Evaluation of The Stability and In Vitro Anti-inflammatory Activity of Partially Purified Bromelain Nanoemulsion

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Abstract. Bromelain is an enzyme belonging to cysteine protease. It is known for topical use as debridement for inflammation treatment. Nanoemulsion of bromelain (NEB) aims to solve this problem and also improve its stability. NEB was made by high emulsification method using homogenizer and sonicator. Nanoemulsion physicochemical stabilities were evaluated. The bromelain as an active compound was isolated from the core of pineapple (Ananas comosus [L.] Merr) which was purified by ammonium sulphate fractionation and then dialysis followed. Nanoemulsion formula 2 was found to be the most suitable formula for encapsulating bromelain with good physicochemical stability after 30 days of storage. This formula showed clear visual appearance with globule diameter of 37.42 nm with polydispersity index of 0.40. The result showed that the fraction of bromelain obtained from each purification step showed an increase in specific activity. The specific activities of fractionation using 20-50% ammonium sulphate and dialysis were 152.05 and 266.58 U/mg, respectively. The purest bromelain fraction was encapsulated into nanoemulsion formula 2. The bromelain fraction’s anti-inflammatory activity was tested based on HRBC membrane stabilization method. The result showed that bromelain dialysis fraction shows the ability as anti-inflammatory agent with stability percentage of 79.76%.

1. Introduction

Pineapple is one of Indonesia’s most leading fruit with a production of 1.73 tons in 2015 [1]. Indonesia exports canned pineapple to various countries and a lot of waste is made such as pineapple peel, cores, stem, and crown. The pineapple wastes were only use for cattle feed even though it contains bromelain. Bromelain is a proteolytic enzyme with sulphydryl group as an active site. Bromelain is used as analgesic, anti-tumors and anti-inflammatory agent [2]. Inflammation is a vascular tissue’s biologic response against the endanger impulse such as pathogen, irritant, and cell damage to initiate the healing process [3]. One of the methods of bromelain’s application as anti-inflammatory agent is via transdermal route. Transdermal is an administration route where active compounds are delivered across the skin to give systemic effect.

The main problem of transdermal route is its penetration rate through the outer skin layer [4]. Nowadays, nanoemulsion is one of the most promising way for transdermal drug delivery. Nanoemulsion can increase the bioavailability due to its big surface area and small free energy. This advantage can prevent the emulsion from creaming, flocculation, coalescence, and sedimentation process [5]. Transdermal drug delivery system can prevent first pass metabolism, estimate activity...
duration, minimize side effect, increase pharmacologic and physiologic response, and increase patient comfort.

Bromelain can be loaded in nanoemulsion by spontaneous emulsification [6]. The small droplet size of emulsion can increase bromelain penetration levels. The nanoemulsion particle size is determined by formula of substance mixture. Accordingly, this study focuses on the formulation, evaluation physicochemical properties, stability, and anti-inflammatory activity of bromelain-loaded nanoemulsions.

2. Methods

2.1. Materials
The bromelain used in this research was isolated from pineapple cores (Ananas comosus [L.] Merr), which purchased from Induk traditional market, Jakarta. All chemical substances and reagents were purchased from Merck.

2.2. Bromelain isolation, ammonium sulphate precipitation, and dialysis
The process of isolation of ammonium sulfate, and dialysis were conducted by the previous method with slight modification [7].

2.3. Determination of bromelain specific activity
The specific activity was determined by dividing the total proteolytic activity with total protein contents. The total protein contents were determined by Lowry method and the proteolytic activities were determined by Kunitz method with some modifications [8].

2.4. Preparation of bromelain-loaded nanoemulsion
The bromelain-loaded nanoemulsion was produced by using spontaneous emulsification method. Water phase such as tween 80, span 60, and aquadest were homogenized at 3000 rpm using homogenizer (Omni-Multimix Inc., Malaysia). Oil phase consist of oleic acid and vitamin E acetate were dissolved in crude palm oil (CPO) and virgin coconut oil (VCO). The oil phase was added into the water phase and then the mixtures were homogenized. Methylparaben and propylparaben were dissolved in propylene glycol and then added into the emulsions. The compositions of emulsions were using the formulation design in Table 1 and these were homogenized at 4000 rpm for 20 min. To make nanoemulsions, the emulsions were sonicated at 24kHz, 27 °C for 60 min by using ultrasound Hielscher UP400s. After physicochemical properties determination and stability test, the most stable nanoemulsion were used as a carrier of bromelain. The bromelain was added to the nanoemulsions and then stirred using magnetic stirrer. The bromelain-loaded nanoemulsions were sonicated for 30 min until the color is clear.

2.5. Evaluation of Nanoemulsion Physicochemical Properties and Stability
Organoleptic evaluation (consists of color, odor, clarity), pH, and cycling test of stability was carried out at 40 °C, 27 °C, 4 °C of nanoemulsion. The organoleptic evaluation was weekly observed for 4 weeks (Agoes, 2012). Density of nanoemulsions was determined by using pycnometer at room temperature. The viscosity of nanoemulsions was determined by using the Brookfield viscometer and spindle 1 with speed of 0.5, 1, 2, 2.5, 4, 10, 20 rpm to get rheology data [9]. The nanoemulsions type was conducted by dilution method, where the nanoemulsions were added into water (1:10 w/w) and oil (1:10 w/w). If the nanoemulsion soluble in water, it will become oil in water type (O/W). While, if the nanoemulsion soluble in oil, it will become water in oil type (W/O). In addition, the particle size of nanoemulsions was carried out by using Zetasizer Nano S (Malvern) at the second week after the nanoemulsion making.
2.6. *HRBC In Vitro Test of Anti-inflammatory Activity*

Human red blood cells (HRBC) were obtained from PMI Depok which already contains anticoagulants. Determination of anti-inflammatory activity was done by Oyedapo method [10]. Some substances were mixed by following the Table 2 and the absorbance was measured using spectrophotometer. The HRBC membrane stability was calculated by following equation,

\[
\% \text{ HRBC membrane stability} = \left( \frac{A_1 - A_2}{A_1} \right) \times 100\%,
\]

where, \(A_1\) = absorbance of aspirin (positive control) and \(A_2\) = absorbance of sample.

**Table 1** Formulation design of nanoemulsion.

| No. | Substance         | Nanoemulsion formula (%b/b) |
|-----|-------------------|-----------------------------|
|     |                   | 1  | 2          | 3          |
| 1.  | Tween 80          | 25 | 35         | 30         |
| 2.  | Span 60           | 25 | -          | 25         |
| 3.  | Oleic acid        | -  | 5          | -          |
| 4.  | *Crude Palm Oil*  | 5  | 5          | 5          |
| 5.  | VCO               | 5  | 5          | 5          |
| 6.  | Propylene glycol  | 5  | 5          | 5          |
| 7.  | Methylparaben     | 0.3| 0.3        | 0.3        |
| 8.  | Propylparaben     | 0.6| 0.6        | 0.6        |
| 9.  | Vitamin E acetate | 0.01| 0.01      | 0.01      |
| 10. | Aquadest          | 54.09| 44.09    | 49.09      |

**Table 2.** Composition of HRBC anti-inflammatory test mixture.

| Blank | Positive control | Sample |
|-------|------------------|--------|
| 2 mL of hyposaline | 2 mL of hyposaline | 2 mL of hyposaline |
| 1 mL of phosphate buffer 0.15 M pH 7.4 | 1 mL of phosphate buffer 0.15 M pH 7.4 | 1 mL of phosphate buffer 0.15 M pH 7.4 |
| 0.5 mL of HRBC suspense (10%v/v) | 0.5 mL of HRBC suspense (10%v/v) | 0.5 mL of HRBC suspense (10%v/v) |
| - | 1 mL of aspirin | 1 mL of bromelain |

incubated for 30 min at 37 °C
centrifuged at 3000 rpm for 30 min
absorbance measurement at λ560 nm

3. **Result and Discussion**

3.1. *Bromelain isolation, ammonium sulphate precipitation, and dialysis*

Bromelain specific activities are shown at Table 2. In line with purification steps, the specific bromelain activities were increased. The highest specific activity of ammonium sulfate salt fraction is in the second fraction which the purification level increases about 2.30 times compared to crude enzyme. The second fraction was dialyzed using membrane semipermeable to omit the salt content in bromelain. The principle of dialysis is diffusion, which the salt and small protein non-bromelain will penetrate throughout the membrane. The result has similar trend with previous study [6, 7], which the specific activity was increased from 63.65 to 266.58 U/mg with the purification levels 4.04 compared to crude enzyme. The dialyzed F2 is used as active compound in nanoemulsion preparation.

3.2. *Evaluation of nanoemulsions physicochemical and stability*

The result of organoleptic evaluation and density determination of nanoemulsions are reported at the Table 4. The density between the three nanoemulsions is found to be quite similar. These three
nanoemulsions are odorless and different in color appearance. The phase separation only occurs in the first formula at the third week. Therefore, the first formula is rejected.

**Table 3.** Bromelain specific activities from each purification step.

| Fraction          | Volume (mL) | Total Protein content (mg) | Total Proteolytic activity (U) | Specific activity (U/mg) | Purification levels (times) |
|-------------------|-------------|---------------------------|-------------------------------|--------------------------|-----------------------------|
| Crude enzyme      | 410         | 3355.84                   | 50.84                         | 66.01                    | 1                           |
| F1 (0-20%)        | 4           | 36.01                     | 0.25                          | 144.41                   | 2.19                        |
| F2 (20-50%)       | 6           | 120.72                    | 0.80                          | 152.05                   | 2.30                        |
| F3 (50-80%)       | 2           | 16.10                     | 0.25                          | 63.65                    | 0.96                        |
| Dialyzed F2       | 8           | 120.76                    | 0.45                          | 266.58                   | 4.04                        |

**Table 4.** Organoleptic and density observation of nanoemulsions.

| Formula | Density Week | Color       | Phase separation | Odor    |
|---------|--------------|-------------|------------------|---------|
| 1       | 1.04 g/mL    | 0           | White            | No      | Odorless     |
|         | 1            | 1           | White            | No      | Odorless     |
| 2       | 1.03 g/mL    | 2           | FG medium white  | No      | Odorless     |
|         | 1            | 1           | FG medium white  | No      | Odorless     |
| 3       | 1.05 g/mL    | 2           | FG NCP transparent white | No | Odorless |
|         | 1            | 1           | FG NCP transparent white | No | Odorless |

The determination of nanoemulsion type was conducted by dilution method in water phase and oil phase (Figure 1). The nanoemulsion is perfectly soluble in water as dispersion medium. This concluded that the nanoemulsion type is water in oil (W/O) and suitable with the purpose of the skin use.

**Figure 1.** Observation of nanoemulsion dilution in; a) water, b) oil.

Based on the data from Zetasizer Nano S, formula 2 has Z-average 37.42 nm with polydispersity index (Pdi) 0.40 (Figure 2). This result fits the previous research which the droplet size for nanoemulsion is about 10-200 nm [11]. The formula 1 has droplets size similar with formula 3 which has big pdi about 0.51 with Z-average 141.1 nm.
pH evaluation results can be seen in the Figure 3. The desired pH of nanoemulsions is about the pH value of skin, 4.5-7.0. The most probable fit for that requirement is the second formula, which pH value is in the middle of the range. The viscosity value of formula 1, 2, and 3 are 600, 200, 800 cps, respectively. It is caused by the different amount of surfactants that used. All of the nanoemulsions have similar flow behavior type that is plastic fluid (Figure 4). Based on the Figure 4, it can be seen that the rheology of nanoemulsion is plastic. In this flow property, the cream could not flow until it sheared stress over the certain yield value. The plastic flow type shows an increasing of shear rate which can cause rearrangement of tween 80 polymer chains into straight chain. This condition can lead the system resistance weaken and the viscosity decrease. Figure 5 shows that the nanoemulsions are stable in stored conditions at 40 °C, 27 °C, and 4 °C.

3.3. HRBC In Vitro Test of Anti-inflammatory Activity
The absorbance in the solution sample indicated a HRBC lysis process in which the inflammation was represented. The stabilization of HRBC is monitored by absorbance value of hemoglobin that produced from HRBC lysis process [10]. The lower absorbance value showed a more stable HRBC.
Based on the data result of anti-inflammatory activities at the Figure 6, bromelain-loaded nanoemulsion has the highest stability HRBC membrane (79.76%).

![Figure 6.](image)

**Figure 6.** In vitro anti-inflammatory test of bromelain nanoemulsion by using HRBC method

4. Conclusion
The specific activity of bromelain can be increased by using ammonium sulfate precipitation and dialysis. The dialyzed F2 bromelain has the highest specific activity. Based on nanoemulsions physicochemical properties and stability test, formulation 2 is the most suitable nanoemulsion to load dialyzed F2 bromelain. The bromelain nanoemulsion has high anti-inflammatory activity.

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