Encapsulation of bromelain in alginate-carboxymethyl cellulose microspheres as an antiplatelet agent

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Abstract. Bromelain isolated from pineapple (Ananas comosus) can be an excellent phytotherapeutic agent for cardiovascular treatment as it can inhibit platelet aggregation. However, if it is used orally, it can be easily degraded in an acidic pH environment due to enzymes secreted during the digestion process. Its instability under a certain condition will reduce its pharmacological activity and, as a result, will reduce its health benefit. Therefore bromelain needs to be encapsulated in a matrix such as alginate-carboxymethyl cellulose (CMC) microsphere cross-linked to Ca$^{2+}$ ion, which will act as a carrier agent. In this research, bromelain encapsulation is done by in situ encapsulation. Particle size analyzer (PSA), Fourier transform infrared spectrophotometer, and scanning electron microscope are used as characterization instruments to investigate the encapsulation of bromelain into the alginate-CMC microsphere. PSA analysis showed that the molecular size of the alginate-CMC microspheres was in the 496.05±2.72 and 629.65±8.70 nm. Encapsulation study using the Bradford method showed that the highest encapsulation ratio was achieved at alginate-CMC ratio of 1.5:0.5 (% w/v:% w/v). These results demonstrated that the alginate-CMC microsphere had potential to be an effective matrix for bromelain encapsulation.

1. Introduction
Bromelain (EC 3.4.22.4) is a proteolytic enzyme found in pineapple plant family Bromeliaceae tissues, including stem, fruit, and leaves (Doko, Bassani, & Jacob, 2018). Pineapple stem has the highest bromelain concentration (Pavan, Jain, Shraddha, & Kumar, 2012) (Putranto, Budianto, & Hudoyon, 2018). Bromelain is effective in treating cardiovascular disease because it can inhibit blood platelet aggregation (Musfiroh, Setiasih, Handayani, Hudiyono, & Ilyas, 2018) (Series & Science, 2020). Bromelain is a safer antiplatelet alternative medicine with lesser side effects compared to synthetic antiplatelet drugs such as aspilet and clopidogrel as it is natural and also non-toxic (Sandhy, Budianto, & Hudiyono, 2018). However, bromelain is sensitive to certain conditions such as high temperature, high acidity, protease enzyme, organic solvents, and some chemicals, which will affect its health benefits and hence its pharmacological applications (Bernela, Ahuja, & Thakur, 2016).

Microencapsulation is a technique used to sustain drug release and reduce or eliminate gastrointestinal irritation (Manjanna, Shivakumar, & Pramod Kumar, 2009). The most common encapsulation material is sodium alginate because it is simple, non-toxic, biocompatible, and low cost (Solanki & Shah, 2016). Its unique property to form a stable gel in aqueous media by the addition of multivalent cations makes this biopolymer very useful for drug delivery and cell immobilization (Agüero, Zaldivar-Silva, Peña, & Dias, 2017). However, the cross-linked alginates are usually fragile.
Therefore, blending with mucoadhesive polymers is one of the most popular approaches because it can improve drug encapsulation and stability (Solanki & Shah, 2016).

Carboxymethyl cellulose (CMC), a water soluble polysaccharide is a potential agent to control drug release and delivery system due to its high biocompatibility and biodegradability (Kim, Park, Gu, & Kim, 2012). Both the alginate and CMC are anionic in nature due to the presence of negatively charged carboxyl groups at pH > 5. These negative charges allow the polymer to shrink in the acidic pH and swell when they are exposed to neutral or basic pH. These properties made these polymers suitable to be applied in oral drug delivery systems (Agarwal et al., 2015).

2. Methods

2.1. Materials and tools
Laboratory equipment used are glass tools, hot plate, and magnetic stirrers, oven, analytical balance, shaker water-bath, analytical balance, pH meter, petri dishes, refrigerator, centrifuge, refrigerated centrifuge, thermometer, freeze dryer, UV-VIS spectrophotometer, Particle Size Analyzer, and IR spectrophotometer, SEM.

Materials used are alginate powder (food grade), CMC powder (food grade), Calcium chloride (CaCl₂), Sodium dihydrogen phosphate (NaH₂PO₄), disodium phosphate (Na₂HPO₄), concentrated HCl, demineralized water, Coomassie Brilliant Blue G-250 (CBB G-250), Phosphoric acid 85%, ethanol p.a, Bovin Serum Albumin (BSA), bromelain (Sigma Aldrich).

2.2. Synthesis of alginate-CMC microspheres
Preparation of the alginate-CMC microspheres were done by ionic gelation method using calcium chloride as crosslinkers. Both alginate and CMC were dissolved in demineralized water at a specific concentration (Table 1). The polymeric solution was extruded as droplets using a syringe (23 G) and poured into 3.75 % calcium chloride solution. The stirrer speed was kept constant at 100 rpm. The formed microspheres were allowed to stand in solution for 15 minutes to be cured then collected by filtration. The microspheres are washed with demineralized water three times and then dried at room temperature for two days. Microspheres formulation are summarized in Tables 1.

| Table 1. Microspheres formulation |
|----------------------------------|
| Formula             | (A) | (B) | (C) | (D) |
| Alginate (g/100 ml) | 1   | 1   | 1.5 | 1.5 |
| CMC (g/100 ml)       | 0.5 | 1   | 0.5 | 1   |
| CaCl₂ (g/100 ml)     | 3.75| 3.75| 3.75| 3.75|

2.3. Synthesis of bromelain loaded alginate-carboxymethyl cellulose microspheres
Bromelain loaded alginate-CMC microspheres were synthesized in the same variation as the empty microspheres. In 50 ml of the alginate solution, CMC powder was dispersed in a certain amount. After the polymer solution is homogeneous, 5 mg of bromelain is added then stirred using a magnetic stirrer for 30 minutes at 750 rpm speed. If air bubbles arise, the solution is sonicated in an ultrasonic water bath for 15 minutes or left at room temperature for 30-60 minutes. The polymer solution was then dropped into 3.75% CaCl₂ solution using a syringe and left for 15 minutes on a stirrer with 100 rpm speed. The microspheres formed were filtered then washed with distilled water 3 times to remove remaining CaCl₂, then filtered using filter paper, spread in a petri dish, dried for 2 days at 40 °C and stored in for further use.
2.4. Characterization of microspheres

2.4.1. Swelling analysis. Dried alginate-CMC microspheres were weighed accurately then immersed in 5 mL of artificial gastric juice (pH 1.2) and artificial intestinal fluid (pH 7.4) for 30 minutes at room temperature. After 30 minutes, microspheres were carefully removed, dried with tissue paper, and then weighed.

\[
\text{Swelling ratio (\%)} = \left(\frac{W_2 - W_1}{W_1}\right) \times 100\%
\]

W1 and W2 represent the dry and wet weight of the beads, respectively.

2.4.2. Encapsulation efficiency. The encapsulation efficiency of each microsphere formula were determined by immersing the weighed microspheres in 10 mL of 0.2 M phosphate buffer at pH 7.4 for 1 day, followed by a sonication process which carried out for 1 hour then centrifuged for 15 minutes at 400 rpm speed. Supernatant were collected then tested using the Bradford method to measure protein content.

2.4.3. Particle size analysis and morphological studies. Particle Size Analyzer (PSA) was used to determine microspheres particle size. Dried microspheres Morphology surface analysis is done by Scanning Electron Microscope (SEM).

2.5. FTIR spectroscopic analysis
Chemical structure and specific chemical groups presence were characterized by ATR-FTIR using Transmittance Mode.

3. Results and discussion
Alginate-CMC microspheres were prepared using ionic gelation method where anionic carboxylic groups in alginate and carboxymethyl cellulose interacted with bivalent calcium ion to form the gel.

3.1. Microspheres characterization

3.1.1. Swelling analysis. Alginate and carboxymethyl cellulose are polyelectrolytes and its swelling is pH dependent. In certain pH its swelling property will be responsive due to the presence of negatively charged carboxyl groups in the polymer backbone. In acidic pH, carboxylic acid groups remain undissociated therefore no net charge is developed in the polymeric network. Once it is exposed to an alkaline medium, the carboxylic acid group converts to negatively charged carboxylate ions caused electrostatic repulsion among different polymer chains, which compel the polymer network to swell.

Microspheres swelling study was carried out in two different pH conditions: 1.2 and 7.4 based on stomach and intestine pH, respectively. The highest swelling for all formulations took place at pH 7.4, while lowest swelling happened at pH 1.2. Microspheres had shown marginal swelling in the acidic environment due to water penetration through microspheres surface pores which caused by the hydrophilic groups. Swelling margin increased along with CMC concentration increase. It is known that increasing CMC concentration will increase solution viscosity as a result, microsphere beads size will get bigger which will allow more water penetration inside.

Interestingly, at higher pH (7.4), the swelling was reduced even if CMC concentration increased while a reverse trend was observed at pH 1.2. CMC concentration increase will increase bead density. The more cross-links are formed, the tighter the microsphere structure will be, which caused water diffusion ability to decrease and affected microsphere's swelling ratio. This phenomenon can be seen from the swelling ratio in formulas B and D.
3.1.2. Encapsulation efficiency. Entrapment efficiency was found to be in the range of 15.15 to 32.22% as shown in (Table 2). Polymers concentration had a profound effect on the loading of bromelain in alginate-CMC microspheres. The drug loading increases along with sodium alginate and sodium CMC concentration increase. However, the drug-loading capacity decreased in the case of D formula and this may be due to less gelation time given after the microsphere formation.

3.1.3. Particle size analysis and morphological studies. Bromelain-loaded microspheres particle sizes were in the range of 496.05 ± 2.72 nm to 629.65±8.70 shown in (Table 2). It was observed that increasing polymers, alginate, and CMC concentration will increase drug particle size. This could be due to viscosity increase at high concentrations of polymer, thus leading large droplet formation when polymer solution added to the calcium chloride solution during ionic gelation process. Increasing the alginate and CMC concentrations causes solution viscosity to increase which contributes to retention of a higher volume fluid at the needle tip, which increases microsphere size.

| No | Formula | Alginate (b/v) | CMC (b/v) | Particle size (nm±SD) | Encapsulation efficiency |
|----|---------|----------------|-----------|-----------------------|-------------------------|
| 1  | A       | 1              | 0.5       | 496.05±2.72           | 15.15 %                |
| 2  | B       | 1              | 1         | 506.00±4.52           | 18.74 %                |
| 3  | C       | 1.5            | 0.5       | 611.05±0.35           | 32.22 %                |
| 4  | D       | 1.5            | 1         | 629.65±8.70           | 21.43 %                |

Formula C was chosen for the SEM test based on its swelling ratio in artificial stomach and intestine environment, particle size, and encapsulation efficiency. Micrograph scans of blank microspheres (before encapsulation) and bromelain encapsulated microspheres (after encapsulation) are shown in Figures 2 and 3. SEM micrographs at low magnification of the blank formulation showed that the microsphere was nearly spherical with little tailing. Micrographs of both microspheres also show indentation on the surface. The surface image of the nanospheres at high magnification shows that the two nanospheres (before and after encapsulation) have very few pores making them ideal as drug delivery systems because they have slow release properties.

Figure 1. Swelling Ratio at pH 1.2 (left) and 7.4 (right) for all microsphere formulas
Figure 2. Blank micrograph of formula C (left) low magnification and (right) high magnification

Figure 3. Micrograph of formula C containing bromelain (left) low magnification and (right) high magnification

3.2. FTIR spectroscopic analysis

The FTIR spectra of the alginate, CMC, alginate-CMC microspheres before and after bromelain encapsulation are presented in Figure 4. The FTIR spectra showed a wide hydroxyl band near 3400 cm$^{-1}$ in all samples, which is due to OH vibrations. The peaks near 1600 cm$^{-1}$ and 1400 cm$^{-1}$ are associated with symmetric and asymmetric vibrations of carboxylate ions in alginate and CMC. The band area in the range of 1200-1000 cm$^{-1}$ are associated with the C-O, C-C, C-O-C, and C-O vibrations of saccharide structure. The peaks of the hydroxyl and carboxyl groups were also observed in cross-linked microspheres before and after encapsulation with bromelain at wavelengths around 3400, 1600, and 1400 cm$^{-1}$.

Based on the figure 4, the FTIR spectra between alginate-CMC microspheres before and after encapsulation shown the same absorption peaks. This indicates that there is no chemical interaction between bromelain and microsphere matrix.
Figure 4. FTIR spectroscopy of alginate, CMC, alginate-CMC microspheres, and alginate-CMC microspheres containing bromelain

4. Conclusion
Alginate-CMC microspheres have been successfully prepared using the ionic gelation method, where calcium chloride acted as a crosslinking agent. Based on the research, it was found that formula C with Alginate: CMC (w/v) composition = 1.5: 0.5 with 3.75% CaCl₂ was the best formula because it had a small swelling ratio at acidic pH and higher at alkaline pH and had the highest encapsulation efficiency value.

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