Dissolution Study of Purified Bromelain from Pineapple Cores (*Ananas comosus* [L.] Merr) Encapsulated in Alginate-Chitosan Microcapsule

Siswati Setiasih*, Asher Reyhan, Sumi Hudiyono, Endang Saepudin

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia

*E-mail: setiasih@ui.ac.id

Abstract. Along with the production of large number of pineapple products, the amount of pineapple waste produced is also higher, including pineapple cores with large amount of proteolytic enzyme called bromelain. Bromelain has many benefits, particularly because of its efficacy in various treatments of diseases, such as platelet anti-aggregation, reducing inflammation associated with infections, sinusitis, osteoarthritis and cancer. However, for oral use, bromelain will be degraded by the presence of proteases and the atmosphere of acidic pH in the stomach, so that bromelain will lose its activity. In this study, the isolated and purified bromelain from pineapple cores was subsequently encapsulated in alginate-chitosan microcapsules as drug delivery medium so that bromelain could reach the intestine without degradation in the stomach. The purification using 20%-50% ammonium sulfate obtained bromelain with specific activity of 5.44 U/mg and purity of 2.85 times. The purified enzyme was subsequently dialyzed and yielded of 8.27 U/mg with the purity level of 4.32 times. The dissolution test of bromelain encapsulated in alginate microcapsules resulted in efficiency of 76.99% which dissolute at pH 1.2 as much as 13.53% and at pH 7.4 of 80.09%, while in chitosan-coated alginate microcapsules, efficiency was obtained for 86.40% with dissolution result at pH 1.2 and 7.4 were 8.59% and 77.35% respectively.

1. Introduction

The pineapple was one of the main commodities in Indonesia which has a production volume amounted to 1,396,153 tons, the third largest in Southeast Asia after the Philippines and Thailand in 2016 [1]. Bromelain is one of proteolytic enzymes that can be isolated from of pineapple. Isolation, separation and purification of the enzyme can be done using methods of chromatography, electrophoresis, ultrafiltration, precipitation, and other methods.

Bromelain also contain peroxidase, acid phosphatase, protease inhibitors, and some organic calcium bonds. This enzyme is made of 212 amino acids with a molecular weight approximately 33 kDa [2]. Bromelain has many benefits including as an anti-inflammatory, anti-thrombotic, fibrinotic, anti-coagulant and anti-platelet aggregation [3]. On its application bromelain is widely used as oral drugs that will be interact with stomach acid before it reaches the digestive intestine.

Bromelain can denature and experience a decrease in activity caused by pepsin enzyme and acid environment in the stomach [4]. Denaturation on bromelain in the stomach can be avoided by means of encapsulation. Microcapsule is one form of encapsulation which has the gradually release profile in...
the gastrointestinal tract, so it can be used as a drug carrier with active substance such as protein [5]. Encapsulation will maintain stability and release of bromelain on gastric environment so that increases bioavailability and could reach intestine.

In this research, bromelain obtained from pineapple core isolation will be purified by ammonium sulfate salt. These salts have the advantage over the other electrolyte compounds, such as has a high solubility, do not denature enzymes, effective precipitation agent, can be used at different pH and the price is not expensive [6]. In addition, ammonium sulfate is easily removed from the enzyme solution through the process of dialysis that can be used as a method for purifying proteins. Purified bromelain then overlaid in alginate microcapsules. Alginate is selected because it is a widely used biomaterial in the biomedical sciences that is biocompatibility and easy to form a gel [7].

Alginate microcapsules coated back using Chitosan, which is interesting polymer compound because it is biodegradable, non-toxic, and eco-friendly [8]. Then the dissolution profile of microcapsules evaluated in gastric and intestinal fluids artificially.

2. Methods

2.1. Bromelain isolation and purification
The isolation and purification of bromelain were conducted by previous method with modification [9]. The modification is in centrifuge condition, which centrifuge condition at 8000 rpm for 15 minutes.

2.2. Microcapsules forming
Firstly, 2% sodium alginate was dissolved in distilled water with a magnetic stirrer until all the sodium alginate powder was dissolved, then added the active ingredient (bromelain enzyme) 1% (v/v) with continuous stirring for 2 hours then added CaCO₃ powder (5% w/v) and stirred until homogeneous. The mixture was then dispersed in paraffin oil containing 1% Tween 80 with a water/oil ratio (30/70, v/v) at a speed of 400 rpm for 15 minutes. After that, 20 mL of paraffin oil were added containing glacial acetic acid with a mole ratio of Ca:acetate acid (1:2) and stirring continued for 60 minutes to dissolve CaCO₃, then the alginate microcapsules gel was added 100 mL of acetate buffer pH 4.5 and stirred with speed 200 rpm for 10 minutes. The microcapsules were cooled for 12 hours at 4-8 °C then the top layer of oil was removed by aspiration. Microcapsules are washed continuously with acetate buffer pH 4.5 and tween 80 until there is no oil left. Microcapsule was taken and transferred into a solution of Chitosan 1% at pH 4 and stirred for 30 minutes, then left for 2 hours and washed using ethanol [10].

2.3. Determination of Bromelain Specific Activities
Referring to research that has been done [11], various enzyme solution fractions obtained from each stage of purification were determined by their proteolytic activity with the modified Kunitz method and the total protein using the Lowry method. Then enzyme specific activity can be calculated by the following formula:

\[ \text{Specific activity (U/mg)} = \frac{\text{total proteolytic activity (U)}}{\text{total protein content (mg)}} \]  

(1)

2.4. Microcapsules characterization
In this study alginate microcapsules containing bromelain and alginate-chitosan microcapsules containing bromelain were characterized using FT-IR instrumentation and SEM. Characterization using FT-IR is done to determine the functional group changes that occur. While the analysis using SEM was done to see the microcapsule morphology.

2.5. Encapsulation Efficiency determination and dissolution test of microcapsules
Before the dissolution test was carried out, the microcapsules obtained were calculated in the
encapsulation efficiency first by weighing 100 mg, and then adding pH 1.2 buffer solutions of 10 ml and stirring for 2 hours, the solution was centrifuged. Then the microcapsules were separated and transferred in a new container and stirred in a phosphate buffer pH 7.4, 10 ml for 2 hours. Stirred solution then centrifuged and the protein content was determined using Lowry method, the bromelain content in the microcapsules was the sum of protein content in 1.2 and 7.4 buffer. Based on previous results, microcapsule efficiency can be determined with equation as follows:

\[
\% \text{Efficiency} = \frac{\text{determined bromelain content (mg)}}{\text{theoretical bromelain loading (mg)}}
\] (2)

Then bromelain containing microcapsules dissolution test was carried out using basket type dissolution equipment. The dissolution test is carried out by filling microcapsules which has been weighed into the cage and dipped in a container containing 500 mL pH 1.2, with temperature at 37 ± 0.5 °C. A container that contains micro-capsules stirred in the dissolution apparatus with rotary speed 100 rpm at 37 °C.

Samples were taken at intervals of 30 minutes for 2 hours. Each sampling taken 10 ml sample solution, and then 10ml stock solution was added back into the dissolution container. After 2 hours, followed by immersion in aqueous phosphate buffer pH 7.4 with sampling time 1 hour up to a total time of 8 hours, then the sampling results at pH 7.4 and pH 1.2 tested to obtain its total protein and specific activities.

3. Result and Discussion

3.1. Bromelain isolation and purification

Data in table 1 shows that it is known that in pineapple core juice solution, specific activity data was obtained at 0.62 U/mg with a protein content of 746.66 mg and proteolytic activity of 467.50 U. After the solution was centrifuged, the crude enzyme obtained was measured for protein content and showed a decrease in protein content to 370.60 mg caused by proteins other than the bromelain which had precipitated. In contrast, proteolytic activity was observed that of the crude enzyme is larger compared to the proteolytic activity of the pineapple core juice to 709.50 U due to the active side of bromelain is not covered by other proteins [12]. So the specific activity also experienced an increase from 0.62 U/mg to 1.91 U/mg.

In the purification stage, the addition of ammonium sulfate salt to the solution causes salting out process which makes the surface tension in the water to rise causing a hydrophobic interaction between protein and water, protein will reduce its surface area to avoid contact with water so that interaction between proteins will cause protein molecule precipitation [13].

The test results shown in table 1 show that specific activity rise from the first fraction to fraction 2 and decreased in the third fraction with the largest specific activity found in fraction 2 of 5.44 U/mg. Based on the specific activity data, then the purity level of each fraction was compared with crude enzymes, the data obtained was that F1 was 1.30 times purer than crude enzymes, F2 with a specific activity of 2.80 times purer, and F3 had less purity than crude enzyme with a value of 0.90 times.

This result shows that the optimum deposition of bromelain enzyme on the addition of ammonium sulfate salts with a ratio of 20% -50%. The results of the fractionation have not been completely pure due to the presence of other proteins that have undergone precipitation by ammonium sulfate. To get a higher level of purity, further purification is done with dialysis.

In the dialysis process, F2 bromelain is added to a semipermeable membrane bag that has been soaked and preheated in a mixture of EDTA solution and sodium bicarbonate at 70 °C which aims to chelate the metal so as not to denature the protein to be purified. The semipermeable membrane used can separate molecules with masses below 14,000 Da, molecules with mass below that can sneak out of the membrane.
Table 1. Results of specific activity measurement of pineapple core juice (PJ) and Crude Enzyme (CE), ammonium sulphate precipitation, and dialyzed fractions.

| Sample          | Total Protein (mg) | Total Proteolytic activity (U) | Specific activity (U/mg) | Purity level (times) |
|-----------------|--------------------|--------------------------------|--------------------------|---------------------|
| PJ              | 746.66             | 467.50                         | 0.62                     | -                   |
| CE              | 370.60             | 709.50                         | 1.91                     | 1                   |
| Centrifuge 8000 rpm, 15 minutes at 4 °C |                    |                                |                          |                     |
| F1 (0%-20%)     | 0.47               | 1.17                           | 2.49                     | 1.30                |
| F2 (20%-50%)    | 1.13               | 6.17                           | 5.44                     | 2.85                |
| F3 (50%-80%)    | 0.33               | 0.48                           | 1.72                     | 0.90                |
| Dialysis        |                    |                                |                          |                     |
| F2 Dialysis     | 0.64               | 5.30                           | 8.27                     | 4.32                |

After dialysis, it can be seen from the data that the specific activity of enzyme F2 has increased to 8.27 U/mg with a purity level of 4.32 times greater than that of coarse enzymes and 1.52 times greater than F2 before dialysis, so that the F2 enzyme after dialysis is used to be encapsulated with alginate-chitosan microcapsules because it has the highest purity level compared to other purification fractions.

3.2. Microcapsules characterization

Scanning electron micrograph (SEM) results from alginate microcapsules and alginate-chitosan containing bromelain were seen in globular shape (Figure 1). Microcapsules size ranged from 135µm to 268µm. The surface of the microcapsules is quite uneven, this is similar to the results of the research conducted before by [14], which is caused by the fact that during the drying stage, the microcapsules lose water causing the microcapsule surface to wrinkle. Both microcapsules look quite dense and on the outside layer of alginate-chitosan microcapsule observed a thin layer coating.

![Figure 1. Micrograph of a) alginate microcapsules 250× magnification and b) alginate-chitosan containing bromelain 500× magnification](image)

Chitosan coated and non-coated chitosan microcapsules were measured using Fourier Transform Infra-Red (FTIR) (Figure 2). Chitosan non-coated alginate microcapsule shows a peak at 1589 cm⁻¹ indicating the C=O of carboxylate bonds, then curve in the range of 3200-3500 cm⁻¹ belongs to the hydroxyl group –OH, and also observed C-O group at a wavenumber of 1050 cm⁻¹.

The Polyelectrolyte complex formed by an electrostatic connection from the assembly of the alginate carboxylate with ammonium from chitosan which caused two polymers have a competent connections. The spectra of chitosan coated alginate microcapsule shows some shifts. The curve of –OH and –NH occurs at 3254 cm⁻¹. Furthermore, the carboxylate group (C=O) moved to 1490 cm⁻¹ as an indication of responses by -COO- of alginate to –NH₂ of chitosan [15].
3.3. Bromelain encapsulation

Table 2 shows the measurement results of bromelain content on alginate microcapsules and chitosan coated alginate microcapsules. These results are then compared with bromelain added to the microcapsule at the formation process resulting in percentage of encapsulation efficiency.

| Microcapsule         | Bromelain content (mg) | Encapsulation efficiency (%) |
|----------------------|-------------------------|-----------------------------|
| Algiante             | 0.33                    | 77                          |
| Alginate-chitosan     | 0.37                    | 86                          |

From the encapsulation data above it can be seen that the alginate microcapsules which are not coated with chitosan have bromelain content of 0.33 mg with an encapsulation efficiency of 77%, it is possible that the bromelain added at the time of making micorcapsules is released during the dispersion process due to high speed stirring or in the process rinsing, the enzyme bromelain which is on the surface is carried away by a rinsing solution.

While the alginate microcapsules coated with chitosan have greater efficiency than the efficiency of the non-coated microcapsules which is 86% with a core content contained of 0.37 mg, the difference in encapsulation efficiency is due to the presence of a protective layer that protects the bromelain enzyme attached to surface of the microcapsules so that they are not carried away during the rinsing stage.

3.4. Microcapsules dissolution profiles

The dissolution test was carried out to see the encapsulated bromelain enzyme release profile on alginate-chitosan microcapsules, carried out at artificial gastric pH and continued with artificial intestinal pH. From the dissolution test, the bromelain enzyme release data is shown in Figure 3.

The data shows that alginate microcapsules that had been coated with chitosan provided better protection against pH 1.2 compared to microcapsules which were not coated with chitosan with dissolution percent of 8.59%. Furthermore, the release of chitosan coated alginate microcapsules that occurred at pH 7.4 was only about 86% likely because the chitosan layer made it difficult for the bromelain enzyme to get out of the microcapsules. From the two microcapsules, the release at pH 7.4 was observed to have a sloping shape, indicating that the release of bromelain coated with alginate microcapsules and alginate-chitosan microcapsules had slow release properties.

Re-coating aim is to strengthen the mechanical and chemical properties of alginate microcapsules, so that it can delay the release of active substances from microcapsules. Chitosan layer is formed due to the electrostatic interaction between carboxyl groups of alginate and amine groups of chitosan which then form a layer of alginate-chitosan complex [16]. The resulting microcapsules are expected to protect bromelain from reaching the intestine without denaturing the stomach.
Figure 3. Microcapsule dissolution profile of alginate non-coated (A) and coated with chitosan (AC) microcapsules.

4. Conclusion

The bromelain enzymes produced from the isolation and purification of pineapple cobs were successfully encapsulated in both alginate microcapsules and chitosan coated alginate microcapsules resulting in higher encapsulation efficiency in alginate-chitosan coated microcapsules. Testing of the second dissolution of microcapsules containing bromelain purification results showed a gradual release rate and can maintain bromelain in acidity of gastric pH. Further studies are needed to obtain dissolution profiles of alginate-chitosan microcapsules with better slow release properties, and reactor design is needed in the process of dispersing sodium alginate solution to obtain smaller and homogeneous microcapsules.

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