In Vivo antiplatelet activity aggregation assay of bromelain fractionate by ethanol from extract pineapple core (Ananas comosus [l.] merr.)

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Abstract. Processed fruit from pineapple is one of largest commodities tropical fruit production in Indonesia and will bring the waste from the skin and core. This study aims to isolate bromelain from the pineapple core (Ananas comosus) are purified by fractionation using ethanol and continued by activity test as an antiplatelets agent by in vivo method using white mice male ddY type with acetosal as positive control. Fractionation of crude enzyme bromelain with ethanol produces highest specific activity on ethanol 30-60% fraction (fraction 2) 3.107 Unit/mg and the protein content 61.25 mg with the degree of purity of 155 times compared to crude enzyme. Antiplatelet aggregation tests from in vivo method shows that faction of bromelain with doses 70 µg/KgBW, 140 µg/KgBW, and 210 µg/KgBW can increase a meaningful bleeding time. The highest percentage of increase shown by the isolate at a dose of 210 µg/KgBW in the amount of 515.10 ± 182.23%, when compared to aspirin (231.20 ± 140.66), the value is relatively higher.

Keywords: Ananas comosus, pineapple core, bromelain, purification, antiplatelet, in vivo

1. INTRODUCTION
Pineapple (Ananas comosus) is a commodity in the trade of tropical fruits which ranks second only to the bananas. Meanwhile Indonesia is the fifth-largest producer after Brazil, Thailand, the Philippines, and China [1]. According to Central Bureau of Statistics (2011), pineapple production in Indonesia increased from 2007 to 2009 is 1.395.566 tons, 1.433.133 tons and 1.558.196 tons.

Research enzyme bromelain in pineapple plants have been started since 1894 until today. Various ways of isolation has been conducted to get the enzyme bromelain with the best enzyme activity [2]. The protease enzymes useful in reducing the clumping of platelets, the formation of plaque in the arteries, and blood clots. All of these effects help in the treatment of cardiovascular disease [3]. Bromelain is widely used in therapeutic applications, among others as antitrombic, fibrinolytic, reversible inhibition of platelet aggregation [4]. Based on this background, research must be done test antiplatelet activity of extracts of pineapple fruit weevil.

The enzyme bromelain has been successfully demonstrated as an antiplatelet agent by in vitro method [5]. Hence, in this study wanted to know more comprehensive effects in the body through the test in vivo, using animal and clinical trials. This approach is employed to test the in vivo findings because it is more suitable to observe the overall effect on the living subject.
2. EXPERIMENTAL

2.1. Preparation of crude extract from pineapple core
Core was cut into pieces and weighed as much as 1000 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. Bromelain purification from crude extract by ethanol precipitation
Bromelain crude extract obtained proceed to the purification process by precipitation method using ethanol. The concentration level was divided into three fractions, fraction 1 (0-30)%, fraction 2 (30-60)% and fraction 3 (60-80)%. Ethanol was added to the crude extract at 4°C. The extracts was kept for ± 12 hours. 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.

2.3. Measurements of protein content and enzymatic activity
The protein content was measured by Lowry method. Standard was prepared using the protein BSA (Bovine Serum Albumine) with a concentration of 800 ppm. 0.1 mL sample was added 0.4 mL of 0.2 M phosphate buffer pH 7 and 5 mL reagent Lowry. Then incubated for 10 minutes at a temperature (4 ± 2)°C. Is then added and incubated 0.5 mL Folin Ciocalteu back for 30 minutes at a temperature (37 ± 2)°C. The solution was measured using a UV-Vis spectrophotometer at a wavelength of 595 nm [6,7,8].
Proteolytic activity was tested by the method Kunitz [9]. Made of control solution and sample. The sample solution is made by adding 0.9 mL 0.05 M tris buffer pH 8 at 0.1 mL sample. Then the substrate in the form of casein was added 1 mL 1% and incubated in a shaker for 30 minutes at a temperature (37 ± 2)°C. Further sampling was disabled with the addition of 3 mL of 10% TCA and incubated again in a shaker for 30 minutes at a temperature (4 ± 2)°C. The control solution was made equal to the sample, but added TCA 10% in advance so that the enzyme off, just after it added its substrate in the form of casein 1%.

2.4. Antiplatelet Aggregation Assays
Animal test that used in this study ddy strain male white mice were divided into five groups: positive control (acetosal), negative control (aquades), dose 1 (70 µg/Kgbw), dose 2 (140 µg/Kgbw), and dose 3 (210 µg/Kgbw).

2.5. Measurements of bleeding time
Bleeding time was measured by the tail of mice injured (cut 2mm), then the tail placed on filter paper to accommodate the blood coming out, note the time until the blood stopped flowing [10].

3. RESULTS AND DISCUSSION

3.1. Bromelain purification from crude extract by ethanol precipitation
The results of proteolytic activity from core solution and bromelain enzyme crude extract are shown in Table 1. The core solution and crude extract enzyme has proteolytic activity 0.967 Unit, and 1.222 Unit, and protein content of 84.11 mg and 61.15 mg of the total volume of solution. The value of the specific activity for both the solution amounted to 0.011 Units/mg and 0.020 Units/mg. From the data it showed an increase of proteolytic activity and specific activity of the enzyme after centrifugation of the solution hump despite the increase in value that occur less significant and purity level of enzyme was still very small. This is due to the enzyme crude extract still contains soluble proteins other than bromelain. Therefore, it is necessary to further purification stages to obtain a more pure enzyme fraction.
Table 1. Effect of ethanol concentration on bromelain precipitation

| Sample                  | Volume (mL) | Protein content (mg) | Proteolytic activity (U) | Specific activity (U/mg) |
|-------------------------|-------------|----------------------|--------------------------|--------------------------|
| Core solution           | 415         | 84.11                | 0.97                     | 0.011                    |
| Crude Enzyme            | 362         | 61.15                | 1.22                     | 0.020                    |
| Fraction 1 (0-30) %     | 15          | 2.33                 | 1.33                     | 0.571                    |
| Fraction 2 (30-60) %    | 46          | 3.30                 | 10.27                    | 3.107                    |
| Fraction 3 (60-80)%     | 24          | 3.45                 | 1.57                     | 0.454                    |
| Remaining filtrate      | 1440        | 7.44                 | 0.00                     | 0.000                    |

Enzyme solution of each fraction were then determined their proteolytic activity and protein levels. Table 1 shows the value of proteolytic activity of each fraction obtained in the fractionation stage. The highest proteolytic activity was obtained in fraction 2 with a value of 10.267 Units and protein levels of 3.30 mg. From the data proteolytic activity and protein levels, the highest specific activity obtained in fraction 2 with a value of 3.107 units/mg with a purity level reached 155 times.

3.2. Antiplatelet aggregation assays

Lengthening of bleeding time showed a decrease in the activity of platelet aggregation induced by inhibition of the cyclooxygenase enzyme, thereby decreasing the synthesis of thromboxane A2. Thromboxane A2 is one of the mediators that influence the process of platelet activation and vasoconstriction when the process is mediated by platelet hemostasis [11].

The results of antiplatelet aggregation assay with parameter bleeding time showed an increased bleeding time of mice were significantly since the 7th day in all groups of test extracts compared to day 0. However, the increase is likely to fluctuate at each measurement (Figure 1). In the calculation, the highest percentage of increase shown by the isolate at a dose of 210 µg/KgBW in the amount of 515.10 ± 182.23%, when compared to aspirin (231.20 ± 140.66), the value is relatively higher. Each isolate of the enzyme indicates increasing time increase bleeding time.

![Figure 1. Bleeding time curve from isolate and control](image-url)
Each fraction of enzyme indicates the increasing of bleeding time. Bleeding time of mice with various dose levels showed no significant difference from each fraction of enzyme.

4. CONCLUSIONS
Bromelain was successfully purified through a series of purification steps, starting from extraction of crude extract and precipitation of bromelain with ethanol. The result of bromelain after precipitation by ethanol gave higher specific activity than before precipitation. In vivo test of bromelain method by measuring bleeding time was divided into five groups of mice. All group of mice showed a positive response by increasing of the length time for each week.

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