinations indicated that the sample was indeed Vannamei shrimp. The determination of the sample aimed to find out the truth of the sample used. Determination was conducted in the next stage so that there was no error in the preparation of the sample that was used in the research.

Enzymes were one of the materials that could be used for health, food, and industry needs. This study used a protein that was obtained from the digestive tract of shrimps. The digestive tract in Vannamei shrimps' head could be a source of enzymes (12). The result of sample obtained 540.0985 g a digestive tract.

Enzyme extraction separated one or more components in a mixture using a solvent with the polarity principle. The polarity principle in extraction applied PBS (phosphate buffer saline) pH 7. Isolation was conducted because the enzyme was in the cells of the shrimp's digestive tract. Therefore, the extraction process was conducted by destroying the

digestive tract of shrimp (13). Vannamei shrimp gastrointestinal enzyme extraction results could be seen in (Table 1).

Table 1. Results of Vannamei shrimps digestion enzyme extraction.

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

Based on the table above, the shrimp's digestive tract obtained was 540.0985 g from 7.11 kg of Vannamei shrimp. Vannamei Shrimps' digestive tract obtained a yield of 7.5963%. These results achieved a higher result when compared with Anhar's result (7). In Anhar research (2018), it had 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 also higher.

Enzyme precipitation was carried out with the aim of the protein deposition and the protein was precipitated to separate it from other molecules besides protein. Enzyme precipitation used in this research was 70% ammonium sulfate. According to Purwanto (2016) stated that higher levels of ammonium sulfate would precipitate a more hydrophilic protein. Therefore, the protein deposited in this process showed that many crude enzyme extracts had proteins with high hydrophilic properties (3).

The crude enzyme extract obtained as much as 1080 ml and then it was centrifuged. The first centrifugation was done to separate proteins and cell debris. Cells that were not expected were still mixed with proteins, so they might be separated from proteins. The second centrifugation process produced a precipitate of 30 ml. 30 ml of pellet contained a lot of protein because it was deposited due to the salting-out process. The withdrawal of water would be taken by salt so that the binding of protein with water became weak and the bond between protein and protein so that the protein became precipitated (3). The deposition of protein caused a high salt level in the sample, so it needed to be separated through a dialysis process.

Dialysis

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

Size Exclusion Chromatography

Gel filtration chromatography was one of the methods used for enzyme separation. Large molecules would pass through the pore of the Sephadex first and smaller particles would go down longer because they were stuck in the pore. Spectrophotometer readings were performed at a wavelength of 280 nm. A wavelength of 280 nm was used to determine whether there was a protein concentration in it. Therefore, it was 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 was that the 14 fractions were considered to have more amylase enzymes compared to other fractions.

The amylase enzyme obtained was expected to have the highest amylolytic activity in breaking down starch into glucose. The results obtained from the dialysis process were in the form of dialysate. The dialysate was obtained in 20 ml. The dialysate contained an enzyme extract that did not contain ammonium sulfate and low molecular protein. The final result of dialysis was identified by using the sulfate test. It was characterized by the absence of white deposits.

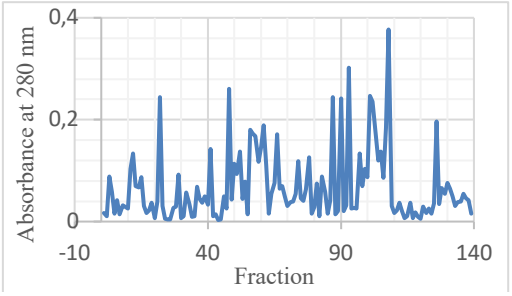

Figure 1. Chromatogram of protein absorption at 280 nm.

Amylase Activity

Amylase enzyme activity values were obtained through calculations integrated into the linear regression formula y = 0.0534 + 0.02165x. The value of y in the regression equation was the difference between the absorbance of blanks and tests, while the x value was obtained from the glucose level collected. The r-value in this study was 0.9968. r-value was expressed as close to 1, and it could be concluded that the correlation coefficient was feasible, which means the points on the calibration curve were close to the slope.

Total enzyme activity was calculated in U (units) per ml of enzyme extract. One unit of an enzyme (U) was defined as the number of ml of the catalyst needed to produce 1 µmol substrate every minute. This showed that the amylase enzyme in the 108th fraction had 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	Enzyme Activity Total (U/mL)	Fraction	Enzyme Activity Total (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 could 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 showed the highest protein content in the sample before the dialysis process. The samples before dialysis contain a lot of protein, it caused by the absence of a separation process. Then, there was a decrease in protein content, especially dialysate, because of the protein precipitation with 70% ammonium sulfate salt. That was not all protein separated from the extracted sample so that the protein content after dialysis got lower yields compared with before dialysis. The reduction of protein level in the 108th fraction was also caused by dilution that occurs during the chromatography process.

Table 3. Protein concentration at each stage of separation.

Stages	Protein (mg/mL)	Amylase Activity Total (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's 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 was concerning to the total protein present (i.e., the specific activity) could be determined and used as a measure of enzyme purity. It was clear that the higher the level of purification, the greater the cost of enzyme activity (15). Anhar (2018) reported that the protein concentration and the amylase activity from salting-out precipitation of 12 kg white shrimps, 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 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 the 108th fraction with an amylase activity of 25.6571 U/mL and specific activity was 30.10 U/mg. This value showed that the protein fraction of Vannamei shrimp digestion had amylase activity, even though it was not high enough. Further investigations on SEC conditions, such as pH, temperature, retention factor, and gel matrix type, were considered to find maximum enzyme activity.

ACKNOWLEDGMENT

This research was supported by Universitas Muhammadiyah Prof DR. HAMKA of Research Institution, which was gratefully appreciated.

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