Inhibitor effect of PCMB and PMSF towards partially purified product from pineapple core (*Ananas comosus* [L.] Merr) using gel filtration column chromatography method

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Abstract. The research aims to isolate, purify, and examine the inhibitors effect of a partially purified product from pineapple core with Palembang type pineapple (*Ananas comosus* [L.] Merr). The isolation process obtained crude enzymes with specific activity 42.60 Units/mg. Purification of crude enzymes using fractionation method with ammonium sulfate obtained bromelain fraction with the highest specific activity at 0–50 % saturation level of ammonium sulfate, which is 230.22 Units/mg with a purity level of 5.40 times from its crude extract. Further purification of 0–50 % ammonium sulfate fraction by gel filtration chromatography method using DEAE-Sephadex G-50 matrix obtained enzyme with specific activity 275.46 Units/mg with purity level 6.50 times from its crude extract. The optimum substrate concentration was at a concentration of 1.0 % (w/v). The purified bromelain has a Michaelis-Menten (Km) constant and the maximum speed of the enzymatic reaction (Vmax) i.e. 0.52 % (w/v) and 0.51 Units/min. The proteolytic activity of bromelain is strongly inhibited by PCMB compound and activated by PMSF at 0.1 mM concentration of 67.27 %.

Keywords: Pineapple core, bromelain, proteolytic activity, specific activity, inhibitors

1. Introduction

Pineapple is a tropical fruit from the *Bromeliaceae* family that widely cultivated in the tropics and subtropical regions. Based on the data of pineapple production development in Indonesia, in 1980 Indonesia has produced 180.64 thousand tons of pineapple and by 2015 pineapple production has reached 1.73 million tons or an increase of 13.46 % per year [1]. The increase in pineapple production is correlated with high pineapple demand for both direct and industrial consumption. The impact of the number of pineapple requests produces a by product or pineapple waste in the form of fruit peels, stems, and bulbs that are plentiful to reach 35–40 % of the pineapple mass. The pineapple waste can be utilized optimally as a source of bromelain which has a higher economical value.

Pineapple fruit contains water, carbohydrates, fats, fiber, sugars, vitamin A, vitamin C, β carotene, a number of proteins, ash, low fiber content, flavonoids, and protease enzyme complexes of bromelain [2]. The enzyme Bromelain has a sulfhydryl group (cysteine-histidine) on its active side [3]. Bromelain is an abundant cysteine of endopeptidase in the stems, leaves and pineapple skin. The main component of bromelain is the sulfhydryl proteolytic fraction [4]. Bromelain can be inhibited by Hg^{2+}, Ag^{2+}, Cu^{2+}, anti-trypsin, estatin A and B and iodoacetic acid [4].
This research used pineapple waste which is pineapple core as bromelain enzyme source. This research includes several steps, i.e. bromelain isolation from pineapple core, purification and characterization. The purification process was conducted using the method of fractionation with ammonium sulphate, subsequent with dialysis process and further purification by gel filtration column chromatography method. Bromelain characterization was observed on several parameters: proteolytic activity of enzyme, protein content, enzyme specific activity, purity level, % yield, determination of Km value, Vmax value, and inhibitor effect on the proteolytic activity of enzyme.

2. Materials and method

2.1. Materials

2.1.1. Pineapple. Pineapple Palembang (Ananas comosus [L] Merr) was purchased from East Jakarta central market. Pineapple core was separated from its skin and meat before used in this research.

2.1.2. Chemicals. The chemicals used were phosphate buffer pH 7.0, ammonium sulphate, Tris HCl buffer pH 8.0, casein, DEAE-Sephadex G-50, HCl, NaOH, TCA (trichloroacetic acid) solution, BSA standard solution (bovine serum albumin), Lowry reagent, Follin ciocalteu 2 N, PMSF (Phenylmethylsulfonyl fluoride), PCMB (4-Chloromeruribenzoic acid), ice cubes, aluminum foil, cellophane pouch, filter paper, and aquades. Other chemical reagents utilized in this research were obtained from Merck and are in analytical grade.

2.2. Extraction of crude enzyme bromelain from pineapple

To obtain crude enzymes, 500 g of pineapple core were cut into pieces, refined in a cold state and filtered with gauze. The filtrate was centrifuged at (5000 rpm, 45 min, 4 °C) to obtain the crude enzyme. The crude enzyme was stored in the refrigerator until used.

2.3. Enzyme fractionation by ammonium sulphate

The crude enzyme was fractionated gradually using ammonium sulfate (0–20 %, 20–50 % and 50–80 %). The protein fraction was then allowed to settle overnight at 4 °C and then centrifuged using a refrigerated centrifuge (Thermo Scientific HANIL COMBIL 514 R). The addition of ammonium sulfate with saturation level S1–S2 % can be calculated by using the formula:

\[ \text{ammonium sulphate (g)} = \frac{533 \times (S2 - S1)}{100 - 0.3 \times S2^2} \times \frac{V (mL)}{1000 \text{ mL}} \]

with S1 is the saturation level of the initial salt (%), S2 is the saturation level of the target salt (%) and 533 is the solubility of ammonium sulfate in 1 L of water at 30 °C.

2.4. Dialysis

The remaining ammonium sulfate was separated by dialysis. Replacement of the buffer was carried out periodically until the enzyme fraction is free of ammonium sulfate. Dialysis was stopped when there is no white sediment of BaSO4. Proteolytic activity and protein content of enzyme fraction were tested.

2.5. Gel filtration column chromatography

The bromelain enzyme fraction was further purified using the DEAE-Sephadex G-50 matrix in a 30 cm column system with diameter of 2.3 cm. Purification begins by introducing a sample of bromelain enzyme into chromatographic column which has been filled by DEAE-Sephadex G-50 matrix then eluted using Tris-HCl 0.05 M buffer pH 8.0 and carried out by 1 mL/min flow rate. The elute was accommodated every 5mL in a vial bottle, then its proteolytic activity was tested by Kunitz method.
2.6. Determination of proteolytic activity of bromelain enzyme
Proteolytic activity of bromelain enzyme was then measured using Kunitz method (1947).

2.7. Determination of protein levels
The protein levels of each enzyme fraction can be measured using the Lowry method (1951).

2.8. Determination of specific activity of bromelain enzyme
Determination of specific activity of enzyme was obtained by comparing the number of enzyme activity (U/mL) with protein levels (mg/mL).

\[
\text{Specific activity (U/mg)} = \frac{\text{Total of proteolytic activity (U)}}{\text{Total of protein levels (mg)}}
\]

2.9. Determination of Km and Vmax
The enzyme kinetic parameters, the Michaelis-Menten (Km) constant and the maximum velocity of the enzymatic reaction (Vmax) can be determined by transforming the Michaelis-Menten equation

\[
v = \frac{v_{\text{max}}[S]}{K_M + [S]}
\]

into Lineweaver-Burk

\[
\frac{1}{v} = \frac{1}{v_{\text{max}}} \frac{1}{[S]} + \frac{1}{v_{\text{max}}} \left(\frac{1}{K_M}ight)
\]

by mapping 1/V to 1/[S]. The enzyme reaction rate of bromelain enzyme was done with variation of casein substrate concentration 0.25; 0.50; 0.75; 1.0; 1.25 and 1.50 (w/v) [4].

2.10. Determination of inhibitor effect towards bromelain enzyme activity
Determination of inhibitor effect was done by addition of 0.1 mL inhibitor with concentration 0.1; 0.2; 0.3; 0.4 and 0.5 mM at optimum casein substrate concentration, then, the proteolitic activity of each fraction was determined and % inhibition and% activation were calculated.

\[
\% \text{Inhibition} = \frac{\text{Proteolitic activity of enzyme fraction without inhibitor} - \text{Proteolitic activity of enzyme fraction with inhibitor}}{\text{Proteolitic activity of enzyme fraction without inhibitor}} \times 100
\]

\[
\% \text{Activation} = \frac{\text{Proteolitic activity of enzyme fraction with inhibitor} - \text{Proteolitic activity of enzyme fraction without inhibitor}}{\text{Proteolitic activity of enzyme fraction without inhibitor}} \times 100
\]

3. Results and discussion
3.1. Extraction and purification of bromelain enzyme
The crude enzyme extract of bromelain from the pineapple core was fractionated with ammonium sulphate, dialyzed, and further purified using gel filtration column chromatography method with DEAE-Sephadex G-50 matrix. Proteolytic activity, protein levels, and specific activity of bromelain enzyme fractions can be seen in table 1. The bromelain crude enzyme extracts increased the specific activity after fractionation using ammonium sulfate salt from 42.60 U/mg to 230.22 U/mg with 5.40 times purity level from the crude enzyme extract and increased again in the process of dialysis to 238.21 U/mg with a purity level 5.60 times from the crude enzyme extract.

Purification by gel filtration column chromatography method was used to separate bromelain enzyme with other proteins based on its molecular size. Samples that have relatively small molecular sizes would be diffused from the solution into the pores of the gel, whereas the relatively large molecules could not penetrate in the pores of the gel so it will come out faster than small molecules as it passes through the gel matrix [5]. Bromelain has a molecular weight between 30,000–36,000 Da [6]. Therefore, the use of DEAE-Sephadex G-50 matrix could separate the protein with molecular weight 1,500–30,000 Da.
Table 1. Specific activity of bromelain enzyme from Palembang pineapple core.

| Sample                              | Protein (mg/ml) | Enzyme activity (U) | Specific activity (U/mg) | Purity levels |
|-------------------------------------|-----------------|---------------------|--------------------------|--------------|
| Crude enzyme                        | 0.28            | 2067.70             | 42.60                    | 1.00         |
| Ammonium sulphate fraction (20–80%) | 0.05            | 238.71              | 230.22                   | 5.40         |
| Dialysis fraction                   | 0.03            | 150.49              | 53.78                    | 5.60         |
| DEAE-Sephadex G-50 fraction         | 003             | 385.50              | 60.67                    | 6.50         |

The elution used in the gel filtration column chromatography was 0.05 M tris-HCl buffer pH 8.0. Elute produced 60 fractions that were accommodated in a vial bottle every 5 mL. Each fraction was measured for absorbance of protein absorption at 280 nm wavelength and tested for its proteolytic activity using Kunitz method. The results of the measurements were plotted in chromatograms as shown in figure 1. The highest specific activity of the DEAE-Sephadex G-50 fraction was found in KR1 which had 275.46 U/mg with a purity level 6.50 times of crude enzyme.

3.2. Determination of Km and Vmax
The determination of Km and Vmax of PA1 fraction. It determined its enzyme activity based on the variation of casein substrate concentration 0.25 %, 0.50 %, 0.75 %, 1.0 %, 1.25 % and 1.50 % (w/v) using the Kunitz method. The rate of casein hydrolysis increased to a substrate concentration of 1.0 % and did not increase significantly when added a substrate with higher concentration. This is because at high concentrations all of the enzyme's active site has been filled by the substrate (already saturated) and the catalytic activity of the enzyme has reached the maximum [5]. The graph of the relation of the enzymatic reaction rate to the substrate concentration is displayed in figure 2.

Michaelis-Menten Constants (Km) value of PA1 fraction is 0.52 % (w/v) and the value of the maximum speed of enzymatic reaction (Vmax) is 0.51 U/min.

3.3. Determination of inhibitor effect towards bromelain enzyme activity
This experiment was to determine the effect of addition of compound 4-(hydroxymercuri)benzoic acid (PCMB) and phenylmethylsulfonyl fluoride (PMSF) to the proteolytic activity of bromelain enzyme.

![Figure 1. Chromatogram of gel filtration column chromatography. (PA: Protein absorption, P: proteolytic activity).](image-url)
Figure 2. (a) The relationship of the enzymatic reaction rate to the substrate concentration and (b) Lineweaver-Burk plot of hydrolysis of bromelain enzyme casein.
(S: substrate concentration, v: enzyme reaction rate).

Table 2. Effect of PCMB dan PMSF towards bromelain enzyme.

| Concentration (mM) | % Inhibition of PCMB | % Activation of PMSF |
|-------------------|----------------------|----------------------|
| 0.0               | -                    | -                    |
| 0.1               | 76.45                | 67.27                |
| 0.2               | 81.74                | 63.42                |
| 0.3               | 91.35                | 35.06                |
| 0.4               | 93.27                | 32.90                |
| 0.5               | 96.40                | 25.45                |

Figure 3. Inhibitor effect of PCMB and PMSF toward bromelain enzyme activity (U/mL).

In table 2 and figure 3, the influence of PCMB on bromelain activity showed a significant decrease in the activity along with the increasing concentrations of PCMB compounds with variations: 0.1; 0.2; 0.3; 0.4 and 0.05 mM. It may decrease bromelain activity due to a conformational change that causes the enzyme's active side to undergo a modification so that the enzyme interaction with the substrate is inhibited. The results of this test indicated that PCMB is a strong inhibitor of bromelain enzyme, in
which the PCMB concentration of 0.1 mM has been shown to have a great inhibitory effect on enzyme
the proteolytic activity, which is 76.45 %.

Based on the literature, PCMB compounds are cysteine protease inhibitors [7], such as bromelain
from pineapple core. In this case, bromelain contains a sulfhydryl group which reacts with heavy metal
Hg²⁺ to the PCMB compound and forms a mercaptide salt which results in the inhibition of an enzyme.

Sulfonyl fluoride has been widely studied as a serine protease inhibitor compound since the early
discovery by Powers et al. [8]. The effect of PMSF on bromelain activity showed a relatively significant
increase of proteolytic enzyme activity. At the concentration of PMSF 0.1 mM, it increased the
proteolytic activity of bromelain up to 67.27 %. This is estimated because of the modification of
conformation that facilitates the interaction of enzymes with substrate. Thus, an increase in proteolytic
activity by PSNF compounds may indicate that bromelain congestive enzymes are not a serine protease
group.

4. Conclusion

The crude enzyme bromelain from Palembang pineapple core (Ananas comosus [L.] Merr) has been
effectively isolated. The gradually purification has a purity level of 6.50 than the crude enzyme.
The optimum condition of casein substrate is at 1.0 % (w/v). The determination of Michaelis-Menten
constants (Km) and maximum velocity of enzymatic reaction (Vmax) obtained values 0.52 % (w/v) and
0.51 U/min. Inhibitor effect of PCMB and PMSF compounds showed a change in proteolytic activity of
enzymes along with increasing high concentration. In addition to the PCMB compound, the proteolytic
activity was decreased along with the increase of concentration. It indicates that the proteolytic activity
was strongly inhibited by PCMB compounds. While on the addition of PMSF compound, the proteolytic
activity was activated at 0.1 mM concentration with 67.27 % activation.

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