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ScienceDirect
Journal of Chromatography B, 850 (2007) 267–276
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

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

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Received 26 June 2006; accepted 22 November 2006
Available online 18 December 2006

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^{TM} 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.

Keywords: *Spirulina platensis*; Purification; C-phycocyanin; STREAMLINE^{TM} 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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doi:10.1016/j.jchromb.2006.11.043

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