Optimization and evaluation of ionically cross-linked alginate-hpmc nanospheres for encapsulation of bromelain as antiplatelet

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Abstract. Thrombus is blood congealment process (platelet) occurred in area of vein and is useful for preventing of bleeding occurrence. The considerable amount of thrombus in blood leads to blocked arteries and angina pectoris. The partial purification of bromelain originated from pineapple core (*Ananas comosus* [L.] Merr) with the specific activity of 124.38 U/mg had inhibition activity to the platelet aggregation of 86.48%. On the other hand, the proteolytic activity of bromelain was relatively stable in the first 4 hours. However, the proteolytic activity significantly decreased in the next 4 hours due to the influence of gastric fluid (pH 1.2). To overcome the problem, bromelain must be encapsulated into alginate-hpmc nanospheres cross-linked by cation (CaCl$_2$). Based on the optimization result of the swelling index and the entrapment efficiency, the nanospheres with the composition of alginate-hpmc 1:1 and 1:2 were the optimal formula and selected to encapsulate bromelain and be characterized by PSA and SEM. Alginate-hpmc nanospheres (1:1) had a particle size of 543.7±4.2 nm. The morphology of nanospheres were almost spherical and had a smooth surface. Moreover, the particle size of alginate-hpmc nanospheres (1:2) was 515.3±26.7 nm and the SEM micrographs showed the spherical nanospheres with slightly rough surface. The swelling degree, entrapment efficiency, PSA, and SEM data will relate to suitability of the nanospheres formulation to orally deliver bromelain.

1. Introduction
Thrombus is blood aggregation formed in area of vein or heart chambers [1]. In the normal condition, thrombus can stop bleeding after injury but the large amount of thrombus causes the formation of platelet aggregation which can lead to blocked arteries [2]. The clogged arteries lead to reduced of blood flow to heart so that the amount of oxygen to heart muscles will decrease leading to heart attack [3].

The abnormal blood congealment process can be treated by bromelain from pineapple. Bromelain can prevent aggregation of platelets by regulating prostaglandin biosynthesis [4]. On the other hand, the proteolytic activity of bromelain is relatively stable in the first 4 hours but the 4 hours later, the proteolytic activity would significantly decreased due to the influence of gastric fluid (pH 1.2) [5]. Whereas, bromelain is well absorbed without lose its activity in intestinal fluid (pH 7.4). Due to the effect of degradation of the digestive system, bromelain must be encapsulated into a delivery system to provide a greater stability in the gastric fluid so that can maximize the therapeutic effect. The often used material of drug delivery system is polymer nanospheres [6]. The potential advantage of polymer
nanospheres is biocompatible, biodegradable, and easily administer using a syringe needle. In addition, the use of polymer has biological safety (non-toxic) for human cells [7].

In the previous studies of Setiasih et al. 2018 [5], the evaluation of bromelain loaded hydrogel chitosan-release profile had been done in the acidic condition. The result of release profile was relatively large in that condition hence to overcome the problem, in this research, the use of other polymers was evaluated. To date, there is no study that utilize alginate-hpmc as bromelain delivery system. It is necessary to conduct research on optimization and evaluation of alginate-hpmc composition as delivery system to determine the optimal formula in encapsulating the isolated bromelain from pineapple core.

Alginate was chosen as polymer matrix because it can form egg box like gel structure after cross-linking with multivalent ions [8]. In addition, alginate in the acidic environment will settle hence the solubility will decline. And, the use of hpmc (hydroxypropyl methylcellulose) is stable over pH range 3.0-11.0 and can reduce the rate of water penetration into matrix polymer [9]. In this study, the commercial bromelain from pineapple steam was used to encapsulate in to nanospheres to determine the optimal formula before the isolated bromelain from pineapple core was encapsulated into the selected nanospheres. The nanospheres was prepared by ionotropic gelation technique using calcium chloride (CaCl$_2$). Then the nanospheres were evaluated for the swelling degree, the entrapment efficiency, the particle size, and the morphology.

2. Materials and Methods

2.1. Materials

Bromelain was obtained from pineapple core, particularly Palembang pineapple purchased in fruit supplier market, West Jakarta (Indonesia). Sodium alginate was purchased from Organic Shop, Indonesia, HPMC (Hydroxypropyl Methylcellulose) K15M (99.5%) and calcium chloride (CaCl$_2$) anhydrous (99.0%) were purchased from Asian Chemical, Indonesia.

2.2. Methods

2.2.1. Extraction and ammonium sulfate fractionation of bromelain. Pineapple cores were cut into smaller size and were crushed by blender in cold condition. Then, the obtained pineapple porridges were filtered by gauze in order to separate between filtrates and residues. The filtrates were centrifuged at 6000 rpm at ±4°C and the resulted supernatants was called crude enzyme. Furthermore, the crude enzyme was fractionated by ammonium sulfate with the level of salt saturation at 50% of the crude enzyme volume. The fractions were stored overnight (±24 hours) at ±4°C and were centrifuged at 6000 rpm for 30 minutes at ±4°C. Precipitates were suspended in cold solution of 0.2 M phosphate buffer at pH 7.0.

2.2.2. Dialysis. Enzyme solutions were put into dialysis membrane and the dialysis membranes contained enzyme were soaked into 100 mL of 0.05 M phosphate buffer at pH 7.0 with stirring continues at ±4°C. Every 2 hours phosphate buffer was replaced with fresh phosphate buffer and precipitates were added 5% (w/v) BaCl$_2$ in acidic solution. The absence of BaSO$_4$ precipitates indicated that dialysis process has finished.

2.2.3. Determination of proteolytic activities and protein contents. The proteolytic activities of enzyme were determined according to the Kunitz method and were measured by UV-Vis spectrophotometer at 280 nm. The protein content of fractions was then determined using the Lowry method [10] with a wavelength of 775 nm.

2.2.4. Preparation of alginate-hpmc nanospheres. The preparation of the alginate-hpmc nanospheres formula containing the bromelain enzyme refers to Patel et al. 2016 [7] method used in situ loading method through an ionic gelation process using calcium chloride (CaCl$_2$) as a crosslinking agent with a
slight modification. Details of the alginate-hpmc formula can be seen in Table 1. In the initial stage, variations in the concentration of alginate 0.5-1.0% (w/v) were made and dissolved with deionized water at room temperature while stirring using a magnetic stirrer at a speed of 700 rpm for 30 minutes. Then, the bromelain enzyme at a concentration of 100 ppm was added to the sodium alginate polymer solution and stirred for 15 minutes using a magnetic stirrer at a speed of 700 rpm. The hpmc was then added with a concentration variation of 0.5-1.0% (w/v) by a direct dispersion into the sodium alginate-bromelain enzyme solution. If during the homogenisation process air bubbles arose then the solution was sonicated in an ultrasonic water bath for 20 minutes. Furthermore, an alginate-hpmc polymer solution containing enzymes was added to the crosslinker agent (CaCl\(_2\)) 3.75% w/v using a syringe. Nanospheres will be formed instantly when in contact with calcium chloride solution. Next, the nanospheres that have been formed were taken by filtering using Whatman No. paper. 42 and dried in the oven at ± 50 °C then the swelling ratio value was determined. Swelling ratio results from nanospheres with various alginate-hpmc compositions loaded with bromelain were then carried out dissolution testing.

| Composition (v/v) Alginate-hpmc | Concentration (% b/v) | Alginate | HPMC |
|-------------------------------|---------------------|----------|------|
| 1:1                           | 1.0                 | 1.0      |      |
| 2:1                           | 1.0                 | 0.5      |      |
| 1:2                           | 0.5                 | 1.0      |      |

2.2.5. Degree swelling study. As much as 20 mg nanospheres loaded bromelain were suspended in 10 ml of simulated gastric fluid (pH 1.2) allowed to swell 30 minutes and intestinal fluid (pH 7.4) allowed to swell for 10 minutes. After nanospheres were suspended carefully removed, blotted dry, and calculated using the following equation 1 [11].

\[
\text{Degree of swelling (%DS)} = \frac{(W_2 - W_1)}{W_1} \times 100\% \tag{1}
\]

Where \(W_2\) is the weight of the dry nanospheres after soaking in a buffer pH of 1.2 and \(W_1\) is the initial weight of the dry nanospheres.

2.2.6. Entrapment Efficiency. As much as 20 mg of nanospheres loaded bromelain was soaked into 10 mL phosphate buffer pH 7.4 for ±24 hours at room temperature then was sonicated at speed of 4000 rpm. The result of supernatant was added to Bradford reagents to determine the bromelain content in the nanospheres through the use of calibration curve (bovine serum albumin as a standard) that was measured at the wavelength of 595 nm using UV spectrometer (U-2900, Hitachi) and calculated using the following formula 2 with slight modification [12].

\[
\% \text{Entrapment efficiency} = \frac{\text{[Bromelain]} \times \frac{1 \text{ g}}{1000 \text{ mg}} \times \frac{1 \text{ L}}{1000 \text{ mL}} \times \text{Volume of Extract} \times (\frac{\text{Total Weight of Nanospheres}_{(g)}}{\text{Weight of Used Nanospheres}_{(g)}}) \times 100 \%}{\text{Weight of Bromelain} \times (\frac{\text{Total Weight of Nanospheres}_{(g)}}{\text{Weight of Used Nanospheres}_{(g)}})} \tag{2}
\]

2.2.7. Determination of in vitro antiplatelet activity. The antiplatelet activity was determined by Born method to the dialysate which had the highest specific activity. In addition, water was used negative
controls, and commercial bromelain was used as a comparison between purified and commercial bromelain. The % platelet aggregation and % inhibition can be calculated using the following equations 3 and 4 [13]:

\[ \text{% Aggregation} = 1 - \left( \frac{\text{Absorbance before addition of ADP}}{\text{Absorbance after addition of ADP}} \right) \times 100\% \] (3)

\[ \text{% Inhibition} = \left( \frac{\text{Platelet aggregation of control} - \text{Platelet aggregation of enzyme}}{\text{Platelet aggregation of control}} \right) \times 100\% \] (4)

2.2.8. Characterization of Nanospheres. The nanospheres were evaluated for particle size, and morphology. The particle size of nanospheres was measured using Horiba-SZ 100z particle size analyzer with (DLS) dynamic light scattering method. The shape and the surface morphology of the alginate-hpmc nanospheres was determined using scanning electron microscope SU3500.

3. Results and Discussion

3.1. Preparation of nanospheres
The used composition in creating nanospheres was 1:1, 2:1, and 1:2 against alginate-hpmc. The preparation of commercial bromelain-loaded alginate-hpmc nanospheres used in-situ loading method (the enzyme was concurrently mixed with the nanospheres-compiler monomer) with gelation technique (the active substance which has been dispersed in a solution containing polymer solution was dropped into solution containing a cross-linker agent). The cross-linker agent used in the gelation technique was calcium chloride (CaCl₂). The use of CaCl₂ as cross-linker agent because CaCl₂ was able to activate the bromelain by stabilizing the tertiary structure of enzyme hence it did not change the three-dimensional structure [14]. The process of the formation of alginate-hpmc nanospheres was that calcium ions would bind to the guluronates and manuronates in the alginate and would close the monomer side chains and then produce a three-dimensional structure known as an egg box [15]. Furthermore, commercial bromelain-loaded alginate-hpmc nanospheres was evaluated the rate of swelling, the percentage of entrapment, the particle size, and the morphology of nanospheres to determine the optimal formula before the isolated bromelain was encapsulated in to nanospheres.

| Ratio of alginate-hpmc | Degree of swelling ( % ± SD ) Acidid pH (1.2) Basic pH (7.4) |
|------------------------|-------------------------------------------------------------|
| 1:1                    | 128.27 ± 2.0 268.70 ± 2.9                                   |
| 2:1                    | 149.88 ± 0.2 362.62 ± 1.6                                   |
| 1:2                    | 134.08 ± 1.1 288.33 ± 7.4                                   |

3.2. Swelling Degree of nanospheres
The determination of swelling index was to evaluate the diffusion mechanisms of water into the polymer matrix and was also related to the process of drug release as it passed through the stomach and intestinal environment artificially. As seen in Table 2, the swelling degree in the acidic pH was lower than that in the basic pH. The carboxylic groups in alginate can’t be protonated hence they can maintain the form of their acidity (-COOH) under the acidic conditions. Whereas, in the basic condition, carboxylic groups are ionized into COO⁻ [16]. The highest swelling index both in acidic and basic environment was obtained in the nanospheres with ratio of alginate-hpmc of 2:1 and the result was 149.88 ± 0.2 % and 362.62 ± 1.6 %. Moreover, the lower of swelling degree was shown in nanospheres with the composition of 1:1 with the result of 128.27 ± 2.0 % in the acidic pH and 268.70 ± 2.9 % in the basic pH. The higher concentration or composition of hpmc caused the surface layer of nanospheres thicker and the formed
pores would be less and smaller. Hence, it could control water penetrated into the nanospheres. Thus it was expected to prevent the release of bromelain in large levels in the stomach and control its release in the intestine.

3.3. **Entrapment Efficiency**

Determination of the entrapment efficiency purposed to evaluate how much active substance (bromelain) was successfully absorbed into the nanospheres. Based on the result, the highest percentage of entrapment was obtained in the nanospheres with the ratio alginate-hpmc of (1:2), 20.81 ± 1.5 %, and the lowest value of entrapment efficiency was 16.07 ± 1.1 % in alginate-hpmc with composition of 1:1 (Table 3). Based on the yield of entrapment efficiency, it could be concluded that the higher alginate concentration resulted in the low percentage of entrapment or the decrease in the entrapment efficiency. It was due to the cross-linking process between calcium ions and alginates could occur immediately so that the incorporation of bromelain into the matrix could take place quickly. In addition, more enzyme was absorbed into the nanospheres and the nanospheres could be formed immediately. Thus, it could prevent the bromelain release from the nanospheres [17]. The decrease in alginate concentration did not inhibit penetration of calcium to the interior of the nanospheres.

| Ratio of alginate-hpmc | Entrapment efficiency (%) ± SD |
|------------------------|-------------------------------|
| 1:1                    | 16.07 ± 1.1                  |
| 2:1                    | 16.99 ± 0.2                  |
| 1:2                    | 20.81 ± 1.5                  |

Table 3. Effect of polymer composition on entrapment efficiency.

| Ratio of alginate-hpmc | Particle size (nm ± SD) |
|------------------------|-------------------------|
| 1:1                    | 543.7 ± 4.2             |
| 2:1                    | 534.0 ± 13.0            |
| 1:2                    | 515.3 ± 26.7            |

Table 4. Particle size of alginate-hpmc nanospheres

3.4. **Particle size analysis**

The size of a nanospheres can be determined through measurements using a particle size analyzer (PSA). The varied formulations would give different particle size value of nanospheres. The largest size of nanospheres was 543.7 ± 4.2 nm in nanospheres with the ratio of (alginate-hpmc) of 1:1. The (2:1) ratio of alginate-hpmc nanospheres had a larger particle size that was 534.0 ± 13.0 nm compared to the alginate-hpmc nanospheres with the composition of 1:2 that was 515.3 ± 26.7 nm. Based on the result (Table 4), the higher used of alginate concentration resulted in a larger particle size. Moreover, the use of compositions between alginate and hpmc which were equally high would obtain a much larger size. This could be concluded that the accumulation of the merging of two polymers with equal concentrations.

![Figure 1](image_url) Morphology of alginate-hpmc nanospheres. Ratio (a and b) 1:1 ; (c and d) 1:2

3.5. **Morphology of alginate-hpmc nanospheres**

The shape and surface morphology of alginate-hpmc nanospheres cross-linked by CaCl$_2$ were observed by a scanning electron microscope. The selected nanospheres to evaluate the morphology were nanospheres with the ratio of 1:1 and 1:2, based on the swelling degree and entrapment efficiency results. The nanospheres morphology of alginate-hpmc (1:1) demonstrated that the nanospheres was almost spherical and had a smooth surface which could be concluded that the 1% w/v concentration of alginate
and hpmc could reduce the hole size [18]. The smooth surface of nanospheres, possibly, was defined that be able to reduce a number of pores which lead to a decrease in the release rate of enzyme from nanospheres. On the other hand, the morphology of alginate-hpmc nanospheres of 1:2 showed proper spherical nanospheres but had a rough surface exhibited slight holes.

3.6. Partial Purification of core bromelain

The result presented in Table 5 showed that the purification of protein was able to increase either the bromelain activity or specific activity. The higher increasing of both the proteolytic activity and the specific activity of bromelain was due to separation of the dissolved solids in the liquid through a centrifugation process and an impurity separation through the process of fractionation by ammonium sulphate saturation of 50% and dialysis (to remove ammonium sulphate in order to prevent the denaturation of protein). The activity of pineapple juice, crude extract, fractionate, and dialysate were studied using casein as a substrate. The activity of pineapple juice was found to be 3.918 U/mL, the crude extract 4.427 U/mL, the fractionate 0.728 U/mL, and the dialysate 1.617 U/mL. The highest specific activity was found in dialysate 124.38 U/mg with 1.18 fold purification. Furthermore, the dialysate was encapsulated into the selected formula of alginate-hpmc nanospheres (1:1 and 1:2) based on the optimal swelling degree and the entrapment efficiency data.

| Table 5. Separation of bromelain from pineapple core |
|-----------------------------------------------------|
| **Sample** | **Protein content (mg/mL)** | **Bromelain activity (U/mL)** | **Specific activity (U/mg)** | **Yield (%)** | **Purification fold** |
| Pineapple juice | 0.057 | 3.918 | 68.74 | - | - |
| Crude extract | 0.042 | 4.427 | 105.4 | - | - |
| Fractionate of 50% (NH₄)₂SO₄ precipitation | 0.022 | 0.728 | 33.09 | 1.64 | 0.31 |
| Dialysate | 0.013 | 1.617 | 124.38 | 4.2 | 1.18 |

| Table 6. Percentage inhibition of platelet aggregation |
|-------------------------------------------------------|
| **Sample** | **Aggregation (%)** | **Inhibition (%)** |
| Negative control (aquades) | 6.73 | - |
| Commercial bromelain enzyme (EC 3.4.22.32) | 1.88 | 72.07 |
| Dialysate | 0.91 | 86.48 |

3.7. Antiplatelet Activity

The in vitro of antiplatelet activity was determined by platelet aggregation assay using a spectrophotometer to evaluate ADP-induced platelet aggregation. The highest percentage inhibition of platelet aggregation was found to be 86.48 % for dialysate (Table 6). The antiplatelet activity of dialysate was higher compared to commercial bromelain enzyme, probably, because the specific activity of dialysate (124.38 U/mg) was higher than commercial bromelain enzyme (≥ 3 U/mg). The difference of inhibition percentage was related to distinction of purification process thus would generate different amino acid composition, molecular size that affected to biology activity. Bromelain had potential as an antiplatelet, because bromelain could act as a plasma protein to degrade the ADP [19].

4. Conclusions

The purity level of bromelain will affect the biology activity. The dialysate showed a higher ability to inhibit platelet aggregation than commercial bromelain. The selected formula of alginate-hpmc nanospheres to encapsulate the isolated bromelain from pineapple core was 1:1 and 1:2 based on the
result of swelling degree in acidic and bases pH and entrapment efficiency. The increase in the concentration of hpmc can reduce fluid penetration into the nanospheres.

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