The effects of PMSF and cysteine addition into partially purified bromelain from pineapple (Ananas comosus [L] Merr) cores to its kinetics behaviour

S Setiasih, M Putri, S Handayani and S Hudiyono
Deparment of Chemistry, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Kampus UI Depok, Depok 16424, Indonesia

Corresponding author’s e-mail: setiasih@ui.ac.id

Abstract. Bromelain found in various tissues in the pineapple plant (Ananas Comusus [L] Merr. The bromelain will be taken from pineapple cores for kinetic study. The effect of addition organic compounds, such as phenylmethylsulfonyl fluoride (PMSF) and cysteine on its kinetics behaviour will be determined by proteolytic activities of the bromelain. The stages of this study were started from isolation of crude enzyme, fractionation with ammonium sulphate salt, dialysis, and ion-exchange column chromatography with CM sephadex C-50 for enzyme purification. The obtained enzyme fractions from crude enzyme, ammonium sulphate, dialysis, and CM-sephadex C-50 ion exchange chromatography have specific activities of 45.39, 238.31, 311.16, and 427.13 U/mg, respectively. The purification stages result in increasing of the bromelain specific activities by 9 times compared to the crude enzyme. The Michaelis-Menten constant (Km) value for the purified bromelain was 0.85 % casein and the Vmax value was 0.41 U/min. Addition of PMSF and cysteine compound effect on its Vmax and Km with various trends. The Vmax value will be decreased by addition of PMSF and increased by addition of cysteine. Thus, proteolytic activity of bromelain from pineapple cores can be significantly increased by the addition of cysteine compounds and inhibited by the addition of PMSF.

Keywords: enzymes, bromelain, pineapple, kinetic study enzymes, chromatography

1. Introduction
Protease enzyme is an enzyme that works to hydrolyze the peptide bonds in proteins into oligopeptides and amino acids. Enzyme is a protein for catalyzing chemical reaction. Enzymes will convert the arrangement of molecules into simpler structures, in particular, and works like keys and locks, including in context only from certain molecular arrangements. The work activity is affected by several factors such as inhibitor, cofactor, coenzyme, temperature, pH, concentration of enzyme and substrate [1].

Pineapple fruit (Ananas comosus) is the largest component containing bromelain protease enzyme, which is 95 % cysteine protease mixture. For the people of Indonesia, Pineapple (Ananas comosus (L) Merr) is one type of fruits commonly known and eat freshly. In the process production, pineapple always produces big enough waste around 60 % and 7 % among them is pineapple core which contains bromelain [2]. Meanwhile, bromelain shows therapeutic effect for human body [3].

In this study, the pineapple part of the weed will bind proteolytic activity and its specific activity after bromelain is fractionated using ammonium sulfate to precipitate the protein, then dialyzed to remove it and purified by ion exchange chromatography column. Furthermore, to determine its stability, the bromelain fraction obtained will be determined the proteolytic activity and bromelain kinetics studies of organic compound addition, such as cysteine and phenylmethylsulfonyl fluoride PMSF.
Table 1. Purification of the crude enzyme by using ammonium sulphate and dialysis on bromelain enzyme

| Fraction                  | Volume (mL) | Total Proteolytic Activity (U) | Total Protein (mg) | Specific Activity (U/mg) | Purification Factor (times) |
|---------------------------|-------------|-------------------------------|--------------------|--------------------------|-----------------------------|
| Crude Enzyme (CE)         | 150         | 2171.50                       | 47.87              | 45.39                    | -                           |
| Fraction 1 (0 %-20 %)     | 3           | 54.65                         | 0.62               | 88.13                    | 1.94                        |
| Fraction 2 (20 %-50 %)    | 10          | 195.4                         | 0.71               | 275.21                   | 6.06                        |
| Fraction 3 (50 %-80 %)    | 8           | 107.47                        | 2.83               | 38.03                    | 0.84                        |
| Residue Enzyme (RE)       | 171         | 1234.05                       | 34.61              | 35.66                    | 0.79                        |
| Fraction Dialysis (FD)    | 8           | 174.00                        | 0.56               | 311.16                   | 6.86                        |

2. Experimental methods

2.1. Isolation, ammonium sulphate precipitation, and dialysis

The isolation, ammonium sulfate precipitation, and dialysis stages were conducted according to the method that has been done with modification [4]. Different circumstance is the centrifuge condition, which was operated at 8000 rpm for 20 minutes. The obtained bromelain fractions were kept for the determination of proteolytic activity and total protein content.

2.2. Specific activity determination

The protein content was determined by Lowry Method. Meanwhile, the proteolytic activities of bromelain fractions were performed by Kunitz method using casein as substrate with modification. The specific activity of the enzyme was obtained by dividing the total units of enzyme activity (U) with the total protein content (mg) [5].

2.3. Enzyme purification by ion-exchange chromatography

The enzyme fraction obtained from the dialysis process was purified by an ion-exchange column chromatography method using CM sephadex C-50. The enzyme sample was fed into the chromatography column and eluted using HCl 0.05M pH 8.0. Further elution was then eluted using Tris-HCl 0.05M buffer pH 8.0, which contains NaCl salt solution with gradual concentration of 0.1M, 0.2M, and 0.3M [6]. The elution results were collected in a fractional reaction tube with a volume of 5 mL/tube. Each collected fraction was determined for its protein content by measuring its absorbance using a UV-Vis spectrophotometer at a wavelength of 280 nm and its proteolytic activity by Kunitz method. Fractions with the highest proteolytic activity are classified to specify their specific activity.

2.4. The effect of various compounds on bromelain activity

The compounds used in this study were PMSF and cysteine. The enzyme solutions, which contain the compounds, were tested for their proteolytic activities with variation concentration of casein substrate to determine Michaelis-Menten constant (Km) value and maximum velocity (Vmax) value. The values were determined by plotting its concentration substrate and velocity data to Lineweaver-Burk plot method.

3. Results and discussion

3.1. Isolation, ammonium sulphate precipitation, and dialysis

The bromelain fraction of ammonium sulfate 20 %-50 % has the highest specific activity with the purity level of 6.06 times compared to the crude enzyme (table 1). The fraction 2 still contain ammonium sulfate salt to be purified, one of the best methods to remove the salt is by dialysis method. The salt and small non bromelain protein will diffuse through cellophane membrane. From the table 1, dialysis fraction has higher purity level than the fraction 2 with specific activity of 311.16 U/mg.
Table 2. Specific activity-specific data of bromelain fraction in the purification step by ion-exchange column chromatography

| Fraction      | Total Proteolytic Activity (U) | Specific Activity (U/mg) | Purification Factor (times) |
|---------------|-------------------------------|--------------------------|----------------------------|
| AP 1 (Fraction 9-16) | 6.87                          | 325.88                   | 7.18                       |
| AP 2 (Fraction 25-39) | 4.47                          | 379.41                   | 8.36                       |
| AP 3 (Fraction 47-55) | 5.46                          | 461.03                   | 10.15                      |

Figure 1. Results of chromatogram of CM sephadex C-50 chromatography column. Separation Conditions: column size 3 x 20 cm, matrix volume 141.3 mL, elution with buffer solution, flow rate 1.5 mL/minute, eluent volume 5 mL/tube.

3.2. Ion exchanger column chromatography using CM sephadex C-50
This purification step is carried out on the fraction of the dialysis result. The stationary phase used in this study is the CM sephadex C-50 resin matrix, which is a weak cation exchange chromatography column (negatively charged resin). From this purification obtained as much as 88 fractions of result of column. Furthermore, the fractions were measured at maximum protein absorbance 280 nm. Furthermore, each fraction is measured for its proteolytic activity based on Kunitz's method. The results of both measurements are described in the form of chromatograms and obtained 3 protein peaks (figure 1).

All fractions around the peaks are combined and determined for its specific activity. Specific activity data of mixture fraction is shown in table 2. The measurement results showed the highest specific activity at the peak fraction of AP3 protein of 461.03 U/mL with purity level of 10.6 times compared to the crude enzyme.

3.3. Enzymatic kinetics
The determination of kinetics parameters was performed on purification isolation enzymes based on Kunitz method under optimum conditions by varying casein substrates (0.25, 0.5, 0.75, 1.0, 1.25, and 1.5) %. In figure 2, we can see the value of proteolytic activity each concentration of casein substrate. The data showed that 1% of the substrate concentration had the highest activity, so that each active side of the enzyme had bound to the substrate optimally. Determined the enzymatic rate of the Michaelis-Menten constant to determine the hyperparabolic relationship between substrate
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Figure 2. Graph of enzymatic reaction rate to substrate concentration

Figure 3. Graph of Lineweaver-Burk plot for the hydrolysis of the casein substrate

Figure 4. Graph of Lineweaver-Burk Plot for addition cysteine concentration and enzymatic rate. In addition, (0–1.0) % of the substrate is accompanied by an enzymatic rate rise, whereas in the addition of 1.25 % to 1.50 % the enzymatic rate is relatively constant, since the active side of the enzyme has been saturated by the substrate.

The Km value of an enzyme can be calculated by the Lineweaver-Burk equation obtained from the Michaelis-Menten equation which then produced a Lineweaver-Burk diagram which can be seen in figure 3, the Lineweaver-Burk Plot for the hydrolysis of the casein substrate by the result of the refractionation bromelain dialysis. The Michaelis-Menten (Km) constant value for the purified enzyme was 0.85 % Casein and the Vmax value was 0.41 U / min.

3.4. Enzymatic kinetics after addition of cysteine and PMSF compounds
Proteolytic activity of bromelain after the addition of cysteine solution showed an increasing trend along with the increase of activator concentration. This is due to alteration of bromelain conformation consisting of sulfhydryl group at its active center establishing an easier way for the substrate to interact with enzyme molecules. Based on the Line-WeaverBurk plot in figure 4, bromelain with addition of 40 µM, 60 µM, and 80 µM have Km value of 0.59, 0.96, and 0.40 % casein and Vmax 0.65, 0.41, 0.64 U/min.
Figure 5. Graph of Lineweaver-Burk Plot for addition PMSF

In spite of the effect of PMSF on bromelain activity showed a decrease of activity along with increasing concentration of PMSF compound with variation 0.05 mM, 0.10 mM and 0.15 mM (figure 5). This decrease in bromelain activity may be due to a conformational change that causes the enzyme's center of activation to undergo a modification so that the enzyme interaction with the substrate is inhibited. The results of this test indicate that PMSF is a strong enough inhibitor against the enzyme bromelain, where at a concentration of 0.15 mM PMSF has been able to provide a relatively large inhibitory effect on proteolytic activity of enzymes. Based on the data, the Michaelis-Menten (Km) constant value for enzymes with the addition of 0.05, 0.10 and 0.15 mM PMSF compound were obtained by 0.42, 0.45 and 0.48 % casein and Vmax value of 0.25, 0.12 and 0.17 U/min, respectively.

4. Conclusions
The bromelain from pineapple cores has been successfully isolated and partially purified by ammonium sulfate precipitation, dialysis, and ion-exchange chromatography using CM-sephadex. The specific activities were sequentially increased by these purification stages and the highest purity level of the bromelain was obtained in AP3 fraction from ion-exchange chromatography. The optimum concentration of casein substrate for bromelain’s proteolytic activity determination is in 1% of casein. The proteolytic activity of bromelain from pineapple cores can be significantly increased by the addition of cysteine compounds and inhibited by the addition of PMSF.

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