Kinetic Studies of Purified Bromelain from Pineapple (*Ananas comosus [L.])Merr)* Core with Hidroxyapatite and CM Sephadex C-50 Ion Exchange Chromatography

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Keywords: Pineapple core, bromelain, specific activity,Michaelis-Menten constant, maximum reaction velocity.

Abstract: Bromelain is a protease enzyme that functions to break peptide bonds. in the health field bromelain can function as anti-inflammatory, anti-thrombotic and fibrinolytic. Here we purposed a new purification step to collect enzyme from pineaplle. The purification of the enzyme from *Ananas comosus* was carried out by precipitation with varying concentration of acetone and followed by column chromatography using hidroxyapatite ion exchage and CM sephadex C-50 resin. The enzyme activities were evaluated using casein as substrate. The highest specific activity of bromelain was gained from acetone fractination as 51,51 U/mg in the range of 50-80% saturation with purity level of enzyme as 11 times from its crude extract. The highest specific activity from last fraction which run on hidroxyapatite ion-exchange column chromatography was 87,49 units/mg with purity level of enzyme resulted 19 times higher compared to the crude extract. Later on, the purification with CM sephadex C-50 resulted in increasing the specific activity to 200 U/mg with purity level of enzyme as 45 times higher from its crude extract. Hydrolysis of various casein concentration with purified bromelain was carried out at optimum reaction condition of pH 7,0 and 37^0C. The results obtained revealed the Km and Vmax value were 0,94% (w/v) and 0,023 U/min respectively. From the various purification steps that have been done, it can be observed the increasing of bromelain specific activity from each stage.

1. INTRODUCTION

Bromelain is a complex mixture of compounds that differ from one another namely thiol endopeptidase and other components that are not yet fully characterized such as phosphatase, glucosidase, peroxidase, cellulase, glycoproteins, carbohydrates, some protease inhibitors, and calcium organically bound, among others. Bromealin is non-toxic compounds and a proteolytic enzyme that can catalyze the hydrolysis reaction of protein. Bromelain is a collective name for proteolytic enzymes or proteases found in tissues including the stem, fruit, and leaves of pineapple plants, the family Bromeliaceae. (A.D Rowan 1990)

Bromelain is usually distinguished as bromelain stem (EC. 3.4.22.32) or bromelain fruit (EC. 3.4.22.33) depending on the source. This proteolytic enzyme has unique functions useful to the food, pharmaceutical and cosmetic industries. As a drug, bromelain has been used for the treatment of various diseases, including thrombosis, rheumatoid arthritis, inflammatory diseases such as atherosclerosis, cancer treatments and others (Ketnawa et al., 2012) (Jeung, A 1980)

In the process of isolation and purification of enzymes, to obtain fractions with proteolytic activity and high purity, it is necessary to know the properties of the enzymes to be isolated. This is needed so that in the process there is no denaturation. Some techniques are often used such as sedimentation, filtering solvent extraction, affinity chromatography, ionic exchange and gel filtration. Although this methods has a high concentration of power but still has a low purity level. (M.A Desai, 2000)

Therefore, in this study protein isolation and purification were carried out using various methods to obtain increased purity and high activity of bromelain from pineapple core.

Putri, N., Hudiyono, S. and Setiasih, S.
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DOI: 10.5220/0009843300002406
In *Proceedings of BROMO Conference (BROMO 2018) - Symposium on Natural Product and Biodiversity*, page 1
ISBN: 978-989-758-347-6

2. MATERIALS AND METHODS

2.1 Materials

The materials used for the process of isolation and fractionation by precipitation include, Phosphate buffers pH 7, prepared from NaH_2PO4 (Merck) and Na_2HPO_4 (Merck), Acetone for precipitation. For ion column exchange chromatography, hydroxyapatite resin and CM sephadex C-50 are required, Tris–HCl buffers, NaCl. Furthermore, for the test of bromelain activity with Kunitz method (Kunitz, 1947) and for the determination of protein content by Bradford method (Bradford, 1976)

2.2 Methods

2.2.1 Preparation of Crude Extract

Core was cut into pieces and weighed as much as 500 gram, then blended and filtered using a filter cloth. Furthermore, core solution that has been obtained centrifuged at 6000 rpm for 15 minutes at 4°C. Supernatant was filtered and the filtrate taken as a crude extract of bromelain

2.2.2 Bromelain Purification from Crude Extract by Acetone Precipitation

Bromelain crude extract obtained proceed to the purification process by precipitation method using acetone. The concentration level was divided into three fractions, fraction 1 (0-20)%, fraction 2 (20-50)% and fraction 3 (50-80)%. Acetone was added to the crude extract at 4°C. The extracts was kept for overnight. Extract was centrifuged with a speed of 6000 rpm for 15 minutes at 4°C. The precipitate obtained re-suspended using 0.2 M phosphate buffer pH 7. The filtrate was resumed to higher concentration levels.

2.2.3 Measurements of Protein Content and Enzymatic Activity

Protein content was measured using Bradford method and the standard used was bovine serum albumin (Bradford, 1976). All measurements were performed in duplicate. All measurements are done in duplicate. While enzyme activity was measured as follows: 1% casein (w / v) at 0.9 M Tris - Hcl buffer (pH 8.0) was used as a substrate. Aliquots of 100 µL of sample were added to a centrifuge tube containing 1.9 mL of casein buffer solution. The mixture was held for 30 minutes in a water bath at 37 ° C. Furthermore, 3 ml of trichloroacetic acid (TCA) solution was added and after 30 minutes at 4 ° C., the mixture was centrifuged at 4000 rpm. for 10 minutes (Kunitz, 1947). The supernatant absorbance was determined at 280 nm using a UV / visible spectrophotometer (Shimadzu UV-2450)

2.2.4 Hydroxyapatite Chromatography

Following acetone precipitation, the sample was applied to a hydroxyapatite column (50 cm × 3,3 cm size), pre-equilibrated with using 0.05 M phosphate pH 7buffer containing at a flow rate of 5 ml/4 min. The enzyme was eluted by a linear gradient of 50–400 mM phosphate buffer. The bound enzyme was eluted by a linear gradient of 50 – 400 mM NaCl in phosphate buffer . Each fraction was assayed for protein and enzyme activity.

2.2.5 CM Sephadex C-50 Chromatography

Fractions with the highest specific activity will be purified by CM sephadex C-50 ion chromatography. The sample was applied to a cm sephadex c-50 column (50 cm × 3 cm size), pre-equilibrated with using 0.05 M Tris - Hcl pH 8 buffer containing at a flow rate of 5 ml/4 min. The enzyme was eluted by a linear gradient of 50– 400 mM Tris-Hcl buffer. The bound enzyme was eluted by a linear gradient of 50 – 400 mM NaCl in phosphate Tris - Hcl buffer . Each fraction was assayed for protein and enzyme activity.

2.2.6 Kinetic Studies

Determination of kinetic parameters such as Michaelis-Menten constant (Km), maximum reaction speed (Vmax) is determined by measuring and distributing enzyme activity data at optimum pH and temperature as a function of substrate concentration, based on the Lineweaver-Burk method (Alves et al., 2014) (Lehninger, 1982).

3. RESULT AND DISCUSSION

3.1 Acetone Precipitation

In this study, acetone (p.a) was used as the enzyme precipitator. Acetone has been widely used for the process of protein deposition and bromelain enzyme (Sharma et al 2014); (Rowan et al., 1990), (Heinicke & Gortner 1957) with high values of specific enzyme activity. The addition of acetone to the

pineapple solution is then stirred until homogeneous to precipitate the protein, including the bromelain enzyme contained in the pineapple. The principle of this protein precipitation process is to reduce water activity in core pineapple solution (Scopes, 1994). The dielectric constant of acetone (20.7) is smaller when compared to the air dielectric constant (80.1). It will be more of the protein contained in the air. Acetone is less polar than air, the more confusing it is from proteins as well because the polar air surface of polarity, mixed with less polar acetone, will be more interested in interacting with the same protein molecules wiped with acetone. Acetone capable of interacting with the hydrophobic part of the protein also further increases the reduced solubility of the mixture and increases the precipitation protein

The largest proteolytic activity, protein content, and specific activity of enzyme were obtained from the third fraction of bromelain enzyme (50-80% saturation level of acetone) with a value of proteolytic activity 7,717 Units and protein content 0.149 mg. Specific activity of 51.513 U / mg. The purity level obtained is 11 times purer than the crude enzyme

3.2 Hidroxyapatite and CM Sephadex C-50 Ion Exchange Chromatography

Ion exchange chromatography is an advanced purification step for separating bromelain from other proteins to obtain enzyme with higher specific activity. Purification by ion exchange chromatography uses the principle of charge difference between proteins and charged groups present in the column matrix. Proteins with the same charge as the matrix will elute first from the column. If the protein charge is different from the matrix, then the protein will interact electrostatically on the matrix and out of the column at different times depending on the strength of the bond

After precipitation with acetone, the sample is loaded onto a ion exchange chromatography columns hidroxyapatite and collected fractions were analyzed for proteins content and proteolytic activity. This chromatography step resulted in a 15 fold purification. The pooled fractions was further purified using CM sephadex C-50 chromatography.

In the second chromatographic step, the pooled active fraction was loaded onto a CM Sephadex C-50 ion-exchange column and the bound enzyme was eluted with a linear gradient of 50-400mM NaCl. The last step chromatography resulted in a 45 fold purification (tabel 1)

The effluent (protein and eluent molecules coming out of the column) is accommodated on tubes that hold 5 mL of effluent on each tube. And all fractions of protein uptake at 280 nm wavelength. This absorbance measure aims to detect and separate spreading proteins in each part of the column chromatographic results (Burgess, 2008). All fractions also measure their proteolytic activity by Kunitz method.

The following Figure shows that Elution profiles of chromatography steps related to the purification of bromelain from pineapple core.

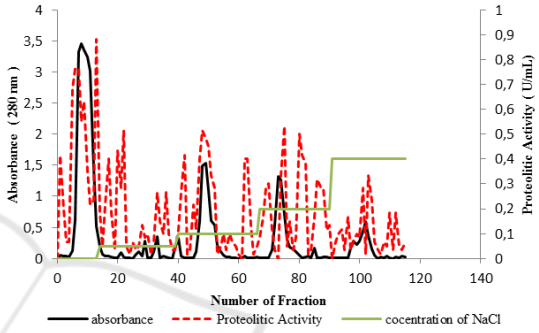

Figure 1: Hidroxyapatite chromatography

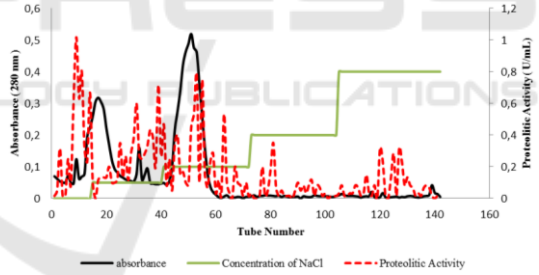

Figure 2: CM Sephadex C-50 chromatography

At the elution stage, the bond between the protein and the resin will be replaced by the ions from the salt eluent, the salt ion ions binding to the resin and the protein molecules will be released out of the column. The protein molecule that binds to the matrix will release more mixture through the lower salt, and the higher the salt concentration, the stronger the binding protein can also get out of the column. Separation occurs in protein molecules based on different times, depending on the strength of the molecular charge attached to the resin.

Table 1 Summary of purification and yields of bromelain

Step	Volume	Proteolitc Activity (U/mL)	Protein Content (mg/mL)	Total		Specific Activity (U/mg)	Fold
				Proteolitc Activity (U)	Protein Content (mg)		
Core solution	350	0,507	0,171	177,45	59,85	2,968	-
Crude Enzyme	200	0,733	0,163	146,60	32,60	4,490	1
Acetone	850	7,717	0,149	6559,45	126,65	51,513	11
Hidroxyapatite	85	2,116	0,031	179,86	2,635	68,258	15
CM Sephadex C-50	45	0,400	0,002	18,00	0,09	200,00	45

Enzyme solution of each fraction were proteinolytic activity and protein levels. Table 1 shows the value of proteolytic activity of each fraction obtained in the purification stage. The highest proteolytic activity was obtained in fraction CM Sephadex C-50 with a value of 200 Units and protein levels of 0,09 mg (figure 3). From the data of proteolytic activity and protein levels, the highest specific activity was obtained by a purity level reached 45 times.

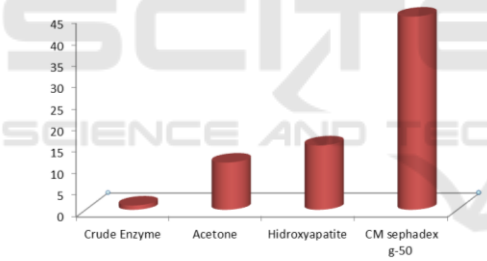

Figure 3 : an increase in purity levels of bromelain after going through various stages of purification

3.3 Kinetic Studies

The Michaelis-Menten (Km) constant, maximum reaction velocity (Vmax) is determined by passing data of activity enzyme at pH and temperature optimum as a function of substrate concentration, based on the Lineweaver-Burk method plot as shown in Figure 4

The Michaelis-Menten (Km) constant is the amount of substrate required to achieve half of the maximum rate of reaction. This constant can also be used to show the enzyme specificity to a substrate (Corzo et al., 2012). While Vmax is the maximum rate of enzymatic reactions in the enzyme state that has been saturated by the substrate. In this study, the Michaelis-Menten (Km) constant for fraction AP1 purification with CM sephadex c-50 was 0.943% (w

/v) casein with a maximum velocity value (Vmax) 0.0234 U / min.

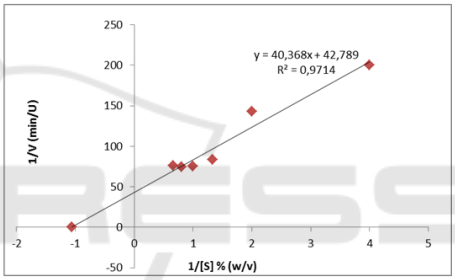

Figure 4 : Lineweaver-Burk Plot for Casein Hydrolysis by Bromelain Fraction AP1 CM sephadex

4 CONCLUSIONS

Bromelain was successfully purified through a series of purification steps, starting from extraction of crude extract and precipitation of bromelain with acetone and purification with chromatography column ion exchange. The result of bromelain after precipitation by acetone and purification with hidroxyapatite and CM Sephadex C-50 gave higher specific activity than before crude enzyme

ACKNOWLEDGEMENTS

This work was funded by Hibah Kompetensi Publikasi Internasional Terindeks Untuk Tugas Akhir Mahasiswa (PITTA), Universitas Indonesia

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