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One-step chromatographic procedure for purification of B-phycoerythrin from *Porphyridium cruentum*

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ABSTRACT

B-phycoerythrin (B-PE) was separated and purified from microalga *Porphyridium cruentum* using one-step chromatographic method. Phycobiliproteins in *P. cruentum* was extracted by osmotic shock and initially purified by ultrafiltration. Further purification was carried out with a SOURCE 15Q exchange column and analytical grade B-PE was obtained with a purity ratio (A_{545}/A_{280}) of 5.1 and a yield of 68.5%. It showed a double absorption peaks at 545 nm and 565 nm and a shoulder peak at 498 nm, and displayed a fluorescence emission maximum at 580 nm. The analysis by sodium dodecyl sulfate −polyacrylamide gel electrophoresis (SDS−PAGE) showed a bulky band between 18 and 20 kDa which could be assigned to subunits α and β and a low intensity band of 27 kDa assigned to γ subunit. Our protocol provides attractive alternative to consider for the purification procedure to obtain analytical grade B-PE at commercial level.

1. Introduction

Phycobiliproteins (PBPs) are a class of chromoproteins found in red algae, cyanobacteria, cryptophytes, and glaucocystophytes [1–5]. According to their spectral properties, PBPs are commonly classified phycoerythrins (PE, λ_{max} = 540–570 nm), phycoerythrocyanins (PEC, λ_{max} = 560–600 nm), phycocyanins (PC, λ_{max} = 610–620 nm), and allophycocyanins (APC, λ_{max} = 650–655 nm) [6,7]. These proteins are an ensemble of photosynthetic complex called phycobilisome and work as accessory pigments to allow light absorption in the cell on the thylakoids of chloroplast [8–10].

Depending on their absorption spectrum, PE can be divided into three main classes R-PE, B-PE, and C-PE [11]. It has been reported that PE is useful for development of fluorescent probes because of its high extinction coefficients, fluorescence quantum efficiency of more than 0.8, and stability as hexamers even at lower concentrations without showing fluorescence decrease [12–15]. Moreover, PE can be used as natural colorants in the food, cosmetic industry and as photo sensitizers in tumor photodynamic therapy [16–23]. In case of PE, commercial scale processing of this material continues to be a daunting task. R-PE is usually extracted and purified from *Porphyra*, *Grateloupia* and *Polysiphonia*, but most of them contained huge amount of polysaccharides which form gel (carbohydrate) and thereby making the purification task most difficult and tedious [2,24–27]. B-PE is the main phycobiliprotein of *Porphyridium* species and it contains three subunits $(\alpha\beta)_6\gamma$ with molecular weights of 19.5 kDa (α and β subunits) and 29 kDa (γ subunit) for a total molecular weight of 263 kDa [10]. This phycoerythrin normally has a double absorption peaks at ~545 and ~565 nm, and a shoulder peak at ~498 nm, and a fluorescence emission maximum at 580 nm [11]. The price of B-PE products varies widely and is dependent on the purity ratio (A_{max}/A_{280}). The cost of analytical grade B-PE (purity ratio higher than 4.0) can be as high as about 50 US$/mg [22]. This high value makes attractive attempt to develop an efficient purification procedure to obtain B-PE with high purity and yield at commercial level. Previous attempt for analytical grade of B-PE has been reported [9,11,28–30]. However most cases are complicated by the need for multiple chromatography steps to obtain highly purified protein. The complexity of the procedures for purification of B-PE is usually characterized for having a high number of stages, low yield and limitation to easy process scale-up, which limits its potential practical implementation at commercial level [22]. Therefore, it is needed to develop an efficient purification procedure to obtain analytical grade of B-PE with high yield at large scale. SOURCE 15Q is an ion exchanger of

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high performance separation medium for fast, preparative purification of biomolecules. It is ideal for intermediate purification steps in industrial processes where high productivity and maintained performance at large scale are important. In the present study, we have improved the efficiency of purification step of the routine methods to obtain B-PE from *P. cruentum*, involving only a one-step chromatography on a SOURCE 15Q ion exchanger column.

2. Materials and methods

2.1. Biological material

The microalga *P. cruentum* were obtained from Laboratory of microalga biotechnology, Yantai University, China. The alga was intensively cultivated in Hemerick culture medium as described before [9,31]. The culture medium and the biomass were separated by centrifuging (CT18RT, Techcomp Instrument Ltd., Shanghai, China) the cell suspension at 10, 000 × g for 10 min. The pellets containing biomass were lyophilized for getting dry biomass.

2.2. Preparation of crude extracts

The extraction was carried out using the method of Bermejo et al. [28] with some modification. Freeze-dried samples (5 g) were resuspended in 50 ml acetic acid-sodium acetate buffer (pH 6.0, 1 M). The slurry was mixed with a variable-speed stirrer at 300 rpm for 1 h and then the slurry was centrifuged at 10, 000 × g for 5 min. The phycoerythrin-rich supernatants were pooled and treated as the crude extract used in the following purification steps (Fig. 1). The following purification procedure included ultrafiltration and chromatography with a SOURCE 15Q anion exchange column.

2.3. Fractionation of water-soluble compounds by ultrafiltration

The crude extract was filtered through PES hollow fiber membrane (GM2540F1073, GE Water &Process Technologies, Shanghai) with a molecular weight cut off (MWCO) of 30 kDa, 50 kDa and 100 kDa (shear force 0.1 MPa, flow rate 300 cm^3/min, inner diam. 0.8 mm, outer diam. 1.2 mm, 4 °C). Deionized water was added constantly until the permeate liquid showed no absorption at 260 nm and 280 nm. The retentate was concentrated and lyophilized to yield 0.20 g of fluffy red solid which was stored 4 °C.

2.4. SOURCE 15Q chromatography

After dissolving 0.20 g lyophilized retentate in 10 ml sodium phosphate buffer (pH 7.2, 20 mM), the solution was futher purified with a SOURCE 15Q (GE, USA) anion exchange column (50 × 16 mm) on a fast protein liquid chromatography (FPLC, AKTA purifier 10, GE, USA). After washing with the same buffer, the column was eluted with a linearly increasing ionic concentration gradient of NaCl (from 0 to 0.5 M) at a flow rate of 2.0 ml/min. The eluate with red color was pooled and analyzed.

2.5. Comparison of the purification efficiency of different chromatography methods

To compare purification efficiency with SOURCE 15Q, the retentate was also purified on Sephadex G-150 (GE, USA) and DEAE cellulose DE52 (GE, USA) column. 0.15 g lyophilized retentate was dissolved in 15 ml sodium phosphate buffer and then divided into three equal portions: sample1, sample2 and sample3. For Sephadex G-150 chromatography, sample1 was passed through Sephadex G-150 media packed in (600 × 25 mm) column equilibrated with 20 mM phosphate buffer pH 7.2. For DEAE cellulose DE52 chromatography, sample2 was applied to a column (100 × 25 mm) of DEAE cellulose DE52, pre-equilibrated with sodium phosphate buffer (pH 7.2, 20 mM). After washing with sodium phosphate buffer (pH 7.2, 20 mM), the column was eluted with a linearly increasing ionic concentration gradient of NaCl (from 0 to 1 M) at a flow rate of 1.0 ml/min. Sample3 was applied to a column (50 × 16 mm) of SOURCE 15Q and the procedure was performed as above.

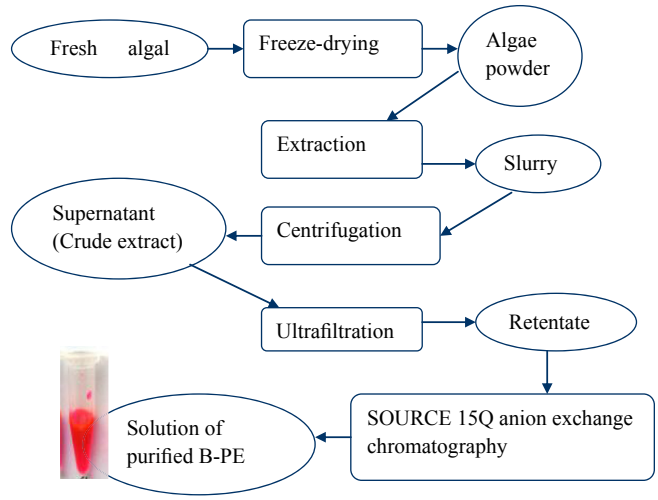

Fig. 1. Schematic diagram for purification of the B-PE from *P. cruentum*.

2.6. Analyses

Protein content was estimated by Lowry's method using bovine serum albumin (BSA) as protein standard [32]. The absorption spectra of purified B-PE were recorded using a UV-VIS spectrophotometer (UV-1700, Shimadzu, Japan) with a 1 cm light path. The purity index of BPE was evaluated spectrophotometrically by the absorbance ratio of A_{545}/A_{280}. The amounts of B-PE in each step of the process were determined with a previously reported equation system by measures of absorbances at 545, 620 and 650 nm according to Marcati et al. [10]. The fluorescence spectrum of purified B-PE was recorded using a fluorescence spectrometer (F-4500, Hitachi, Japan).

The homogeneity and structure integrity were analyzed by native polyacrylamide gel electrophoresis (PAGE) using a vertical electrophoresis cell (Mini-Protean II, Bio-Rad, USA) with a separating gel of 10% and a stacking gel of 5% as described before [33]. After electrophoresis, the protein band corresponding to B-PE was identified firstly by the pink color. Then, protein bands were staining with 0.2% (w/v) Coomassie brilliant blue R250.

The molecular weights of the subunits was analyzed by performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in a Bio-Rad Miniprotean II vertical slab gel apparatus using Laemmli-buffer system according to H. Schägger and von Jagow [34]. Electrophoresis was carried out using a 12% polyacrylamide slab gel with 0.1% (w/v) SDS and 5% polyacrylamide stacking gel. Protein bands were detected by staining with 0.2% (w/v) Coomassie brilliant blue R250. The molecular weights of the subunits were assayed by calibration with protein molecular weight marker (Broad Range, TaKaRa).

Results presented here are the average of three independent experiments and the mean ± standard deviation (SD) was less than ± 5%.

3. Results and discussion

3.1. Extraction of B-PE

P. cruentum is a sea water microalga well known for its capacity to produce B-PE in large quantities. In the present study, typical growth conditions for *P. cruentum* were employed. After 25 days of culture, the biomass achieved was 8 g of wet biomass per L and the result is similar to that reported by Ruiz-Ruiz et al. [9]. After harvesting the cell mass, osmotic shock was used to extract the initial protein. The disruption technique can be easily scaled up and is much simple and efficient as compared with the methods that use ultrasound, freeze-thawing or the addition of chemical compounds like TritonX-100, lysozyme and rivanol [28]. In the experiment, the crude extract was obtained and analyzed by a UV-VIS spectrophotometer, the purity ratio (A_{545}/A_{280}) was 0.9. The apparent absorption spectrum of crude extract at 545–565 nm which was the absorption peak for B-PE, indicated a high content of B-PE in *P. cruentum*. Absorbance at 260 and 280 nm corresponded to a mixture of nucleic acid and protein (Fig. 2).

3.2. Fractionation of water-soluble compounds by ultrafiltration

Three membranes, with MWCO of 30 kDa, 50 kDa and 100 kDa, were initially screened for their effect on the retention of B-PE in crude extract. The results revealed the retention rate of B-PE were 98.9%, 98.1% and 90.5% for the 30 kDa, 50 kDa and 100 kDa cut-off membranes, respectively. The purity ratios of the B-PE retentate obtained by ultrafiltration, when using membranes with MWCO 30 kDa, 50 kDa and 100 kDa were 1.3, 1.7 and 1.6, respectively. Consider that the retention rate after ultrafiltration with a MWCO 50 kDa membrane was almost not reduced and the purity ratio were higher than those with a MWCO 30 kDa and 100 kDa, membrane with MWCO, 50 kDa is suitable for the ultrafiltration process. Ultrafiltration, which is currently gaining importance in the processing of biomolecules/liquids, can be applied to large-scale production and is simple to carry out. Moreover, this technique is a very efficient separation technology without addition of chemicals and is normally carried out at ambient temperature, which minimizes thermal damage to the product [35]. Marcati et al. reported that a first ultrafiltration using a membrane with a MWCO 300 kDa yielded high molecular weight polysaccharides and the resulting 300 kDa permeate was then run through a second MWCO 10 kDa membrane to recover B-PE with a purity ratio of 2.3 [10]. Ruiz-Ruiz et al. reported that ultrafiltration with a MWCO 300 kDa membrane could successfully remove most of polyethyleneglycol (PEG) of B-PE solution [9]. In our study, the B-PE fraction was obtained with a purity ratio of 1.7 and a retention rate of 98.1% when crude extract ultrafiltration with a MWCO 50 kDa membrane (Table 1). Fraction by ultrafiltration could increase the purity ratio of B-PE approximately 2 fold, compared with the initial purity ratio of the crude extract.

Table 1
Purification of B-PE from *P. cruentum*.

Purification step	Crude extract	Ultrafiltration (MWCO 50 kDa)	SOURCE 15Q chromatography
Total protein content (mg)[a]	305.0 ± 12.8	168.1 ± 7.4	59.5 ± 2.6
B-PE content (mg)[b]	83.2 ± 3.2	81.6 ± 2.9	57.0 ± 2.1
B-PE from total proteins (% w/w)	27.3 ± 0.9	48.5 ± 1.3	95.8 ± 3.4
B-PE recovery (% w/w)[c]	100	98.1 ± 5.2	68.5 ± 3.7
Purity ratio (A_{545}/A_{280})	0.9 ± 0.1	1.7 ± 0.1	5.1 ± 0.4

Data are expressed as the mean ± SD (n = 3).
[a] Total protein was determined by Lowry's method.
[b] B-PE content was determined with equation system by measures of absorbances at 545, 620 and 650 nm.
[c] B-PE recovery is expressed relative to the original amount of B-PE in the crude extract.

3.3. SOURCE 15Q chromatography and characterization of B-PE

The B-PE fraction was further purified with a SOURCE 15Q anion exchange column on FPLC. The column was eluted with a linear gradient of NaCl in the same buffer at a flow rate of 2 ml/min, resulted in high purity ($A_{545}/A_{280} = 5.1$) B-PE with a yield of 68.5% (Table 1). The absorption spectra of the purified B-PE had a double absorption peaks at 545 and 565 nm, and a shoulder peak at 498 nm. No absorbance peak was found at 620 and 652 nm indicated the absence of phycourobilin, C-PC or APC in the purified B-PE sample, while low absorption in the region of 280 nm suggested

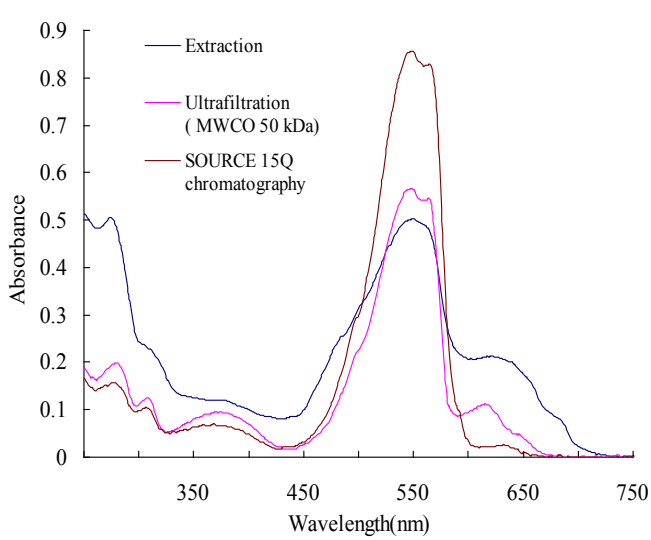

Fig. 2. Absorption spectrum of B-PE from *P. cruentum* at each step of purification.

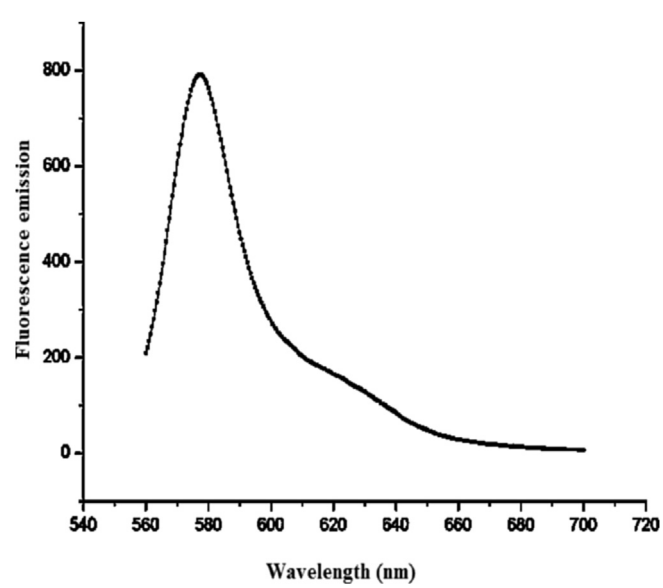

Fig. 3. Fluorescence emission spectrum of purified B-PE from *P. cruentum*.

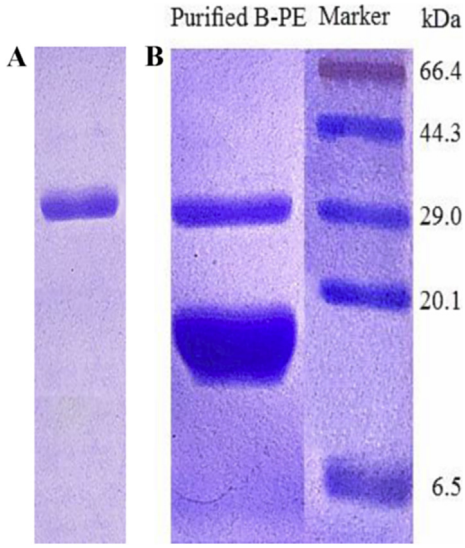

Fig. 4. (A) Native polyacrylamide gel electrophoresis (PAGE) analysis of the purified B-PE from *P. cruentum*. (B) SDS–PAGE analysis of the purified B-PE from *P. cruentum*.

high purity of the PE in the eluate (Fig. 2). Fluorescence emission spectrum of purified B-PE from *P. cruentum* showed maximum fluorescence at 580 nm (Fig. 3). These spectra agree well with those published references for pure B-PE [9,11,12,30,36,37]. The homogeneity and structure integrity of the B-PE obtained from the SOURCE 15Q column were confirmed by the native PAGE which showed only one band (Fig. 4A). The analysis by SDS–PAGE revealed a broad band between 18 and 20 kDa which could be assigned to subunits α and β and a low intensity band of 31 kDa assigned to γ subunit (Fig. 4B), which consistents with the value reported previously [9,28,38,39].

3.4. Comparison of the purification efficiency of different chromatography methods

To compare purification efficiency of SOURCE 15Q, the sample was also purified on media of Sephadex G-150 and DEAE cellulose DE52. Purification efficiency of different chromatography methods is summarized in Table 2. The media of Sephadex G-150 and DEAE cellulose DE52 were screened from several of frequently-used media in previous studies, with relative good purification efficiency. As seen from the table, the B-PE recovery and purity ratio are highest on SOURCE 15Q. The result suggested that purification efficiency of SOURCE 15Q was higher than the other two kinds of media. Moreover, SOURCE 15Q can be applied on FPLC, which can be readily scaled to industrial production of kilograms of purified protein in columns with volumes of many liters. The conventional chromatography methods employed in the purification of B-PE from *P. cruentum* involve gel filtration (Sephadex), DEAE chromatography, expanded bed adsorption (EBA) chromatography, often in combination [11,12,18,30]. By our protocol, using only a one-step SOURCE 15Q chromatography method, purity ratio of the B-PE of *P. cruentum* was enhanced by a factor of 2.6. Compared with the studies on the purification of B-PE implementing more complex procedures, this protocol reduced the processing time, which is advantageous to the preservation of the native conformation of B-PE.

Table 2
Purification efficiency of different chromatography methods.

Media	SOURCE 15Q	Sephadex G-150	DEAE-cellulose 52
Protein recovery (% w/w)[a]	35.2 ± 1.8	31.6 ± 1.5	27.9 ± 1.1
B-PE recovery (% w/w)[b]	68.5 ± 3.7	58.3 ± 2.7	52.6 ± 2.6
Purity ratio (A_{545}/A_{280})	5.1 ± 0.4	3.8 ± 0.2	3.2 ± 0.2

Data are expressed as the mean ± SD (n = 3).
[a] Protein recovery is the ratio between proteins eluted after chromatography and the total protein loaded, as measured by Lowry's method.
[b] B-PE recovery is the ratio between total B-PE eluted from chromatographic columns and total B-PE loaded, as determined with equation system by measures of absorbances at 545, 620 and 650 nm.

4. Conclusion

In this study, involving osmotic shock, ultrafiltration, and one-step SOURCE 15Q chromatography method for extraction and purification of B-PE, analytical grade B-PE was obtained with a purity ratio (A_{545}/A_{280}) of 5.1 and a yield of 68.5%. The efficiency of this procedure was confirmed by the absorption spectrum, fluorescence spectrum, native PAGE, and SDS-PAGE. Moreover, the procedure is a feasible alternative for industrial scale-up since it can adapt to continuous process and is simple to carry out in large scale. Thus, our protocol represents attractive alternatives to consider for the purification stage to obtain analytical grade B-PE at commercial level.

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