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Efficient separation and purification of allophycocyanin from *Spirulina* (*Arthrospira*) *platensis*

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Abstract Allophycocyanin (APC) is a minor component of phycobiliproteins in cyanobacteria and red algae. This paper describes a simple and inexpensive extracting method for isolating APC from *Spirulina* (*Arthrospira*) *platensis* with high efficiency. The crude phycobiliprotein extract was pretreated by ammonium sulfate fractionation. Then, by adding hydroxylapatite into crude phycobiliprotein extract dissolved in 20 mM phosphate buffer (pH 7.0), APC was selectively adsorbed by hydroxylapatite but C-phycocyanin (C-PC) was not. The hydroxylapatite was collected and APC was extracted from the crude phycobiliprotein extract. Then, the enriched APC was washed off from the hydroxylapatite using 100 mM phosphate buffer (pH 7.0). In this simple extracting method it was easy to remove C-PC and isolate APC in large amounts. The absorbance ratio A_{650}/A_{280} of extracted APC reached 2.0. The recovery yield was 70%, representing 4.61 $mg \cdot g^{-1}$ wet weight. The extracted APC could be further purified by a simple anion-exchange chromatography with a pH gradient from 5.6 to 4.0. The absorbance ratio A_{650}/A_{280} of the purified APC reached 5.0, and the overall recovery yield was 43%, representing 2.83 $mg \cdot g^{-1}$ wet weight. Its purity was confirmed by native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-PAGE.

Keywords Allophycocyanin · Purification · Hydroxylapatite · Anion-exchange chromatography · *Spirulina platensis*

Introduction

Phycobiliproteins exist mainly in cyanobacteria, red algae, and cryptomonads and function as macromolecular light-harvesting pigment proteins (Chen et al. 2003; Glazer 1985; Grossman et al. 1993; MacColl 1998; Wedemayer et al. 1992; Zhang et al. 1999). Based on their spectral properties and chromophores, phycobiliproteins can be divided into four classes: allophycocyanin (APC), phycocyanin (PC), phycoerythrin (PE), and phycoerythrincyanin (Bermejo et al. 2003; Gantt 1981; Glazer 1981). The major phycobiliproteins in cyanobacteria and red algae are PC or PE, while the APC content is much less. APC is purified as a trimer, and its monomer is composed of two subunits—α and β—each of which is covalently attached to a tetrapyrrol chromophore called phycocyanobilin (Glazer 1994). The absorption spectrum of the trimer has a maximum of 650 nm and a shoulder at 620 nm, while the absorption maximum of the monomer is 615 nm (MacColl 2004; MacColl et al. 1980).

APC is widely used in biochemical techniques as a fluorescent protein probe, particularly for flow cytometry (Shapiro et al. 1983; Yeh et al. 1987). It has been also reported that APC has antioxidant (Ge et al. 2006) and anti-enterovirus properties (Shih et al. 2003) and other applications (Sekar and Chandramohan 2008). Although APC is a useful protein, its application is somewhat limited by the difficulty of purifying large amounts of the protein.

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Previous studies on purifying phycobiliproteins have mainly focused on methods of purifying PE (Galland-Irmouli et al. 2000; Rossano et al. 2003; Bermejo et al. 2003; Benavides and Rito Palomares 2004, 2006; Liu et al. 2005) or PC (Minkova et al. 2003, 2007; Benedetti et al. 2006; Patil et al. 2006; Chen et al. 2006; Zhu et al. 2007; Silveira et al. 2007), which are major phycobiliproteins in cyanobacteria or red algae, while publications on APC purification are much less (Bermejo et al. 1997; Minkova et al. 2007; Zhang and Chen 1999). Conventional methodologies for purifying phycobiliproteins often involve a pretreatment step and several chromatography steps. The methods of APC purification usually involve a combination of gel filtration chromatography and ion-exchange chromatography (Bermejo et al. 1997; Zhang and Chen 1999). Although the methods are good, the gel filtration step is time-consuming and not suitable for separation of large amounts of material. Furthermore, the yields of the published purification methods are usually not calculated, only focusing on improvement of the purity of the APC.

Cyanobacteria are good resources for APC purification, but there are two problems. First, the properties of APC and C-phycocyanin (C-PC) in cyanobacteria are very similar; second, the abundance of APC in cyanobacteria is very low. Thus, separating the phycobiliproteins and purifying large amounts of APC are a big challenge. Ge et al. (2005, 2006) have expressed recombinant APC in *Escherichia coli*, but the recombinant APC was the apo-protein without chromophores. Thus, it is desirable to establish efficient methods for purifying APC.

Spirulina (*Arthrospira*) *platensis*, which is widely cultured in many countries, is an inexpensive source for purifying phycobiliproteins (Bermejo et al. 1997). It has been estimated that the gross biomass of *S. platensis* produced in China was about 1,000 tons (Niu et al. 2007). Moreover, the phycobiliprotein content in *S. platensis* is high and can be extracted easily (Silveira et al. 2007). Our goal was to develop an efficient method to purify APC from *S. platensis*. To achieve this goal, several problems needed to be resolved: first, how to extract the low abundance APC and separate the APC from C-PC in the crude extract; second, how to improve the purity ratio of the enriched APC. However, APC has low abundance in *S. platensis* as in other cyanobacteria, constituting less than 10% of the total phycobiliproteins, most of which is C-PC (Bermejo 1994; Bermejo et al. 1997). Here, we describe a simple method which can extract APC with high efficiency. With a further chromatography step, pure APC can be purified.

Materials and methods

Spirulina platensis was kindly provided by the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China. Cells were grown in 5-L liquid Zarrouk medium at 30°C, with aeration provided by bubbling (Chen et al. 1996). *S. platensis* in log phase was collected.

DEAE-Sepharose Fast Flow and the materials used in polyacrylamide gel electrophoresis (PAGE) were from Pharmacia (Sweden), and the protein markers used in sodium dodecyl sulfate (SDS)-PAGE were from Fermentas (Germany). Hydroxylapatite was prepared as described (Bernardi 1973). All other chemicals of analytical grade were purchased commercially.

APC purification

Pretreatments

Spirulina platensis was suspended in 0.02 M phosphate buffer (pH 7.0). Then, cells were frozen at −20°C overnight and subsequently thawed to 20°C and this freeze–thaw was repeated three times. The solution was centrifuged at 11,000 rpm for 15 min at 4°C and the blue supernatant was collected, which was the crude extract.

The crude extract was fractionated with ammonium sulfate at 30% and 60% (*w*/*v*). When the ammonium sulfate powder was added to 30% saturation, the solution was allowed to stand for 4 h at 4°C and then centrifuged at 11,000 rpm for 15 min at 4°C. The blue supernatant was collected, and subsequently, the solid ammonium sulfate was added to 60% saturation. With the same centrifuge process, the precipitates were obtained. Then, they were dissolved in 20 mM phosphate buffer (pH 7.0) and were dialyzed overnight against the same buffer.

Extraction with hydroxylapatite

Hydroxylapatite pre-equilibrated with 20 mM phosphate buffer (pH 7.0) was added into the dialyzed APC solution and stirred. Then, the hydroxylapatite on which the APC was adsorbed was collected and poured into a column. After being washed with two volumes of equilibrating buffer, the hydroxylapatite was eluted with 100 mM phosphate buffer (pH 7.0) and the APC was washed off from the hydroxylapatite and collected.

Anion-exchange chromatography

The APC extracted with hydroxylapatite from the crude phycobiliprotein extract was dialyzed against 20 mM acetate buffer (pH 5.6). The dialyzed APC was applied to a DEAE-Sepharose Fast Flow column (1.6 cm×20 cm), which had been pre-equilibrated with 20 mM acetate buffer (pH 5.6) containing 0.05 M NaCl. After being washed with two volumes of the same buffer, the column was eluted with 20 mM phosphate buffer containing 0.05 M NaCl with

a pH gradient (pH 5.6–4.0) at 1 mL min^{-1}. The eluate was monitored at 280 nm and was collected in 2-mL fractions.

Determination of purification

Absorption spectrum and fluorescence emission spectrum

The absorption spectrum was determined using a V-550 spectrophotometer (Jasco, Japan) between 250 and 700 nm. The purity of the APC was evaluated based on the absorbance ratio, A_{650}/A_{280}. The fluorescence emission spectrum was determined using a FP-6500 spectrofluorometer (Jasco, Japan) with an excitation wavelength of 620 nm. All spectra were measured and recorded at room temperature.

Native PAGE and SDS-PAGE

Native PAGE and SDS-PAGE were performed using a vertical slab discontinuous gel electrophoresis apparatus (Bio-Rad, USA). SDS-PAGE was performed with a 5% stacking gel and 15% separation gel. For the native PAGE, a 7.5% separation gel was used. The gels were stained with Coomassie Brilliant Blue R-250.

Results

After fractional ammonium sulfate precipitation between 30% and 60% saturation, most of the phycobiliproteins were precipitated, although there was an 8% APC loss, calculated from the absorption spectra. The absorption spectra of the crude extract and the phycobiliprotein solution after fractional precipitation showed a major peak at 620 nm (Fig. 1) representing C-PC. The crude extract $A_{650}/A_{280\ ratio}$, which indicates the purity of APC, was only 0.3 (Fig. 2a), and after ammonium sulfate precipitation, the ratio was 0.5. Their fluorescence spectra are also shown in Fig. 1. At an excitation wavelength of 620 nm, the fluorescence emission spectra of the crude extract and the phycobiliprotein solution after ammonium sulfate precipitation both had two peaks at 646 and 661 nm, the former of which represented the C-PC and the latter the APC.

The obtained phycobiliprotein solution after fractional ammonium sulfate precipitation was first dialyzed against 20 mM phosphate buffer (pH 7.0), and then the pre-equilibrated hydroxylapatite was added to the solution. The hydroxylapatite selectively adsorbed APC in this buffer but C-PC remained in the solution. Then, the hydroxylapatite with the adsorbed APC was collected from the solution and poured into a column. Because a very small amount of C-PC might be somehow attached to the hydroxylapatite, the hydroxylapatite was first washed with 20 mM phosphate buffer (pH 7.0) to remove the attached C-PC. After this treatment, the phycobiliproteins adsorbed by hydroxylapatite were nearly all APC. The APC was then washed off from the hydroxylapatite with 100 mM phosphate buffer (pH 7.0). Thus, after hydroxylapatite extraction, C-PC was removed and APC was separated. The purity ratio (A_{650}/A_{280}) of the obtained enriched APC reached 2.0, which could indicate that the APC washed off from the hydroxylapatite was not contaminated by C-PC. The recovery yield was 70%, representing 4.61 mg g^{-1} wet

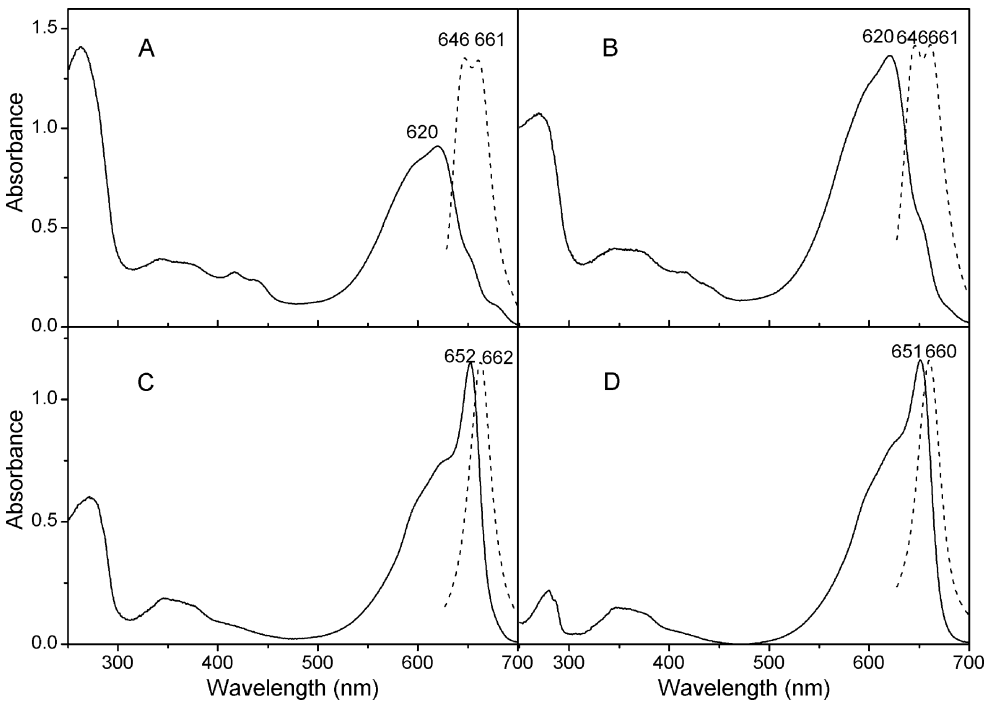

Fig. 1 Absorption spectra and fluorescence emission spectra (*dashed line*) of samples during separation and purification. **a** The crude phycobiliprotein extract. **b** The phycobiliprotein solution after ammonium sulfate precipitation. **c** Enriched APC by hydroxylapatite extraction. **d** Pure APC after anion-exchange chromatography. The exciting wavelength was 620 nm

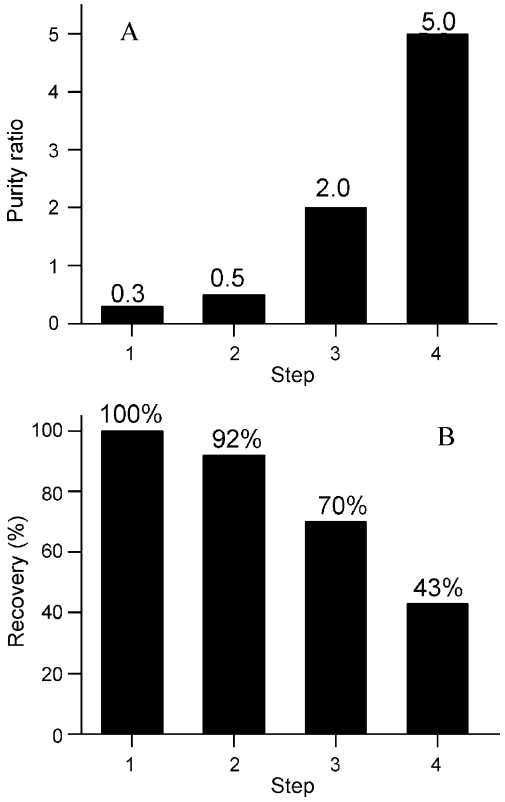

Fig. 2 Purity ratio (**a**) and recovery (**b**) of APC during separation and purification. *1* crude extract, *2* the phycobiliprotein solution after ammonium sulfate precipitation, *3* enriched APC by hydroxylapatite extraction, *4* pure APC after anion-exchange chromatography. The APC contents in different solutions were estimated from the height of the absorbance at 650 nm

weight. Its absorption spectrum had a sharp peak at 652 nm and a shoulder at 620 nm (Fig. 1c). At an exciting wavelength at 620 nm, the fluorescence emission spectrum had only one maximum at 662 nm, which indicated that C-PC had been removed (Fig. 1c).

The APC had been separated efficiently by hydroxylapatite extraction, and it could be further purified by anion-exchange chromatography with a gradient of pH from 5.6 to 4.0 to obtain pure APC without any other contaminant protein. After DEAE-Sepharose Fast Flow chromatography, one main eluting peak containing APC was obtained (Fig. 3). The purity ratio A_{650}/A_{280} of the purified APC reached 5.0, which achieved the standard of pure APC (Padgett and Krogmann 1987). Thus, the obtained APC can be considered pure, and its purity was further demonstrated by electrophoresis (see below). The total recovery yield of pure APC at the end was 43% of the crude extract (Fig. 2b), representing 2.83 mg g^{-1} wet weight. The APC contents were estimated from the height of absorbance at 650 nm. The absorption maximum of the purified APC was 651 nm, and the fluorescence emission maximum was 660 nm at an excitation wavelength of 620 nm, which are the typical spectral properties of APC (Fig. 1d).

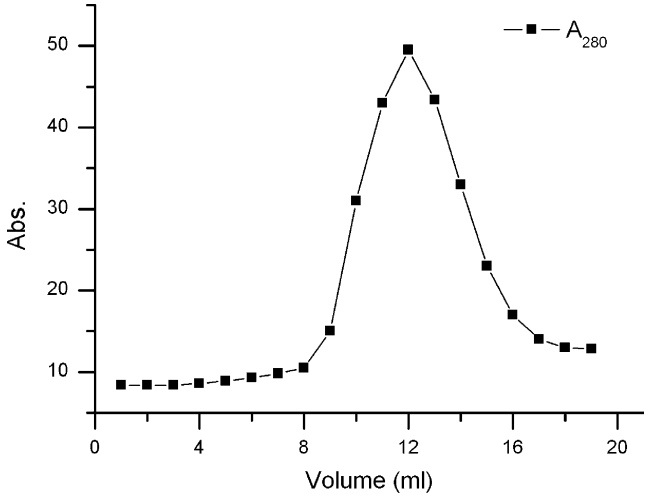

Fig. 3 Elution curve of the APC by anion-exchange chromatography

The purity of the purified APC was also inspected by native PAGE and SDS-PAGE. The extracted APC showed two main bands, corresponding to its two subunits, α and β. At the same time, some minor contaminant protein bands could also be seen. The purified APC produced only a single band on native PAGE in a 7.5% separation gel, while it produced two bands corresponding to α and β subunits in SDS-PAGE (Fig. 4), demonstrating that the APC after anion-exchange chromatography was pure. The

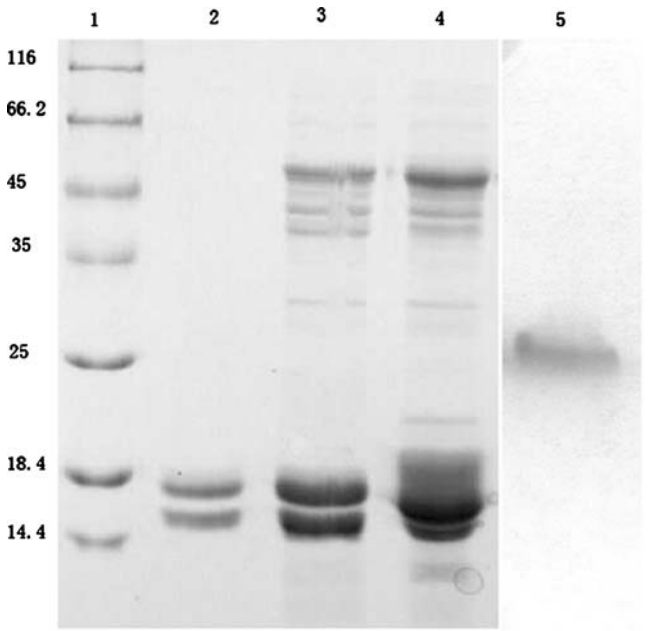

Fig. 4 Native PAGE and SDS-PAGE of the APC from *S. platensis*. *Lanes 1–4* are SDS-PAGE of the APC from each purification step: *lane 1*, marker; *lane 2*, APC purified by ion-exchange chromatography; *lane 3*, APC extracted by hydroxylapatite chromatography; *lane 4*, APC after ammonium sulfate precipitation. The molecular weights of subunits α and β correspond to the 15.7- and 17.6-kDa bands, respectively. *Lane 5*, native PAGE of the purified APC from *S. platensis*

molecular weights of α and β subunits were determined to be 15.7 and 17.6 kDa, respectively.

Discussion

The use of APC is somewhat limited by the available amount of purified APC, so how to purify natural APC with high efficiency remains an important question. The difficulty of purifying APC with high efficiency is due to the low abundance and similar properties of APC to C-PC. The methods for APC purification in published papers usually involve several techniques such as precipitation with ammonium sulfate, ion-exchange chromatography, and gel filtration chromatography. Gel filtration is a very useful method in phycobiliprotein separation and purification, but it is time-consuming and not suitable for large-scale separation. A recently reported non-chromatographic, rivanol-sulfate method has been introduced and is fairly efficient with 35% recovery yield (Minkova et al. 2007). Our hydroxylapatite extraction method is also easy and very efficient, with the recovery yield reaching as high as 70% in the separating step.

Hydroxylapatite–protein interaction is a complicated process, as both calcium ions and phosphate groups act as active functional groups. Proteins bind to hydroxylapatite with their amino groups and carboxyl groups at the protein surface. This complicated interaction provided us a unique method for protein purification. As APC and C-PC differ in their surface charge properties, their abilities in binding to hydroxylapatite are different. Our preliminary experiments showed that phycobiliproteins differ in their capacities to be adsorbed by hydroxylapatite in different phosphate buffers. Based on this, we introduced this simple and efficient method using hydroxylapatite extraction.

In a proper buffer, hydroxylapatite selectively adsorbed only APC but not C-PC. It is a solid–liquid two-phase extraction process. In this extraction process, the low-abundant APC is not only separated from C-PC but also enriched. Furthermore, the efficiency of the hydroxylapatite extraction method is greatly improved compared to the traditional hydroxylapatite chromatography method. Hydroxylapatite chromatography is useful in separating phycobiliproteins, but in the traditional hydroxylapatite chromatography process, hydroxylapatite first adsorbs both APC and C-PC, then the phycobiliproteins are sequentially eluted using the wash buffer and collected, respectively. Since APC is only a small percentage of the total phycobiliproteins adsorbed in the hydroxylapatite column, each time hydroxylapatite chromatography is performed only a small amount of APC can be separated. In the hydroxylapatite extraction process, the phycobiliprotein adsorbed by the hydroxylapatite is only APC, so the efficiency is improved. Moreover, this hydroxylapatite extraction method has the potential for APC separation in large amounts. Thus, in this simple and easy hydroxylapatite extraction step, APC is efficiently prepared. The purity ratio of APC after hydroxylapatite extraction reached 2.0, and only some minor bands could be seen in the SDS-PAGE. The APC obtained in the hydroxylapatite extraction reached the standard for food and biotechnological use.

Hydroxylapatite extraction has been demonstrated to be simple and efficient. For some scientific research, pure APC is needed. Thus, a further anion-exchange chromatography treatment could be combined with the hydroxylapatite extraction step. Ion-exchange chromatography has many advantages, such as rapidness and simplicity of purification. However, ion-exchange chromatography with ion strength gradient elution would bring in high ionic strength which would be a problem in further experiments. In previous studies, it had been shown that phycobiliproteins are stable over a wide pH range (Liu et al. 2005; Zhou et al. 2005; Gysi and Zuber 1979). As a result, a pH gradient elution was used in the anion-exchange chromatography to purify APC. We have reported anion-exchange chromatography with pH gradient elution for R-PE purification (Liu et al. 2005). In our experience, this method, using anion-exchange chromatography with pH gradient elution, is efficient in purifying most of the phycobiliproteins including PE, PC, and APC. It also avoids the problems that high ionic strength might cause in subsequent processes. With this purification step, the purity ratio of the APC reached 5.0. In addition, the native PAGE and SDS-PAGE results and spectra characteristics all suggest that the APC is pure. The overall recovery of the separation process was up to 43% from the crude extract, representing 2.83 mg g^{-1} wet weight.

In conclusion, *S. platensis* has been shown to be a good source for extraction and preparation of APC. This paper presents a simple and inexpensive method to purify APC efficiently from *S. platensis*.

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