Characterization of phycocyanin from Spirulina fusiformis and its thermal stability

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Abstract. Microalgae have some pigments which are potential to be used as functional colorant for foods and cosmetics industries. Here, Spirulina fusiformis is one of the major sources of a blue pigment phycocyanin which has commercial and biotechnological value for biomedical research, as well as a natural colorant for food and cosmetic industries. This study aims at investigating the characteristic of isolated C-phycocyanin from Spirulina fusiformis and its stability under different temperature. A food grade phycocyanin with purity of 2.0 (A620/A280) was obtained successfully from Spirulina fusiformis cultured in seawater-based modified medium and purified by ammonium sulfate precipitation. No significant changes were observed in the spectrum absorbance of C-phycocyanin at temperature up to 60°C over 30 minutes of incubation, and was observed to significantly denaturation at 70°C and higher temperature. Incubation of the pigment at relatively high temperature resulted in a decrease in the spectrum intensities of the C-phycocyanin in a temperature-dependent manner. Taken together, our results suggest that the optimal condition for preserving the stability of food grade phycocyanin isolated from Spirulina fusiformis is taking it at low temperature.

1. Introduction
Presently, the utilization of synthetic colorants are quite common in food cosmetic, nutraceuticals and pharmaceutical industries [1]. However, evidences linked the synthetic colorants to a number of potential health problem, most notably certain types of cancer and hyperactivity [2]. Therefore exploration of microalgae pigments as promising reactor to produce natural colorants become an attractive options [3]. Exploitation varieties of colorants from microalgae has been carried out for replacing the synthetic colorants by their natural counterparts. There are some pigments that have been utilizing as natural colorants, such as astaxanthin (red), β-carotene (orange), lutein (yellow), chlorophyll (green), and phycocyanobilin (blue) [4]. Among them, blue color found rarely commercial available. Spirulina, a representative of microalgae, has well known as an excellent source of blue pigment phycocyanin (further referred as C-phycocynin) [5-6]. Therefore, exploitation of natural blue colorant from Spirulina group is recently a promising options.

C-phycocyanin is protein complex of phycobilisome, an organelle responsible for photosynthesis in microalgae [7]. C-phycocyanin from Spirulina plantesis has been used as a cosmetic and food colorant [1]. The use of C-phycocyanin has increased in pharmaceutical industries due to its antioxidant
activity, anti-inflammatory, stimulant for antibody production [8], and anticancer activity [9-11]. Due to its unique fluorescent properties, C-phycoerythrin is utilized as a tracer in numerous bioassay [7]. Blue pigment C-phycoerythrin has more benefits to be used as natural blue colorant since it has aesthetic value due to their potential to give a bright blue color to the product and providing functional activity for health as well [1]. However, the application of C-phycoerythrin in food and other products is limited due to the sensitivity of thus pigment to heat treatment which results in protein degradation and vanishing of the blue pigment [12]. Those processes are irreversible [13] and prevent the use of C-phycoerythrin pigment in the products that require high temperature in their products processes [14]. The pure C-phycoerythrin has been obtained from *Spirulina fusiformis* using the ammonium sulfate precipitation [15]. Here, we describe the thermal stability of C-phycoerythrin from *Spirulina fusiformis* for the first time, based on the principles of the procedure for C-phycoerythrin from *Spirulina plantesis* developed by Martelli et al. [14]. Since each species of *Spirulina* contains unique composition of phycobiliprotein [7].

2. Methods

2.1. Raw material

*Spirulina fusiformis* (from Biology Department, Universitas Padjadjaran), Sodium hydrogen phosphate (NaH$_2$PO$_4$, tech grade, Bratachem), Sodium hydroxide (NaOH, p.a., CV Menara Agung Abadi), Ammonium sulfate ((NH$_4$)$_2$SO$_4$, tech grade, Bratachem), Sodium chloride (NaCl, p.a., Bratachem) and deionized water. All tech grade raw materials were used after further purification.

2.2. Extraction and purification of C-phycoerythrin

Extraction was carried out based on the principle and procedure developed by Minkova et al. [15] and Martelli et al. [14] with some modification. Extraction was conducted by mixing biomass-solvent ratio at 0.02 g mL$^{-1}$. Firstly, the fresh biomass (2 g) was rinsed with extraction buffer (0.1M sodium phosphate buffer PH 7.0) and re-suspended in 100 mL of the same buffer then continued with sonication for cells disruption. The extraction was continued by incubating the mixture for 15 hours for completely recovery of C-phycoerythrin. After that, the sample was collected by centrifugation at 1050xg for 15 min. The purification of C-phycoerythrin was determined by ammonium sulfate precipitation. Here, the precipitate obtained from the extraction steps were separated from the supernatant. The supernatant was saturated to 25% with ammonium sulfate and centrifuged at 1050 x g for 15 min. The blue eluate was kept and saturated to 50% with ammonium sulfate and centrifuged at 1050 x g for 15 min. The processes were subjected to another two rounds of ammonium sulfate precipitation. Each of the blue precipitate from each rounds were dissolved in extraction buffer and kept for analysis. C-phycoerythrin concentration ($C_{PC}$) was calculated using Eq. (1) [12].

$$C_{PC} = \frac{(OD_{620} - 0.474OD_{652})}{5.43}$$  \hspace{1cm} (1)

Where $C_{PC}$ is the C-phycoerythrin concentration in mg mL$^{-1}$, $OD_{620}$ is the optical density of the sample at 620 nm, and $OD_{652}$ is the optical density at 652 nm.

The purity of the C-phycoerythrin fraction was measured spectrophotometrically by the ratio of the absorbance at 620 nm divided by 280 nm by the procedure developed by Abalde [16] modified by us, using Eq. (2).

$$EP = \frac{OD_{620}}{OD_{280}}$$  \hspace{1cm} (2)

Where EP is the extract purity, $OD_{620}$ is the optical density of the sample at 620 nm, and $OD_{280}$ is the optical density at 280 nm.
2.3. Thermal stability of C-phycocyanin
The thermal stability of C-phycocyanin from *Spirulina fusiformis* was studied by incubating the samples at different temperature at variation 25°C, 50°C, 60°C, and 80°C in a water bath. Samples were taken at 30 min for C-phycocyanin thermal analysis. The stability of the C-phycocyanin as analyzed by spectrum analysis of the C-phycocyanin at range 300-700 nm.

3. Results and Discussion

3.1. Characteristic of C-phycocyanin *Spirulina fusiformis*
The characteristics of C-phycocyanin were determined by evaluating the absorption spectrum of the samples as can be seen in Figure 1.

![Figure 1](image)

*Figure 1.* Absorption spectra of crude extract and pure C-phycocyanin obtained by ammonium precipitation that were recorded from 300-800 nm. All spectra were measured at room temperature

A strong absorption maximum occurred at 620 nm, and other two smaller ones appeared, respectively, at 348 nm and 268 nm, for the C-phycocyanin extract purified step 1 to 3. Absorption spectrum of the crude extract and purified extract of C-phycocyanin from each steps showed that *Spirulina fusiformis* contain C-phycocyanin (peak at 620 nm). No peak allophycocyanin was observed at the spectrum absorption of the purified C-phycocyanin (particularly showed by the shoulder at 650 nm). This results fitted to previous findings for the absorbance maximum of four main classes of phycobiliprotein: allophycocyanin $\lambda_{\text{Amax}}$ 650–655 nm, phycocyanins $\lambda_{\text{Amax}}$ 615–640 nm, phycoerythrin $\lambda_{\text{Amax}}$ 565–575 nm and phycoerythrocyanin 577 nm whereas they emit light at 660 nm, 637 nm, 577 nm and 607 nm respectively [17].

The sequential treatment of the crude extract with ammonium sulfate increased the C-phycocyanin purity as summarized in Table 1.
**Table 1.** Data of purification of C-phyocyanin from *Spirulina fusiformis*

| Step of purification | A\textsubscript{280} | A\textsubscript{652} | A\textsubslash A\textsubscript{280} Ratio (EP) | Concentration of C-phyocyanin (C\textsubscript{PC}) (mg/mL) |
|----------------------|-----------------|-----------------|----------------|-----------------|
| Crude extract        | 1.05            | 0.27            | 0.55            | 0.079           |
| Step 1               | 0.29            | 0.14            | 0.40            | 1.36            |
| Step 2               | 0.24            | 0.13            | 0.41            | 0.062           |
| Step 3               | 0.17            | 0.07            | 0.35            | 0.064           |

Pure C-phyocyanin extract from *Spirulina fusiformis* was performed with the 25% saturation of the crude extract with ammonium sulfate to eliminate the allophyocyanin content and continued the precipitation with the 50% saturation with ammonium sulfate to completely remove the C-phyocyanin-contaminating protein. The $A_{620}/A_{280}$ ratio from each steps of purification are showing an increase in C-phyocyanin purity. The sequential precipitation with ammonium sulfate increase the purity index from 0.53 to 1.36 which considered to be food grade [18].

### 3.2. Thermal stability of C-phyocyanin *Spirulina fusiformis*

The effect of temperatures on C-phyocyanin stability was evaluated by incubating the pure extract of C-phyocyanin obtained from each steps at variation temperature of 25°C, 50°C, 60°C, 70°C, and 80°C (Figure 2.). This study demonstrated that C-phyocyanin has the potential to maintain its spectrum intensity up to 60°C, but started to decrease at 70°C and higher temperature, indicating the thermal labile of the pigment during heating. This results supported earlier study on C-phyocyanin degradation at a higher temperature ranging from 26°C to 74°C [12]. Given the high temperature, has reported cause the protein denaturation and have a significant impact on the color maintenance of C-phyocyanin [19]. The alteration of C-phyocyanin spectrum at 620 nm designate the changes of structure conformation of C-phyocynin [13].

![Figure 2. The effect of temperature increase on C-phyocyanin spectrum](image-url)

Naturally, one phyocyanin ($\alpha\beta$)$_3$ often carry nine phyecyanobilins and six phycourobilins [20]. Temperature increase causes the unfolding reaction on bilin protein-C-phyocyanin. Native structure of phyocyanin contain bilin with aliphatic chain. The absence of peak absorbance of C-phyocyanin
spectrum at 620 nm, and the existance of peak absorbances at range 325-380 reveal the alteration of bilin structure orientation from lineer to cyclic form which impacted to the alteration of 3D structure of C-phycocyanin [21].

4. Conclusion
Extraction is one of the most important processes to reach high purity through precipitation. The sequential purifications by saturating the extracts with ammonium sulfate 50% spotted the strong peak absorbance at 620 nm, indicating the C-phycocyanin content in the Spirulina fusiformis. The last supernatant obtained from the third steps provide a pure C-phycocyanin at ratio absorbance of A_{620}/A_{280} is 2.06 which classified as food grade category. The temperature of 70°C and higher, therefore, reduce the thermal stability of the C-phycocyanin for applications in the high temperature-required industries.

5. References
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