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Chromatographic purification of biliproteins from *Spirulina platensis* High-performance liquid chromatographic separation of their α and β subunits

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Abstract

A fast preparative two-step chromatographic method for purification of C-phycocyanin and allophycocyanin from *Spirulina platensis* is described. Both biliproteins were homogeneous as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis yielding two closely migrating bands. Separation of α and β subunits from C-phycocyanin and allophycocyanin was also accomplished by a chromatographic procedure under denaturing conditions. The phycocyanobilin chromophore from both biliproteins has been isolated, tested for purity by reversed-phase high performance liquid chromatography and spectroscopically characterized. © 1997 Elsevier Science B.V.

Keywords: Spirulina platensis; Biliproteins; Proteins; Phycobilisomes

1. Introduction

Biliproteins are proteins with linear tetrapyrrole prosthetic groups (bilin) that, in their functional state, are covalently linked to specific cysteine residues of the proteins. They form light-harvesting antenna complexes of cyanobacteria (blue–green algae) and of two groups of eukaryotic algae, the red algae and the cryptomonads. These complexes, called phycobilisomes (PBS), are attached to the outer surface of thylakoid membrane. The biliproteins are broadly classified into three groups based on the spectroscopic properties: phycoerythrin (PE), λ_{max} 540–570 nm, which has the red chromophore phycoerythrobilin; phycocyanin (PC), λ_{max} 610–620 nm, which contains either a mixture of the phycocyanobilin (PCB) and phycoerythrobilin chromophores or just phycocyanobilin, depending on the species of origin; and allophycocyanin (APC), λ_{max} 650–655 nm, with phycocyanobilin as prosthetic group [1,2]. A few cyanobacteria possess a fourth type of biliprotein in place of PE [3], the phycoerythrocyanin (PEC), which has a phycocyanobilin and a phycobiliviolin tetrapyrrole chromophores, λ_{max} 568 nm, 585 (shoulder). The PBS consist of a core of two or three cylinders composed of APC aggregates and a number of rods made up of discs of trimeric or hexameric units of either the same or different types of biliproteins connected in a hemidiscoidal array, (for reviews, see [4–10]). Antenna systems of some cyanobacteria, i.e. *S. platensis*, are composed by only C-PC and APC at an approximate 10:1 ratio [11].

The introduction of biliproteins as fluorescent tags of cells and macromolecules [12] was followed by its

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widespread application in highly sensitive fluorescence techniques such as fluorescence-activated cell sorting, flow cytometry, fluorescence immunoassay and fluorescence microscopy [13]. APC and C-PC have been shown to be particularly useful using either tunable dye lasers or higher wavelength helium–neon lasers [14,15]. The fact that the excitation and emission maxima for APC lie in the red is particularly important because of the lack of interfering emissions from most biological materials in this region of the spectrum, however the widespread use of APC has been somewhat limited by the tedious preparation of adequate amounts of the purified biliprotein.

These biliproteins are comprised of two subunits, α and β, with open chain tetrapyrroles covalently attached by a cysteine thioether bond [16]. APC carries one phycocyanobilin on each subunit, C-PC carries one phycocyanobilin on the α subunit and two phycocyanobilin on the β subunit [17]. 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, the separations are long and in some instances under conditions where they undergo some oxidative degradation. Other HPLC procedures performed with trifluoroacetic acid (TFA) [17] resulted in partial precipitation of the sample with preferential loss of the β subunit.

On the other hand, it is interesting for us to determine the purity of the phycocyanobilin chromophore extracted from both biliproteins, since its absorption may be in the same spectral region as the amide I band, which is employed for determination of biliproteins secondary structure by FT-IR.

Here, we report the purification of adequate amounts of C-PC and APC by only two chromatographic steps from *S. platensis*, a blue-green alga that grows easily along the Mediterranean coast serving as an inexpensive source material of these biliproteins. Moreover, we describe a quick reversed-phase HPLC method, under denaturing conditions, for separation of α and β subunits from both biliproteins and an uncomplicated method for phycocyanobilin purity test.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile and methanol were obtained from Lan-Scan Analytical Sciences (Dublin, Ireland). Sephadex G-100 from Pharmacia (Uppsala, Sweden) and DEAE-cellulose DE-52 from Whatman (Maidstone, UK). Molecular mass standards (14 400–94 000) were from Sigma diagnostics (St. Louis, MO, USA). All other chemicals were purchased from Sigma and used without further purification.

2.2. Biliproteins purification

Cells of *S. platensis* were grown in the southeast coast of Spain and generously provided by Imade S.L. (Granada, Spain) as frozen cells. For purification of biliproteins, cyanobacterial cells stored at −20°C were used as starting material. Frozen cells were removed from the growth medium by centrifugation. In a typical experiment 50 g (wet weight) were resuspended in 50 ml of 1 *M* acetic acid–sodium acetate buffer (pH 5.0). The slurry was sonicated for 10 min under stirring, transferred to ultracentrifuge 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 70% saturation, allowed to stand for 2 h, and then centrifuged at 20 000 *g* for 15 min. The pellets were resuspended in 1 ml of 100 m*M* acetic acid–sodium acetate buffer (pH 5.0) and dialyzed overnight against 2 l of the same buffer (Fig. 1).

The dialyzed biliproteins-containing solution was applied to a column (60×2.5 cm) of Sephadex G-100, preequilibrated with the same acetate buffer, eluted at a flow-rate of 50 ml/h until the eluate became blue and collected in 3-ml fractions. The blue fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and UV–visible absorption spectroscopy. The first seven fractions were C-PC rich, then three fractions in which the two biliproteins appear mixed and the last four fractions were APC-rich solutions.

The C-PC- and APC-rich tubes were pooled

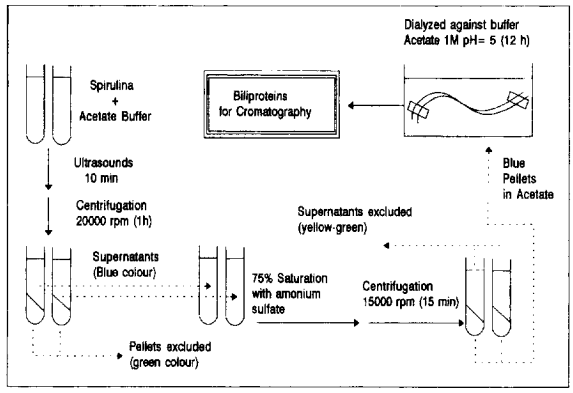

Fig. 1. Initial stages for biliprotein purification.

separately and brought to 70% saturation with $(NH_4)_2SO_4$, and allowed to stand for 1 h prior to centrifugation at 20 000 *g* for 15 min. The pellets were resuspended in 5 m*M* sodium phosphate buffer (pH 7.0) and then dialyzed overnight against 4 l of the same phosphate buffer. Dialyzed solutions analyzed by SDS–PAGE showed additional impurity bands together with those typical of biliprotein subunits (Fig. 2). For C-PC-rich solutions a A_{620}/A_{277} ratio 4 was obtained. In APC-rich solutions both, a A_{650}/A_{280} ratio 3.5 and a 0.72 constant A_{620}/A_{650} ratio have been obtained.

Each dialyzed solution was applied to a column (15×2.5 cm) of DEAE-cellulose DE-52 preequilibrated with 5 m*M* sodium phosphate buffer (pH 7). After washing with 1 bed volume of starting buffer, both columns were developed with 0.29 *M* sodium phosphate buffer (pH 7.0). C-PC is eluted from the column with C-PC-rich solution. On the other column, the 0.29 *M* eluate was rejected, and immediately afterwards APC was eluted with 0.40 *M* sodium phosphate buffer (pH 7.0). The flow-rate was 70 ml/h. The blue eluates from each biliprotein were

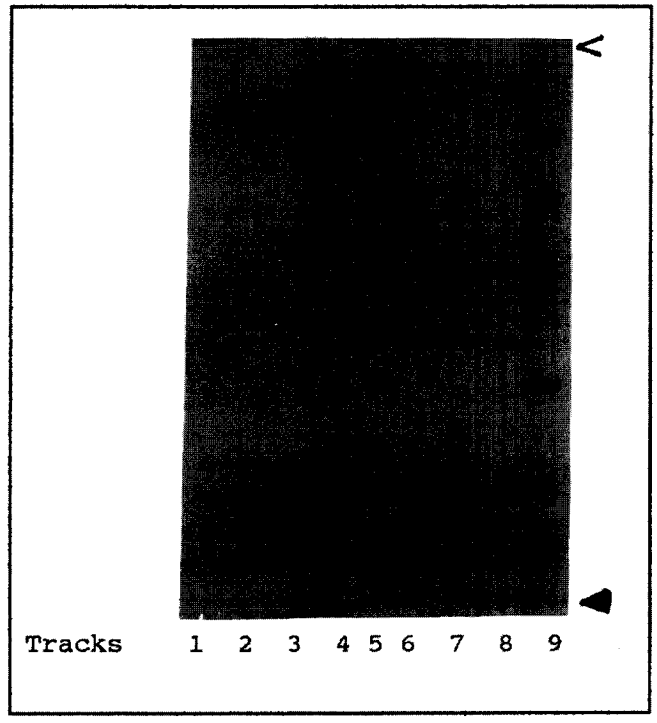

Fig. 2. SDS–PAGE from gel permeation step in Sephadex G-100. From left to right, the tracks are: 1–4, C-PC-rich solutions; 6–8, APC-rich solutions; 5 and 9 marker proteins. The gel origin is shown by the open arrow, and the front by the closed arrow. The molecular weight of the known markers from top were: phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

pooled, brought to 70% saturation with $(NH_4)_2SO_4$ and left to stand overnight in the dark at 4°C before centrifugation. The pellets of purified biliproteins were resuspended in small volumes 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 and β subunits are detected on analysis of purified biliproteins by SDS–PAGE (Fig. 3), indicating that the linker polypeptides were effectively removed in the purification procedure.

2.3. Bilin purification

C-PC and APC mixture from Sephadex G-100, were suspended in methanol (biliproteins–methanol, 1:4, v/v) containing 1 mg/ml $HgCl_2$ and dark incubated for 24 h at 40°C. After methanolysis, the solution was centrifuged at 20 000 *g* for 10 min, and the violet supernatants pooled. A 10-ml volume of methylene chloride–1-butanol (2:1, v/v) was added with mixing to the methanolic solution, and then 20 ml of water were added. After phase separation occurred, the organic phase containing bilin was washed with several 20 ml portions of water and evaporated under vacuum to dryness.

2.4. SDS–PAGE

Electrophoresis was carried out according to Laemmli [18] using a 12.5% (w/v) polyacrylamide slab gel, 1.5-mm thick, containing 0.1% (w/v) SDS

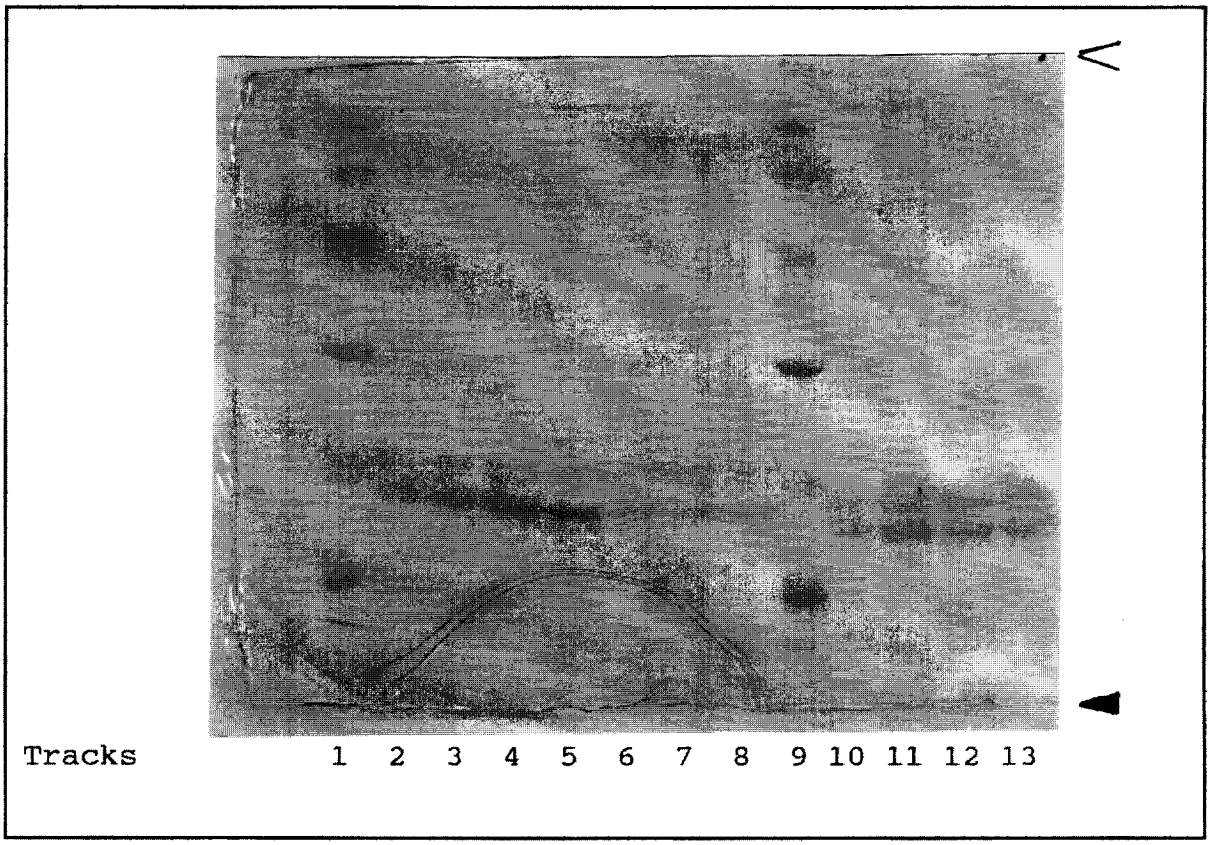

Fig. 3. SDS–PAGE of purified biliproteins from DEAE-cellulose DE-52 exchange ionic chromatography. From left to right, the tracks are: 2–8, purified C-PC showing subunit bands; 10–13, purified APC; 1 and 9 marker proteins. The gel origin is shown by the open arrow, and the front by the closed arrow. The molecular weight of the known markers from top were: phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

with a stacking gel of 4% acrylamide and 0.1% bis-acrylamide. Samples were preincubated with 2% (w/v) SDS, 10% (v/v) glycerol, 4.5% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue and 60 m*M* Tris (pH 6.8), for about 5 min at 95°C. Gels were run at room temperature and visualized by staining for 12 h 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.

2.5. High-performance liquid chromatography

HPLC chromatography was performed using a Merck–Hitachi (Darmstadt, Germany) Model L-6220 pump with a Model interface D-6000 for photodiode array detection (DAD)-system manager and HPLC manager. Data were acquired using a Merck L-4500 photodiode array detector; spectra were collected from 250–700 nm at 200 ms intervals. Biliprotein (20 μl of 0.5–1 mg/ml) in sodium phosphate buffer 20 m*M* (pH 6.5) (0.1% β-mercaptoethanol), were injected into a Hiperils Butil-300 (Supelco, Barcelona, Spain) column previously equilibrated in 40% acetonitrile (0.1% β-mercaptoethanol) (buffer A) and 60% sodium phosphate buffer 10 m*M*, pH 6.5 (0.1% β-mercaptoethanol) (buffer B). Protein subunits were eluted from the column at a flow-rate of 1 ml/min according to the following program: 0–5 min, buffer A–buffer B (40:60, v/v); 5–40 min, linear gradient to 100% buffer A; 40–50 min linear gradient to buffer A–buffer B (40:60, v/v). All samples were spun for 5 min in a microcentrifuge immediately prior to injection.

Phycocyanobilin from C-PC and APC, was dissolved in methanol (0.5 mg/ml) and injected into the same column, previously equilibrated in methanol–acetonitrile–water (48:32:20, v/v/v). The bilin was eluted from the column at a flow-rate of 1 ml/min following a linear gradient to methanol–acetonitrile (60:40, v/v) in 60 min.

2.6. 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 0.08–0.1 g/l. Fluorescence emission spectra were recorded on a Shimadzu (Kyoto, Japan) RF5001 spectrofluorometer. Solutions were sufficiently diluted so that reabsorption of fluorescence was not detected. For steady state polarized fluorescence measurements the spectrofluorometer was equipped with polarizers in the excitation and emission paths. The degree of polarization was calculated as $P=(I_{VV}-GI_{VH})/(I_{VV}+GI_{VH})$, 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.

3. Results and discussion

3.1. Biliprotein purification

Sonication and $(NH_4)_2SO_4$ addition to 70% saturation, result in a precipitate which contains C-PC and APC. In preliminary studies, we tested columns with different bed volumes, 2.5×20, 40, 60 and 80 cm length and different flow-rates in the range 30–90 ml/h. Fractions of 3 ml were collected. These fractions were analyzed by UV–Vis spectroscopy and SDS–PAGE. The better resolution was obtained with 60-cm length columns. Likewise, the optimum flow-rate was 50 ml/h. In these conditions, SDS–PAGE showed that three fractions contain biliprotein mixture of roughly 50%. The other coloured fractions showed enriched C-PC or APC solutions, though other contaminant proteins appear in all fractions (see Fig. 2).

The results obtained by size exclusion chromatography indicate that purification of biliproteins from *S. platensis* is not only reached with a chromatographic process but it also needs an ionic exchange process. Before proceeding with the ionic change, the pH and the initial ionic strength (*I*) have to be selected by a preliminary test with a continuous and simultaneous gradient of both pH and *I*, and with the simplest continuous gradient of *I* only. At pH 7 we found that C-PC elutes at 0.29 *M* sodium phosphate buffer concentration and APC at 0.4 *M* approximately. The method has been improved using two independent columns and different departure solutions. For C-PC

purification, C-PC-rich fractions from Sephadex G-100 were pooled, added into the column and eluted with 0.29 *M* sodium phosphate buffer. Similar steps were done for APC purification, with APC-rich tubes and 0.4 *M* sodium phosphate buffer.

Both biliproteins were homogeneous as determined by SDS–PAGE yielding two bands (Fig. 3) of closely similar amounts. By comparison with standards, the molecular masses were of 21 500 and 19 000, corresponding to the α and β subunits of C-PC, and 19 600 and 17 700 for α, β subunits of APC, respectively. By comparing absorption and fluorescence spectra from selected pooled fractions with the published spectra of pure C-PC and APC [12,19,20] we observe an excellent fitting of the data.

At pH 6.5–8.0, monomer and trimer of C-PC exist in equilibrium, but the protein is primarily in a monomeric form at concentrations less than 8 μg/ml [21,22]. The absorption and fluorescence spectra of C-PC in trimeric and monomeric forms show that the spectra are slightly affected by the aggregation changes in the protein [23]. However, a dramatic increase in the excitation polarization fluorescence spectra has been shown when the aggregation state of the biliproteins changes from trimers to monomers [24]. At 1.0 *M*, sodium perchlorate and sodium thiocyanate have the ability to dissociate C-PC to a homogeneous solution of monomers but not to dissociate monomers into the two subunits [25]. Visible absorption, fluorescence emission and excitation polarization fluorescence spectra of solutions of C-PC in 20 m*M* sodium phosphate buffer, pH 7.0 (Fig. 4A), and C-PC in the same buffer with 1 *M* sodium perchlorate (Fig. 4B), have been recorded. The comparison of the excitation polarization spectra revealed a clear rise in the degree of polarization when the solution of C-PC is added with 1.0 *M* sodium perchlorate. Moreover, the spectrum shape and the values of degree of polarization of C-PC in 20 m*M* sodium phosphate buffer (pH 7.0) are similar to those earlier published for trimeric C-PC from *Mastigocladus laminosus* [24]. Thus, purification by ionic exchange provides C-PC solutions in trimeric aggregation state, a basic unit of the C-phycocyanin rod in phycobilisome.

On the other hand, the dissociation of trimeric APC into monomeric APC causes the absorption maximum to shift from 650–615 nm and the emis-

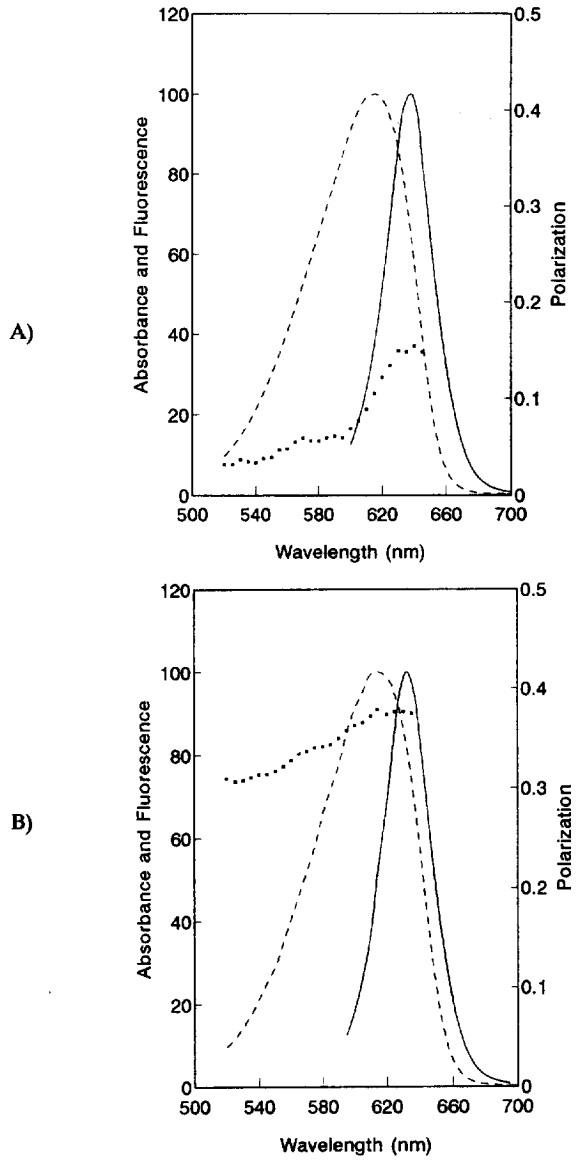

Fig. 4. Spectral characteristics of (A) C-PC in 20 m*M* sodium phosphate buffer, pH 7.0. (B) C-PC in 1 *M* sodium perchlorate, 20 m*M* sodium phosphate buffer pH 7.0. (- - -) Absorption spectra. (———) Fluorescence emission spectra. Samples were excited at 617 nm and the slits were 1.5 nm. Absorption and fluorescence spectra were normalized since were only used on a comparative basis. (· · ·) Fluorescence excitation polarization spectra monitored at 655 nm. Samples were excited in the range 520–645 nm, the slits were 3 nm. The polarization values shown in the figure are the means of five values and were calculated for each 5 nm. All spectra were recorded at room temperature.

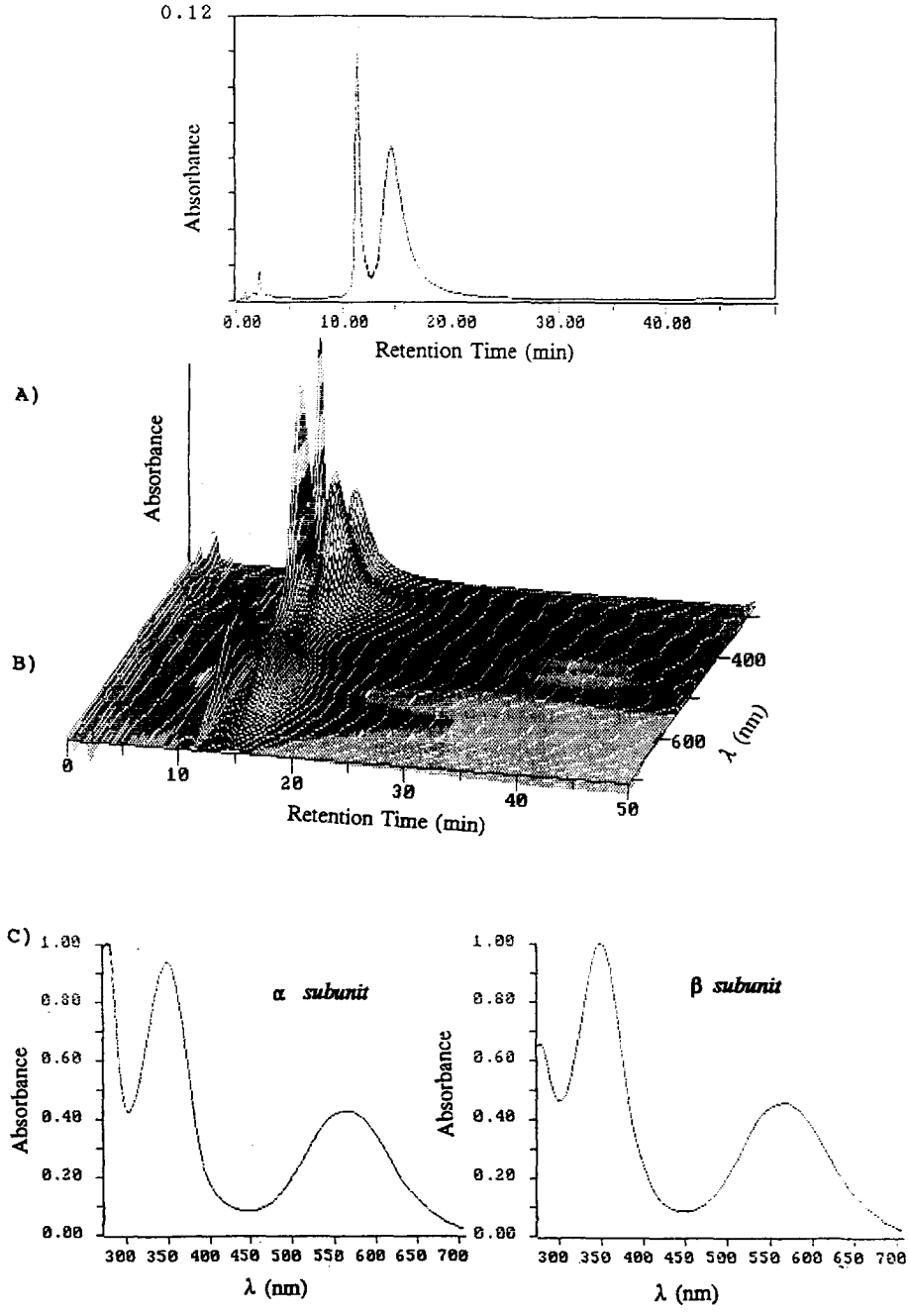

Fig. 5. (A) Cyanobacterial *Spirulina platensis* C-PC subunit separation. The elution profile was monitored at 350 nm, one of the peaks of visible absorbance of peptide-linked PCB chromophore. (B) Three dimensional chromatogram. The elution profile was monitored over the range 250–700 nm. (C) Photodiode-array spectrum of the peak corresponding to the retention times of α subunit (11.7 min) and β subunit (14.6 min).

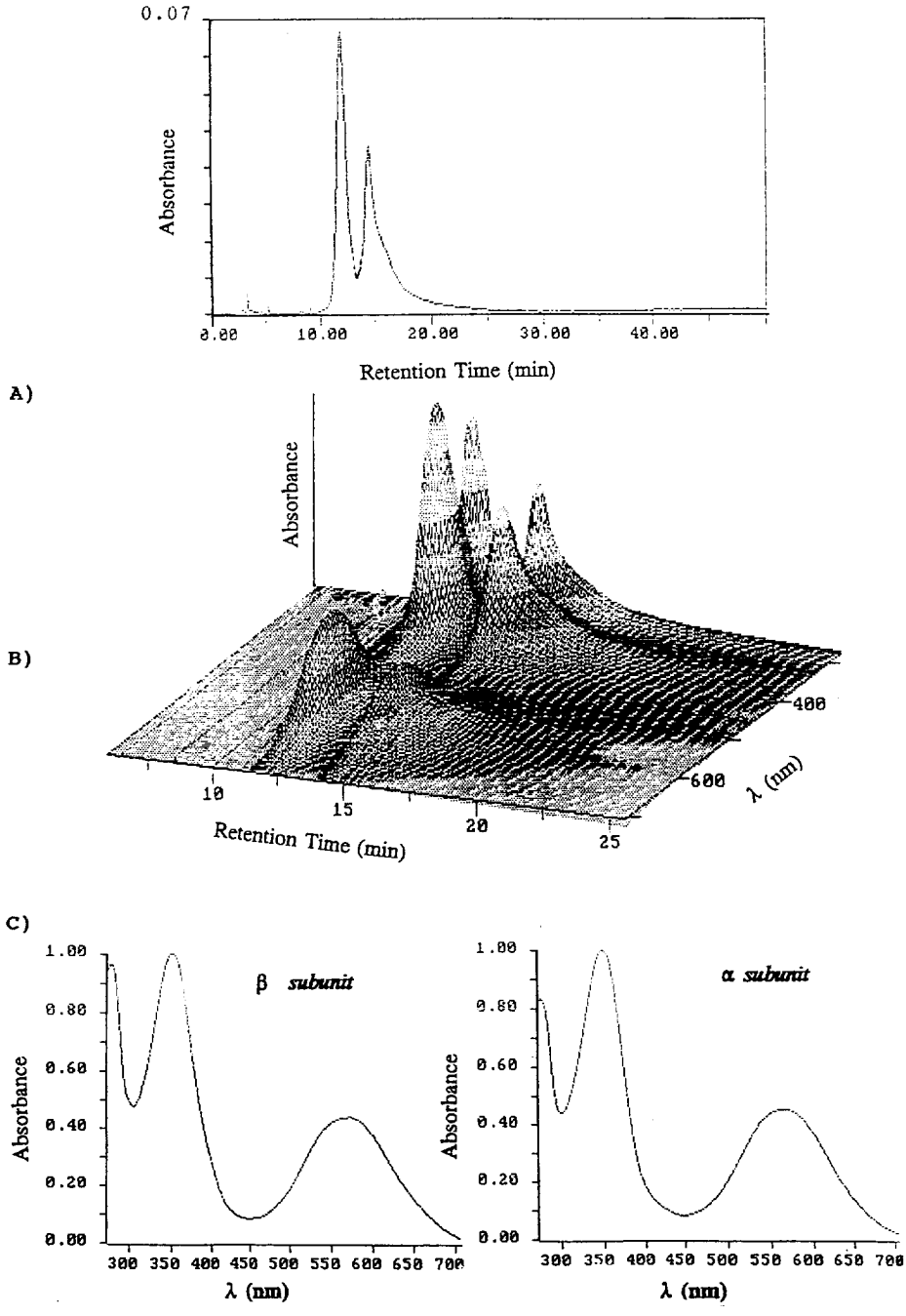

Fig. 6. (A) Cyanobacterial *Spirulina platensis* APC subunit separation. The elution profile was monitored at 350 nm, one of the peaks of visible absorbance of peptide-linked PCB chromophore. (B) Three dimensional chromatogram. The elution profile was monitored over the range 250–700 nm. (C) Photodiode-array spectra of the peak corresponding to the retention times of α subunit (11.9 min) and β subunit (14.4 min).

sion maximum to shift from 660–641 nm [20,26]. The purified APC showed no absorbance and fluorescence peak other than that corresponding to the trimeric APC. Thus, the APC purified in our experiments retained its native trimeric structure.

3.2. Separation of α and β subunits of C-PC and APC

The separation conditions were developed with well-characterized C-PC and APC. Denaturation of the proteins was reached with the simple addition of 0.1% β-mercaptoethanol to the sample and mobile phase. The α and β subunits of C-PC and APC from *S. platensis* are very similar to each other in molecular mass, moreover in APC each subunit carries a single phycocyanobilin group and these characteristics make the separation of the subunits difficult. We have tested several gradients with different slopes, all performed with acetonitrile and sodium phosphate buffer. The gradient described in Section 2 was found to give the best baseline separation of the α and β subunits of both biliproteins. This is illustrated in Fig. 5 for C-PC. The α subunit, which carries a single phycocyanobilin chromophore, elutes at 11.7 min and is followed by the β subunit (14.6 min), which carries two phycocyanobilin. By denaturing C-PC with β-mercaptoethanol, the effects of local protein environment of the chromophore absorption spectra can be essentially removed [27]. Under these conditions, the visible regions of the absorption spectra of the α and β subunits are virtually identical, with the intensity of the β subunit being twice that of the α subunit. Since the absorbances at 553 nm are normalized in the Fig. 5C, the 280 nm absorption corresponding to total proteins is approximately doubled in value in the spectrum of α subunit.

Under the conditions described above, APC was resolved into two components of equal phycocyanobilin content and very similar spectrum profiles, with retention times of 11.9 and 14.4 min (Fig. 6). Since each subunit carries a single phycocyanobilin, the retention times are close and their whole absorption spectra are similar.

3.3. Phycocyanobilin characterization

To determine whether the phycocyanobilin chromophore was pure for protein secondary structure determination, purified phycocyanobilin was dissolved in methanol and injected into the column. Elution with a linear gradient to methanol–acetonitrile (60:40, v/v) provided a single peak with a retention time of 10 min. The absorption spectrum for this peak is showed in Fig. 7.

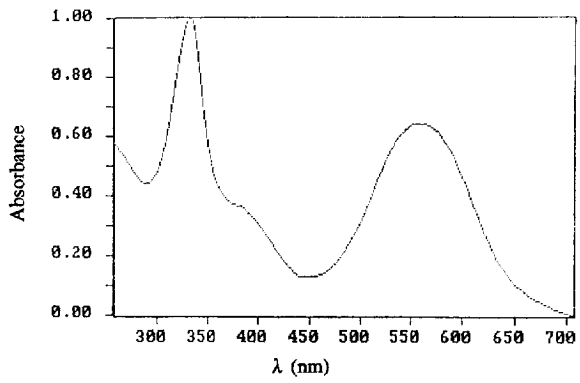

Fig. 7. Photodiode-array spectrum of the peak corresponding to the retention time of PCB. The elution profile was monitored over a range 250–700 nm.

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