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# Large-scale recovery of C-phycocyanin from *Spirulina platensis* using expanded bed adsorption chromatography

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**Abstract**

C-phycocyanin was purified on a large scale by a combination of expanded bed adsorption, anion-exchange chromatography and hydroxyapatite chromatography from inferior *Spirulina platensis* that cannot be used for human consumption. First, phycobiliproteins were extracted by a simple, scaleable method and then were recovered by Phenyl-Sepharose chromatography in an expanded bed column. The purity (the $A_{620}/A_{280}$ ratio) of C-phycocyanin isolated with STREAMLINE™ column was up to 2.87, and the yield was as high as 31 mg/g of dried *S. platensis*. After the first step, we used conventional anion-exchange chromatography for the purification steps, with a yield of 7.7 mg/g of dried *S. platensis* at a purity greater than 3.2 and with an $A_{620}/A_{650}$ index higher than 5.0. The fractions from anion-exchange chromatography with a level of purity that did not conform to the above standard were subjected to hydroxyapatite chromatography, with a C-PC yield of 4.45 mg/g of dried *S. platensis* with a purity greater than 3.2. The protein from both purification methods showed one absolute absorption peak at 620 nm and a fluorescence maximum at 650 nm, which is consistent with the typical spectrum of C-phycocyanin. SDS-PAGE gave two bands corresponding to 21 and 18 kDa. In-gel digestion and LC-ESI-MS showed that the protein is C-phycocyanin.
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*Keywords:* *Spirulina platensis*; Purification; C-phycocyanin; STREAMLINE™ column; Anion-exchange chromatography; Hydroxyapatite chromatography

## 1. Introduction

Phycobilisomes are a type of large supramolecular aggregate that attach to the thylakoid membrane of some blue-green and red algae, and have a function in light catching and energy migration [1–4]. These antenna complexes are composed of phycobiliproteins (a family of hydrophilic, brilliantly colored, and stable fluorescent pigment proteins), which include phycoerythrin (PE), phycocyanin (PC), allophycocyanin (APC) and phycoerythrocyanin (PEC). These proteins are water-soluble and have similar three dimensional structures. The basic building block is composed of an (αβ)-monomer. Three (αβ)-monomers are arranged around a 3-fold symmetry axis and form an $(\alpha\beta)_3$-trimer. Each subunit contains one or more phycobilin chromophores (phycobilins). All the phycobilin chromophores bind to specific cysteines in the polypeptide chains by thioether bonds [5,6]. Usually, a PC solution appears dark cobalt blue, PE bright pink, and APC brighter aqua blue, with an absorption maximum between 610 and 620 nm, 540 and 570 nm, and 650 and 655 nm, respectively [5,7].

Because of their excellent spectroscopic property [5,6], stability, high absorption coefficient and high quantum yield, a wide range of promising applications of phycobiliproteins in biomedical research, diagnostics and therapeutics has become possible [1,8–10]. The commonest use of these proteins is as fluorescent tags of cells and macromolecules in highly sensitive fluorescent techniques [5,6]. They have already been widely used as a light-sensitive agent in tumor photodynamic therapy, and are potential substitutions for Photofrin, which is purified from animal blood [11]. Recent studies have also shown their applicability in immunomodulating and anticarcinogenic activities [1]. Phycobiliproteins are attractive because they are not harmful to humans if applied externally or ingested. Another interesting application of the biliproteins is their use as natural

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dyes in food and cosmetics, replacing synthetic dyes, which are generally toxic or unsafe [1].

Phycobiliproteins have wide spread applications and great economic potential. Nevertheless, the use of this biliprotein has been limited by the tedious preparation of large amounts of the purified protein. Conventional methods for purification of phycobiliproteins involve two steps: pretreatment of the sample to liberate the intracellular material, making a crude extract ready for an isolation step in which the phycobiliproteins are separated using conventional processes [12–14]. These schemes involve a combination of several techniques, such as centrifugation, precipitation in ammonium sulfate, ion-exchange chromatography, gel-filtration and chromatography on hydroxyapatite [1,5,7,9,15–17]. These methods are generally time-consuming, complex, and difficult to scale up [17].

*Spirulina* is a genus of filamentous cyanobacteria that includes 13 species of simple, single-celled alga that thrive in warm, alkaline fresh-water bodies, and represent a rich source of protein. Dried spirulina contains up to 70% protein by weight [18], which is comparable to the levels in high-protein vegetables, such as soybeans (in which proteins represent 35% of the dry mass). *Spirulina platensis* has been the subject of much attention and has been developed as a nutritious food for humans. The gross mass of *S. platensis* in China, estimated to be about 1000 tons, represents one-third of the world's total amount. However, large-scale cultivation of this alga is usually influenced by some biotic and abiotic factors, such as bacteria, protozoa and heavy-metal pollution, which result in large amount of inferior *spirulina* [19] that cannot be used as human food according to the criterion of 'Food grade spirulina powder' in China [20]. Considering this fact, we used those inferior algae to isolate C-phycocyanin, which has a great potential for use as fluorescent tags, food additives, cosmetics additives, etc.

In this study, we carried out the purification of C-phycocyanin from *S. platensis*, taking advantage of the adsorption properties of Phenyl-Sepharose in expanded bed mode. This method reduces the amount of protein pretreatment required, and makes the capture of target proteins from cell homogenate feedstock possible [21–23], thus achieving a higher product recovery in a shorter time. In the first step, phycocyanins were recovered by the adsorbent while the cell debris, particulates and contaminants in the extract pass through with the upward flow. Next, the eluates from the STREAMLINE$^{TM}$ column were loaded into an anion-exchange column or a hydroxyapatite column, where C-PC and APC were separated effectively. The purity of the C-PC was monitored at each stage of purification by SDS-PAGE and absorption spectroscopy.

## 2. Materials and methods

### 2.1. Materials

*S. platensis* was provided by Shandong Lonsun Bioengineering Company (Shandong, China) as dried powder.

Phenyl-Sepharose (STREAMLINE$^{TM}$ Phenyl), Q-Sepharose and the STREAMLINE$^{TM}$ column (STREAMLINE$^{TM}$ 25, 100 cm × 2.5 cm) were purchased from Amersham Biosciences Corp. (New Jersey, USA).

All solutions mentioned were made with Milli-Q-prepared water and contained 0.02% (w/v) sodium azide unless specified otherwise.

### 2.2. Methods

#### 2.2.1. The preparation of hydroxyapatite

The preparation of hydroxyapatite was as described [24]:

500 ml of $CaCl_2$ (2 M) and 550 ml of $KH_2PO_4$ (2 M) were mixed at a rate of about 5 ml/min. The mixture was stirred with a magnetic stirrer bar and then KOH (2 M) was added to control the pH. When the pH value decreased, more KOH was added. This step was repeated until the pH was maintained at 9. Finally, sodium phosphate buffer (1.00 mM, pH 7.0) was used to rinse the freshly prepared hydroxyapatite repeatedly to remove the fine particles. The hydroxyapatite precipitation was stored at room temperature for use later.

#### 2.2.2. Extraction of phycobiliproteins

A 4 g sample of *S. platensis* dried powder was added to 120 ml of 0.50 M ammonium sulfate. The mixture was kept in the dark at 4 °C for 12 h to allow lysis of the cells due to the hypotonicity of the mixture. The mixture was then centrifuged at 10,000 × *g* at 4 °C for 10 min. The blue supernatant was recovered and 120 ml of 0.50 M ammonium sulfate was added to the precipitate and left in the dark for another 12 h. The crude extract was collected by centrifugation and the above steps were repeated. All the supernatants were combined. The volume was measured and the quantity of C-PC in the supernatant was determined according to the following equation [25]:

$$\text{C-PC (mg mL}^{-1}) = \frac{A_{615\text{nm}} - A_{730\text{nm}} - 0.47(A_{652\text{nm}} - A_{730\text{nm}})}{5.34}$$

The complete purification protocol is shown in Fig. 1.

#### 2.2.3. Elution of S. platensis C-PC from STREAMLINE$^{TM}$ column with $(NH_4)_2SO_4$ solution

A STREAMLINE$^{TM}$ column loaded with 50 ml of the Phenyl-Sepharose as expanded adsorbent was prepared and equilibrated with 0.50 M ammonium sulfate. The crude extract containing 0.50 M ammonium sulfate was pumped upwardly into the column at room temperature. The flow-rate was adjusted to maintain the degree of expansion ($H/H_0$) as high as 2. The phycobiliproteins in the extract were captured by the adsorbent, while the cell debris, particulates and most contaminant proteins were eluted by the upward flow. Afterwards, the adsorbent with the captured phycobiliproteins was washed upwardly with 0.50 M ammonium sulfate to remove loosely bound and unbound proteins until the effluent from the column was clear. After washing in the expanded mode, the upward flow was stopped and the bed was allowed to settle in the column. The adaptor of the STREAMLINE$^{TM}$ column was moved down towards the surface of the settled bed. Then the bound phycocyanins were recovered using 0.20, 0.10, 0.05 M of ammonium sulfate solutions and distilled water successively, at a rate of

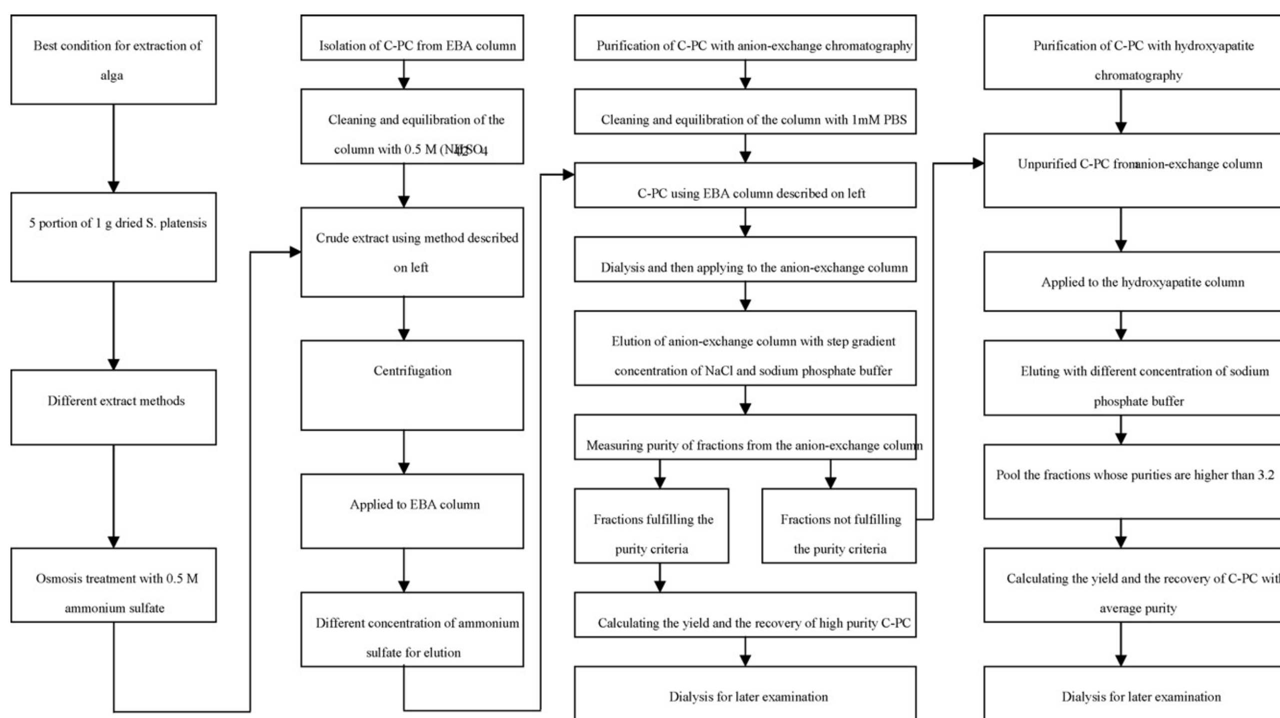


Fig. 1. C-PC extraction, isolation and purification procedures.

5 ml/min in the downward direction. The eluates were collected and their volume and absorbance at designated wavelengths (280, 615, 620, 652, 730 nm) were measured. In this way, the purity ($OD_{620}/OD_{280}$), concentration and quantity of isolated C-PC were determined [23,25,26]. The absorption spectra of the eluates from the STREAMLINE$^{TM}$ column with different concentrations of ammonium sulfate and distilled water were measured. Next, a cleaning procedure was carried out. Firstly, the column was washed upward with three bed volumes of 0.5 M NaOH solution containing 1 M NaCl at a flow-rate of 30 cm/h. Then, three bed volumes of distilled water, three bed volumes of 30% (v/v) isopropanol, three bed volumes of 25% (v/v) acetic acid, and three bed volumes of distilled water were applied successively at a flow-rate of 100 cm/h upwardly in expanded mode. Finally, the column was equilibrated with five bed volumes of 0.50 M ammonium sulfate in the upward direction to prepare the column for a new experiment.

### 2.2.4. *Purification of C-phycocyanin*

We used the phycocyanin rich solution from the expanded bed adsorption (EBA) step to obtain pure C-PC by means of anion-exchange chromatography followed by hydroxyapatite chromatography.

The fractions eluted with 0.20, 0.10, 0.05 M ammonium sulfate and distilled water were dialyzed against a suitable volume of 0.001 M sodium phosphate buffer (pH 7.0) and applied downwardly to the anion-exchange column (20 cm × 1 cm) loaded with 10 ml of Q-Sepharose. Then, the adsorbent with the captured C-PC was washed with ten bed volumes of 0.001 M sodium phosphate buffer (pH 7.0) containing 0.10 M NaCl downwardly to remove loosely bound or unbound proteins. After that, 0.010 M sodium phosphate buffer containing 0.10 M NaCl, 0.010 M sodium phosphate buffer containing 0.15 M NaCl and 0.015 M sodium phosphate buffer containing 0.15 M NaCl were pumped into the column in succession to elute C-PC. The eluates with a blue color were collected, respectively. Both the volume and absorbance at designated wavelengths (280, 615, 620, 652, 730 nm) of all eluates were measured in order to determine the quantity and purity of the C-PC. The eluates whose purity ($A_{620}/A_{280}$) was higher than 3.2 and with an $A_{620}/A_{650}$ index higher than 5.0 were combined. The other fractions with a blue color were combined and dialyzed against 0.001 M sodium phosphate buffer (pH 7.0). After dialysis, the solution was applied in the downward direction to a hydroxyapatite column (1 cm × 8 cm) equilibrated with the starting buffer, 0.001 M sodium phosphate buffer (pH 7.0). The phycobiliproteins were adsorbed by the upper portion of the hydroxyapatite and the non-adsorbed substances passed through the column. After washing with three bed volumes of starting buffer, the column was developed with 15 mM and then 30 mM sodium phosphate buffer, pH 7.0, each containing 0.20 M NaCl. The blue eluate was collected for measurement of the volume and absorbance to determine the quantity and the purity of C-PC.

The purified C-PC was dialyzed overnight at 4 °C against 0.001 M sodium phosphate buffer, and then stored at 4 °C for later use.

### 2.2.5. *Identification of purified C-phycocyanin*

The C-PC purified by anion-exchange chromatography and hydroxyapatite chromatography was analyzed by electrophoresis, UV–vis absorption spectroscopy, and steady-state fluorescence.

Table 1
Quantity and purity of the crude extract with different extraction conditions

| Extraction conditions | Volume (ml) | $OD_{280}$ | $OD_{620}$ | $OD_{650}$ | Purity ($OD_{620}/OD_{280}$) | Quantity (mg) |
|---|---|---|---|---|---|---|
| Sonication | 810 | 1.299 | 0.870 | 0.371 | 0.67 | 104 |
| 0.5 M of ammonium sulfate | 980 | 0.907 | 0.652 | 0.274 | 0.72 | 96.5 |
| Freeze-thawing | 720 | 1.521 | 0.893 | 0.396 | 0.59 | 94.9 |
| Osmotic shock with distilled water | 738 | 1.287 | 0.892 | 0.387 | 0.69 | 97.8 |
| 0.5 M sodium acetate buffer (pH 5.0) | 570 | 0.980 | 0.617 | 0.252 | 0.63 | 52.4 |

The absorption spectra from 250 to 750 nm were measured using a UV/VIS spectrophotometer (UV757 CRT, China) in increments of 2 nm with a 1 cm light-path. A fluorescence spectrometer (F-4500 Hitachi, Japan) was used to record the fluorescence spectra of the purified C-PC in increments of 0.5 nm.

SDS-PAGE was performed in a vertical slab gel apparatus using discontinuous gels as described [27]. The polyacrylamide gel was 1.0-mm thick and contained 0.1% (w/v) SDS. Equal volumes (50 μl) of Laemmli sample buffer and protein solution were mixed and then heated for 10 min at 95 °C. A 30 μl sample of the mixture and 10 μl of molecular mass standards were loaded onto the gel (15% polyacrylamide, 0.1% (w/v) SDS slab gel with a stacking gel of 5% polyacrylamide). Samples were separated at room temperature using a constant voltage of 80 V and visualized by staining with 0.2% (w/v) Coomassie Brilliant blue R-250 in methanol/acetic acid/water (4:1:4, by vol.) for 30 min, and destained in ethanol/acetic acid/water (25:8:67, by vol.). The gel was then stained with silver as described [28]. The following proteins were used as molecular mass markers: phosphorylase b (94.0 kDa), albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.0 kDa) and α-lactalbumin (14.4 kDa).

The bands of interest in SDS-PAGE gel were excised and digested in gel with trypsin as [29]. A 20 μl of digested sample was analyzed using an ion-trap mass spectrometer model LCQ DECA XPplus MS (ThermoFinnigan, San Jose, CA, USA). The results were correlated with the SEQUEST sequence database and analyzed by Bioworks software.

#### *2.2.6. Detection of heavy-metal in C-PC*

The material we applied to obtain C-PC was inferior algae which cannot been used as human food according to the criterion of 'Food grade spirulina powder' in China. Thus, it is essential to detect the concentration of toxic heavy-metals in purified C-PC. We selected Pb, Cr, Cd, As, and Hg as analytes. To detect the concentrations of Pb, Cr, and Cd, flame atomic absorption spectrometry (Model AA6800, Shimadzu Japan) was used. The concentrations of Hg, and As were recorded by ICP-MS (Agilent 7500 series).

## 3. Results and discussion

### *3.1. Extraction*

In general, the extraction method is a key step for the maximum recovery of phycobiliproteins in the natural state from alga [34]. We investigated different methods, such as sonication, freeze-thawing and osmolysis for the initial protein extraction. The quantities and purities of the crude extracts using different methods were given in Table 1. The yields from those methods were similar, except the method that used treatment with 0.5 M acetic acid-sodium acetate buffer (pH 5.0). More importantly, extraction using 0.5 M of ammonium sulfate gave the highest purity of C-PC. Sonication freed a large number of contaminants from the *S. platensis* cells, which resulted in high viscosity in the crude extract. The osmotic shock without ion influenced the subunit integrity of phycobiliprotein. After considering the two aspects of yield and purity, we adopted the extraction method using 0.5 M of ammonium sulfate. For the exhaustive recovery of C-PC from the algae, we repeated the treatment for three times. After that, the blue extracts were pooled.

The extract was centrifuged at 10,000 × *g* for 10 min to obtain a cobalt blue supernatant that was used in subsequent EBA chromatography. The volume of this crude extract was measured and the absorption spectrum was recorded, (Fig. 2a) which showed a maximum absorption at 260 nm, corresponding to a mixture of proteins and nucleic acid. The absorbance at 280 nm was greater than that at 620 nm, which indicated the presence of proteins besides C-PC. Using the equation given in Material and Methods, it was calculated that the crude extract contained 354 mg of C-PC.

It is widely known that the phycobiliproteins are highly conservative proteins in cyanobacteria, however, the aggregation state of this protein shows great variations, such as dimeric, trimeric and hexameric forms [31,32]. The aggregation state of these pigment proteins in solution is affected strongly by pH, ionic strength, and protein concentration. At neutral pH, moderate ionic strength and a moderate concentration of protein, the highest possible aggregation state is the trimer. In this state, the conformation and the spectral properties of phycobiliproteins are well maintained [33]. So, the extraction method that we adopted provided suitable conditions for maintenance of the C-PC conformation.

### *3.2. Isolation and Purification*

The STREAMLINE™ column is designed for using expanded bed adsorption, which is expanded by the upward liquid flow when feeding crude extract without the need for prior clarification. The adsorbent particles are suspended in equilibrium due to the balance between particle sedimentation velocity and upward flow. When the crude extract of *S. platensis* is applied to the column with an upward flow, phycobiliproteins are captured by the adsorbent while cell debris, particulates and contaminants in the extract pass through the column unhindered.

It has been reported that flow disturbance and unequal distribution of a sample are usually caused by its high viscosity in the EBA system [34]. It is clear that the increase in sample viscosity leads to the decrease of sample diffusion, which influences the adsorption of proteins to the resin. On the other hand, excessive sample dilution causes a decrease of C-PC yield due to the poor adsorption of the protein to the resin. Thus, the C-PC concentration in the crude extract was controlled at about 1 mg $ml^{-1}$. Since the adsorption capacity of the resin is usually higher in the dynamic mode than in the static [9], another significant parameter is the expansion degree ($H/H_0$). As has been pointed out in recent research, a ratio of expanded bed volume to the settled bed volume of 2 to 2.2 is ideal [33]. Yield decreased with increase of expansion degree, because the distance between gel particles and the proteins increase, which causes less adsorption to the resin. On the other hand, when the expansion degree is too low, the chance of resin catching proteins become very slight. In our research, an expanded bed with twice its settled height (obtained by a flow-rate of 11 ml $min^{-1}$ or 190 cm $h^{-1}$) was used. At the flow-rate mentioned above, no unequal distribution or channeling phenomena was observed. The volumes and the purities of fractions eluted with an ammonium sulfate step gradient and distilled water from the STREAMLINE$^{TM}$ column are listed in Table 2.

Absorption spectra of eluates from the STREAMLINE$^{TM}$ column (Fig. 2b–e) showed one absolute peak at 620 nm, and a

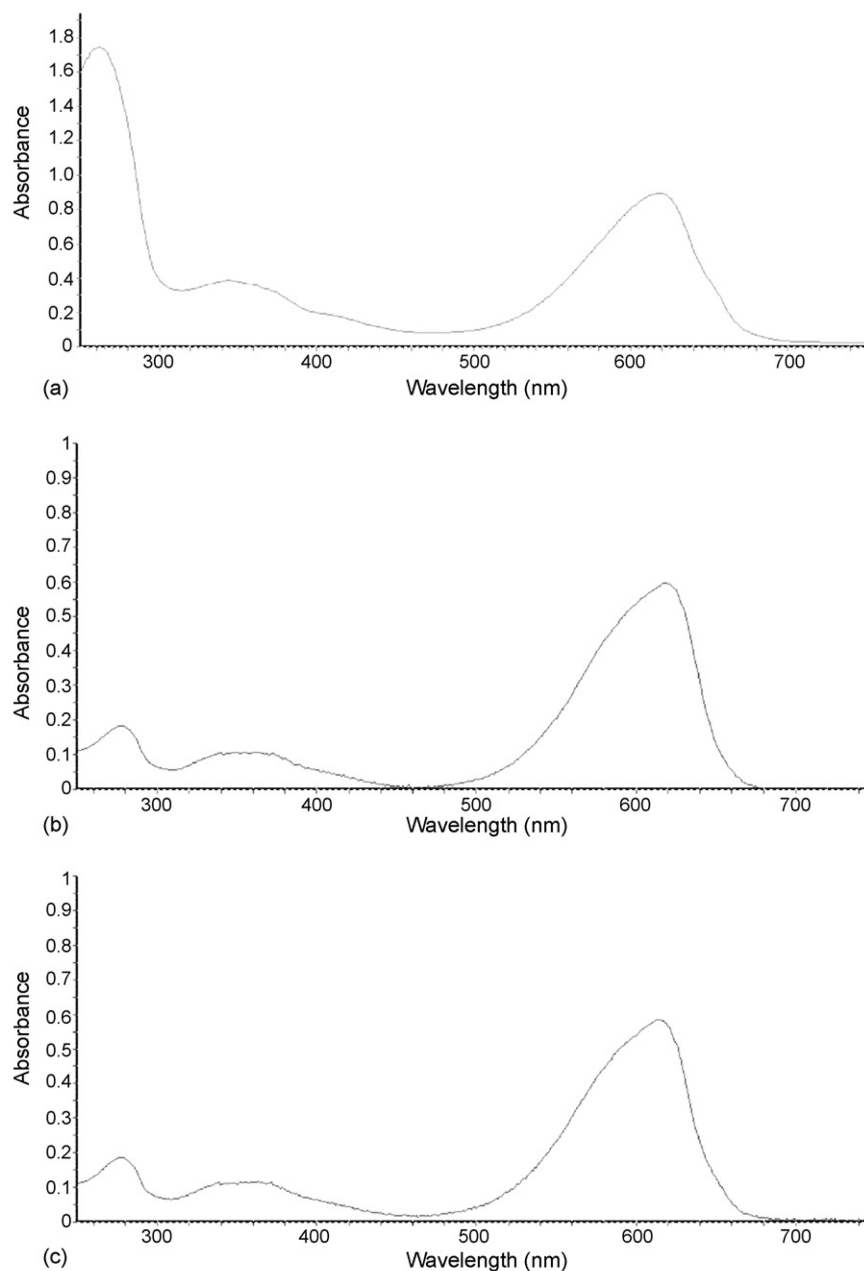


Fig. 2. Absorption spectra of the crude extract and eluates with different concentrations of ammonium sulfate: (a) the crude extract, (b) the eluate with 0.20 M $(NH_4)_2SO_4$, (c) the eluate with 0.10 M $(NH_4)_2SO_4$, (d) the eluate with 0.05 M $(NH_4)_2SO_4$, (e) the eluate with distilled water.

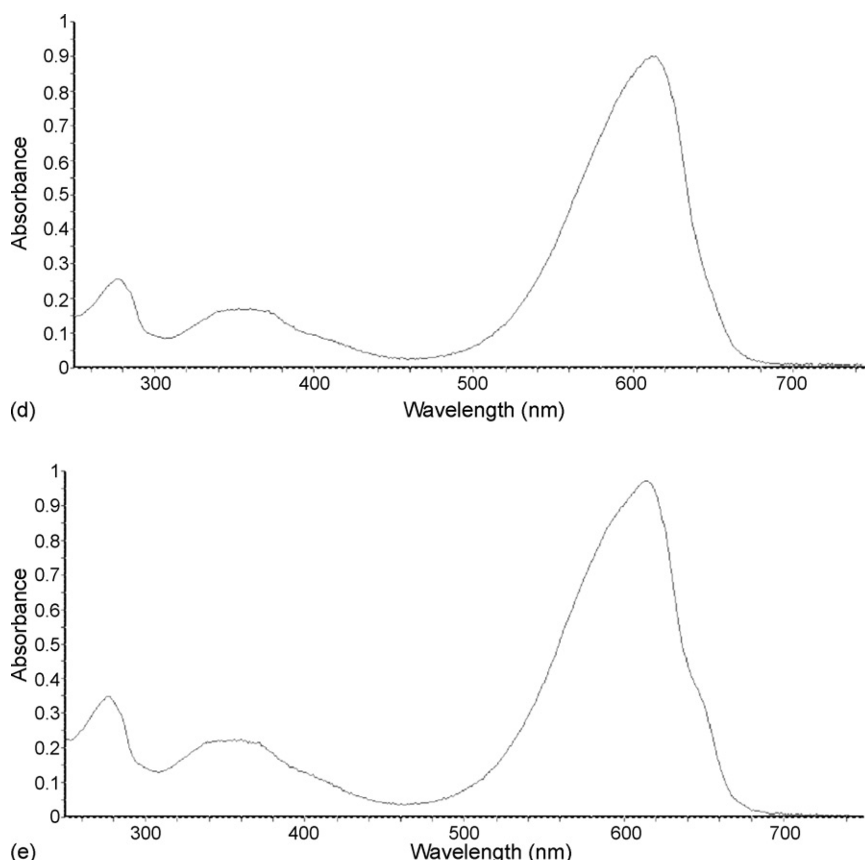


Fig. 2. (Continued).

significant loss of absorption in the near-UV region compared with the spectrum of the crude extract. Although this characteristic absorption peak and spectrum shape conformed to the typical spectrum of C-PC, contamination with APC could be detected. In addition, the purity values ($A_{620}/A_{280}$) were about 3, which is four times higher than that of the crude extract ($A_{620}/A_{280} = 0.69$), which dose not meet the purity standard of C-PC.

Isolation of C-PC with the expanded bed adsorption described here is much simpler. When using the STREAMLINE$^{TM}$ column, only a simple and low-cost treatment is necessary before separation in the column. Many steps and procedures of concentration and partial isolation, such as precipitation in ammonium sulfate, gel-filtration and, etc., used in conventional chromatographic purification protocols are eliminated. One cycle of the isolation process by EBA chromatography, including equilibration (30 min) + loading (60 min) + washing (50 min) + elution (60 min), took only about 3.5 h.

The fractions recovered from the STREAMLINE$^{TM}$ column were assayed by anion-exchange chromatography in packed bed modes. In an attempt to elute pure C-PC, discontinuous ionic strength gradients were applied, as well as pH gradients. The pH gradient protocol was not successful. Pure C-PC was recovered by an ionic strength step gradient. Elution with 0.010 M sodium phosphate buffer (pH 7.0) containing 0.10 M NaCl, 0.010 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl, and 0.015 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl were used successively. The volumes and the absorption at the designated wavelengths (280, 615, 620, 652, 730 nm) of each fraction were measured for the determination of C-PC quantity (Table 3).

The C-PC fractions whose spectroscopic purity ratios ($A_{620}/A_{280}$) was higher than 3.2 with an $A_{620}/A_{650}$ index of higher than 5.0 were pooled, lyophilized and stored in the dark at −20 °C. The UV–vis absorption spectrum and fluorescence spectrum of pure C-PC solution are shown in Fig. 4. It is well

Table 2
Quantity and purity of the eluates eluted with 0.20, 0.10, 0.05 M ammonium sulfate and distilled water from the STREAMLINE$^{TM}$ column

| $(NH_4)_2SO_4$ (M) | Volume (ml) | $OD_{280}$ | $OD_{620}$ | $OD_{650}$ | Purity ($OD_{620}/OD_{280}$) | Index of $OD_{620}/OD_{650}$ | Quantity (mg) |
|---|---|---|---|---|---|---|---|
| 0.20 | 298 | 0.181 | 0.592 | 0.135 | 3.27 | 4.39 | 29. 9 |
| 0.10 | 220 | 0.171 | 0.576 | 0.156 | 3.37 | 3.69 | 20.9 |
| 0.05 | 148 | 0.247 | 0.889 | 0.256 | 3.60 | 3.47 | 21.9 |
| 0.00 | 340 | 0.337 | 0.967 | 0.37 | 2.87 | 2.61 | 51.0 |

Table 3
Quantity and purity of the eluates eluted with different concentration of NaCl from ion-exchange column

| $(NH_4)_2SO_4$ (M) | Na-phosphate (mM) | NaCl (mM) | Volume (ml) | $OD_{280}$ | $OD_{620}$ | $OD_{650}$ | Purity ($OD_{620}/OD_{280}$) | Index of $OD_{620}/OD_{650}$ | Quantity (mg) |
|---|---|---|---|---|---|---|---|---|---|
| 0.20 | 10 | 10 | 43 | 0.085 | 0.281 | 0.047 | 3.31[a] | 5.98[a] | 2.13 |
| | 15 | 10 | 118 | 0.053 | 0.191 | 0.03 | 3.60[a] | 6.37[a] | 4.01 |
| | 15 | 15 | 75 | 0.131 | 0.469 | 0.14 | 3.58 | 3.35 | 5.64 |
| 0.10 | 10 | 10 | 33 | 0.133 | 0.452 | 0.072 | 3.40[a] | 6.23[a] | 2.67 |
| | 15 | 10 | 38 | 0.074 | 0.271 | 0.034 | 3.66[a] | 7.97[a] | 1.90 |
| | 15 | 15 | 48 | 0.246 | 0.933 | 0.284 | 3.79 | 3.29 | 7.19 |
| 0.05 | 10 | 10 | 54 | 0.213 | 0.775 | 0.126 | 3.64[a] | 6.15[a] | 7.80 |
| | 15 | 10 | 107 | 0.058 | 0.203 | 0.019 | 3.50[a] | 10.1[a] | 4.37 |
| | 15 | 15 | 53 | 0.119 | 0.389 | 0.145 | 3.27 | 2.68 | 3.43 |
| 0.00 | 10 | 10 | 31 | 0.186 | 0.609 | 0.12 | 3.27[a] | 5.08[a] | 3.23 |
| | 15 | 10 | 52 | 0.139 | 0.518 | 0.096 | 3.73[a] | 5.40[a] | 4.69 |
| | 15 | 15 | 58 | 0.465 | 1.672 | 0.758 | 3.60 | 2.21 | 14.2 |

[a] The eluate whose purity is more than 3.2 and the $OD_{620}/OD_{650}$ index is higher than 5.

known that absorbance at 280 nm is due to the total concentration of proteins in solution. Absorbance at 620 nm indicates the concentration of C-PC, and that at 650 nm corresponds to APC. A sharp increase in a single peak at 620 nm suggested the absorbance maximum of C-PC; a decrease in absorbance at 280 nm suggested the removal of other proteins. There was no absorbance peak at 650 nm, suggesting the absence of APC from the purified sample.

The C-PC fractions from the anion-exchange column whose purity didn't conform to the above criteria were combined and then purified by passage through the hydroxyapatite column. The volumes and the absorption at designated wavelengths (280, 615, 620, 652, 730 nm) of eluates developed with 0.015 M and 0.030 M sodium phosphate buffer (pH 7.0), each containing 0.20 M NaCl, were recorded (Table 4). Hydroxyapatite chromatography had the advantage of separating C-PC from APC effectively. Unfortunately, the purity ($A_{620}/A_{280}$) of C-PC was lower than that purified with anion-exchange chromatography, and the $A_{620}/A_{650}$ index was less than 5. This was perhaps because the long time needed for chromatography on hydroxyapatite leads to partial denaturing of C-PC. The fractions whose spectroscopic purity ratios ($A_{620}/A_{280}$) was higher than 3.2 were pooled, lyophilized and stored in the dark at −20 °C.

### 3.3. Recovery evaluation and purity test

The C-PC recovery, expressed as a percentage of the amount of C-PC obtained from each treatment step of the total amount of C-PC in the crude extract containing 354 mg C-PC, is given in Table 5. Following isolation with the STREAMLINE™ column, the recovery of C-PC was 35%; 8.7% of high purity C-PC was recovered by the anion-exchange chromatography, and an additional 5% C-PC with average purity had been recovered by the hydroxyapatite chromatography. It is difficult to obtain a high level of recovery if there are many steps in the process. Here, we used two chromatography steps to achieve high purity C-PC and hydroxyapatite chromatography to obtain additional yield of C-PC with average purity.

The marine algae contain many polysaccharides, which often block conventional chromatographic column. Hence, there are many difficulties in large-scale isolation and purification of phycobiliproteins. The yield of phycobiliproteins using the conventional methods is very low. For example, the yield of C-PC from *S. platensis* is just 0.05 mg/g dried *S. platensis* previously. Only 1 mg of purified R-PE can be obtained from 74 g of frozen *Polysiphonia urceolata* [35]. However, using the STREAMLINE™ column combined with anion-exchange chromatography, a C-PC yield of more than 7.7 mg/g of dried *S. platensis* can be obtained. The yield described here is more than 150 times that reported earlier using methodologies that do not involve EBA chromatography. Recently, Bermejo et al. reported a 12% yield of pure C-PC using STREAMLINE™-DEAE anion-exchanger in expanded bed mode [33]. While the method we adopted has a lower recovery in the EBA step, which maybe caused by the partial denaturing and disaggregating of C-PC when the algal powder was produced in the factory, considering the quantity of C-PC purified from hydroxyapatite, the total recovery we obtained is up to 13.7%, higher than that reported previously [33,35]. On the other hand, purification with anion-exchange chromatography is better than that with molecular exclusion chromatography used by Bermejo in terms of time

Table 4
Quantity and purity of the eluates from hydroxyapatite column

| Na-phosphate (mM) | NaCl (M) | Volume (ml) | $OD_{280}$ | $OD_{620}$ | $OD_{650}$ | Purity ($OD_{620}/OD_{280}$) | Index of $OD_{620}/OD_{650}$ | Quantity (mg) |
|---|---|---|---|---|---|---|---|---|
| 15 | 0.2 | 62 | 0.296 | 0.894 | 0.226 | 3.02 | 3.96 | 9.4 |
| 30 | 0.2 | 71 | 0.456 | 1.472 | 0.312 | 3.23[a] | 4.72 | 17.8 |

[a] The eluate whose purity is more than 3.2.

Table 5
The purity, yield and the recovery of C-PC in various stages

| Purification step | Purity ($OD_{565}/OD_{280}$) | Index of $OD_{620}/OD_{650}$ | Quantity (mg) | Yield (mg per g) | Recovery (%) |
|---|---|---|---|---|---|
| Crude cell-free extract | 0.69 | 2.15 | 354 | | |
| Eluate after STREAMLINE$^{TM}$ column | ≥2.87 | ≥2.61 | 124 | 31 | 35 |
| C-phycocyanin purified by anion-exchange chromatography | 3.64 | 6.15 | 30.8 | 7.7 | 8.7 |
| R-phycoerythrin purified by hydroxyapatite chromatography | 3.23 | 4.72 | 17.8 | 4.45 | 5.0 |

4 g *S. platensis* dried powder was used in the preparation of C-PC.

consumption and process scale-up. It should be emphasized that, here, recovery was more important than the purity of the product, since the proposed procedure is intended to replace the low yield methods used in C-PC recovery.

The purity of C-PC was checked by calculating the ratio of absorbance at 620 nm against that at 280 nm. The ratio of absorbance at 620 nm to that at 650 nm was higher than 5.0, indicating the high purity of C-PC and the absence of APC. These indexes have been considered as good indicators of the purity level of phycocyanin. The purity ($A_{620}/A_{280}$) of C-PC in each step was determined and is given in Table 5. The purity of the crude extract was as low as 0.69, because many more proteins, including C-PC, were extracted from the biomass. After isolation by the STREAMLINE$^{TM}$ column, the ratio ($A_{620}/A_{280}$) of each eluate increased to at least 2.87, and much of the contaminants were eliminated. The following anion-exchange chromatography or hydroxyapatite chromatography yielded a purity higher than 3.2, which is the commonly accepted criterion for purity [17]. According to Reis et al. [7], phycocyanin with a purity between 1 and 4 can be used in the food and cosmetic industries. Thus, all C-PC purified here could be applied in such industries.

The variation of C-PC purity was monitored also by SDS-PAGE in 15% polyacrylamide gels and staining with Coomassie R-250 and then silver (Fig. 3). In Fig. 3, the bands located near 20 kDa in lanes 2 to 5 should be assigned to the combination of C-PC and APC (intense bands), and other bands (less intense) correspond to contaminant proteins. Unfortunately, the α and β subunits of C-PC and C-APC have very similar molecular mass (21.5 and 19.0 kDa, and 19.6 and 17.7 kDa, respectively). Consequently, their mobility in SDS-PAGE is very similar and it is difficult to distinguish them by this simple method. Subsequently, after anion-exchange chromatography or hydroxyapatite chromatography, only two bands with similar intensities (lane 6 and 7), which should be the α and β subunits of C-PC, were detected.

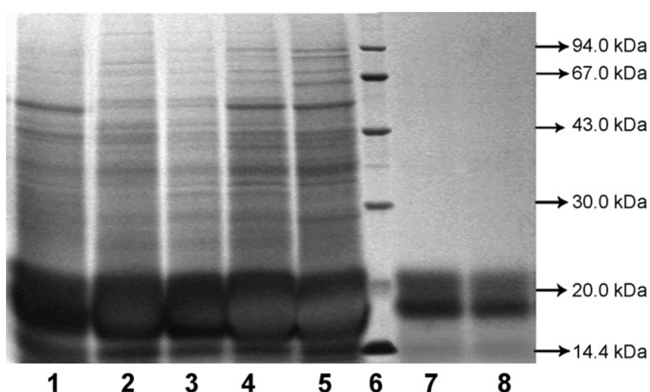


Fig. 3. SDS-PAGE analysis of the fractions collected during the various isolation and purification steps from left to right: lane 1, crude extract of alga. Lane 2, protein fraction from the STREAMLINE$^{TM}$ column with 0.20 M $(NH_4)_2SO_4$. Lane 3, protein fraction from the STREAMLINE$^{TM}$ column with 0.10 M $(NH_4)_2SO_4$. Lane 4, protein fraction from the STREAMLINE$^{TM}$ column with 0.05 M $(NH_4)_2SO_4$. Lane 5, protein fraction from the STREAMLINE$^{TM}$ column with distilled water. Lane 6, molecular weight markers. Lane 7, pooled fractions from anion-exchange chromatography. Lane 8, fraction from hydroxyapatite chromatography.

### 3.4. C-PC identification

The SDS-PAGE analysis of purified C-PC showed two bands of similar intensity at positions corresponding to 21 and 18 kDa, respectively (lanes 6 and 7 in Fig. 3). These values are in good agreement with those reported earlier [2,16,30,31].

C-PC purity was also tested by spectroscopic characterization. Fig. 4 shows the absorption and fluorescence spectra

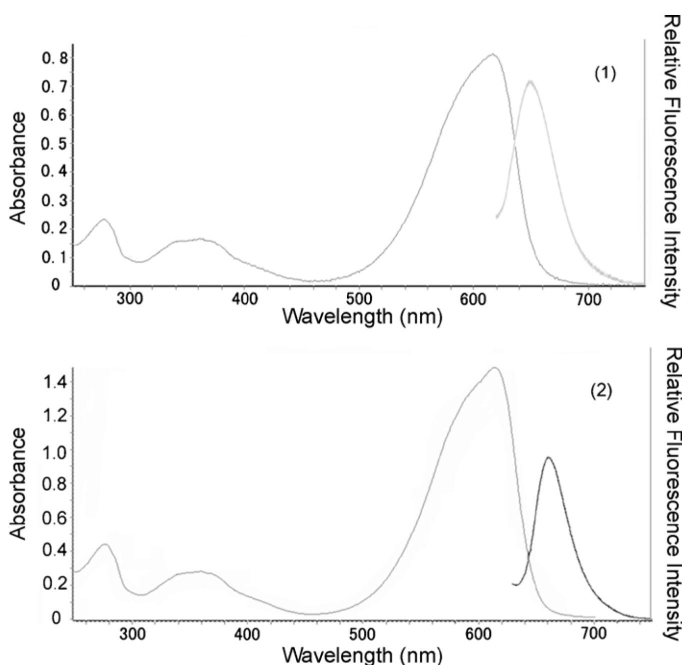


Fig. 4. Absorption and fluorescence spectra of the purified C-PC solution: (1) from the anion-exchange column (0.08 mg/ml) and (2) from the hydroxylapatite column (0.25 mg/ml).

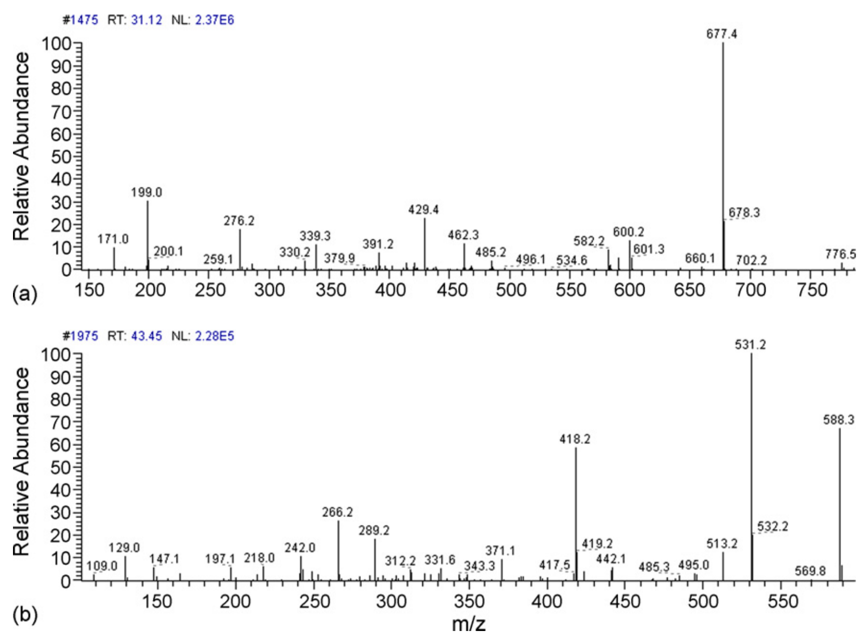


Fig. 5. Characteristic spectrum of matched peptide fragments with C-PC. (a) Showed the spectrum of matched peptide fragments of –VVSQADTR– from α subunit. (b) Indicated the spectrum of matched peptide fragments of –AGLEAAK– from β subunit.

of C-PC solutions purified by either anion-exchange chromatography (0.08 mg/ml) or hydroxyapatite chromatography (0.25 mg/ml), which agree well with those published for pure C-PC [5]. The fluorescence emission maximum was at 650 nm with an excitation wavelength at 620 nm. Because the absorption spectra of the trimeric and monomeric C-PC forms are affected only slightly by the aggregation state of the protein [13], it is not possible to determine the aggregation state of C-PC by these spectra.

Through in-gel digestion and identification using LC-ESI-MS, two peptide fragments matched with the amino acid sequence of α and β subunits of C-PC (Fig. 5a and b) were detected. The matched peptide fragments located at α 8–15 (VVSQADTR) and β 36–42 (AGLEAAK). The results of LC-ESI-MS analysis demonstrated that the protein purified was C-PC.

### 3.5. Detection of heavy-metal in C-PC

Considering the risk of heavy-metal, five elements (Pb, Cr, Cd, Hg, and As), the most potential of arising environment contamination in purified C-PC were investigated. The recorded concentrations of Pb, Cr and Cd by flame atomic absorption spectrometry were 1.95, 0.43 and 0.18 ppM, respectively. The concentrations of Hg and As were determined to be 0.047 and 0.354 ppM using ICP-MS. These values were conformed to the National Criteria of People's Republic of China for 'Food grade spirulina powder' [20]. Therefore, C-phycocyanin obtained from the inferior quality of *S. platensis* did not have heavy-metal contamination and could be used safely as fluorescent tags, food, cosmetics, etc.

## 4. Conclusion

Using the purification protocol presented here, we were able to obtain pure C-PC solutions from *S. platensis.*

The advantage of this new procedure is avoiding the need for prior removal of the suspended solids, which would normally result in the blockage of packed beds. The adoption of this EBA technique greatly reduces the complexity of downstream chromatography processing, simplifies the product isolation process, decreases process time, and increases product yield. Moreover, this new method can be used for recovery of phycocyanin on a large scale.

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