Effect of alginate concentration on the physicochemical characteristics and entrapment efficiency of bioactive compounds in microencapsulated bekasam extract

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Effect of alginate concentration on the physicochemical characteristics and entrapment efficiency of bioactive compounds in microencapsulated bekasam extract

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Abstract

Bekasam extract functions as an effective agent for reducing cholesterol and hypertension levels. One of the practical methods to utilize this extract is through microencapsulation. During the manufacturing of consumables, microcapsules can be incorporated into foods and beverages as functional additives. Therefore, this study aimed to investigate microencapsulation methods of bekasam extract, specifically by using ionic gelation with sodium alginate as a coating at varying concentrations of 0.50%, 0.75%, 1.00%, and 1.25%. The results showed that the diameter of the microcapsules produced ranged from 3.71-3.92 mm, and the yield was 21.83-40.47%. The microcapsules contained 6.3-10.08% peptides and 37.89-46.39 ppm of Lovastatin with an entrapment efficiency of 17.31-27.69% and 59.08-72.76%, respectively. The microcapsules made with alginate (1.25%) had higher levels of Lovastatin and peptides.

1. Introduction

Hypercholesterolemia is a condition characterized by higher-than-normal cholesterol levels (>240 mg/dL) and is considered one of the contributing factors to coronary heart disease. It is the main risk factor for cardiovascular diseases which often lead to death (Mohd Isa *et al*., 2021). In Indonesia, about 18% of the population suffers from hypercholesterolemia (Jampormase *et al*., 2016), and only 35.9% of individuals aged above 15 years have a normal total cholesterol level (Shafira *et al*., 2020). One of the methods to reduce cholesterol levels in hypercholesterolemia is inhibiting synthetic cholesterol by decreasing the activity of the HMG-CoA reductase enzyme (Rinto and Suhartono, 2016).

Bekasam is an Indonesian fermented fish product (Anggrahini *et al*., 2019) that can potentially reduce cholesterol due to bioactive compounds, such as peptides and Lovastatin, capable of inhibiting the HMG-CoA reductase activity (Rinto *et al*., 2023). Bioactive peptides are also antihypertensive because they can inhibit the activity of angiotensin converting enzyme (ACE) (Wikandari *et al*., 2012), which increases blood pressure (Wikandari and Yuanita, 2016). Bekasam has many health benefits, but its distinctive appearance, taste, and pungent smell are detested by some people. One means of obtaining bekasam benefits is by microencapsulating extract as an intermediate product in food manufacturing. Microcapsules can be incorporated into foods and beverages as functional additives. Microencapsulation is considered the best method to protect bioactive components (Ranveer *et al*., 2022). This is easy to use and the coating material offers an enhanced resistance, which prevents evaporation or degradation during storage.

Microencapsulation techniques include air suspension, coacervation, centrifugation, pan coating, dry spraying, and ionic gelation (Sugindro *et al*., 2008). Among all, ionic gelation is the most widely used due to its good biocompatibility properties, ease of application, lack of need for organic solvents, and relatively low cost (Saraei *et al*., 2013). This employs different coating materials, such as chitosan, carrageenan, and alginate (Liouni *et al*., 2007). Alginate is the most commonly used to produce good, biocompatible, non-toxic, and easy-to-use microcapsules (Burgain *et al*., 2011). This polymer can preserve the stability of bioactive compounds against heat, pH, oxygen, and other factors during processing and storage (Goh *et al*., 2012).

Several studies have been conducted on the

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manufacture of microcapsules with alginate coating, including those for entrapping carbamazepine, cumin oil, and *Leuconostoc mesenteroides* ssp. (Hermana *et al*., 2015). These show that alginate can be used to produce microcapsules for liquid, paste, or microorganisms, as well as bekasam extract.

2. Materials and methods

2.1 Materials

The materials used in this study were Minnows/carp fish (*Rasbora argyrotenia*) obtained from *Indralaya* traditional market in South Sumatra, Indonesia. Sodium alginate, calcium chloride, sodium hydroxide, phenolphthalein, formaldehyde, methanol, and Whatman No. 1 filter paper were purchased from Sigma-Aldrich. Also, the instruments employed included a centrifuge (Hettich Universal 320R, Germany), water bath (Memmert, Germany), hot plate stirrer (B-One-AHS-12A, Germany), micropipettes of 0.5-10 µL (Eco pipped by Capp, Germany), 10-100 µL, and 100-1000 µL (Dumo, India), a UV-Vis spectrophotometer (Shimadzu UV-1800), and HPLC with Column C-18.

2.2 Study method

This study used a completely randomized design method with four treatment levels of alginate concentration of 0.50% (A1), 0.75% (A2), 1.00% (A3), and 1.25% (A4), each of which was repeated three times.

2.3 Preparation and extraction of bekasam

Minnows/carp fish (*Rasbora argyrotenia*) weighing 250 g were obtained, and then cleaned through the removal of the viscera and washing. Afterwards, rice and salt were added at 15%, respectively, and fermented in a container at 28-30°C for seven days. To extract bekasam, 10 g of it was homogenized with 40 mL distilled water for 30 mins. The homogenate was filtered with Whatman No. 1 filter paper to produce filtrate (1) and residue. The residue was further macerated by adding distilled water (50 mL) and stirring for 30 mins at room temperature. The extract was filtered with Whatman No. 01 filter paper to produce another filtrate (2). The mixture of filtrates 1 and 2 was centrifuged at 6,000 rpm and 4°C for 15 mins to produce supernatants and precipitate. The supernatant was evaporated using a water bath to collect bekasam extract (Rinto *et al*., 2017).

2.4 Identification of Lovastatin and peptides in bekasam extract

The statin group of compounds from the extract was identified using High-Performance Liquid Chromatography (HPLC). This employed methanol as the mobile phase and C-18 as the column, while measurements were conducted at a wavelength of 238 nm (Rinto *et al*., 2019). The identification of the peptides was carried out in two stages. First, the peptides' molecular weight was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with resolving gel (18%) in Tris-HCl (1 M, pH 8.8). The second involved identifying the structure of their amino acid components. In the SDS-PAGE step, protein fractions were put in an electrophoresis gel containing Tris buffer (24.8 mM), glycine (192 mM), and SDS (0.1%) at pH 8.3. To estimate the standard molecular weights of the peptides from the sample, low-range protein ladder markers were added to the gel, which was stained with silver staining after electrophoresis.

To identify the amino acid structure, the peptide band from the SDS-PAGE result, which inhibited HMG-CoA reductase, was cut, extracted, purified, and sequenced (Proteomics International Pty Ltd, Australia). The sequencing process involved protein digestion with trypsin, and analysis of peptides through electrospray ionization mass spectrometry (Agilent 1260 Infinity HPLC system and Agilent 6540 mass spectrometer). The tryptic peptides yielded were inserted into the C18-300 SB column of a 5 m size (Agilent), and their spectrum was analyzed for identification using Mascot sequence matching software (Matrix Science) with Ludwig NR. Finally, the peptide structure was modeled using SWISS-MODEL https://swissmodel.expasy.org/ and RasMol (Rinto *et al*., 2017).

2.5 Microencapsulation methods of bekasam extract

Bekasam extract microcapsules were prepared by a modified ionic gelation method comprising two polymers. Based on the formulation presented in Table 1, Na-Alginate and tragacanthin were soaked in distilled water for 3 mins at 60°C, then homogenized using a homogenizer with a speed of 2000 rpm. The extract was added to the solution and homogenized again at 2000 rpm for 3 mins. The solution was drawn into a syringe equipped with a 22.5 G needle and dropped into a 0.5 M $CaCl_2$ solution at 10°C to produce microcapsules. The microencapsulated mixture was left to solidify for 30 mins before being filtered.

Table 1. Microencapsulation formulation of *bekasam* extract (%).

	A1 (%)	A2 (%)	A3 (%)	A4 (%)
Bekasam extract	30±0.001	30±0.001	30±0.001	30±0.001
Gum tragacanth	0.50±0.001	0.50±0.001	0.50±0.001	0.50±0.001
Na-alginate	0.50±0.001	0.75±0.001	1.00±0.001	1.25±0.001
Distilled water	100±0.001	100±0.001	100±0.001	100±0.001

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2.6 Organoleptic assay

The organoleptic assay in this study included the characterization of bekasam extract microcapsules in terms of color, odor, shape, and diameter.

2.7 Peptide assay

The peptide levels contained in the extract were determined by formol titration. A sample of 0.25 g was dissolved with 4.75 mL distilled water in a 250 mL Erlenmeyer flask. This was further dissolved with 10 mL of distilled water and a phenolphthalein indicator of 0.5 mL. Titration was performed with 0.1 N NaOH until pink color appeared. About 1 mL of formaldehyde solution (40%) was added to the sample and titrated again with NaOH until pink color appeared. The concentration of peptide was calculated with the following equation:

$$\%N = ab \times N\ NaOH \times Ar\ N \times fp$$

Where a = Volume of titration, b = Sample (g) and fp = thinning factor.

2.8 Data analysis

The statistical data were analyzed using Analysis of Variance (ANOVA) with a 95% confidence interval. Once the results showed significant differences, the Least Significant Difference (LSD) is used. Descriptive data analysis was conducted to describe the results of each variable in the test.

3. Results

3.1 Identification of statin compounds and peptides from bekasam extract

The HPLC analysis conducted showed that the statins contained in the extract belonged to the Lovastatin group. There are four statin standards, including Compactin, Pravastatin, Lovastatin, and Simvastatin. Extracted compounds were identified as Lovastatin because its retention time was similar to that of the Lovastatin standard, as indicated in Figure 1. Besides, potential anticholesterol peptides acting as HMG-CoA reductase inhibitors were identified to consist of 57 amino acids:

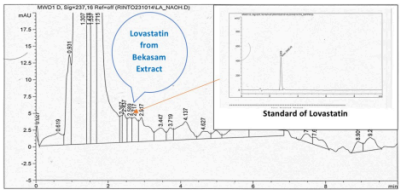

Figure 1. Histogram of Lovastatin retention time using HPLC.

Aspartic acid – alanine – threonine – alanine – alanine – valine – aspartate – alanine – valine – phenylalanine – serine – serine – isoleucine – glutamine – alanine – serine – leucine – alanine – lysine – alanine – alanine – glutamic acid – valine – valine – Alanine - Phenylalanine - Leucine - Asparagine - Lysine - Glutamic Acid - Alanine - Isoleucine - Glutamic Acid - Alanine - Isoleucine - Alanine - Aspartic Acid - Threonine - Methionine - Lysine - Lysine - Threonine - Isoleucine - Isoleucine - Aspartic Acid - Isoleucine - Aspartic Acid – Asparagine - Glutamate - Lysine - Leucine - Alanine - Alanine - Aspartic Acid - Aspartic Acid - Methionine - Arginine.

They can be presented in the order: "D-A-T-A-A-V-D-A-V-F-S-S-I-Q-A-S-L-A-K-A-A-E-V-V-A-F-L-N-K-E-A-I-E-A-I-A-D-T-M-K-K-T-I-I-D-I-D-N-E-K-L-A-A-D-D-M-R". Bekasam peptides consist of 32 (56%) hydrophobic amino acids and 25 (44%) hydrophilic amino acids. The 3-dimensional structure of the peptides detected with the RasMol program is shown in Figure 2.

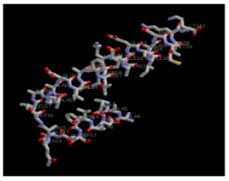

Figure 2. The 3-dimensional structure of the HMG-CoA reductase inhibitor peptide from bekasam.

3.2 Average diameter of microcapsules from bekasam extract

The average size of microcapsules from each formulation was determined by measuring their diameter. This was carried out using a micrometer screw by taking 40 microcapsules from each treatment. The results are presented in Figure 3, which shows different formulations produce microcapsules of varying diameters. The average diameter of microcapsules with alginate concentrations 0.5% (A1), 0.75% (A2), 1.00%

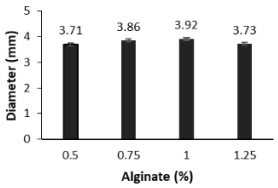

Figure 3. Average diameter microcapsules of bekasam extract.

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(A3), and 1.25% (A4) was 3.71 mm, 3.86 mm, 3.92 mm, and 3.73 mm respectively. Different alginate concentrations did not significantly influence the diameter size ($p>0.05$). Therefore, alginate addition at 1.25% had no significant effect on the diameter of bekasam extract microcapsules.

3.3 Organoleptic of microcapsules

Based on organoleptic observations, the microcapsules of bekasam extract were spherical, colored white to yellowish, and had a specific bekasam odor. The results can be seen in Figure 4 and Table 2. According to Figure 4, microcapsules from 0.75% alginate have an imperfect round form and are porous when dried. Those from 100% (A3) and 1.25% (A4) have a round form and are slightly flat. Moreover, alginate concentration influenced the microcapsules' color.

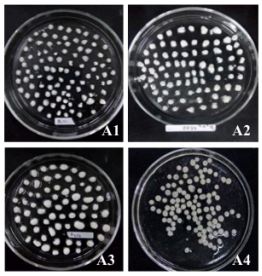

Figure 4. Microcapsules of bekasam extract with different alginate concentrations. A1: 0.50% alginate, A2: 0.75% alginate, A3: 1.00% alginate, A4: 1.25% alginate.

3.4 Yield of microcapsules

The microcapsules' yield was calculated based on their weight in comparison to the total weight of the solid components (coating polymers and substances encapsulated), expressed as a percentage (Purnomo *et al*., 2014). This calculation was used to estimate the number of wasted materials, and to determine the efficiency and effectiveness of the encapsulation process (Hasrini *et al*., 2017). Microcapsules yields are influenced by the ratio of core material to coating agent, the agent's characteristics form, and the encapsulation method used. Figure 5 shows the average yield of microcapsules, which increases along with the concentration of the forming polymer. This is consistent with the report by Supriyadi and Rujita (2013) that the yield of microcapsules of essential galangal oil increased with an elevation in polymer concentration.

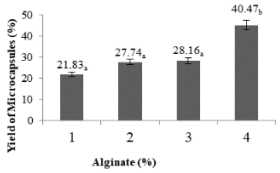

Figure 5. The yield of microcapsules from bekasam extract. Values with different superscripts are statistically significantly difference ($p < 0.05$).

3.5 Peptide levels

A peptide is a molecule formed from two or more amino acids connected through amide or peptide bonds. In this study, peptide levels in the microcapsules increased with elevating the concentration of alginate as a coating agent. The average peptide levels can be seen in Figure 6, indicating that an increase in alginate concentration augments peptide content, but not significantly (5% level). This is suspected because the peptide can diffuse out of alginate matrix. Peptides in fish fermentation products emanate from the degradation of protein by proteolytic enzymes produced by lactic acid bacteria (LAB) (Koesoemawardani and Neti, 2009).

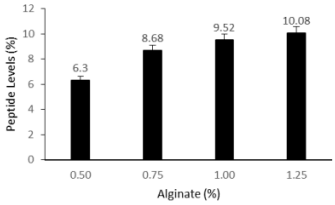

Figure 6. Peptide levels of the microencapsulate.

Table 2. The organoleptic observations result of of bekasam extract microcapsules.

Alginate Concentration	The organoleptic observations result of bekasam extract microcapsules			
	Color		Odor	
	Wet	Dry	Wet	Dry
0.50%	White	White	Specific of bekasam	Specific of bekasam
0.75%	White	Rather yellowish white	Specific of bekasam	Specific of bekasam
1.00%	White	Rather yellowish white	Specific of bekasam	Specific of bekasam
1.25%	Rather yellowish white	Yellowish white	Specific of bekasam	Specific of bekasam

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3.6 Lovastatin content

The Lovastatin levels in the microcapsules ranged from 37.89 to 46.39 ppm and the greatest level was yielded by the highest concentration of alginate. The average level of Lovastatin is presented in Figure 7, which shows a simultaneous increase in Lovastatin with increasing alginate concentration. Increasing the concentration of alginate as a coating material ought to elevate lovastatin levels in extract microcapsules. However, the ANOVA result showed the addition of alginate did not significantly affect lovastatin levels (5%). This indicates that even though lovastatin levels increase with elevating alginate concentration, the increase was not significant.

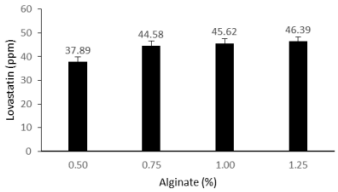

Figure 7. Lovastatin content in the microcapsules.

3.7 Entrapment efficiency

Entrapment efficiency is a calculation to determine the success rate of the microencapsulation process by comparing the levels of the active substance before and after encapsulation. Higher efficiency correlates proportionally to a better ability of the coatings to protect the core material. Entrapment efficiency is interpreted in the form of percentage (%) and the results can be seen in Figure 8.

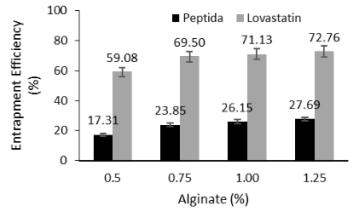

Figure 8. Entrapment efficiency of peptide and lovastatin in the microcapsules.

The results showed that the efficiency of encapsulation was influenced by the alginate concentration used. Efficiency value increased along with an increase in alginate concentration. This was because the high polymer concentration can affect the encapsulation efficiency. The polymer with high concentration settles faster on the dispersion phase surface, thereby preventing the core substances from diffusing out (Rafati *et al*., 1997).

4. Discussion

Microcapsules prepared with alginate 0.5% (A1), 0.75% (A2), and 1.00% (A3) tend to be large but flat and porous. Suciati *et al*. (2011) reported that the concentration of sodium alginate used was very small, leading to imperfect cross-linking and an inability of the microcapsules to maintain their shape. However, alginate 1.25% (A4) yielded microcapsules with a round and non-flat form, which caused a decreased diameter. The difference in diameter can be attributed to various factors, including the size of the needle used (Rokka and Rantamaki, 2010), its distance to the crosslinking agent (Solanki *et al*., 2013), and the viscosity value (Krasaeokoopt *et al*., 2004).

Moreover, the difference in alginate concentration will affect the form, color, and odor of microcapsules produced as presented in Table 1 and Figure 4. The microcapsules prepared from alginate 0.5% (A1) had an imperfect round form and were porous. This was caused by the alginate concentration being extremely small, leading to imperfect crosslinking and difficulty in maintaining shapes (Suciati *et al*., 2011).

A higher concentration of alginate increases the microcapsules' color and odor of *bekasam* component, indicating that the higher concentration led to more effective extract entrapment. This was supported by the efficiency of peptides and lovastatin entrapment which increased with the addition of alginate as presented in Figure 8.

The yield of microencapsulation was significantly different at the level of 5%. The concentration of 1.25% alginate has a significant effect on yield values since the microcapsules prepared from 0.5%, 0.75%, and 1.0% alginate are not formed perfectly. Therefore, it can be concluded that alginate concentrations of 0.50 - 1.00% are unable to entrap bekasam extract maximally due to an insufficient amount of the coating polymer.

According to Gombotz and Wee (1998), proteins encapsulated in an alginate matrix can diffuse out through the polymer network pores. The calcium alginate microcapsules analyzed with electron microscopy showed pores ranging from 5-200 nm. This is supported by the observation of Azzarnia *et al.* (2008) that microencapsulation of aminopeptidase (PepN) from *Lactobacillus rhamnosus* S93 with alginate coating

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yielded an entrapment efficiency of 30.2%. Furthermore, the value increased by 88.6% when chitosan was added as the second coating material (Azzarnia *et al*., 2008).

The load on proteins also affects the diffusion rate of alginate. Proteins with a high pH and positive overall charge have the potential to interact with negatively charged alginate polymers, inhibiting the gel's diffusion. Those with low pH can be released more quickly than alginate matrix (Gombotz and Wee, 1998). Bekasam extract has a pH of 4.45 and it was included in low-pH proteins to promote the release of peptides from the alginate tissue matrix, leading to low peptide levels in the microcapsules produced.

Lovastatin is a statin compound capable of reducing cholesterol levels because it can inhibit HMG-CoA reductase which is a key enzyme for cholesterol synthesis. The dose of Lovastatin used generally ranges from 20-60 mg/day and can reduce the levels of Low-Density Lipoprotein (LDL) by around 30-41%. The highest levels of Lovastatin in the microcapsules produced were 46.39 ppm or 46.39 mg/kg. These levels were still insufficient for the substitution of hypercholesterolemia drugs. However, as the Lovastatin in bekasam extract microcapsules is natural, it has less impact than the synthetic variant and can be consumed in the long term. This means the microcapsules can be used as an intermediate product for functional foods.

Entrapment efficiency of the peptides in bekasam extract microcapsules ranged from 17.31 to 27.69%. The ANOVA conducted at a 5% level showed no significant difference in entrapment efficiency. This is due to the low level of peptides that can be enclosed in the microcapsules, caused by peptides diffusing out of the gel network pores (Gombotz and Wee, 1998). Azzarnia *et al*. (2008) supported this result by reporting that the entrapment efficiency of aminopeptidase (PepN) from *Lactobacillus rhamnosus* S93 coated with alginate was 30.2%. However, the microencapsulation of PepN with a double coating of alginate and chitosan can increase entrapment efficiency by up to 88.6%. In another study, the encapsulation of stevia plants (*Stevia rebaudiana*) extracted using water and overlaid with alginate had an efficiency of 69.8%. This indicates that applying the same solvent during extraction and encapsulation can yield an efficiency above 50% (Arriola *et al*., 2016).

Entrapment efficiency of Lovastatin ranged from 59.08 - 72.76% and tends to elevate with increasing concentration of alginate as a coating material. Based on the ANOVA conducted at a 5% level, the difference in entrapment efficiency of Lovastatin was insignificant. This shows that the increase in alginate treatment concentration had no significant effect on the efficiency of microcapsules. Compared to the solvent diffusion emulsion method, which produced an entrapment efficiency of 42.3-51.2%, the microcapsules generated from ion gelation had a significantly higher value.

5. Conclusion

The results showed that microcapsules prepared with 1.25% alginate concentration yielded the highest entrapment efficiency for peptide and Lovastatin. Additionally, they exhibited the most desirable characteristics, such as being in good form.

Conflict of interest

The authors declare no conflict of interest.

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