Improvement stability of phycocyanin from *Spirulina platensis* encapsulated by water soluble chitosan nanoparticles

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**Abstract.** *Spirulina platensis* is a microalga containing protein and other nutrients, also pigments which has several advantages in food and pharmaceuticals. Phycocyanin is a blue pigment from *Spirulina* sp. composed by α and β polypeptide with phycocyanobilin. Phycocyanin can be extracted by ultra-sonication method. However, this pigment can be easily degraded because of its instability caused by pH, temperature, and light. One of methods for maintaining the stability is encapsulation by coating material. Water soluble chitosan (WSC) is known as a low-molecular weight and low toxic coating material dissolve in water. The purposes of this study were to determine phycocyanin extraction with different time and evaluate the stability of phycocyanin in WSC nanoparticles. Phycocyanin-WSC nanoparticles were prepared with three different ratio of WSC to phycocyanin i.e 1:1, 1:0.75, 1:0.5 (w/w). Extraction phycocyanin using ultrasonicator for 15 min showed the highest concentration (1.28 mg/mL) and yield (2.56%). The smallest size and narrow polydispersities of phycocyanin-WSC nanoparticles was achieved with ratio of WSC to phycocyanin of 1:0.75 (w/w). Encapsulation of phycocyanin in WSC nanoparticle enhanced the stability at 50 °C for 90 min. Encapsulation can be used for any supplement application.

**Keywords:** Chitosan; phycocyanin; *Spirulina platensis*; ultrasonication.

1. Introduction

*Spirulina platensis* is a microalga containing of 55-70% protein, 15-25% carbohydrate, 18% essential fatty acids, vitamins, minerals, pigments such as chlorophyll, carotene, xantophyll, and phycocyanin which has several advantages in food and medicine sector [1]. Phycocyanin is the most pigment in *S. platensis* at 6.7-11.7% of the total protein 50-70% which has a brilliant blue colour and water soluble. It composed of α and β polypeptide subunits which has molecular weight of 12-24 kDa on α subunit and 15-22 kDa on β subunit [2]. Phycocyanin can be extracted by ultrasonication with its high frequency vibration, thus damages the cell wall and release the compounds [3].

Phycocyanin also has been used as a colorant for health drink, beverages, confectionary, and cosmetics [4]. On the other hand, it has many biology activities, such as anticancer, anti-inflammatory, antibacterial, antioxidant, and nerve cell protector [5] and anti-*Plasmodium falciparum* 3D7 [6]. However, the application is limited due to its sensitivity. This pigment is only stable on pH 5.5-6, dark
condition, low humidity, and low temperature storage. Phycocyanin will be degraded at temperature stored of over 47°C [7]. Denaturation of phycocyanin is showed by losing its colour[8]. Our approach for overcome this problem is the encapsulation of phycocyanin in biodegradable polymer such as chitosan.

Chitosan is a natural polysaccharide consists of 50% N-glucosamine and N-acetyl-glucosamine which is derived from crustacean shells, such as crabs, shrimps, and lobsters [9]. It can be used in the pharmaceutical and medical fields because it has low toxic, biodegradable, biocompatible, antibacterial, antifungal, and anticancer properties [10]. However, chitosan has low slubility in a physiological solution caused the limiting of their application. Depolymerization of chitosan can be done to produce an oligochitosan and low molecular weight chitosan which can improve its solubility in neutral aqueous solution [11]. Some chitosan depolymerization methods include a combination H₂O₂ treatment with heating process, microwave, UV, and potassium nitrite [12]. Depolymerized chitosan is known as a water soluble chitosan (WSC). WSC nanoparticles is an encapsulation technique which prepared based on ionic gelation. WSC nanoparticles can be used as a nano-carrier system for protein delivery [13]. The purposes of this study were to determine the time of sonication in phycocyanin extraction and evaluate stability of phycocyanin in WSC nanoparticles. Improving stability of phycocyanin can increase its application in food and medicine sector, for instance as supplements.

2. Materials and Method

2.1. Materials
*Spirulina platensis* was obtained from Center of Brackish Water Aquaculture of Jepara-Indonesia and cultured at Laboratory of Biotechnology, Department of Aquatic Product Technology, IPB University. Commercial chitosan (DD 81%) was purchased from Bio Chitos factory, Bogor-Indonesia, Hydrochloric acid was purchased from Emsure, sodium tripolyphosphate and ammonium sulphate were purchased from Merck.

2.2. Methods

2.2.1. *Spirulina platensis* Cultivation
*S. platensis* was cultivated using organic media namely GA organic fertilizer (Premium). Before cultivation all the equipments and materials were sterilized using UV light for 45 minutes with fertilizer added into it after using UV. *S. platensis* seeds were added for 20% of the total volume of cultivation. The cultivation was conducted in indoor with continuous aeration and light intensity of 3250 lux (40 W), a temperature of 28°C and pH of 8.5-11. The measurement of OD values was done every two days using spectrophotometer at a wavelength of 670 nm to reach a value of 0.5. The biomass was harvested using nylon 500 mesh after OD values reached 0.5.

2.2.2. Extraction phycocyanin from *Spirulina platensis*
Phycocyanin extraction was referred to [14] with the modification. Phycocyanin was carried out by extracting of *S. platensis* wet biomass with sodium phosphate buffer, pH 7 (1:20, w/v) using ultrasonic (DSA) for 10, 15, and 20 min at temperature 4°C and frequency 42 kHz. Samples were then centrifuged at 10,000 g at 4°C for 20 min. Supernatant was precipitated with ammonium sulphate 50% and centrifuged at 4000 g at 4°C for 20 min. The resulting precipitate was dissolved in 3 mL of deionized water followed by dialysis (dialysis membrane bag, MWCO 12-14kDa) against running for two days. Phycocyanin obtained was measured on concentration, yield, and purification index. The concentration, yield, and purify index of phycocyanin were estimated using equations

\[
PC = \frac{(OD_{620} - 0.474(OD_{652}))}{5.34} \\
Yield = \frac{PC \times V}{DB}
\]
2.2.3 Depolymerization of chitosan

Chitosan was depolymerized using UV light for producing water-soluble chitosan (WSC) which refers to [15] with some modifications. Chitosan was dissolved in 1% hydrochloride acid (HCl) (1:9, w/v), then depolymerized using UV light for 30 and 60 min with three repetitions of each treatment. The sample was precipitated and neutralized using isopropyl alcohol (IPA), then filtered using nylon 40 mesh. The resulting natant was dried at room temperature. The solubility of WSC was analysed by dissolving in water. The deacetylation degree of WSC was measured using FTIR spectrometer following by [16] equation:

\[
Purify\ index = \frac{A_{620}}{A_{280}}
\]  

(3)

2.2.4 Encapsulation phycocyanin in water soluble chitosan nanoparticle

Encapsulation of phycocyanin was prepared by mixing phycocyanin and WSC solutions. Sodium tripolyphosphate was added respectively as a cross-linker. The WSC solution (1 mg/mL) was prepared by dissolving oligochitosan in distilled water, then stored at 4°C for a day to be fully hydrated. Then, 1 mL of phycocyanin solution in deionized water was added slowly to 1 mL of WSC (1 mg/mL) under stirring at 25°C. Sodium tripolyphosphate (2 mg / mL) was added 0.5 mL respectively, then adjust the pH to 7 with 1% HCl, and added 0.5 mL of PEG. The ratio of WSC to phycocyanin were 1: 1, 1:0.75, 1: 0.5 with abbreviation EPC1, EPC0.75, and EPC0.5, respectively. The particle size of phycocyanin loaded WSC nanoparticles was determined using a dynamic light scattering (Malvern Mastersizer range, Germany).

2.2.5 Thermal stability study of phycocyanin

Encapsulation stability was evaluated by heating up the sample with 50°C during 0, 15, 30, 60, 90 min. Encapsulate and free phycocyanin solution was diluted five times in water after heated. After that, scanning from 500 to 700 nm was carried out by UV-Vis spectrophotometer.

3. Result

3.1. Effect of sonication on the yield of phycocyanin from Spirulina platensis

Phycocyanin was extracted from S.platensis wet biomass using an ultrasonicator (42 kHz) with different time. Result on phycocyanin concentration, yield, and purify index is presented in Figure 1. The highest phycocyanin concentration, yield, and purify index was obtained by extraction time 15 min which 1.28 mg/mL, 2.56%, and 0.58, respectively. In addition, purify index was increased after precipitation from 0.58 to 1.33. This indicates that the samples were more purified after precipitation than extraction.

![Figure 1](image1.png)

Figure 1. Effect of time extraction on concentration, yield (a), and purify index (b) of phycocyanin.
3.2. Characteristic of water soluble chitosan

Figure 2 shows FTIR spectrum of chitosan and WSC. There are some functional groups differences between chitosan and WSC. Chitosan has β-1,4-glycosidic in 896 and 1152 nm\(^{-1}\) and O-H bond in 2879 nm\(^{-1}\), whereas WSC has alkyl group in 2926.34 nm\(^{-1}\). It is indicated that depolymerization made the changing of functional group between chitosan and WSC. According to Figure 2, chitosan had 81% deacetylation degree and WSC had 90% deacetylation degree. In addition, water soluble chitosan was dissolved well in neutral aqueous solution. This indicates that depolymerization of chitosan could increase deacetylation degree and improve solubility in water.

![Figure 2. Fourier Transform Infrared (FTIR) Spectrum of chitosan and WSC.](image)

3.3. Characteristics of phycocyanin in water soluble chitosan nanoparticles

The ratio between WSC and phycocyanin were 1:0.5, 1:0.75, and 1:1 with abbreviation EPC1, EPC0.75, EPC0.5, respectively using ionic gelation method. Therefore, the effect of those ratio on particle size and polydispersities index of phycocyanin-WSC nanoparticle were investigated. Variations of phycocyanin concentration at a constant WSC concentrations of 1 mg/mL was evaluated.

Figure 3 shows the intensity and size between three ratio. The particle size decreased as PC concentration increased from 0.5 to 0.75 mg/mL, then increased as PC concentration increased from 0.75 to 1 mg/mL. The ratio between WSC to phycocyanin 1:0.75 (EPC0.75) showed that it had the smallest size (457.3±35.2 nm). The polydispersities index decreased from 0.423 to 0.263 as PC concentration increased from 0.5 to 0.75 mg/mL, then increased from 0.263 to 0.409 as increasing PC concentration from 0.75 to 1 mg/mL. Therefore, we chose this ratio to the next stability analyzation using 50\(^\circ\)C temperature with different time between 0-90 min. Figure 4 shows absorbance differences between free phycocyanin and phycocyanin-WSC nanoparticles.

Figure 4 shows absorbance differences between free phycocyanin and phycocyanin-WSC nanoparticles. As expected, the absorbance intensity of phycocyanin was slightly decreased after encapsulated in WSC nanoparticles. The absorbance maxima of phycocyanin in aqueous solution was 620 nm, and absorbance of phycocyanin in WSC nanoparticles was slightly red-shifted to ~625 nm. This suggested that the phycocyanin was encapsulated in WSC nanoparticles.
3.4. Stability of phycocyanin-WSC nanoparticle and free phycocyanin

Phycocyanin-WSC nanoparticle and free phycocyanin absorbance were measured using UV-Vis spectrophotometry. The samples were heated at 50°C during 0-90 min aiming to see how the differences between them. Absorbance spectrum can be seen in Figure 5.

Free phycocyanin (PC) has higher absorbance value than phycocyanin-WSC nanoparticle that presented in Figure 4. Free phycocyanin hasn’t stable yet at 50°C during 90 minutes signed by deduction of absorbance value every minutes spent in heated temperature presented in Figure 5b than
phycocyanin-WSC nanoparticle that showed in Figure 5a. It was indicated that more minutes to spend in heating temperature damaged the purified phycocyanin.

Figure 5. Absorbance spectrum between (a) phycocyanin-WSC nanoparticles (EPC) and (b) free PC during heated at 50°C for 90 min.

4. Discussions
In this study, phycocyanin was extracted using ultrasonificator for 10, 15, and 20 minutes at 42 kHz frequency. It showed that extraction during 15 minutes had the highest values of concentration, yield, and purify index which were 1.28 mg/mL, 2.56%, and 0.58. Phycocyanin extraction significantly was more effective in frequency of 42 kHz and optimum yield was obtained in 20 minutes extraction [14]. There were phenomena may involve during extraction which were diffusion of solvent through cell walls and after the cells broke, the cell contents will be washed out.

Purify index values after extraction was 0.58 and after precipitation was 1.3319. Purify index of crude extract phycocyanin was 0.97 to 1.43 after precipitation [17]. The use of ammonium sulfate as the purification step exhibited high efficiency in removing of protein contamination. Purify index was more than 0.7 indicated that it was food grade material, if more than 3.9, it was classified as reactive grade, and if more than 4, it was classified as analytical grade [18].

Chitosan had 81% deacetylation degree, whereas oligochitosan had 90% deacetylation degree and lower molecular weight than chitosan. According to the Biopolymer Protein Standard (1994), they had fulfilled the standard quality criteria (≥ 70%). Depolymerization process helped accelerate the main chain β-glycosidic termination of chitosan and obtained lower molecular weight [19]. It showed by disappearance of peak 896 nm and 1152 nm in oligochitosan which represent β-1,4 glycosidic.

Nanoparticles are solid drug conduction with 1-1000 nm average diameter. Lowest size was showed in EPC0.75 which size was 457.3±35.2 nm with highest intensity. Nanoparticles size ranges from 20 to 500 nm consist of numerous distinct entitles, each with characteristic features which enables for their application. Polydispersity is the ratio of standard deviation to mean droplet size and indicates uniformity of droplet size. The higher polydispersity index, then the lower uniformity of droplet size in formulation [20]. The polydispersity index of the optimized formulation in EPC0.75 found to be 0.263±0.05 indicating homogenous distribution of particles in the formulation.

Absorbance spectrum showed differences between the encapsulation and free phycocyanin. The longer time phycocyanin under 50°C, then the absorbance spectrum would be decreased and phycocyanin would be losing its color. The degradation rate increased dramatically between 47 to 64°C and it precipitated out of solution, then also losing its color [4]. Different from the encapsulation absorbance that showed stability and there weren’t any significant degradation as showed on it. The encapsulation conferred enhanced protection of free phycocyanin against heated temperature compared with free phycocyanin.
5. Conclusions
Extraction-assisted with ultra-sonication for 15 min produced the highest yield, concentration, and purity index of phycocyanin from *Spirulina platensis*. The smallest size and narrow polydispersities of phycocyanin-WSC nanoparticles was achieved with ratio of WSC to phycocyanin of 1:0.75 (w/w). Encapsulation of phycocyanin in WSC nanoparticles enhanced the stability at 50°C for 90 min.

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