Preparation, characterization and release profile of chitosan alginate freeze dried matrices loaded with mangostins

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Abstract. Freeze drying or lyophilisation method was selected for preparing chitosan-alginate matrices loaded with the extract of mangosteen pericarp for oral administration. The objective of this research was to obtain chitosan-alginate matrices for colon targeted drug delivery system that had a high content of mangostins by using a freeze drying method. Various compositions of matrices consisting of chitosan, alginate and mangostins have been used to study the effect of alginate and mangostin content on the release property of freeze dried matrices. Sharp X-ray diffraction peaks of the crystalline phase in pure chitosan and pure alginate, vanished in the chitosan-alginate matrices. The infrared spectroscopy spectra of matrices showed that mangostins were entrapped in the matrices. Release of mangostin from the chitosan-alginate freeze dried matrices was affected by the proportions of alginate and mangostins in the formulations. The in-vitro release assays in simulated gastrointestinal fluids showed the mangostin was burst released from the chitosan-alginate matrices prepared by freeze drying method. The chitosan-extract-alginate matrix with mass ratios of 1:0.1:0.5 showed low release of mangostin in simulated gastric fluid, but high release in simulated intestinal and simulated colonic fluids. The freeze drying method facilitates high bioactive loading, and with a proper proportion of chitosan and alginate, it should be possible to obtain matrices that can be used for colon targeted oral drug delivery.

1. Introduction
Mangosteen (Garcinia mangostana L.) is a fruit tree of the Guttiferae family. Mangosteen is known to contain bioactive mangostin compounds, extracted from the pericarp, fruit, stem, and leaves, which have been long used as a traditional herbal medicine in various countries in the continent of Southeast Asia. The secondary metabolites contained in mangosteen, known as xanthones with mangostins as the major components, possess therapeutic activities such as antioxidant, antitumoral, anti-inflammatory, antiallergy, antibacterial, antifungal, and antiviral [1-3]. Mangostins, mainly α-mangostin and γ-mangostin, in the mangosteen pericarp extract was also reported to have cytotoxic properties as well as the ability to induce apoptosis of colon cancer and mammary cells [4-7]. The mangostins have been proven to inhibit microbes such as Bacillus subtilis and Staphylococcus aureus, which explain the topical uses of mangosteen pericarp extract to treat infection with traditional medicine [8]. In order to transport the active compounds to target area inside the body, for example, in the colon, the active compounds need to be protected from the acidic gastric fluids.

Matrices or microparticles of polymers are well studied, carrier and protector for delivering drugs or active compounds via oral administration. Chitosan is known as biodegradable, biocompatible and
non-toxic biopolymers [9-11] that has good mucus membrane in the gastrointestinal tract [12]. Other biopolymer, alginate, is also known as a carrier in an oral delivery system due to its stability in the acidic gastric acid solution of stomach [13]. The combination of chitosan and alginate has been used in the formulation of controlled or extended drug release [14,15].

In this research, the formulation of chitosan-alginate matrix loaded mangosteen extract was done using the freeze drying method, which is also known as lyophilization method. It is a process which consists of removing water from a frozen sample by sublimation and desorption under vacuum condition [16]. Lyophilization may become a prospective method for matrix formulation with high encapsulation efficiency since it does not involve washing or filtration process that could leach and degrade the active substances [17]. The preparation of chitosan-alginate matrices loaded with mangosteen extract has not been commonly studied. Likewise, the characteristics as oral delivery system and the release property of chitosan-alginate matrix that prepared by the freeze drying method are unknown. Therefore, the objectives of this research were to obtain the formulation of chitosan-alginate matrix that had a high mangostins loading using a freeze drying method and to characterize its release property in simulated gastrointestinal fluids, for colon-targeted drug oral administration.

2. Materials and methods

2.1. Materials

The mangosteen pericarp was obtained from the Depok area at West of Java. Standard α-mangostin (98%) was obtained from Aktin Chemicals, China. The chitosan was white having MW of 80-120 kDa, viscosity of 100-200 MPa.s, purchased from Chimultiguna, Indramayu. Tween 80 was obtained from Brataco Chemical, Indonesia, while the other ingredients such as alginate powder, ethanol, and ethyl acetate were obtained from Merck.

2.2. Extraction process of mangostins

The maceration of mangostins from the mangosteen pericarp followed the modified procedure reported previously [18]. The pericarp was washed carefully, after that drained under sunlight for 5 days and drained in the oven for 2 days, then reduced its size by grinding to powder form. The maceration was done using ethanol 96% for 7 days, with mangosteen powder to ethanol ratio of 1:3 (w/v), and stirred up periodically. Then the mixture was filtered, and ethanol was evaporated using a rotary evaporator (EYEELA N-1000) to obtain a viscous ethanol extract. The next step was fractionation of the extract by a mixture of water and ethyl acetate with a ratio of 1:1 (v/v). The ethyl acetate fraction was separated, concentrated, and dried using a rotary evaporator to obtain dry paste of mangosteen extract.

2.3. Chitosan-alginate matrices freeze dried preparation

Six different matrices were made, five of them vary in the amount of extract or alginate used in the matrix formula, while one of them was a blank or a non loaded matrices. All of the matrices were prepared by freeze drying method [19]. The chitosan solution was made by dissolving 1 g of chitosan in 50 ml 2.5% acetic acid (v/v). Mangostin extract was dissolved in ethanol with a ratio of 0.1 g per 1 ml and then was added to the chitosan solution. The emulsion mixture was stirred using a four-blade impeller at 1500 rpm for 10 minutes. Then, 0.5 mL of 2% (v/v) tween 80 solution was added dropwise to the mixture using a syringe and stirred using a four-blade impeller at 1000 rpm for 5 minutes to form a homogeneous emulsion. The solution of sodium alginate was made by dissolving the powder in water, in 0.1 g per 12.5 ml ratio. The alginate solution was then added into the chitosan mixture emulsion, and stirred using a four-blade impeller at 1800 rpm for 20 minutes. The resulting mixture was refrigerated until frozen and then lyophilized using EYELA FDU-2100 freeze dryer. The formed matrices were ground into powder with size of less than 1 mm.
2.4. Mangostin analysis
The quantitative determination of xanthone content, given as total mangostin equivalent, in the ethyl acetate fraction extract as well as in the matrices of chitosan-alginate were performed using UV spectrophotometry analysis (Spectrophotometer Bel UV-VIS UV-M51). The absorbance data of \( \alpha \)-mangostin standard solution (4-20 mg/L) was obtained at the wavelength of 316 nm [20].

2.5. Matrix morphology test
The powder X-ray diffraction patterns of pure chitosan, alginate and all freeze dried matrices were recorded using SHIMADZU XRD 7000 MAXIMA-X with Cu tube. The crystalline or amorphous character of pure chitosan or alginate and freeze dried matrices can be identified based on the powder X-ray diffraction patterns measured. All samples were analyzed between 2\( \theta \) angles of 10\( ^{\circ} \) and 80\( ^{\circ} \) using the voltage, the current and the time per step of 40 kV, 30 mA and 1s, respectively. The Fourier Transform Infra-Red (FTIR) analysis was conducted to determine the structure of matrices formed using freeze drying method, as well as to validate the presence of mangostin entrapped in the matrices. The Thermo Scientific Nicolet 1S5 with iD5 ATR optic was used to analyze bioactive compounds from natural sources in the wavelength ranged from 4000 to 400 cm\(^{-1}\).

2.6. Encapsulation efficiency and loading capacity of matrices
The encapsulation efficiency and loading capacity were obtained by quantifying the mangostins present in matrices and the amount matrices formed, using the following formulas:

\[
\text{Encapsulation efficiency (\%) = } \frac{\text{mass of mangostin present in matrix (mg)}}{\text{mass of mangostins used (mg)}} \times 100 \tag{1}
\]

\[
\text{Loading (\%) = } \frac{\text{mass of mangostin present in matrix (mg)}}{\text{total mass of matrices (mg)}} \times 100 \tag{2}
\]

2.7. In-vitro mangostin release test in simulated gastrointestinal fluids
The release profiles of mangostin from chitosan-alginate matrices were obtained sequentially using three simulated gastrointestinal fluids as the release media. The matrix samples in the form of particles were put inside a dialysis membrane tube and then the tube was immersed in the simulated gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.4) and simulated colonic fluid (SCF, pH 6.8), in sequence. The in-vitro release test was conducted by immersing 20 mg particles in 60 mL of simulated fluids and incubated at 37 \( ^{\circ} \)C. Each volume sample of simulated fluids taken for analysis was 5 mL, the samples were taken periodically in 2, 4, 6, 8, and 24 hours, and the \( \alpha \)-mangostin content was determined by UV-spectrophotometry analysis. The mangostins released was measured as \( \alpha \)-mangostin equivalent. The profile release was obtained by plotting the \( \alpha \)-mangostin cumulative release from the matrix as a function of immersion time in simulated gastrointestinal fluids.

3. Result and discussion

3.1. Quantification of mangostin in ethyl acetate fraction extract
The extraction was performed by macerating 300 grams of mangosteen pericarp powder against 900 ml of absolute ethanol analytical grade, which was followed by fractionation in ethyl acetate. The weight of dried extract obtained was 22.01 grams, which was equal to the extraction yield of 7.34\% (w/w). The mangostin content in the extract, quantified using UV-vis spectrophotometry, was 61.04\% (w/w).

3.2. Matrix formulation, loading capacity and encapsulation efficiency
Six different matrices were prepared, five of them vary in the amount of extract or alginate used in the matrix formula, while one of them was a blank matrix without loaded mangostin. The data of those
matrices are shown in Table 1. The matrix yields were above 91%, not affected by the proportion of chitosan, alginate, and extract in the matrix preparation.

Table 1. Matrix formulation and characteristics.

| Matrix | Extract: chitosan (g/g) | Chitosan: alginate (g/g) | Matrix yield (% w/w) | Encapsulation efficiency (% w/w) | Theoretical loading (% w/w) | Actual loading (% w/w) |
|--------|-------------------------|--------------------------|----------------------|---------------------------------|----------------------------|-----------------------|
| Blank  | 0.0:1                   | 1:0.1                    | 91.91                | -                               | -                          | -                     |
| M1     | 0.1:1                   | 1:0.1                    | 98.48                | 80.72                           | 5.41                       | 4.36                  |
| M2     | 0.1:1                   | 1:0.5                    | 98.81                | 99.48                           | 3.86                       | 3.84                  |
| M3     | 0.1:1                   | 1:1.0                    | 91.06                | 86.55                           | 3.22                       | 2.79                  |
| M4     | 0.3:1                   | 1:0.5                    | 97.48                | 78.33                           | 10.51                      | 8.23                  |
| M5     | 0.5:1                   | 1:0.5                    | 97.41                | 83.23                           | 15.76                      | 13.12                 |

The theoretical loading capacity of mangostin in the matrix was calculated based on the quantitative data of compounds used in matrix preparation and the equation (2). In order to obtain the matrices actual loading value, the matrices were destructed using procedure reported by [21] with modification. The matrix was immersed in synthetic gastric fluid for six hours, stirred and the mangostin released was analyzed. The actual loading measured by destruction was lower than the theoretical loading value. This might due to the mass loss during the formation of matrices, either in the process of raw materials mixing, homogenizing, or freeze drying. The encapsulation efficiency of matrices was calculated based on the value of actual loading and the equation (1), as shown in Table 1. The highest encapsulation efficiency was reported in matrix M2, where the alginate amount was half of the amount of chitosan. It seemed that the interaction between chitosan and alginate was quite stable even until the end of the freeze-drying process, where the water molecules involved were sublimed. The matrix M2 also had the smallest difference between theoretical loading and actual loading. Increasing amount of extract in the matrices, from 0.1/1 to 0.5/1 mass ratio extract to chitosan, results in increasing actual loading value, from 3.8% to 13.1%. The results from the increasing amount of alginate were decreasing actual loading. It seems the matrix of chitosan and alginate can have a high loading capacity when it is prepared using the freeze drying method.

3.3. Characteristics and morphology of matrices

The XRD or X-ray diffraction test was performed to determine the structure of the matrices, since the matrices prepared using the freeze drying method can be crystalline or amorphous [19]. The samples tested were the blank or chitosan-alginate matrices without mangosteen extract, M2 and M5 matrices, pure chitosan and alginate that were used the matrices preparation. The intensity of the diffracted x-ray as the function of intensity is shown in Figure 1. Based on the XRD graphs, there are two crystalline peaks with the angle of 20 as much as 20.45 and 29.49 in pure chitosan and one crystalline peak with a 20 angle of 20.47 in pure alginate, so that the structure of both materials was semi-crystalline. Meanwhile, for the empty/blank, M2 and M5 matrices, there was no crystalline peak, so it can be concluded that the matrices formed by freeze drying method had amorphous structure.

The characteristics of the matrices formed were analyzed using Fourier Transform Infra Red spectroscopy or FTIR to obtain a spectral graph that represents the functional groups present in the matrices. The assay was carried out in a range of wavenumber of 4000 to 400 cm⁻¹ for FTIR analysis of natural material compounds. The samples tested were variations of Blank, M1, M2, M3, M4, and M5 matrices. In the range from 2800 to 3000 cm⁻¹, there are peaks that represent the presence –CH stretch bond in mangostin [22]. Peak at 1600-1700 cm⁻¹ represent the C=C stretch bond in benzene ring [23] in mangostins. These peaks are present in the spectra of M1, M2, M3, M4, and M5 matrices, but none in the spectra of blank matrix. Thus, it justifies that mangostins are entrapped in the matrices of M1-M5.
Figure 1. XRD graphs of chitosan (C), alginate (A), and matrices with chitosan to mangostin extract mass ratio of 1:0.1 and 1:0.5 for M2 and M5, respectively.

Chitosan and alginate are polysaccharide compounds, it is characterized by the appearance of the peaks in a wavenumber of about 1031 cm$^{-1}$ (C-O-C stretch) and about 1086 (C-C stretch). In addition, the emergence of peaks around the 3250 cm$^{-1}$ wavenumber was caused by the formation hydrogen bonds between the -OH and -CH groups of the chitosan compound, as well as to the -C=O and -OH groups of sodium alginate [24].

Figure 2. FTIR spectral graphs of blank and various formulation matrices as shown in Table 1.

It can be concluded that, from the FTIR spectra, the mangostins were entrapped in the matrices of chitosan-alginate. All matrices had similar IR spectra that might indicate the structures of matrices prepared by freeze drying method were similar. The sublimation of water does not affect the interaction/bond that presents between chitosan and alginate.
3.4. In-vitro release study

The in-vitro release profiles of mangostins loaded in chitosan-alginate matrices having different compositions are shown in Figure 3, indicating the pH sensitivity of the matrices sequentially contacted with simulated the gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.4), and simulated colonic fluid (SCF, at pH 6.8). In this study, a matrix sample was immersed inside a dialysis tube containing SGF for 3 hours, transferred to SIF and kept for 4 hours, and lastly transferred to SCF and kept for 17 hours. Cumulative release of mangostin of all matrix samples for 24 hours in gastrointestinal fluid was less than 100%. This may be because the freeze dried matrix of chitosan-alginate effectively restrains mangostin in its microstructure and delays its release.

As can be seen in Figure 4, the rate of release in the first two hours in SGF medium pH 1.2, faster than in other periods with the release medium pH 6.8 and 7.4. The electrostatic interaction between alginate and chitosan that occurs during matrix formation seems to break down, so that when immersed in a pH 1.2 solution, the mangostin diffuse out. One interesting thing is to increase the mass ratio of the alginate to chitosan from 0.1: 1 to 0.5: 1, the release of mangostin increases by a half, whereas usually the coating of chitosan with alginate will prevent release in a pH 1.2 solution. Similar effects of adding alginate to the chitosan matrix are also reported by [25], where the release of mangostin increases in simulated gastric fluids. Rapid sublimation of frozen water from the chitosan alginate matrix forms a cavity that facilitates absorption of the release solution. If the solution fills the cavities in the matrix, the chitosan-alginate interactions break down rapidly, so that the mangostin easily diffuses outward. This might indicate the release of active compounds from the matrices is controlled by diffusion process, not swelling of chitosan-alginate matrices that control the release [14]. Although chitosan is known to have a fairly good solubility in acidic solutions [26]. Composition of matrix M2 with a mass ratio of chitosan to alginate as much as 1:05 was selected to vary the mangosteen extract loaded.

Figure 5 shows that the chitosan-alginate matrix loaded with higher mangostin will release more mangostin. Figure 5 shows that the higher chitosan-alginate matrix loaded with mangostin will release more mangostin. The release trend of mangostin in simulated gastrointestinal fluid media remains the same, which is quickly released in gastric fluid and slowed in intestinal and colonic fluids. Freeze drying method seems to be able to increase the loading of mangostin into the chitosan alginate matrix. For this matrix application in colon targeted drug delivery, the proper loading and composition of chitosan and alginate can be designed.
Figure 4. Mangostin release profile from chitosan/alginate matrices in: M1 (1:0.1), M2 (1:0.5), M3 (1:1). The release media in 0-3 hour: SGF, 3-7 hour: SIF, and in 7-24 hour: SCF.

Figure 5. Mangostin release profile from chitosan/extract ratio: M2 (1:0.1), M4 (1:0.3), M5 (1:0.5). The release medium in 0-3 hour: SGF, 3-7 hour: SIF, and in 7-24 hour: SCF.

Conclusion
Chitosan-alginate matrices made by freeze drying method have a relatively high encapsulation efficiency (average 85%) for the composition of chitosan mass ratio to alginate between 1:0.1 to 1:1. This relatively easy method managed to load mangostin up to 13% (weight). The morphology of the matrix through XRD diffraction pattern shows that the interaction of chitosan and alginate and the removal of frozen water through sublimation leads to the loss of the crystalline phase of the chitosan-alginate so that the existing structure of the matrix is the amorphous phase. Based on the FTIR spectroscopy analysis, it was found that based on the vibration peak of the existing functional groups, all matrices show functional groups derived from chitosan and alginate, as well as functional groups indicating the presence of mangostin in the matrices. Freeze drying method succeeded in making matrices that have homogeneous morphology, no separation of chitosan and alginate structures. The release profile of mangostin from the chitosan-alginate matrix appears to be affected by the pH condition of the release solution medium. At low pH release occurs rapidly but then slows at higher pH within a period of immersion for 24 hours in simulated gastrointestinal fluids. Sublimation of
frozen water during the freeze drying process causes the formation of cavities in the matrix which helps accelerate the release of mangostins from the matrix.

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