In vitro antiplatelet activity tests conducted on bromelain extracted from the pineapple plant and purified by ion-exchange chromatography using a diethylaminoethyl cellulose column

V D Rahmawati, S Setiasih and S Hudiyono

Department 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. The present study is aimed at isolating and purifying the bromelain enzyme extracted from the cores of the fruit of the pineapple plant (Ananas comosus). The process of purification of the crude enzyme extract included, in sequence, fractionation through ammonium sulfate precipitation, dialysis, and ion-exchange chromatography. Fractionation of the crude enzyme extract with ammonium sulfate resulted in an increase of the specific activity. The fraction with the highest specific activity was that obtained by precipitation with 50–80 % ammonium sulfate. For this fraction, the specific activity was 8.683 U/mg and the degree of purity of the enzyme was 160 times greater than that of the crude enzyme extract. Further purification by column chromatography using diethylaminoethyl cellulose (DEAE-C) as ion-exchange medium produced six fractions (out of 92) characterized by peaks in proteolytic activity. In particular, the highest proteolytic activity (10.530 U/mg) was displayed by the fifth such fraction. The platelet aggregation inhibitory activity of the DEAE-C-purified bromelain extract was assayed through the Born’s method that uses platelet-rich plasma and asetosal as positive control. The aggregation percent of this bromelain extract was measured to be 49.70% and its inhibition percent 46.89 %.

Keywords: Ananas comosus, pineapple core, bromelain, specific activity, purification, chromatography, ion-exchange

1. Introduction
According to data from the World Health Organization, Indonesia ranks as the fourth country in the world in terms of the number of people living with diabetes, after India, China, and the United States. In 2008, as many as 17.3 million people died worldwide from cardiovascular disease, and this number is expected to rise to 23.3 million by 2030 [1]. Cardiovascular disease is also the leading cause of death in Indonesia, accounting for 37% of the total deaths caused by disease [2].

Diabetes is a condition characterized by an amount of glucose in blood that exceeds the normal limits (hyperglycemia). This condition can lead to complications such as disorders of the blood vessels (cardiovascular disorders) and changes in thrombocyte function; specifically, diabetes may increase the tendency of thrombocytes to aggregate [3]. Thrombocyte or platelet aggregation is part of the coagulation system in dealing with disrupted body system. For instance, when the blood vessel endothelium is damaged, platelet activation will occur as a form of body’s system in doing its homeostasis [4].

The bromelain enzyme is an endopeptidase comprising a sulphhydryl group in its active site. Basically this enzyme is found in the tissues of the pineapple plant (also known as ananas sativus), which is part of the Bromeliaceae family. Herdyastuti [5] found that the highest bromelain content is found in the shaft of the pineapple core.
2. Materials and methods

2.1. Materials

Cores of the fruit of the pineapple plant (Ananas comusus), phosphate buffer pH 7, ammonium sulfate, Tris HCl buffer (0.05 M, pH 8), casein 1%, HCl 0.1 M, NaOH 0.1 M, NaCl 2 M, trichloro acetic acid 10%, bovine serum albumin, Lowry protein assay kit, Folin–Ciocalteu reagent 1N, sodium citrate, distilled water, demineralized water, adenosine diphosphate (ADP), and platelet-rich plasma.

2.2. Methods

2.2.1. Extraction of pineapple cores. The core of a pineapple fruit was grinded using a domestic juicer in the absence of water or buffer. The resulting mixture was centrifuged at 6000 rpm at 4 °C for 15 min. The supernatant was cooled to 4 °C and the proteins present in it were precipitated using ammonium sulfate in the following ranges: 0%–20%, 20%–50%, and 50%–80%. Ammonium sulfate was added slowly to the extract under constant agitation and, after salt addition was completed, the mixture was kept under stirring at 4 °C for an additional 15 min. The resulting suspension was centrifuged at 6000 rpm at 4 °C for 15 min. The supernatant was discarded and the precipitate was dissolved in ammonium sulfate that can be dissolved with a potassium phosphate buffer at pH 7.0. This sample was then desalted by dialysis.

2.2.2. Purification of bromelain by ion-exchange chromatography. The chromatographic procedure was carried out using a low-pressure chromatographic system operating at room temperature and at a flow rate of 0.5 mL min⁻¹. The ion exchange medium, diethylaminoethyl cellulose (DEAE-C), was suspended in water, degassed, and packed without compression into columns (20.0 cm x 1.6 cm) to give a bed volume of 2.0 mL. The columns were equilibrated with buffer. To study the influence of the buffer on enzyme adsorption, a pre-purified bromelain solution was prepared for each buffer to be used to feed the column. After injection of the sample, the column was washed with the loading buffer until the absorbance value at 280 nm was close to zero. Elution was performed increasing the ionic strength of the loading buffer with NaCl at various (increasing) concentrations (0.25, 0.50, 0.75, and 1.0 M). After each measurement, in vitro test using Born’s method is performed.

2.2.3. Antiplatelet activity test. All of an enzyme with highest specific activity at each purification phase will determine antiplatelet activity. About 70 μL of an enzyme solution was diluted with 560 μL of platelet-rich plasma; the resulting mixture was incubated at 37 °C for 2 min while being vortexed at low speed. The absorption of this solution at a wavelength of 600 nm was measured before and after the addition of ~70 μL ADP solution which acts as platelet aggregator. Please note that the positive control in this test consisted of a mixture in which the 70-μL solution of enzyme was replaced by the same volume of asetosol solution (1 mg/mL). On the other hand, the negative control included a 70-μL solution of distilled water replacing the enzyme solution.

3. Results and discussion

3.1. Isolation and purification of the enzyme

Core solution and the crude extract have a proteolytic activity values from isolation phase to fractionation are shown in table 1. The proteolytic activity of the bromelain extract is defined as the difference in absorbance at 280 nm between a tube containing a control solution and one containing the enzyme solution. In fact, the mentioned wavelength of 280 nm corresponds to the maximum absorbance of the tyrosine amino acid, which derives from the hydrolysis of casein, the substrate of the proteolysis reaction [6,7]. The amount of protein from each fraction was determined using the approach introduced by Lowry et al. [8], based on the formation of colored complexes resulting from the reaction between the alkali copper with tryptophan and tyrosine residues of bromelain.

At the protein fractionation stage, four fractions were obtained based on the degree of saturation namely fraction 1 (0%–20%), fraction 2 (20%–50%), fraction 3 (50%–80%) and the residual fraction. All fractions displayed a degree of proteolytic activity. Fraction 2 had the lowest proteolytic activity, because the protein amount in it was higher than it is in any of the other fractions, so that value of the specific proteolytic activity was lower than that of any of the other three fractions.
### Table 1. Data of isolation and fractionation phase.

| Fraction    | Treatment          | Proteolytic Activity (U/mL) | Protein Amount (mg/mL) | Specific Activity (U/mg) |
|-------------|--------------------|------------------------------|------------------------|--------------------------|
| Crude       | Core Solution Filter| 9.028                        | 63.270                 | 0.142                    |
| Enzyme      |                    |                              |                        |                          |
| Extract     | Crude Extract      | 3.366                        | 62.100                 | 0.054                    |
| Protein     | FR1                | 0–20%                        | 2.600                  | 0.971                    |
|             | FR2                | 20–50%                       | 1.116                  | 3.625                    |
|             | FR3                | 50–80%                       | 3.100                  | 0.357                    |
|             | FR4                | Residual Fraction            | 2.483                  | 8.977                    |
| Dialysis    | D1                 | 1.633                        | 0.180                  | 9.028                    |

**Figure 1.** Ion-exchange chromatogram of the fraction obtained using 50–80% ammonium sulfate as protein precipitant. The matrix used was DEAE-C. (P: absorption peak, FE: peak of proteolytic activity)

At the stage of fractionation, fraction 3 (50%–80%) displayed a value for the specific proteolytic activity of 8.683 U/mg, thus an increase in purity of 160 times greater than the crude enzyme extract, which has specific proteolytic activity of 0.142 U/mg. This fraction 3 showed the highest increase in purification. Fraction 4 was the final remaining filtrate and has the highest amount of protein compared to other fractions, but shows the lowest specific activity.

Then the fraction with the highest specific proteolytic activity was dialyzed to remove ammonium sulfate from it. As a consequence of the dialysis process, the volume of the sample increased from 10 mL to 13 mL, causing a dilution of the enzyme in the buffer solution. In particular, the protein amount dropped from 0.357 mg/mL to 0.180 mg/mL. The specific activity of the enzyme fraction subjected to dialysis increased from 8.683 U/mg to 9.028 U/mg.

After undergoing dialysis, the fraction was purified by ion-exchange chromatography. Absorbance measurements at 280 nm were performed for each fraction. The proteolytic activity of each fraction was subsequently determined by the Kunitz method [9]. The values for the absorbance at 280 nm and for the specific proteolytic activity obtained are reported versus the fraction number in the chromatogram in figure 1.

As can be evinced from the data reported in figure 1, six peaks of proteolytic activity were identified, with the highest specific proteolytic activity measured for the fifth peak (FE5), corresponding to a value of 10.530 U/mg and a purity level 192 times higher than that of the crude enzyme.
3.2. Antiplatelet activity
In this study, the antiplatelet activity of a sample was quantified by way of a parameter called the platelet aggregation percent, which measures the ability of platelets to form aggregates, and of a parameter called the inhibition percent, which measures the ability of antiplatelet agents to inhibit the aggregation of these blood components. The in vitro test to determine the extent of platelet aggregation is based on the turbidimetry method proposed by Born [10]. In this method, the absorption of light by the plasma solution is measured before and after the addition of aggregator compounds. The results of these experiments on antiplatelet activity are summarized in table 2. Inhibition percent and aggregation percent have an inverse relation with each other: the more effective the antiplatelet agent, the smaller the aggregate percent and the greater the inhibition percent[11].

Based on the data obtained, the ability of the antiplatelet fraction of the DEAE-C fraction obtained the antiplatelet aggregate percentage of 49.70 %. This proves that the purer the enzyme fraction, the lower the platelet aggregation. The difference between the percentage of aggregation platelet from the fraction purified by DEAE-C and negative control is 43.88 %. Table 2 also shows the inhibition percent of every enzyme extract fraction.

Table 2. Results of antiplatelet test.

| Fraction                  | Treatment   | % Aggregation | % Inhibition |
|---------------------------|-------------|---------------|--------------|
| Distilled water           | Control (-) | 93.58         | -            |
| Crude Enzyme              | Extraction  | 89.6          | 4.25         |
| Fraction 3 (Dialysate)    | Dialysate Result | 65.95  | 29.53        |
| DEAE-Cellulose Fraction   | Ion-Exchange | 49.7           | 46.89        |
| Asetosal                  | Control (+) | 9.7           | 89.85        |

4. Conclusions
The crude bromelian extract isolated from the cores of pineapple (Ananas comusus) has a specific activity of 0.142 U/mg. After undergoing the purification process, fractions with saturation of 50–80% showed an increase in specific proteolytic activity to 8.683 U/mg. This means that the fraction has 160-fold increase in purity compared to crude enzyme extract. Subsequent purification using DEAE-C produced six fractions associated with peaks of proteolytic activity. The highest specific proteolytic activity was obtained at 10.530 U/mg, thus an increase in purity that occurred was 166 times that of crude enzyme extract. All bromelian fractions displayed antiplatelet behaviour, as they hampered platelet aggregation, but the extent to which they inhibit platelet aggregation is still lower compared to aspirin, which is used as a positive control.

Acknowledgements
The authors would like to thank Enago (www.enago.com) for the English language review.

References
[1] World Health Organization 2017 Cardiovascular Diseases (CVDs) Key Facts (Geneva: WHO)
[2] Hussain M A, Al Mamun A, Peters S A E, Woodward M and Huxley R R 2016 J. Epidemiol. 26 515–21
[3] Schneider D J 2009 Diabetes Care 32 525–7
[4] Periayah M H, Halim A S and Mat Saad A Z 2017 Int. J. Hematol. Oncol. Stem Cell Res. 11 319–27
[5] Herdyastuti N 2006 Berkala Penelitian Hayati 12 75–77
[6] Febriani K, Wahyuni I, Setiasih S and Hudiyono 2017 AIP Conf. Proc. 1862 030095
[7] Setiasih S, Wahyuni I and Hudiyono 2017 Proc. International Conference on Biosciences & Medical Engineering (ICBME) (Johor: UTM Johor Malaysia) pp 163–170
[8] Lowry O H, Rosenbrough N J, Farr A L and Randall R J J 1951 J. Biol. Chem. 193 265–75
[9] Kunitz M 1947 J. Gen. Physiol. 30 291–310
[10] Born G V R 1962 Nature 194 927
[11] Praharaningsih E 2006 Effect of Precipitant on The Isolation Process of Bromelain from Pineapple on the Proteolytic Activity of Enzymes in Casein Hydrolysis (Depok: Universitas Indonesia) Undergraduate Thesis