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# Chromatographic purification and characterization of B-phycoerythrin from *Porphyridium cruentum* Semipreparative high-performance liquid chromatographic separation and characterization of its subunits☆

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**Abstract**

A fast preparative two-step chromatographic method for purification of B-phycoerythrin from *Porphyridium cruentum* is described. This biliprotein was homogeneous as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis yielding three closely migrating bands corresponding to its three subunits. Baseline separation of its α-, β- and γ-subunits was achieved by a reversed-phase HPLC gradient semipreparative method with a $C_4$ large-pore column and a solvent system consisting of 0.05% trifluoroacetic acid (TFA) in water and 0.05% TFA in acetonitrile. B-Phycoerythrin in different aggregation states and its subunits have been spectroscopically characterized. Hexameric B-phycoerythrin has similar secondary and tertiary structure than dissociated B-phycoerythrin determined by circular dichroism. 

*Keywords:* *Porphyridium cruentum*; Phycoerythrins; Biliproteins; Proteins

## 1. Introduction

The biliproteins form light-harvesting antenna complexes of cyanobacteria, red algae and cryptomonads. The spectroscopic properties of individual biliproteins depend mainly on the chemical nature of the bilins they carry [1–5]. The biliproteins can be divided into three main classes depending on their visible absorption properties: phycoerythrins (PEs), $\lambda_{max}$ 540–570 nm, which have the chromophores phycoerythrobilin (PEB) and phycourobilin (PUB); phycocyanins (PCs), $\lambda_{max}$ 610–620 nm, which contain either a mixture of the phycocyanobilin (PCB) and PEB chromophores or just PCB, depending on the species of origin; and allophycocyanins (APCs), $\lambda_{max}$ 650–655 nm, with PCB as prosthetic group. A few cyanobacteria possess a fourth type of biliprotein in place of PE, the phycoerythrocyanin (PEC), which has a PCB and a phycobiliviolin tetrapyrrole chromophore, $\lambda_{max}$ 568 and 585 nm (shoulder) [6,7].

The unicellular red algae *Porphyridium cruentum*

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has been shown to contain four biliproteins in the following approximate relative amounts: APC (5%), R-phycoerythrin (R-PC) (11%), b-phycoerythrin (b-PE) (≈42%) and B-phycoerythrin (B-PE) (≈42%). APC shows an absorption maximum at 650 nm and a shoulder at 620 nm. R-PC exhibits absorption maxima at 618 and 655 nm. B-PE has absorption maxima at 563 and 545 nm and a shoulder at 498 nm, its $A_{545\ nm}/A_{495\ nm}$ ratio is about 2.5. The spectral properties of b-PE differ to those of B-PE, i.e., the absorption maxima of b-PE at 543 and 563 nm closely agree with those observed for B-PE, but the shoulder at 498 nm is absent in b-PE [8,9].

The introduction of biliproteins as fluorescent tags for cells and macromolecules [10] was followed by its widespread application in highly sensitive fluorescence techniques [11]. B-PE has been shown to be particularly useful due to its large absorption coefficient and great fluorescence properties just as the high quantum yield and high Stokes' shift. Therefore, B-PE is a valuable candidate in the design and characterization of light-sensing elements in biosensors [12]. Another interesting application of the biliproteins is their use as natural dyes in foods and cosmetics, replacing the synthetic dyes [13–15]. Once again, B-PE is the most valuable of the biliproteins due to its intense and unique pink color. Nevertheless, the use of this biliprotein has been somewhat limited by the tedious preparation of adequate amounts of the purified protein, so it is of significance to search for new methods of preparative purification.

Highly purified B-PE is a heteropolymer with the $(\alpha\beta)_6\gamma$ polypeptide composition [1,16]. B-PE α-subunit contains 164 amino acids and β-subunit contains 177 amino acid residues. Two PEB groups are attached through single thioether bonds to the α-subunit and three PEB groups to the β-subunit, one of them is bound through two thioether linkages. The five PEB groups of the α and β-subunit are attached to the cysteine residues at position α-84, α-184a, β-84, β-155, and β-50/β-61. The amino acid sequence of the γ-subunit is unknown, but it carries four bilins, two PEBs and two PUBs. The B-PE structure is a disc formed by two αβ-trimers assembled face-to-face with the γ-subunit located in a central hole [9,17,18].

The characterization of biliproteins with respect to subunit composition, bilin type and content, and amino acid sequence requires separation of the subunits. Several methods performed under denaturing conditions have been employed in the past, but all share one disadvantage, that the separations are long, and that in some instances and under certain conditions they undergo some oxidative degradation [19]. Once again, the research for quick semipreparative new methods of subunit separation can be profitable. On the other hand, its use as natural dyes in some kind of heat-treated foods or in micellar solubilisates, makes it convenient to obtain smaller aggregates than in hexameric B-PE [20].

We report here the purification of adequate amounts of pink biliprotein, B-PE, by being only two chromatographic steps from the red algae *P. cruentum*, as an inexpensive source of this protein. Moreover, we describe a quick semipreparative reversed-phase high-performance liquid chromatography (HPLC) method for the separation of its α-, β- and γ-subunits. The procedure described in this report is rapid, because high sample recovery requires less than a milligram of protein and the subunits are isolated in a suitable form for back characterization. The separation work is accompanied with spectroscopic characterization of B-PE (in different aggregation states) and its subunits.

## 2. Experimental

### 2.1. Chemicals

HPLC-grade acetonitrile was obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). Trifluoroacetic acid (TFA) was purchased from Fluka and the materials used for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Sephadex were from Pharmacia (Uppsala, Sweden). Preswollen microgranular DEAE-cellulose DE-52 was from Whatman (Maidstone, UK). Molecular mass standards were from Sigma diagnostics (St. Louis, MO, USA). All other chemicals were purchased from Sigma and used without further purification.

### 2.2. Biliprotein purification

All buffers mentioned in this paper contained 0.01% sodium azide unless otherwise specified. Cells of *P. cruentum* were generously provided by the Chemical Engineering Department of Almeria University (Spain) as frozen cells. In a typical experiment 50 g (wet mass) were resuspended in 50 ml of 1 *M* acetic acid–sodium acetate buffer (pH 5.5). The slurry was sonicated for 10 min under stirring, transferred to ultracentrifugue tubes, and centrifuged at 80 000 *g* for 1 h. The procedure was repeated again with the pellets, and the supernatants from both centrifugations pooled. Solid $(NH_4)_2SO_4$ was added to 65% saturation, followed by incubation for 2 h, and centrifugation at 20 000 *g* for 15 min. The pellets were resuspended in 1 ml of 50 m*M* acetic acid–sodium acetate buffer (pH 5.5) and dialyzed overnight against 2 l of the same buffer.

The dialyzed biliprotein-containing solution was applied to a column (17×2.5 cm) of DEAE-cellulose DE-52, preequilibrated with 50 m*M* acetate buffer (pH 5.5). After washing with 90 ml of 50 m*M* acetate, pH 5.5, the column was developed with 100 ml of 0.25 *M* acetic acid–sodium acetate buffer, pH 5.5. After this, the column was washed with 100 ml of 0.35 *M* acetic acid–sodium acetate buffer, pH 5.5. The flow-rate was maintained at 100 ml/h and the fractions were of 3 ml.

The pink fractions were analyzed by SDS–PAGE and UV–visible absorption spectroscopy. These fractions were brought to 65% saturation with $(NH_4)_2SO_4$ and allowed to stand for 1 h prior centrifugation at 20 000 *g* for 15 min. The pellets were resuspended in 20 m*M* sodium phosphate buffer and dialyzed exhaustively against the same buffer.

The PEs solution was applied to a column of Sephadex G-100 (60×2.5 cm) preequilibrated with 0.02 *M* phosphate buffer (pH 7.0). Gel filtration was performed at a constant flow-rate of 60 ml/h. The selected pink eluates were pooled, brought to 65% saturation with $(NH_4)_2SO_4$ and left to stand overnight in the dark at 4°C before centrifugation. The pellets of purified B-PE were resuspended in a small volume of 5 m*M* sodium phosphate buffer (pH 7.0), dialyzed overnight at 4°C against the same buffer and freeze–dried for storage, until utilization. Typically, only the bands corresponding to the α-, β- and γ-subunits are detected on analysis of purified B-PE by SDS–PAGE (Fig. 1).

### 2.3. SDS–PAGE

Electrophoresis was carried out in a vertical slab gel apparatus (Miniprotean III; Bio-Rad) according to the tricine buffer system described by Shägger and von Jagow [21] using a 16.5% polyacrylamide slab gel containing 0.1% (w/v) SDS with a stacking gel of 4% polyacrylamide. Samples were preincubated with 4% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue, and 50 m*M* Tris, pH 6.8, for about 5 min at 95°C. Gels were run at room temperature, and visualized by staining for 30 min with 0.1% (w/v) Coomassie Brilliant Blue R-250, 40% methanol (v/v) with 7% (v/v) acetic acid and destained in dilute acetic acid. For calibration, marker proteins were used.

### 2.4. High-performance liquid chromatography

HPLC was performed using a Merck–Hitachi

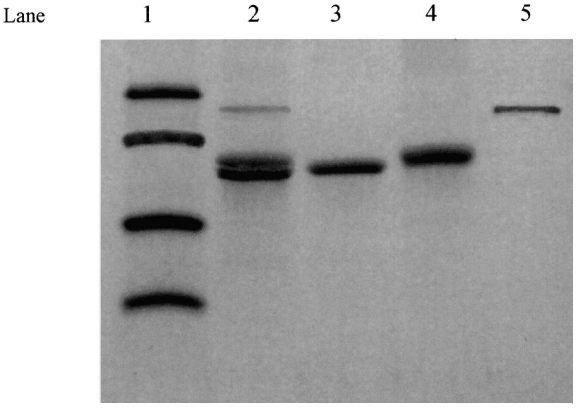


Fig. 1. SDS–PAGE of purified B-PE and its subunits. From left to right, the lanes are, 1: marker proteins; 2: purified B-PE after Sephadex G-100; 3: B-PE α-subunit; 4: B-PE β-subunit and 5, B-PE γ-subunit. α-, β- and γ-subunits were after HPLC (for experimental details, see text). The molecular masses of the markers from top were: carbonic anhydrase (30 000), trypsin inhibitor (21 100), cytochrome (12 400) and aprotinin (6500).

(Darmstadt, Germany) Model L-6220 chromatograph. B-PE (20 μl of 0.5–1 mg/ml) in sodium phosphate buffer 20 m*M* (pH 7.0) were injected into a Vydac $C_4$ column (150×4.6 mm), purchased from The Separations Group (Hesperia, CA, USA), previously equilibrated with a solution formed by 5% of buffer A (0.05% TFA in acetonitrile) and 95% of buffer B (0.05% TFA in water). B-PE subunits were eluted from the column according to the following program: 0–3 min, buffer A–buffer B (5:95, v/v); 3–8 min, linear gradient to 40% buffer A; 8–28 min, linear gradient to 60% buffer A; 28–31 min, linear gradient to 95% buffer A; 31–34 min, constant composition at 95% buffer A and 34–40 min linear gradient to 5% buffer A (initial conditions). The flow-rate was 0.8 ml/min and the effluent was monitored at 226 nm. The eluted subunits were collected manually as they elute from the column and then freeze–dried for further analysis. All samples were spun for 5 min in a microcentrifuge immediately prior to injection.

### *2.5. Spectroscopic methods*

Absorption spectra were recorded on a Perkin-Elmer (Beaconsfield, UK) Lambda-16 UV–Vis spectrophotometer with a 1-cm light path. Protein concentrations for these experiments were in the range of 0.1–0.8 g/l. Fluorescence emission spectra were recorded on a Shimadzu (Kyoto, Japan) RF5001 spectrofluorometer. For steady state polarized fluorescence measurements the spectrofluorometer was equipped with polarizers in the excitation and emission paths. The anisotropy was calculated as $A=(I_{VV}-GI_{VH})/(I_{VV}+2GI_{HH})$, where $G=I_{HV}/I_{HH}$, a correction factor for the polarization due to the optics in the instrument. Protein concentrations were chosen so that reabsorption of the emission was negligible. All spectra were recorded at room temperature.

Time-resolved fluorescence intensity was measured in the single-photon-counting mode with an Edinburg Instrument (UK) spectrofluorometer, Model FL900. The data were obtained with a free running $H_2$ discharge flashlamp operating at 7.0 kV, at 0.40 bar and a frequency of 40 kHz. The lamp pulse was recorded at the same wavelength as that of the excitation. The bandwidth of the monochromators was 10 nm. Accumulation was stopped when 10 000 counts were stored in the peak channel for the total fluorescence intensity decay. The quality of the fittings was judged by the reduced $\chi^2$ method and the weighted residuals were checked for random distribution.

Circular dichroism (CD) spectra were made at 25°C on a Jasco (Tokyo, Japan) Model J-710 spectropolarimeter equipped with a thermostated cell holder and a Neslab (Newington, NH, USA) Model RTE-110 water circulating bath. 1 mm and 5 mm path-length quartz cells were used for measurements in the far- and near-ultraviolet region, respectively. The results are expressed as mean residue ellipticity $[\Theta]$, which is defined as $[\Theta]=100\Theta_{obs}/lc$, where $\Theta_{obs}$ is the observed ellipticity in degrees, $c$ is the concentration in residue moles per liter and $l$ is the length of the light path in centimeters.

## 3. Results and discussion

### *3.1. B-Phycoerythrin purification*

Sonication and $(NH_4)_2SO_4$ addition to 65% of saturation resulted in a precipitate which contains R-PC, APC, B-PE and b-PE. This biliprotein mixture shows an absorption spectrum with maxima at 545 and 618 nm, and shoulders at 650, 563, 498, 375, 310 and 280 nm corresponding to biliproteins and other contaminant proteins (Fig. 2).

Before performing ion-exchange chromatography, the pH and the initial ionic strength ($I$) were determined by preliminary tests using continuous and simultaneous gradients of both pH and *I*. Fig. 3A shows the elution curve after DEAE-cellulose DE-52 chromatography. The results indicate that an ionic strength discontinuous gradient was sufficient to separate the phycoerytrins, since the R-PC and APC were eluted at 0.05 *M* acetic acid–sodium acetate buffer, pH 5.5 (peak 1), whereas B- and b-PE were eluted at 0.25 *M* acetic acid–sodium acetate buffer, pH 5.5 (peak 2). The absorption spectrum of PE mixture after the ionic exchange step shows absorption maxima at 545, 375 and 280 nm and shoulders at 310, 498 and 563 nm (Fig. 2). It should be noted in this spectrum the absence of significant absorption at longer wavelengths indicating that R-PC and APC

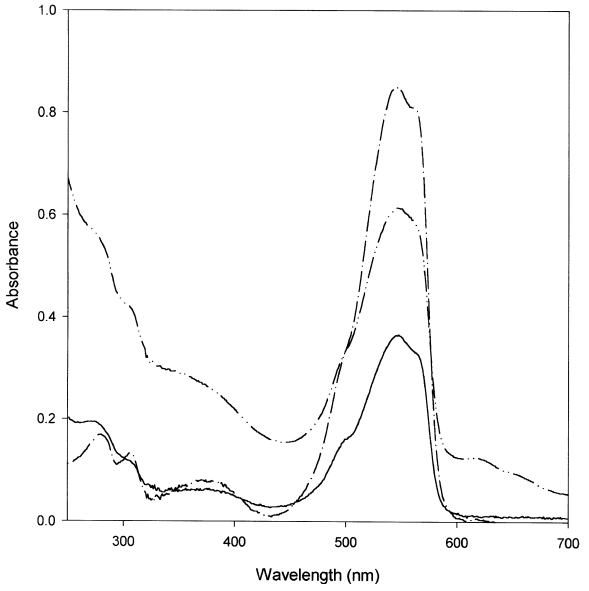


Fig. 2. Absorption spectra of (- ·· - ·· -) biliprotein mixture from *P. cruentum* after initial stages of purification, (———) eluted PEs mixture after ion-exchange chromatography and (- · - · -) purified B-PE after gel filtration chromatography on Sephadex G-100.

have been removed from the initial protein mixture. Nevertheless, the $A_{545}/A_{280}$ ratio of around 1.9 indicates the presence of contaminant proteins other than phycoerythrins.

The considerable difference between the molecular mass of B-PE and b-PE lead us to select an additional chromatographic process of gel filtration. In preliminary studies, we tested columns with different heights and flow-rates. The best resolution was obtained with 60 cm columns and a flow-rate of 50 ml/h. Fractions of 3 ml were collected. Fig. 3B shows the elution profiles at 280 nm and 545 nm, as well as the $A_{545}/A_{280}$ ratio from Sephadex G-100 chromatography. As can be seen, the six first pink fractions have the $A_{545}/A_{280}$ ratios established for PE purity [6].

SDS–PAGE shows that the five first pink fractions eluted yield each one three bands corresponding to B-PE subunits (Fig. 1). The α-subunit contains 164 amino acid residues and a calculated molecular mass (including the two PEB chromophores) of 18 991, consequently its mobility is larger than of the β-subunit that contains 177 residues and has a molecu-

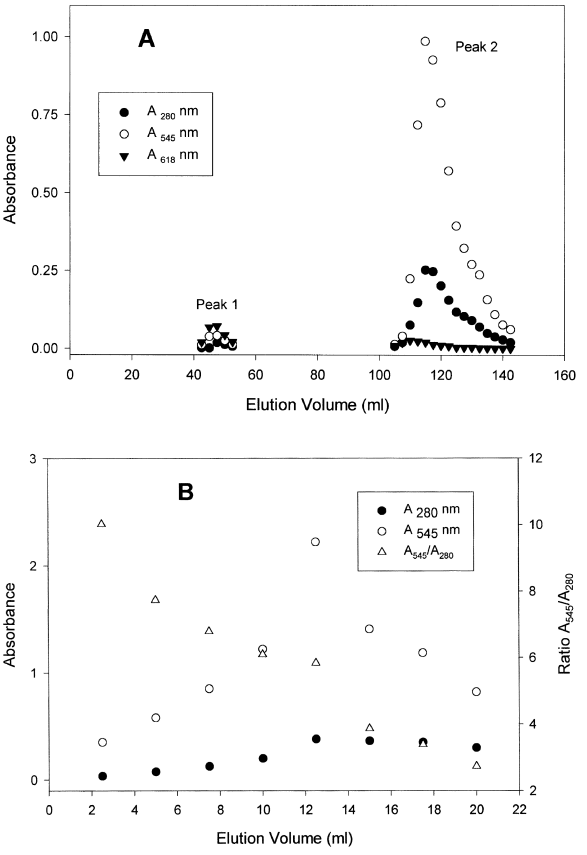


Fig. 3. (A) Elution curve of the fractions from *Porphyridium cruentum* after DEAE-cellulose DE-52. Peak 1: 50 m*M* acetate buffer (pH 5.5). Peak 2: 0.25 *M* acetate buffer (pH 5.5). Absorbances were at 280 nm (·), 545 nm (○) and 618 nm (▼). (B) Elution curve of selected pink fractions from DEAE-cellulose DE-52 (between 100 and 150 ml of the elution volume) after Sephadex G-100. Absorbances were at 280 nm (·), 545 nm (○) and $A_{545}/A_{280}$ ratio (△).

lar mass of 20 315 including three PEBs [6], both are present in similar amounts. The third band corresponds to the γ-subunit, a minor component of apparent molecular mass about 27 000 [18,22,23]. By comparison with standards, we obtained molecular masses of the α-, β- and γ-subunits of about 16 500, 18 000 and 27 000, respectively. These values are in agreement with the previously reported values estimated by SDS–PAGE [18,22,23]. The band intensities are in concordance with a heteropolymer with polypeptide composition $(\alpha\beta)_6\gamma$.

The absorption spectrum in Fig. 2 from the above mentioned isolated fractions agree well with the

spectra of pure B-PE published elsewhere [8,18,22,24]. Likewise, the $A_{545}/A_{280}$ ratio from these selected fractions was larger than 3.5. Moreover, it shows the $A_{545}/A_{495}$ ratio of 2.5, as established for purity criterion of B-PE [8]. Consequently, we conclude that the purification method described above provides B-PE solutions in hexameric aggregation state, the basic unit of the B-PE rod in phycobilisomes [1].

The original work of Glazer and Hixson for B-PE purification was a three chromatographic steps scheme [8]. More recently this methodology has been simplified to two chromatographic steps [25,26]. Nevertheless, none of them used discontinuous ionic strength gradient in the common ion-exchange step. Hence, the main goal of our scheme is that the ion-exchange step was achieved by a discontinuous ionic strength gradient.

In the pH range of 5 to 7 and low ionic strength, phycoerythrins show the aggregation state $(\alpha\beta)_6\gamma$. Chaotropic salts at 1.0 *M* concentration, e.g., sodium perchlorate, have the ability to dissociate C-PC and APC to a homogeneous solution of monomers but not to dissociate monomers into its subunits [27], as has been showed by us for C-PC and APC from *Spirulina platensis* [28]. Fig. 4 shows visible absorption, fluorescence emission and anisotropy spectra of B-PE solutions in 20 m*M* sodium phosphate buffer, pH 7.0 and B-PE in the same buffer including 1 *M* sodium perchlorate. The absorption spectra of B-PE in both media are different, since the dissociation of hexameric B-PE causes the loss of the absorption maximum at 563 nm for conversion in a shoulder, and a decrease in the ratio $A_{545}/A_{280}$. The fluorescence emission maximum in both media is the same, 574 nm, but the fluorescence intensity of hexamers is larger than B-PE in phosphate buffer, pH 7.0 added of 1 *M* sodium perchlorate. In general, the fluorescence spectra of biliproteins are modulated by fluorescence resonance energy transfer (FRET) between the several chromophores that they have in their different subunits [29], therefore when the aggregation state of the biliproteins changes from hexamers to monomers, energy transfer decreases and a dramatic increase in the fluorescence excitation anisotropy spectra must be observed, as has been showed for C-PC from *Mastidoglastus laminosus* [30] and C-PC from *S. platensis* [28]. The anisotropy

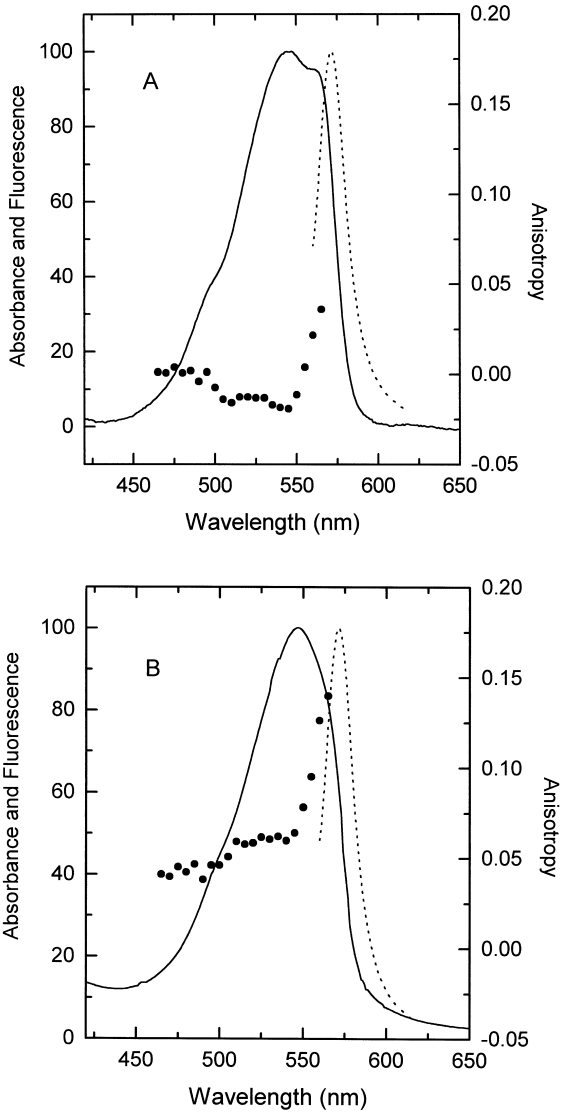


Fig. 4. Spectral characteristics of purified B-PE: (A) in 20 m*M* sodium phosphate buffer, pH 7.0 and (B) in 1 *M* sodium perchlorate, 20 m*M* sodium phosphate buffer, pH 7.0. (———) Absorption spectra. (-----) Fluorescence emission spectra ($\lambda_{ex}$= 540 nm; $\Delta\lambda_{ex}=\Delta\lambda_{em}$=1.5 nm). Absorption and fluorescence spectra were normalized since were only used on a comparative basis. (····) Fluorescence excitation anisotropy spectra ($\lambda_{em}$=595 nm, $\Delta\lambda_{ex}=\Delta\lambda_{em}$=3 nm and excitation wavelength range=460–560 nm). The anisotropy values shown are the means of five values and were calculated for each 5 nm. All spectra were recorded at room temperature.

spectrum shape of B-PE in 20 m*M* sodium phosphate buffer, pH 7.0 is similar to those earlier published for hexameric phycoerytrins from other red algae [31–33], however in 1.0 *M* sodium perchlorate solution, the anisotropy spectrum shows characteristic larger values than anisotropy from native B-PE, as can be seen in Fig. 4.

In the B-PE crystals, the electron density inside the central cavity of the hexamer is distinctly higher than outside, which indicates the location of the γ-subunit [9,17]. Therefore, dissociation of hexamers must generate (αβ) monomers and free γ-subunit. To check B-PE dissociation, a solution of B-PE in 1.0 *M* sodium perchlorate, 0.02 *M* phosphate buffer, pH 7.0, was added to a 50×1.5 cm column of Sephadex G-75 (preequilibrated with 1.0 *M* sodium perchlorate, 0.02 *M* phosphate buffer, pH 7.0) and eluted by means of the same buffer at 16 ml/h flow-rate. Fig. 5 shows the visible absorption spectra of the first and the last pink fractions obtained. The spectrum of the first fraction shows a shoulder corresponding to the PUB chromophore in the γ-subunit at 498 nm. This shoulder disappears smoothly in the following fractions until it vanishes in the last five fractions that only show the 545 nm absorption maximum characteristic of the PEB chromophore, suggesting the presence of only (αβ) monomers in agreement with the spectra from α- and β-subunits (see Fig. 9A) and spectrum from b-PE (that lacks of the γ-subunit) [8,9]. This methodology might be used for no-denaturing-low-pressure (αβ) monomer and γ-subunit preparative purification and we are presently analyzing this possibility. The results of this analysis will be presented elsewhere.

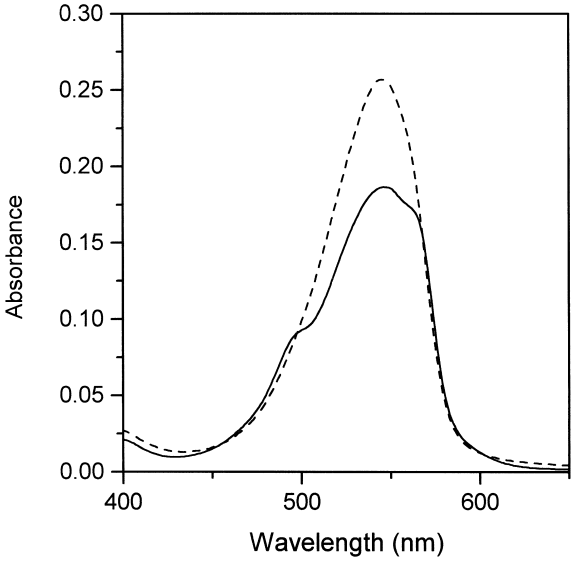


Fig. 5. Visible absorption spectra of the first (——) and the last (------) pink fractions of B-PE in 1.0 *M* sodium perchlorate, 0.02 *M* phosphate buffer (pH 7.0) after gel filtration on Sephadex G-75. For chromatographic conditions, see text.

CD studies have been realized with B-PE to obtain structural information of the biliproteins in different aggregation states. CD bands in the far-UV (170–250 nm) contain information about the secondary structure and are employed to monitor changes in this structure in the course of structural transitions. When the α-helix structure dominates, the CD spectra shows an intense negative band about 222 nm and another reasonably intense negative band at 208 nm. Moreover the CD of the α-helix dominates with an intense positive band at 192 nm [34–36]. Fig. 6 shows that native and dissociated forms of B-PE have a similar secondary structure dominated by α-helix, whereas acid media (i.e., B-PE in 0.01 *M* HCl, pH 2.0) result in an important loss of secondary structure. The near-UV CD spectrum can be used as a finger print of the protein [34–36]. CD bands in the near-UV are observed when aromatic side chains are immobilized in a folded protein and thus transferred to an asymmetric environment. The CD of the aromatic residues is very small in the absence of ordered structure. Fig. 6 show clearly that a 0.01 *M* HCl (pH 2.0) solution produces the loss of the well-defined tertiary structure of the native protein. Acid media induce the protein denaturation, demonstrated by the loss of the secondary structure and the disruption of the tertiary structure. Nevertheless, dissociate forms of B-PE seem to maintain the gross conformational features of the native protein as given by both far-UV and near-UV data. The CD spectrum in the 250–350 nm region of B-PE in 1.0 *M* sodium perchlorate, 0.02 *M* phosphate buffer, pH 7.0, still maintains the overall features of the spectrum of the native species in phosphate buffer only, even though the intensity of the CD signal is somewhat reduced (14%), as shown in Fig. 6B.

Nanosecond time resolved fluorescence from B-PE in both, 20 m*M* sodium phosphate buffer, pH 7.0 and 1.0 *M* sodium perchlorate solutions in the same

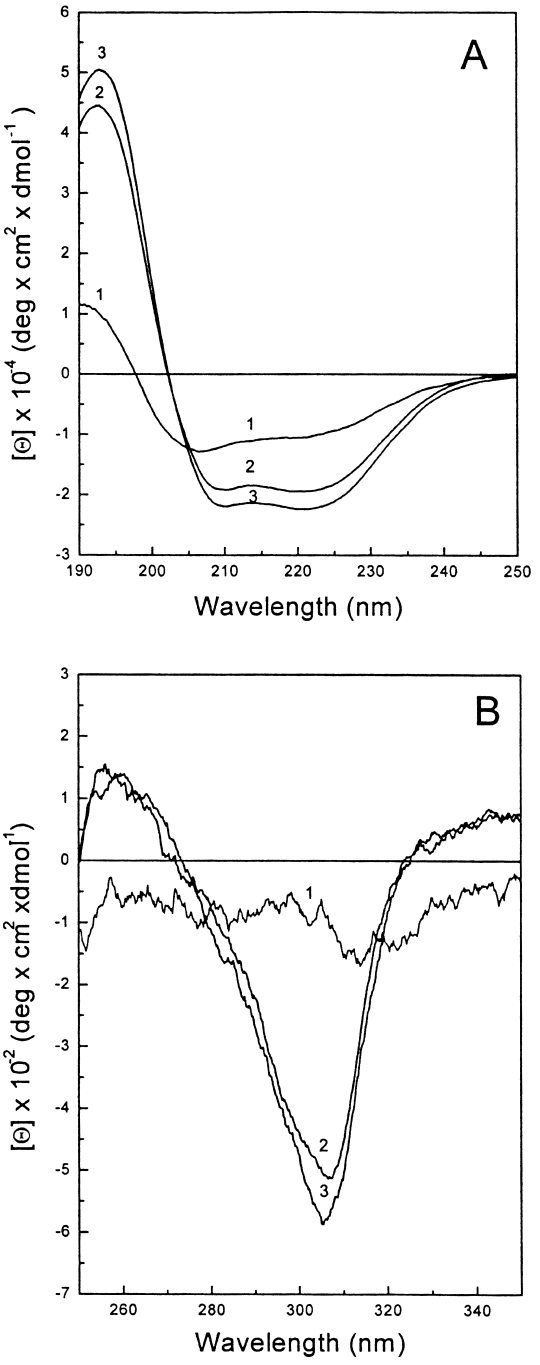


Fig. 6. (A) Far-UV and (B) near-UV CD spectra of B-PE in: (1) 0.01 *M* HCl, pH 2.0, (2) 1.0 *M* sodium perchlorate, 20 m*M* sodium phosphate buffer, pH 7.0, (3) 20 m*M* sodium phosphate buffer, pH 7.0. B-PE concentration was 0.1 mg/ml and 0.3 mg/ml for far-UV and near-UV CD, respectively.

buffer, have been recorded. Fig. 7 shows, as an example, the instrumental response, fluorescence decay and weighted residuals plots from B-PE in 20 m*M* sodium phosphate buffer, pH 7.0. The two fluorescence decay profiles have been well fitted with tri-exponential functions. The low $\chi^2$ values, as well as the random distributions about the zero line of the weighted residuals indicate how good the fit is. The best fitting values of lifetimes, normalized weighting coefficients and fitting statistical parameters resulting from tri-exponential analyses of B-PE solutions are shown in Table 1. In 20 m*M* sodium phosphate buffer, pH 7.0, the lifetime of 2.77 ns corresponds to fluorescence lifetime of PEB chromophore [37], whereas the lifetimes of 0.37 ns and 1.54 ns would correspond to different chromophores strongly or

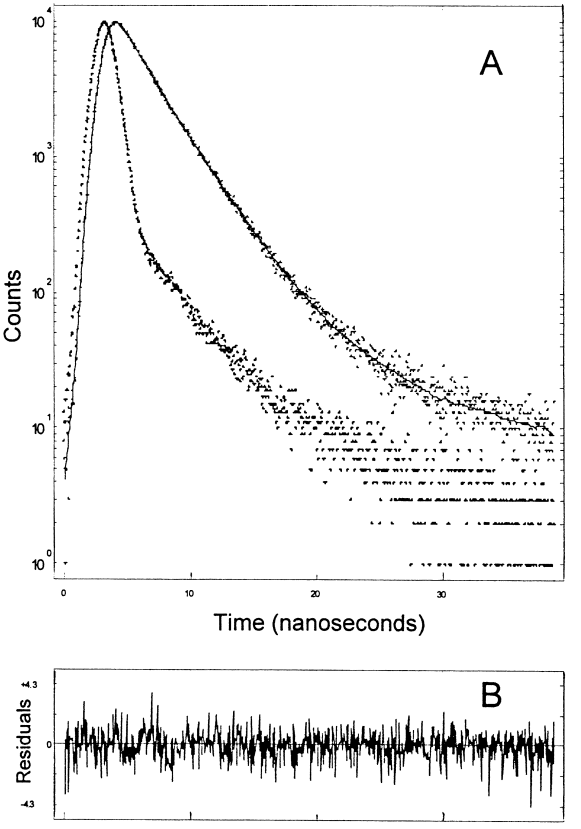


Fig. 7. (A) Fluorescence intensity decay of B-PE in 20 m*M* sodium phosphate buffer, pH 7.0 ($\lambda_{ex}$=550 nm, $\lambda_{em}$=576 nm, $\Delta\lambda_{ex}$=$\Delta\lambda_{em}$=5 nm), curve fitting based on a tri-exponential model (line), and lamp pulse ($\lambda_{ex}$=$\lambda_{em}$=550 nm, $\Delta\lambda_{ex}$=$\Delta\lambda_{em}$=5 nm). (B) Residuals. $\chi^2$=1.02. For further parameters, see Table 1.

Table 1
Fluorescence lifetimes, normalized weighting coefficients and fitting statistical parameters resulting from tri-exponential analysis of hexameric and dissociated B-PE solutions

| Sample | $\tau_1$ (ns) | $\tau_2$ (ns) | $\tau_3$ (ns) | $\alpha_1$ | $\alpha_2$ | $\alpha_3$ | $\chi^2$ (ns) |
|---|---|---|---|---|---|---|---|
| Hexameric B-PE | 0.37 | 1.54 | 2.77 | 3.40 | 20.82 | 75.78 | 1.02 |
| Dissociated B-PE | 0.39 | 1.64 | 2.60 | 4.61 | 29.76 | 65.63 | 1.03 |

weakly coupled that are involved in multitude of pathways for energy transfer [18] and that our nanosecond device is unable to resolve. Similar patterns can be seen for the nanosecond fluorescence decay from B-PE in 1.0 *M* sodium perchlorate, 20 m*M* sodium phosphate buffer, pH 7.0, although the lifetime of 2.60 ns is slightly smaller than the 2.77 ns lifetime corresponding to the PEB chromophore, probably due to quenching by larger solvent chromophore exposition in the dissociated biliprotein. On the other hand, the intermediate lifetime of 1.64 ns is slightly greater than the intermediate lifetime from hexameric B-PE, perhaps by the uncoupling of some chromophores when the hexameric structure is disrupted. Pioneering fluorescence studies on biliproteins demonstrated that there are two functional types of chromophores in phycoerythrins. The sensitizing (s) chromophores transferred excitons with a high efficiency to the fluorescing (f) chromophores, which fluoresced with fairly high quantum yield [33]. In our B-PE solutions the two shortest lifetimes can be the exciton transfer from s to f chromophores and the largest lifetime is the decay by purely radiative events from f chromophores.

### *3.2. Separation and characterization of α-, β- and γ-subunits of B-PE*

Although various types of reverse phase columns including silica based $C_4$ or $C_{18}$ columns were tested for B-PE subunit analysis, it was found that the Vydac $C_4$ column gave a better result in our laboratory. The separation was comparable to that obtained by Paddgett and Krogmann [38] with a $C_{18}$ and other authors with $C_4$ columns [19,39]. Nevertheless, our methodology have the advantage to result in semipreparative separations of the α-, β-, and γ-subunits, although the eluates of several experiments must be withdrawn, due to the low proportion of γ-subunit in native B-PE.

The separation conditions were developed with well-characterized B-PE. Denaturation of the protein simply was reached by the addition of 0.05% TFA to the mobile phase. We have tested several gradients with different slopes all performed with acetonitrile and water. The gradient described in Section 2 was found to give the best baseline separation of the α-, β- and γ-subunits of B-PE (Fig. 8). The α-subunit, which carries two PEB chromophores elutes at 19.2 min and is followed by the β-subunit (25 min) which carries three PEB chromophores. The first part of the chromatogram shows a partial resolution of at least three γ-subunits species (11.4, 11.6 and 11.8 min). In this instance three distinct isomeric components are partially resolved in the γ-subunit region of the chromatogram whereas SDS–PAGE only shows one band corresponding to γ-subunits of B-PE. There are reports of the presence of these isomers of B-PE

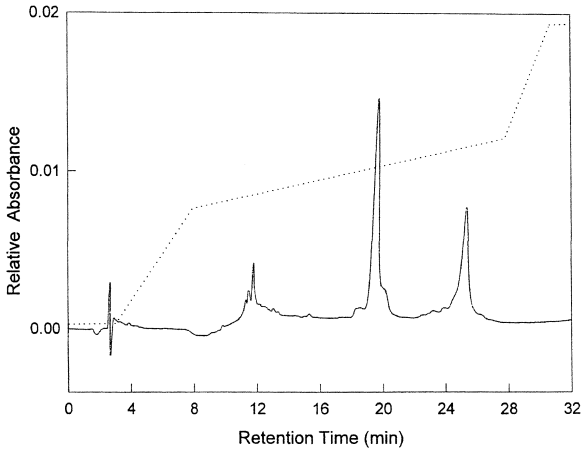


Fig. 8. Semipreparative HPLC separation of the subunits from *P. cruentum* B-PE. The elution profile monitored at 226 nm is shown. The identity of each peak was determined from the visible absorption spectra, the known distribution and content of PEB and PUB and SDS–PAGE. Dotted line symbolizes the gradient in percentage of acetonitrile (for quantitative details, see Section 2.4). The order of subunit elution is γ, α, β. The elution profile shows a partial resolution of at least three γ-subunit species.

from *P. cruentum* [23] and similar results were obtained by Swanson and Glazer [19]. To recover the protein-containing volume of each isolated subunit, the eluates from selected retention times were pooled. The retention time range was 18.5 to 20 min for α-subunit, 24.5 to 25.5 min for β-subunit and 11 to 12 min for γ-subunit. The sample recovery was high and the subunits were isolated in a suitable form for spectroscopic analysis and SDS–PAGE. Fig. 1 shows the bands for B-PE α-, β- and γ-subunits recovered after HPLC experiments. The subunit molecular masses determination agrees with the corresponding bands from B-PE.

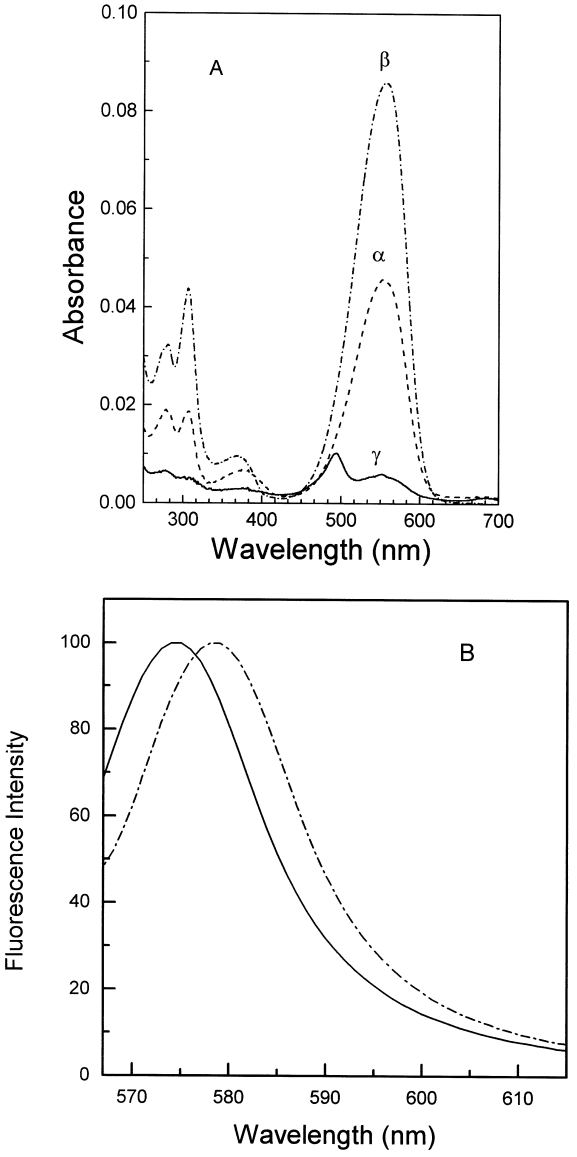


Fig. 9. Spectral characteristics of B-PE subunits recovered after semipreparative reversed-phase HPLC in water–acetonitrile (0.05% TFA). (A) Absorption spectra and (B) fluorescence emission spectra, (——) α-subunit and (------) β-subunit ($\lambda_{ex}$= 525 nm, $\Delta\lambda_{ex}$=10 nm and $\Delta\lambda_{em}$=20 nm). In absorption spectra the concentrations were the corresponding at the elution volumes for α- and β-subunits. For the γ-subunit one must withdraw the elution volume of three experiments.

Fig. 9 shows the spectroscopic differences between B-PE subunits. The α- and β-subunits have very similar absorption spectra, these show absorption maxima at 280 nm (total protein peak), 310, 375 and 550 nm. The absorption maximum at 550 nm is typical of the PEB chromophore, the only one present in these subunits. The ratio $A_{310}/A_{280}$ was approximately 1 for the α-subunit whereas for the β-subunit was around 1.5, due to the different number of bilin chromophores that each subunit carries. The absorption spectrum of γ-subunit shows another absorption maximum at 490 nm corresponding to the PUB chromophore, that is only present in this subunit type. The fluorescence spectra of α- and β-subunits (Fig. 9B) show fluorescence emission maxima at 576 for α-subunit and 580 nm for β-subunit.

The methodology shown in this report facilitates the purification of adequate amounts of B-PE by means of two chromatographic steps from *P. cruentum*. The procedure is fast with high sample recovery. In addition, we describe a quick semipreparative reversed-phase HPLC method for separation of its α-, β- and γ-subunits and a preparative low-pressure method for (αβ) monomers purification. Both separations could facilitate the screening of phycobiliproteins for interesting structural features. Moreover, the subunits are isolated in a suitable form for amino acid sequence determination and isolation of bilin peptides.

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