Improvement of Stability and Antioxidant Activities by Using Phycocyanin - Chitosan Encapsulation Technique

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Abstract. Encapsulation is a coating process to improve the stability of bioactive compounds. Phycocyanin has been encapsulated using chitosan in microcapsules form to keep its stability. This study aims to determine the optimum conditions of the encapsulation process using the extrusion method thorough characterization of the physicochemical properties of the microcapsules, antioxidant activity test using DPPH, in vitro release performance and evaluate the storage stability against temperature. The results showed that Na-TPP provided better encapsulation performance than Na-citrate as crosslinker at 3% chitosan content. The study of antioxidant activity also showed that at 3% chitosan concentration resulted highest antioxidant activity. The morphological analysis of microcapsules showed that the beads have compact spherical shape with diameter range of 900-1000 µm. In vitro release study demonstrated a quick release in an acidic environment (SGF) during 2 hours experiments and slow release under alkaline conditions (SIF) for 8 hours experiments under constant temperature at 37°C. The encapsulation also showed that phycocyanin was more stable against temperature changes during storage.

Keywords: Encapsulation, stability, chitosan, phycocyanin

1. Introduction
Antioxidants are compounds that can counteract free radicals and an electron donating compound or a reductant. Antioxidants also are compounds that can inhibit the oxidation reaction, to scavenge free radicals and highly reactive molecules, which can inhibit cell damage [1]. Phycocyanin is a group of pigments that are bound to protein (biliprotein). Besides the potential as a natural dye, phycocyanin also known to have healing abilities, such as antioxidants [2] and anticancer [3]. But the application of phycocyanin very limited because it is vulnerable to light and temperature [4]. Phycocyanin can be damaged at temperatures above 30°C [5], and a solution of phycocyanin undergo color fading by 30% after 5 days of storage and become clear after 15 days at a temperature of 35°C [6]. Therefore, the need for technique to improve the phycocyanin stability but still maintain its antioxidant activity is required. Encapsulation is a process of coating a core material by using a specific encapsulation materials [7]. Encapsulation can be used to protect the drug from environmental influences (humidity, light, heat), as well as to control the release of antioxidants to the targeted medications [8]. In the process of encapsulation, it needs proper matrix with the core material to be encapsulated.
Chitosan has a structure similar to cellulose and capable of forming a gel that serves as the matrix in drug delivery [9]. Chitosan has many advantages such as not toxic, unstable during use, and can be used as a matrix to extract plant [10]. This study focuses on encapsulation of phycocyanin using chitosan to improve the stability of phycocyanin in order to retains its antioxidant activity.

2. Research methodology

2.1. Encapsulation by using Na-Citrate Crosslinker

Chitosan solution of 3% (w/v) was prepared by using 1% acetic acid. At the ratio of 1:1, the solution was stirred until homogeneous and dripped slowly through a 23G syringe to Na-Citrate 4% (w/v) crosslinker solution. After 45 min, the encapsulated product filtered and rinsed with distilled water. The rinse water and crosslinker solution were used to determine the encapsulation efficiency [11] and the load of encapsulation [12]. Do the procedure for other Na-citrate crosslinker concentration.

\[
EE = \frac{\text{mass of phycocyanin} - \text{mass of uncoated phycocyanin}}{\text{mass of phycocyanin}} \times 100\% \quad (1)
\]

\[
\text{Load} = \frac{\text{phycocyanin mass in the encapsulation}}{\text{mass of encapsulated product}} \times 100\% \quad (2)
\]

Determination of mass loss and phycocyanin in encapsulation (% Load) using a UV-Vis Spectrophotometer [11].

2.2. IR analysis

The interaction of physical and chemical bonds in the encapsulation process was observed using Fourier Transform Infrared Spectroscopy (FTIR) on each sample.

2.3. Determination of antioxidant activity

Each encapsulated phycocyanin was varied at concentration of 40-800 mg/mL in distilled water. Each solution was pipeted at 2 mL and added to 2 mL DPPH 50 μM. The mixture was homogenized and incubated for 30 minutes without being exposed to the light [13]. The pure phycocyanin was treated with the same procedure and used as a positive control. The percentage of inhibition was calculated according to equation 3:

\[
\% \text{ Inhibition} = \frac{A_o - A_s}{A_o} \times 100\% \quad (3)
\]

where:

A_0 = Absorbance of blank and A_s = Absorbance of samples

% Inhibition was used to determine the IC50 (anti-oxidant activity). The best antioxidant was used to determine the stability and the release test.

2.4. Phycocyanin release test

A total of 50 mg of encapsulated phycocyanin was added to 50 mL of simulated gastric fluid (SGF). The pH was set constant at 1.2 by using HCl buffer. The encapsulated phycocyanin was kept for 2 hours in acid solution before it was transferred to simulated intestinal fluid (SIF) solution. The SIF solution was prepared by using phosphate buffer at pH 7.4. The mixture was kept for 8 hours under stirring at 100 rpm at 37°C. The measurements was done by taking 4 mL solution every hour and analyzed by using UV-Vis spectrophotometer to determine the concentration of released phycocyanin.
2.5. Determination of product stability

The stability test was performed by exposing the beads to various temperature. 25 mg of sample was kept in a vial bottle at a temperature of 25, 35, 45, and 55°C with no light condition [14]. The observations were made every day during 3 days of experiment.

3. Results and Discussion

3.1. Effect of Na-Citrate and Na-TPP Crosslinkers

The encapsulation of phycocyanin was conducted by using Na-citrate and Na-TPP crosslinkers. Figure 1 shows that the beads of encapsulation by using Na-citrate crosslinker have irregular shape and softer texture. In other hands, Na-TPP crosslinker gives more uniform shape and hard texture. The encapsulation process of citric crosslinker is faster than the linking process by Na-TPP, but beads from Na-TPP crosslinker has higher mechanical properties due to stronger ionic bonds between chitosan and tripolyphosphate. In this case the charge density of tripolyphosphate is the main affect for this mechanical properties [15]. The encapsulation efficiency (EE) of both cross linkers are 40% and 60.9% for citrate and tripolyphosphate, respectively (Table 1).

At variation of chitosan concentration, the encapsulation efficiency and load show a positive correlation. This result is in agreement with Yan [14] who reported that higher encapsulation efficiency was achieved at higher encapsulation concentration. Table 1 also shows that the bead sizes are influenced by chitosan concentration, and this is supported by Goncalves [16] who stated that the size of encapsulation particles was in the range of 1-1000 µm [8].

| Encapsulation | EE (%) | LOAD (%) | Shape                  | Size (µm) |
|---------------|--------|----------|------------------------|-----------|
| Chitosan 2%   | 51.8   | 16.9     | Spherical, Uniform     | 987.8     |
| Chitosan 2.5% | 56.6   | 17.5     | Spherical, Uniform     | 902.4     |
| Chitosan 3%   | 60.9   | 22.1     | Spherical, Uniform     | 1000      |

3.2. FTIR analysis

The FTIR analysis was aimed to determine the presence of functional groups in the encapsulated phycocyanin [17]. Figure 2 shows the similarities peak between phycocyanin and chitosan at wave numbers between 3200-3500 cm⁻¹ which is identified as N-H bonds. Peak at a wavelength of 2850-3000 cm⁻¹ indicates a C-H bond and peak at a wavelength of 1000-1150 cm⁻¹ indicates the presence of C-O bond. At wavelength of 1600-1800 cm⁻¹ shows the C = O bond wherein the spectra peak of phycocyanin appears at wavelength of 1550-1600 cm⁻¹. At this wavelength, there was no evidence of C = C bonds meaning that there are no presence of chitosan. A shifted peak at wavelength of 1000-1150 cm⁻¹ into 1200-1250 cm⁻¹ showed the presence of electrostatic interaction between chitosan and phycocyanin. FTIR analysis also showed that no new peaks appeared which indicates that no new bonds were formed during encapsulation process and only physical interactions i.e. electrostatic interactions affecting the encapsulation [18].
Figure 1. Results of encapsulation using: (a) Na-TPP, (b) Na-Citrate, (c, d) Encapsulation 2%, (e, f) Encapsulation 2.5%, and (g, h) Encapsulation 3%

Figure 2. Results of FTIR Analysis: (a) Chitosan, (b) Phycocyanin, (c) Encapsulation of 3%, (d) Encapsulation of 2.5%, and (e) Encapsulation 2%
3.3. Antioxidant activity test

Testing antioxidant activity was determined through DPPH method and measured by using UV-Vis spectrophotometer. Measurements were carried out to determine the absorbance of DPPH remaining after the sample was added. The absorbance of DPPH will be used to determine the percentage of inhibition of free radical (% inhibition) and the result is shown in Table 2.

| No | Sample                  | IC50 (ppm) |
|----|-------------------------|------------|
| 1  | Encapsulation 2%        | 978.87     |
| 2  | Encapsulation 2.5%      | 899.25     |
| 3  | Encapsulation 3%        | 639.54     |
| 4  | Phycocyanin (control)   | 97.44      |

According to the Table 2, the IC50 of pure phycocyanin as a control is 97.44 ppm and the highest antioxidant activity was achieved at at 3% encapsulation (IC50 = 693.44 ppm). This experiment also shows that encapsulation will affect the stability of phycocyanin.

3.4. Phycocyanin Release Test in Encapsulation

Figure 3 shows release of phycocyanin in the simulated stomach and intestinal. In SGF, the quick release was found between 1 to 2 hours and could achieve 53% release. The release begins with the formation of swelling in the first hour, and continued by formation of gel in 1-2 hours later which resulted in quick release of phycocyanin. In another hand, the testing in SIF during 3-10 hours experiments, the slow release was found and only increased 4.5%. In SIF, the hardening process occurs and the bead does not swell perfectly. This is because chitosan has less protonated amine groups, and therefore causing low-level release [20].

3.5. Encapsulation stability against temperature exposure

Determination of stability used the beads with 3% chitosan concentration. According to Burgess and Hickey [8], Encapsulation can be used to protect the drug from environmental influences (humidity, light, heat, and oxidation). Figure 4 shows that the encapsulated phycocyanin is more stable against temperature than phycocyanin without encapsulation treatment. In this case, the encapsulation process may formed a semipermeable membrane walls[21]. The thin membrane walls can protect the high temperatures of the environment. This is supported by the half-life constants in the degradation kinetics (Table 3).
The constant degradation has a proportional relationship to the temperature [22,23]. The value of $k$ increases by increasing temperature, indicating that higher temperature will stimulate faster degradation rate of phycocyanin. At temperature of 25°C, the smallest $k$ value was observed than $k$ at temperature of 35-55°C.

![Figure 4. Stability of the encapsulated phycocyanin](image)

Table 3. Half-life time of phycocyanin

| Sample                  | Constant Degradation (k) | Half-life (t_{1/2}) |
|-------------------------|--------------------------|---------------------|
| Phycocyanin at 25°C     | 1.405x10^{-3}            | 493,2384            |
| Encapsulation at 25°C   | 0.750x10^{-3}            | 924,0000            |
| Phycocyanin at 35°C     | 3.500x10^{-3}            | 197,4350            |
| Encapsulation at 35°C   | 1.510x10^{-3}            | 458,9403            |
| Phycocyanin at 45°C     | 16.35x10^{-3}            | 42,38530            |
| Encapsulation at 45°C   | 7.700x10^{-3}            | 90,00000            |
| Phycocyanin at 55°C     | 32.850x10^{-3}           | 21,09580            |
| Encapsulation at 55°C   | 13.800x10^{-3}           | 50,21730            |

4. Conclusion
Phycocyanin encapsulation using the extrusion method obtained Na-TPP as the best crosslinker. The morphology encapsulation of Na-TPP based encapsulation was round, hard and uniform, with a value of encapsulation efficiency was 60.9778% and the maximum load was 22.19%. FTIR analysis showed that no new bond formation during encapsulation process. The highest antioxidant activity was shown at chitosan concentration of 3% with IC50 of 639 ppm. In vitro study showed that 53% of encapsulated phycocyanin could be released while a slow release has been observed at simulated intestinal pH.

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