Evaluation of dissolution of purified bromelain from pineapple core extract (Ananas comosus [L.] Merr) in the form of chitosan-coated microspheres

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Abstract. The aim of this study is to coat the bromelain from pineapple’s core into chitosan microspheres crosslinked by glutaraldehyde to maintain its activity until it reaches the intestines. The crude enzyme was first purified by ammonium sulfate precipitation method, followed by the dialysis. The bromelain fraction of the dialysis has a specific activity of 294.44 U/mg with a purity level of 15.68 fold compared to the crude enzyme. Coating bromelain using glutaraldehyde crosslinked microsphere showed an efficiency of 87.14 %. The dissolution test results showed the release rate of bromelain in artificial gastric fluid is 9.35 % and in artificial intestinal environment is 79.92 %. The result of this study indicates that chitosan-coated microspheres are good enough as bromelain coatings for slow-release matrices.

1. Introduction
Pineapple (Ananas comosus [L.] Merr) is the one of the tropical plant cultivated in Indonesia. In the food industry, usually pineapple core are not used and becomes waste. Bromelain is a proteolytic enzyme contained in almost all pineapple’s part such as skin, stems, and core [1]. Purified bromelain purified has a therapeutic effect such as fibrinolytic, anti-thrombotic, and anti-inflammatory properties and has been used in animal and human studies [2]. In the stomach fluid, bromelain will experience a decrease in activity after four first hours [3]. This is due to the deactivation and degradation of bromelain by gastric enzymes and acidic gastric acid pH [4]. In previous studies, the activity of bromelain experiences disruption in the stomach fluid and has activity in the intestinal environment [5]. Therefore, in application as an oral medication, bromelain needs to be coated in order to be released on target. In addition to hydrogel, microspheres could also be other ingredient of the drug encapsulation material [6].

2. Experimental

2.1. Materials
Tools needed in this research are beaker glass, measuring gourd, measuring glass, cooler box, refrigerated centrifuge, pH meter, blender, UV-Vis spectrophotometer, thermometer, oven, micropipette, tip, vial bottle, cuvette, FTIR spectrometer, analytical balance, SEM, refrigerator, magnetic stirrer, hot plate, centrifuge tube, and dissolution apparatus. While, for necessary materials for doing the research were pineapple core, buffer phosphate, ammonium sulfate, Folin-Ciocalteu reagent, bovine serum albumin, CuSO₄·5H₂O, K-Na tartrate, Na₂CO₃, NaOH, Aquadest, buffer tris-HCl, casein TCA, chitosan powder, magnesium silicate, Al powder, glutaraldehyde, EDTA, aquademin, glacial acetic acid, BaCl₂, and HCl.

2.2. Crude enzymes preparation
In this study, bromelain was isolated from the Palembang pineapple core obtained from the Pasar Induk Kramat Jati, East Jakarta. The core pineapple was cut into small dice-shape and destroyed with blender. Core enzyme solution was filtered and centrifuged at 6000 rpm, 4 °C for 20 minutes. This filtrate is called crude enzyme. Set aside some ml of core pineapple solution and crude enzyme. Protein content test was performed with Lowry method and proteolytic activity test with Kunitz method [7-9].

2.3. Purification of crude enzymes
The crude enzyme filtrate in a cold state was then purified by multilevel fractionation using ammonium sulfate with interval concentrations variation of 0–20 %; 20–50 %; and 50–80 % [9]. Furthermore, the highest specific activity fraction, followed by dialysis purification method was conducted to remove ammonium sulfate.

2.4. Synthesis of chitosan microspheres with crosslinker glutaraldehyde
Chitosan 4 % (w/v) in acetic acid solution was mixed with magnesium silicate and aluminum powder to synthesize the chitosan microspheres with simple coacervation method [10].

2.5. Determination of crosslinking degree and swelling ratio
The crosslinking degree of the microspheres can be determined [11]. Cross-linked swelling microsphere behavior was explored by placing at 5 mL reservoir containing gastric fluid artificial (pH 1.2) and intestinal environment artificial (pH 7.4) [10].

2.6. Encapsulation bromelain to chitosan microspheres with post loading method
Immersed the microspheres into a bromelain solution for 24 hours. Furthermore, the microspheres which has been soaked overnight was washed using a phosphate buffer pH 7. After that, it was allowed to dry and became encapsulated chitosan microspheres. To determine the % efficiency, the encapsulated chitosan microspheres was rinsed with phosphate buffer 0.2 M pH 7 to determine the untrapped bromelain.

2.7. Dissolution of bromelain test
The dissolution test was carried out by loading the encapsulated microsphere into a dissolution apparatus. Sample was taken with interval of 30 minutes for 2 hours in artificial gastric fluid. Meanwhile, the filtrate was taken 10 mL. After two hours, the microspheres were transferred into the artificial intestinal environment with an interval of 30 minutes in the first two hours and 60 minutes in the next hour until eight hours. Each sample was tested to Kunitz and Lowry methods.

2.8. Microspheres characterization
Chitosan, non-covalent chitosan microspheres, crosslinked glutaraldehyde chitosan microspheres and encapsulated chitosan microspheres were characterized using an instrument Fourier Transform Infrared (FTIR) to determine the functional group changes. Furthermore, an analysis to see the morphology was performed using Scanning Electron Microscopy (SEM) for crosslinked glutaraldehyde microspheres before encapsulation, after encapsulation, and dissolution.

3. Results and discussion
3.1. Synthesis of chitosan microspheres with crosslinker glutaraldehyde
Chitosan was dissolved in acid condition using 5 % acetic acid because it is an amide with pKa 6.5. It can be protonated at low pH and dissolve in acidic atmosphere. The addition of aluminum powder to form artificial pores supports the impregnation of crosslinked chitosan microspheres with an amine crosslinking group [10]. This process is a way of making microspheres using a simple coacervation method, which utilizes the physicochemical properties of insoluble chitosan in basic pH medium, but precipitates when has a contact with an alkaline solution.

Furthermore, the immersion water was removed and the microspheres were re-immersed using a 2.5 % glutaraldehyde crosslinking agent for 24 hours. Also, the synthesized chitosan microspheres without addition of aluminum powder and magnesium silicate (chitosan microspheres variation b) and non-covalent chitosan microspheres was used as comparison. The dried microspheres can be seen in figure 1.

Figure 2a shows that chitosan powder has an absorption-uptake characteristic of a particular wave number. The characteristic lies in the widespread uptake of the wave number 3550-3200 cm⁻¹ for the
Figure 1. Microspheres synthesized with (a) variation a (with addition of magnesium silicate and aluminum powder), (b) variation b (without addition of magnesium silicate and aluminum powder) and (c) noncovalent microspheres.

Figure 2. (a) FTIR spectra with (b) reaction scheme

vibration of the hydroxyl group (O-H) stretching and the vibration of the overlapping N-H amine group and the wave number 1570-1515 cm⁻¹ for the N-H bending vibration. In the wave number 1649 cm⁻¹ is the absorption resulting from the vibration of the remaining C=O acetyl group remaining on the chitin chain. Chitin has C=O chain and N-H chain. This shows that the chitosan used was not in 100% purity level. Then, the wavenumber 1154.22 cm⁻¹ shows the vibration of the bridge C-O-C, which is the typical absorption of the saccharide structure [12].

3.2. Reaction of crosslinking

Chitosan has several functional groups in the structure i.e. the primary amine group (-NH₂) and the hydroxyl group (-OH). In crosslinking reaction, chitosan with glutaraldehyde is reacted to an acidic atmosphere resulting in a nucleophilic addition reaction. It occurs when the free electron pair of the chitosan primary amine attacks the carbonyl group on glutaraldehyde, which has a positive partial. Then, there will be tetrahedral dipolar intermediates. The proton on N will be transferred to O and produce the neutral compound of carbinoamine. The crosslinking scheme can be seen in figure 2b. 5% acetic acid as solvent will make the tetrahedral carbinolamine to undergo protonation of the acid catalyst to the hydroxyl group. So, causing the electron pair on nitrogen to push the release of water to
produce iminium ion. Then, the H· ion will be released from nitrogen into a neutral imine product with a new double bond of C=N. A series of crosslinking processes between chitosan and glutaraldehyde is a formation reaction of schiff bases [13].

3.3. Crosslinking degree and swelling ratio
The degree of crosslinking and swelling ratio are the parameters to test the physical properties of the microspheres. Microspheres immersed in a crosslinking agent have more rigid physical properties than non-covalent microspheres. The process of determining the degree of crosslinking is by soaking the microspheres with 5 % acetic acid for 24 hours. The indicator of crosslinking is the ratio of the dry mass of the microsphere after being immersed by the solvent to the dry mass of the microsphere before being immersed by the solvent.

Based on the results of cross-linked determination, the microspheres chitosan variety a has a lower crosslinked degree than variety b due to the presence of magnesium silicate on the surface which blocks the chitosan part for cross-linking (table 1).

The ability of microspheres in absorbing liquid phase to coat can be known by conducting swelling ratio test. The microspheres immersion is related to the ability of amine groups present in protonated chitosan due to the presence of H· concentration in the solution. The amine chitosan group will form the -NH₂ group, causing repulsion in the polymer chain and there is stretching on the polymer chain. Microspheres that have a lower degree of crosslinking will have a higher swelling ratio and will be considered by the selected microspheres.

3.4. Isolation of crude enzyme
The temperature in this process was 4 °C to inactivate the activity of other protease enzymes that can damage bromelain. The pineapple core solution was then centrifuged using a refrigerated centrifuge at 6000 rpm for 20 minutes.

Determination of protein content was done by Lowry method. The principle of this method is to sharpen the color produced in the biuret method, where Cu (II) -protein will be formed and in the alkaline atmosphere Cu (II) will be reduced to Cu (I). Furthermore, the determination of proteolytic activity was performed using Kunitz method. Proteolytic activity is the ability of a bromelain to hydrolyze or break down casein the substrate under its optimum condition [14]. The obtained specific activity based on test results of protein enzyme levels and proteolytic activity test is shown in table 2.

**Table 1.** The degree of crosslinking and swelling ratio microspheres with glutaraldehyde variety a, b, and non covalent.

| Microspheres          | Average % crosslinking degree ± SD (n = 5) | Average % Swelling Ratio pH 1.2 ± SD (n = 5) | Average % Swelling Ratio pH 7.4 ± SD (n = 5) |
|-----------------------|---------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| variety a             | 94.53 ± 0.67                                | 116.45 ± 4.08                                 | 53.60 ± 2.18                                  |
| variety b             | 96.79 ± 0.36                                | 72.34 ± 3.80                                  | 10.43 ± 0.57                                  |
| non covalent          | -                                           | 122.71 ± 12.08                                | 141.13 ± 7.03                                 |

**Table 2.** Specific activity of bromelain each purification.

| Sample                  | Volume (mL) | Total of proteolytic activity (U) | Total of protein content (mg) | Specific activity (U/mg) | Level of purity (fold) |
|-------------------------|-------------|----------------------------------|------------------------------|--------------------------|------------------------|
| Core Solution           | 550         | 467.5                            | 169.4                        | 2.760                    | -                      |
| Crude Enzyme            | 100         | 326.7                            | 17.4                         | 18.776                   | 1                      |
| Ammonium sulfate fractionation method |           |                                   |                              |                          |                        |
| F1 (0-20)%              | 4           | 25.532                           | 0.552                        | 46.2536                  | 2.46                   |
| F2(20-50)%              | 6           | 99.798                           | 0.594                        | 168.01                   | 8.95                   |
| F3 (50-80)%             | 1.8         | 3.089                            | 0.0648                       | 47.669                   | 2.53                   |
| F4                      | 100         | 25                               | 2.1                          | 11.905                   | 0.63                   |
| Dialysis method         |             |                                  |                              |                          |                        |
| F5 Dialysis             | 5.1         | 94.605                           | 0.3213                       | 294.44                   | 15.68                  |
Figure 3. The microsphere characterization 100x magnification with SEM instrument (a) before and (b) after encapsulation

Pineapple core and crude enzyme solutions have a specific activity of 2.76 U/mg and 18.78 U/mg. Table 2 shows an increase of enzyme activity after centrifugation.

3.5. Fractionation enzyme with ammonium sulfate
The principle of fractionation ammonium sulfate is salting out. Interactions between proteins are stronger than protein interactions with the solvent and makes the proteins can be separated from high salt concentrations [15]. The protein molecules are triggered to interact and form an aggregate in lower solubility. The variation in saturation level aims to determine the appropriate ammonium sulfate concentration to precipitate bromelain.

The solution, which has been reserved for ± 18 hours was centrifuged (6000 rpm, 20 min, ± 3 °C). The obtained pellets were then reconstituted at 0.2M phosphate buffer pH 7.0. The second fraction level of ammonium sulfate (20–50 %) showed the highest fraction with specific activity of 168.01 U/mg and the purity level 8.95 times compared with the crude enzyme. Then, carried out further purification step that is dialysis method.

Dialysis causes a decrease in bromelain protein content from 0.396 mg to 0.321 mg as a result of the release of a small non-bromelain protein from the cellophane membrane bag. This leads to an increase in the specific activity of bromelain to 294.44 with a purity rate of 15.68 times compared to crude enzymes. Furthermore, the results of this dialysis will be coated using chitosan microspheres.

3.6. Encapsulation of purified bromelain into chitosan microspheres
The chitosan microspheres variety a was used to coat the purified bromelain. The selection was based on swelling test results at pH 7.4 for 30 minutes. Variety a gave a larger swelling ratio i.e. 53.60 % compared to variation b i.e. 10.43 % (see table 1). A larger swelling ratio will give the effectiveness of coating bromelain into a microsphere. Meanwhile, although the noncovalent chitosan swelling was the largest among the variation i.e. 141.13 %, the microspheres cannot be used. This was decided after the assay was applied into artificial gastric orientation (pH 1.2) for two hours, which all microspheres were completely destroyed.

Bromelain was coated for 24 hours then washed with 2 ml of 0.2 M phosphate buffer pH 7.0 for measuring the amount of enzymes un-entangled in the chitosan microspheres. Thus, the percentage efficiency of bromelain coating of post loading method i.e. 87.14 ± 0.59 %. According to figure 3, a fairly obvious difference is observed between the microspheres before and after encapsulation. Microsphere that have not yet been incorporated in bromelain have a more subtle morphology than microspheres that have been incorporated in bromelain. In addition, on the microsphere surface after insertion of bromelain there are many streaks are arranged uniformly. It indicates the presence of bromelain entering the microsphere with the occurrence of swelling on the microsphere.

Furthermore, characterization was done using FTIR. Spectrum is presented in figure 4. Meanwhile, the spectra between microspheres of chitosan cross-linked glutaraldehyde before and after insertion of bromelain showed the same absorption peaks. This information indicated that there is no chemical interaction between microspheres and purified bromelain. However, there is a physical interaction in the form of hydrogen bonds shown in sharper intensity changes at about 3500-3080 cm⁻¹.
Figure 4. Spektra IR microspheres before and after encapsulation.

Figure 5. Proposed interactions chitosan-coated microspheres application with amino acid residues from bromelain following proposed interactions between chitosan and amino acid residue fragments from bromelain by previous studies in figure 5.

3.7. In vitro dissolution test of bromelain

Dissolution is a process of the release of a drug, which aims to determine protein levels and proteolytic activity of bromelain escapes from chitosan microspheres. The profile can be seen from taking sample every 30 minutes for two hours in gastric fluid artificial and every 30 minutes then continue every 60 minutes up to 480 minutes in an intestinal environment artificial. This study used the basket dissolution method. The release rate of bromelain from the cross-linked glutaraldehyde chitosan microsphere is presented in figure 6. The figure shows that bromelain successfully escaped from the microspheres with the release rate i.e. 9.53 % in artificial gastric fluid and the release rate in the artificial intestinal environment i.e. 79.92 % made the total release of bromelain after ten hours was 89.45 %.

The proteolytic activity of each pH based on the sampling time interval was also measured and presented in figure 7 for artificial gastric (pH 1.2) and transferred to the artificial intestinal environment (pH 7.4). It can be concluded that bromelain has the highest proteolytic activity when it was in the intestinal environment after eight hours of in-vitro test process of 1.083 U/mL. The
fluctuative of proteolytic activity of enzyme is caused by the general properties of the enzyme, which works at the optimum pH and optimum temperature. The concentration of the enzyme also causes proteolytic activity. The destruction of the microspheres is marked by the large number of holes in which it causes enzymes to escape from the microspheres. The longer the microspheres are immersed in the artificial intestinal environment (pH 7.4) with the aid of a speed of 100 rpm, will cause the microspheres to be further damaged (figure 8). Accordingly, the more bromelain would be released in the intestines.

4. Conclusions

The crude bromelain enzyme from the pineapple core was successfully isolated with specific activity of 18.78 U/mg. Purification by fractionation method yielded the bromelain with purity level 8.95 fold i.e. 168.01 U/mg and dialysis method yielded bromelain with purity 15.68 fold i.e. 294.44 U/mg (ammonium sulfate concentration 20–50 %) compared with crude enzyme. Bromelain coated chitosan microsphere with an efficiency of 87.14 ± 0.59 %. Chitosan microspheres used as coatings have a crosslinking degree of 94.53 % and the swelling ratio at pH 7.4 of 53.60 %. A dissolution profile in artificial gastric fluid is 9.35 % for two hours and in artificial intestinal environment is 79.92 %. The highest proteolytic activity is performed in 600 minutes in an artificial intestinal environment (pH 7.4). The results of this study indicate that chitosan-coated microspheres are good enough as bromelain coatings for slow-release matrices.

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