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Purification of phycoerythrin from *Porphyra yezoensis* Ueda (Bangiales, Rhodophyta) using expanded bed absorption

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Abstract R-phycoerythrin was purified by means of phenyl-sepharose expanded bed absorption and DEAE-sepharose ion-exchange chromatography from *Porphyra yezoensis*, one of the largest and important aquaculture species in China. Final R-phycoerythrin preparation was characterized by purity ratio above 4 and the homogeneity in native PAGE, respectively. The results of absorption spectrum, fluorescence spectrum and SDS-PAGE were in agreement with previous reports on R-PE. The yield of R-phycoerythrin was 0.82 mg g^{-1} wet leafy gametophyte of *P. yezoensis*. This method is a high-protein recovery technology while reducing processing time, and is suitable for the large batch production of R-phycoerythrin, which will enhance the value of *P. yezoensis* in China, especially the inferior *P. yezoensis* which can not be used for flake processing.

Keywords *Porphyra yezoensis* · R-phycoerythrin · Hydrophobic interaction chromatography · Expanded bed absorption · Ion-exchange chromatography · Streamline column

Introduction

Phycobiliproteins (PBPs) are hydrophilic, brilliantly colored, and stable fluorescent pigment proteins (Apt et al. 1993; Santiago-Santos et al. 2004). The different cofactors that are covalently bound to these PBPs collectively absorb at wavelengths between 500 and 650 nm at normal growth temperatures to control input of solar energy into the photochemical reaction centers (Ailie et al. 2008). The PBPs in intact phycobilisome (PBS) are only weakly fluorescent (Viskari and Colyer 2003; Zhao et al. 1995). When the PBPs are released from the cells, they become highly fluorescent in a region of the spectrum that is well separated from the autofluorescence of other biological cell matter (Viskari and Colyer 2003).

Due to their excellent spectroscopic properties, stability, high absorption coefficient and high quantum yield, PBPs can be used as highly sensitive fluorescent tags of cells and macromolecules in biomedical research (Isailovic et al. 2004). They have been used as photosensitizers for the treatment of tumors (Huang et al. 2002; Li et al. 2000). Recent studies have also shown their applicability in immunomodulation (Román et al. 2002). In addition, they have potential as natural colorants for use in food, cosmetics, particularly as substitutes for chemical synthetic dyes, which are generally toxic or unsafe (Akhilender et al. 1999; Bermejo et al. 2003a).

However, the applications of this kind of pigment-protein encounter the bottle-neck arising from the high price, which can be attributed to difficulties in their purification. Although some researchers are attempting to transfer related genes into genetically engineered microbes to express PBP on a large scale, this has not yet been achieved. At present, the main approach of obtaining PBP is still purification from the natural organism. Thus, looking for cheap biomaterial and studying the proper purification procedure is an attractive endeavor.

Expanded bed adsorption (EBA) chromatography is an alternative bioseparation technique and suitable for direct

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protein recovery from cell lysis or homogenization without the need for prior clarification. Due to the weaker interaction between proteins, EBA has been considered to be a "gentle" method for protein purification (Ghosh and Wang 2006; Queiroz et al. 2001). There are many reports on the purification of biomolecules such as serum proteins, nuclear proteins, hormones, recombinant proteins, and enzymes using this technique (Queiroz et al. 2001). The main advantages are not only high speed, high yield, and no requirement of sample clarification, no extensive equilibration of matrix, but also the production of partially concentrated product ready for the next purification step, usually chromatography on a packed-bed column (Wang 2002; Bermejo et al. 2006; Soni et al. 2006). The advantages are savings in equipment and operating costs (Bermejo et al. 2003b; 2006) compared with previous methodologies. These advantages led us to exploit EBA as a main purification step for R-PE recovery in this paper.

The rhodophytes genus *Porphyra* encompasses several species used for mariculture in Eastern Asia, and is a kind of highly consumed seaweed (Masahiro et al. 2009). These algae, rich in dietary fiber, minerals, carbohydrates, proteins, etc. (Fleurence 1999; Kuda et al. 2005), have become one of the most profitable aquaculture products with production of over 18,000 metric tons dry biomass yearly and an annual market value of over US$ 1.3 billion (FAO 2006). The widely cultivated species of *Porphyra* in China are *P. haitanensis* Chang and Zheng, an endemic species cultivated in the south of China, and *P. yezoensis* Ueda, cultivated mainly in the north of China. The gross mass of *P. yezoensis* in China is estimated to be about 12,000 t, and not all *P. yezoensis* produce is fit for the flake processing. How to use these inferior *Porphyra* is a worthwhile study. One of the feasible processes may be as the resource for R-PE purification.

Materials and methods

Porphyra yezoensis was collected from the seashore of Huiquan Bay, Qingdao, China. The alga was washed with Milli Q water for three times and dried by filter paper for use later.

Phenyl-sepharose (STREAMLINETM Phenyl), DEAE-sepharose and the Streamline column (STREAMLINETM 25, 100×2.5 cm) were purchased from Amersham Biosciences Corp. (New Jersey, USA). All buffers and reagents used in purification were prepared in Milli Q water with added 0.01% sodium azide.

Protein extraction

Twenty-eight grams of leafy gametophyte of *P. yezoensis* was dipped in 300 mL 10 mM phosphate buffer (pH 6.8). The alga-buffer mixture was fragmented in a triturator for 30 min and then subjected to freeze–thaw cycles of −25 and 4°C shocks. The resulting slurry was firstly filtrated with gauze and the pellet was treated with the same procedure for three times. All the supernatants were pooled and the powdered $(NH_4)_2SO_4$ was gradually added in to achieve a final concentration of 0.50 M. The crude extract was kept in 4°C overnight and then centrifuged at 3,000 g for 10 min. The volume was measured and the quantity of R-PE in the supernatant was determined according to Kursar et al. (1983).

R-PE isolation

The Streamline column was filled with the necessary volume of adsorbent (phenyl-sepharose) and a suitable volume of starting buffer (0.50 M ammonium sulfate solution) was then pumped upwards through the bed to equilibrate the column. Then, the crude R-PE extract was applied at room temperature. The expanded bed run was conducted as previous description by Niu et al. (2006). The volume and absorption spectrum of each eluate from the Streamline column were measured for determination of the quantity (Kursar et al. 1983) and the purity (OD565/OD280) of isolated R-PE (Wang 2002; Wang et al. 2002).

R-PE purification

The eluates from Streamline column were combined and dialyzed against Milli Q water. Afterwards, they were pumped into the anion-exchange column (20×1 cm) loaded with 15 mL DEAE-Sepharose downwardly. The procedure of Niu et al. (2007) was used with some modifications: For washing buffer, we changed to 10 mM NaAc (pH 4.2) to wipe off PC absorbed by DEAE-sepharose. Then the column was developed with 50 mM phosphate buffer (pH 6.8) containing increasing step gradient of NaCl from 0 M to 0.20 M at a flow-rate of 2.5 mL min^{-1}. The eluate with red color was collected and the volume was measured. Both the UV–visible absorption spectrum and the fluorescence spectrum of eluate were recorded at room temperature (Wang 2002; Wang et al. 2002).

Spectroscopic measurements

Absorption spectra were recorded with a UV–Vis spectrophotometer (UV757 CRT, China) in increments of 2 nm with a 1-cm light path in quartz cells. A fluorescence spectrometer F-4500 (Hitachi, Japan) was used to record the fluorescence spectrum of the purified R-PE in increments of 5 nm. All spectra were recorded at room temperature. The amounts of R-PE in the different biliprotein-containing solutions were calculated from measurements of the absorbance at

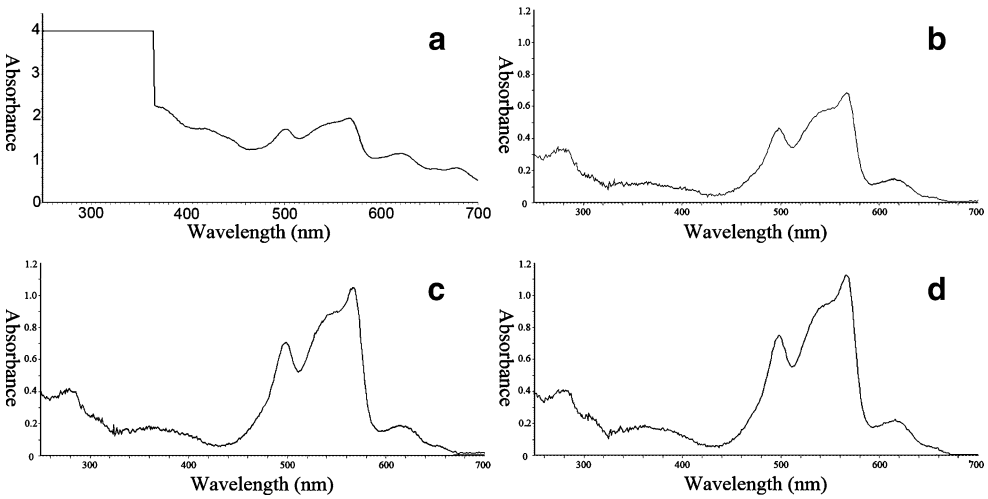

Fig. 1 Spectra of the fractions collected during R-PE isolation from leafy gametophyte of *P. yezoenis*. **a** Crude extract in 0.5 M $(NH_4)_2SO_4$. **b** Eluate with 0.20 M $(NH_4)_2SO_4$. **c** Eluate with 0.10 M $(NH_4)_2SO_4$. **d** Eluate with 0.05 M $(NH_4)_2SO_4$

498.5, 614 and 651 nm using the following equation (Kursar et al. 1983):

$$\mathrm{R-phycoerythrin(mgL^{-1})} = 155.8\mathrm{OD}_{498.5} - 40.0\mathrm{OD}_{614} - 10.5\mathrm{OD}_{651}.$$

Nondenaturing gel electrophoresis

To test the aggregation state of the purified protein, native R-PE solution without urea or treated with 4 M urea at boiling water for 10 min were assayed respectively using a mini-protean electrophoresis unit (BioRad, USA) with a stacking gel of 5% and a running gel of 12.5%. All the samples applied on the gel contained 20 mM Tris–HCl (pH 6.8), 4% glycerol, 0.009% bromophenol blue. After electrophoresis, the protein band corresponding to R-PE was identified firstly by intrinsic fluorescence under UV illumination. Then, protein bands were stained with 0.2% Coomassie blue R-250 in methanol:acetic acid:water (4:1:4, *v*/*v*/*v*) and recorded with a Gel Doc. Imaging system (BioRad, USA).

Denaturing gel electrophoresis

SDS-PAGE was performed in a vertical slab gel apparatus using discontinuous gels according to Schägger and von Jagow (1987). Fifty microliters of Laemmli sample buffer was added into 50 μl of PBP solution and the mixture was incubated in boiling water for 5 min. Twenty microliters of the samples and 5 μl of molecular mass standards were applied onto the 12.5% polyacrylamide gel containing 0.1% (*w*/*v*) SDS with a stacking gel of 5% polyacrylamide. Samples were separated using a constant voltage of 120 V at room temperature. The resolved SDS gel was soaked in 20 mM zinc acetate solution for 5 min at room temperature (Brekelman and Lagarias 1986). The bilin fluorescence was observed under UV illumination. Then, it was stained with 0.2% Coomassie blue R-250 in methanol:acetic acid:water (4:1:4, *v*/*v*/*v*) and scanned with a Gel Doc Imaging system (BioRad, USA).

Results and discussion

Extraction

In general, the extraction method is an absolutely pivotal step for the maximum recovery of phycobiliproteins in the natural state from an alga (Apt et al. 1993). Xiao et al. (2006) evaluated different wall-breaking methods on extracting R-PE from *P. yezoensis* and showed that freeze–thaw resulted in higher purity but lower yield of PE than any other methods. For the exhaustive recovery of R-PE from the algae, we use a triturator to fragment the

Table 1 Quantity and purity of the eluates eluted with 0.20 M, 0.10 M, 0.05 M ammonium sulfate from the streamline column

$(NH_4)_2SO_4$ (M)	Volume (mL)	OD_{280}	OD_{498}	OD_{565}	OD_{614}	OD_{620}	Purity (OD_{565}/OD_{280})	Quantity (mg)
0.20	92	0.339	0.47	0.684	0.146	0.142	2.0	6.5
0.10	130	0.410	0.709	1.045	0.187	0.183	2.5	13.9
0.05	57	0.395	0.747	1.119	0.214	0.208	2.8	6.4

*Twenty-eight grams of wet leafy gametophyte of *P. yezoensis* was used in the preparation of R-PE

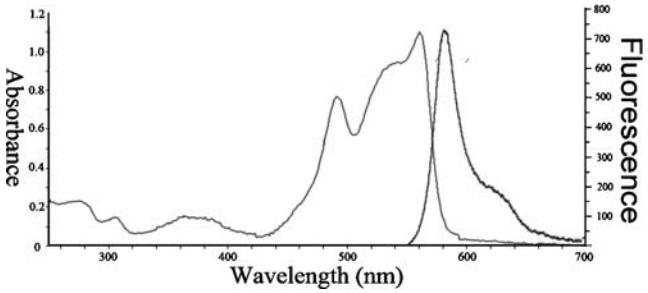

Fig. 2 The absorption and fluorescence spectrum of purified R-PE from DEAE-sepharose column

firm cell layer of *P. yezoensis* in 10 mM phosphate buffer (pH 6.8). Then, the slurry was subjected to freeze–thaw for protein extraction. Considering the extraction ratio, we repeated the extraction treatment three times. It should be emphasized this pretreatment method is much simpler than others that use ultrasound or the addition of chemical compounds (lysozyme, rivanol, acetone, Triton X-100 …; Saxena 1988; Tchernov et al. 1999).

When the extract was collected, solid ammonium sulfate was added to achieve the final concentration of 0.50 M for subsequent EBA chromatography. The volume of this crude extract was measured and the absorption spectrum recorded (Fig. 1a). The absorption located at 260 nm corresponds to a mixture of proteins and nucleic acid. The absorbance at 280 nm was higher than that at 565 nm, which indicated the presence of additional contaminant proteins besides R-PE. The high absorbance between 300 nm and 360 nm in Fig. 1a indicated other biomolecule contaminants whose nature has not been identified. According to the equation above, R-PE quantity in every solution was calculated out and the results are listed in Table 1.

Isolation of R-PE using EBA method

The EBA method has been used widely to purify PBPs from algae recently, for it overcomes the blocks of chromatographic column due to high polysaccharide contents of algae such as *P. haitanensis* (Niu et al. 2007). Another point leading to the success of hydrophobic interaction chromatography (HIC) in PBPs purification is the non-chaotropic nature and compatibility of $(NH_4)_2SO_4$. The proper concentration of this salt is helpful for binding of PBPs on hydrophobic resin. We have standardized and optimized the purification of R-PE by HIC, taking advantage of binding the protein to the phenyl-sepharose in the presence of high concentration of $(NH_4)_2SO_4$ salt and eluting of target protein through decreasing $(NH_4)_2SO_4$ concentration. For R-PE purification from *P. yezoensis*, we selected the binding concentration of 0.50 M and the eluting concentration of 0.2 M to 0.05 M. The volumes and the purities of fractions eluted with decreasing gradient of ammonium sulfate solution from the Streamline column are listed in Table 1 and the absorption spectra are shown in Fig. 1b–d.

The total quantity and the yield of R-PE after Streamline column were 26.9 mg and 0.96 mg g^{-1}, respectively. Moreover, the spectroscopic ratio (OD_{565}/OD_{620}) of the eluates from the streamline column was up to 2. This was very important for further purification. After the partial purification and concentration with the HIC steps, R-PE could be recovered successfully by anion-exchange chromatography.

Three absolute peaks at 498, 565, and 620 nm and a shoulder at 540 nm were recorded. A significant loss of absorbance in the near-UV region disappeared compared with the crude extract spectrum. Although the characteristic peaks at 498 nm and 565 nm, and spectrum shape conformed to the typical spectrum of R-PE (Galland-Irmouli et al. 2000; Rossano et al. 2003), the absorbance peak located at 620 nm indicated the PC contamination.

Isolation of R-PE with the expanded bed adsorption described here was very simple. When using the Streamline column, only a simple and low-cost pretreatment was necessary before separation on the column. One cycle of the isolation process by EBA chromatography, including equilibration (20 min)+loading (50 min)+washing (30 min)+elution (50 min), took only about 2.5 h.

Table 2 Purification of R-phycoerythrin

Fraction	Volume (mL)	OD_{280}	OD_{498}	OD_{565}	OD_{620}	Purity(OD_{565}/OD_{280})	Relative purity		Yield (mg g^{-1})	Recovery
							(OD_{565}/OD_{498})	(OD_{620}/OD_{565})		
Aqueous	800	–	1.706	1.975	1.152	–	1.2	0.58	6.06	–
Phenyl-sepharose	279	–	–	–	–	2.0–2.5	1.45–1.49	0.18–0.21	0.96	16%
DEAE-sepharose	230	0.204	0.640	0.917	0.021	4.50	1.4	0.02	0.82	13.5

– Indicated the value was not determined or could not be determined

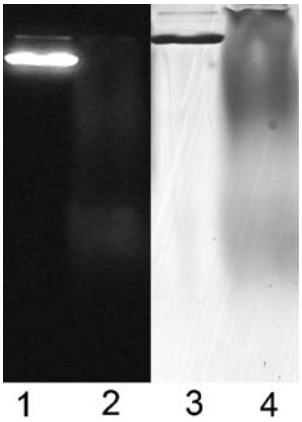

Fig. 3 Native PAGE of R-PE from *P. yezoenis*. From *left* to *right*, the lanes from *1* to *2* were the results under UV illumination and *3* to *4* were stained with Coomassie blue R-250. R-PE from *P. yezoenis* was analyzed electrophoretically in 20 mM Tris buffer without urea (lanes *1* and *3*) or treated for 5 min at 100 °C with 4 M urea (lanes *2* and *4*)

Purification of R-PE from *P. yezoensis*

The fractions recovered from the Streamline column were combined and purified by anion-exchange chromatography loading DEAE-Sepharose in a packed-bed column. For purification of PBPs from EBA system, ion-exchange chromatography was the most common method applied. However, not all PBPs from different organisms would be purified using the same ion-exchange resin or the same protocol of column developing. For example, both R-PE from *P. urceolata* (Niu et al. 2006) and *P. haitanensis* (Niu et al. 2007) could be purified using Q-Sepharose, but the buffer to eliminate PC contaminant and the conditions in detail for R-PE eluting were different. When *P. yezoensis* was used, we had to turn to DEAE-Sepharose and optimized the procedures for R-PE. To obtain the pure R-PE, continuous ionic strength gradients were applied, as well as pH gradients. However, the purification of R-PE was not achieved using the pH gradients. Through the optimization tests, 20 mM NaAc solution (pH 4.2) was selected to remove the PC, whereas pure R-PE was recovered with a continuous ionic strength gradient of NaCl from 0 M to 0.15 M in 30 mM sodium phosphate buffer (pH 6.8). The purified R-PE presented an intense orange fluorescence with emission maxima at 578 nm when excited with at 565 nm. Intense red color and fluorescence properties are a consequence of the interactions between multiple covalently bound chromophores and apoprotein. The UV–Vis absorption spectrum and fluorescence spectrum of pure R-PE solution are shown in Fig. 2. The absorption peak at 620 nm was removed and no PC could be detected by spectrometry.

R-PE recovery and purity analysis

It is widely known that absorbance at 280 nm is due to the total concentration of proteins in the solution and that at 565 nm indicates the concentration of R-PE, that at 620 nm corresponds to PC. Decrease in absorbance at 280 nm suggested the removal of other proteins and the ratio of A_{565}/A_{280} is considered as a good indication of the purity level of PE. The purity ratio (A_{565}/A_{280}) of the eluate from the Streamline column was 2.0 to 2.8, which is lower than 3.2 the average criterion for PBP purity (Galland-Irmouli et al. 2000). The A_{565}/A_{280} ration of eluate from anion-exchange column increased to 4.5 and the absorbance attributed to PC was no longer found. That is, R-PE from *P. yezoensis* could be purified with these two chromatographic steps.

According to the equation, $\text{R-PE}=155.8\text{OD}_{498.5}-40.0\text{OD}_{614}-10.5\text{OD}_{651}$ (Kursar et al. 1983), the yield of R-PE whose purity was more than 3.2 was 0.82 mg g^{-1} leafy gametophyte of *P. yezoensis*. In comparison with the traditional method for R-PE purification, which requires 74 g of frozen alga to obtain 1 mg of purified R-PE (Wang et al. 1996), the yield was improved greatly. The R-PE recovery, expressed as a percentage of the amount of R-PE obtained after each purification step to the total amount of R-PE in the crude extract is shown in Table 2. From the crude extract of *P. yezoensis* containing 170 mg R-PE, we recovered 22.9 mg pure R-PE using the method described here. The recovery was 13.5% which is lower than that reported in other species, such as *Polysiphonia urceolata* with a recovery of 22% (Niu et al. 2006) or *Porphyra haitanensis* with a recovery of 23% (Niu et al. 2007). Thus, the paradox is that a similar extraction method and a similar

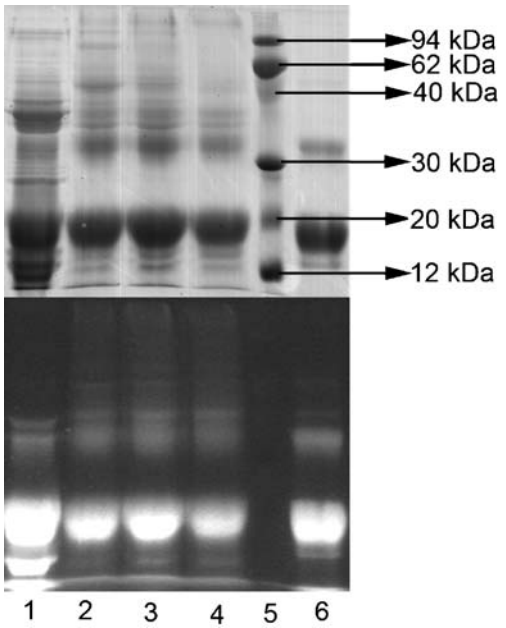

Fig. 4 SDS-PAGE analysis of the fractions collected during the purification steps. Lanes: *1* crude extract, *2* eluate with 0.20 M $(NH_4)_2SO_4$, *3* eluate with 0.10 M $(NH_4)_2SO_4$, *4* eluate with 0.05 M $(NH_4)_2SO_4$, *5* molecular weight markers, *6* purified R-PE using ion-exchange chromatography. The *upper* one shows results of Coomassie blue R-250 staining and the *lower* one indicates the biliprotein with the assistance of zinc acetate

EBA isolation system give different recovery. An explanation may be the complicated components and high content of PC in *P. yezoensis* crude extract which leads to the difficulty in R-PE purification. After the Streamline column was eluted with decreasing concentration of $(NH_4)_2SO_4$, there was still much biliproteins attached to the resin. When the eluate eluted with distilled water from the Streamline column was applied to the ion-exchange column, no R-PE solution, whose purity ratio (OD_{565}/OD_{280}) was higher than 3.2, could be obtained.

Purity of R-PE is also estimated by means of following indexes: A_{565}/A_{498} and A_{620}/A_{565}. The ratio of $A_{565}/A_{498} \leq 1.5$ indicated the identity of the R-PE. R-PE has a strong secondary absorption peak at 498 nm, where B-PE exhibits only a slight shoulder. If the ratio of A_{565}/A_{498} is less than 1.5, the R-PE is not significantly contaminated with B-PE (Rossano et al. 2003). Our R-PE solution other than the crude extract of R-PE did not contain B-PE. This could be explained by the absence of B-PE in *P. yezoensis*. Secondly, the index of $A_{620}/A_{565} \leq 0.005$ indicated the purity of the R-PE preparation with respect to PC (Rossano et al. 2003). Our A_{620}/A_{565} ration of the R-PE eluent was only 0.02. These fractions could be used as food or cosmetics additives.

Electrophoresis assay

The aggregation state of the R-PE samples were determined by native PAGE, and the bands were visualized by illumination with UV light (Fig. 3). It clearly shows one band (lane 1) before staining and only one single protein band was found after Coomassie blue R-250 staining (lane 3). A very faint smear band lower than the native R-PE sample can be seen the in the presence of 4 M urea (lane 4), which indicated that the R-PE disassembled into oligomer and then further into subunits in the presence of urea. It is widely known that the aggregation state of this protein shows great variations such as dimeric, trimeric, and hexameric forms (Duerring et al. 1991; Patel et al. 2005). Thus, the R-PE purified here was homogenous and existed only as a hexamer complex.

The procedure of R-PE purification was also monitored by SDS-PAGE in the presence of 2-mercaptoethanol in 12.5% polyacrylamide gels stained with Coomassie R-250 (Fig. 4). In crude extract, there were many bands suggesting the presence of contaminant proteins other than R-PE, which could be successfully removed in successive isolation in Streamline column. The fraction eluted from DEAE-sepharose presented two main bands in the gel, which correspond to the γ subunit (33 kDa) and the overlapped α and β subunits (17 and 19 kDa). These values are in agreement with the previously reported values estimated by SDS-PAGE (Thomas and Elisabeth 1981; Rossano et al. 2003; Isailovic et al. 2004). However, the gel treated with zinc acetate revealed four protein bands. Beside the lower two corresponding to γ and α/β subunits, the upper two low-intensity bands located between 30 and 60 kDa might be the aggregates of linker peptides with the bilin-subunit of R-PE. The use of zinc enhances the fluorescence of bilin-containing protein and improves the sensitivity of biliprotein detecting in gel (Brekelman and Lagarias 1986), and the ease to perform made it the method of our choice.

In conclusion, our results indicate that the method described here reduced the processes time and achieved a higher product recovery compared with the traditional chromatographic method and a scaleable procedure for the purification of R-PE from *P. yezoensis* is established.

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