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Purification and characterization of phycocyanin from the blue-green alga *Aphanizomenon flos-aquae*☆

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Abstract

Aphanizomenon flos-aquae (AFA) is a blue-green alga and represents a nutrient-dense food source. In this study the presence of phycocyanin (PC), a blue protein belonging to the photosynthetic apparatus, has been demonstrated in AFA. An efficient method for its separation has been set up: PC can be purified by a simple single step chromatographic run using a hydroxyapatite column (ratio A_{620}/A_{280} of 4.78), allowing its usage for health-enhancing properties while eliminating other aspecific algal components. Proteomic investigation and HPLC analysis of purified AFA phycobilisomes revealed that, contrary to the well-characterized *Synechocystis* and *Spirulina* spp., only one type of biliprotein is present in phycobilisomes: phycocyanins with no allo-phycocyanins. Two subunit polypeptides of PC were also separated: the β subunit containing two bilins as chromophore and the α subunit containing only one.

Keywords: *Aphanizomenon flos-aquae*; Cyanobacteria; Phycocyanin; Phycocyanobilin; Purification

1. Introduction

Aphanizomenon flos-aquae (AFA) is a fresh water unicellular blue-green alga (cyanobacterium) that spontaneously grows in copious amounts in Upper Klamath Lake (Oregon, USA). It is a nutrient-dense food source and is consumed for its health-enhancing properties [1,2]. Besides some controversial studies [3,4], several scientific reports have documented the therapeutic value of this microalga [5,6], although the molecular mechanisms responsible for its beneficial properties are not clear. Recent studies have demonstrated that in the microalga *Spirulina platensis*, a blue protein called phycocyanin (PC) belonging to the photosynthetic apparatus has antioxidant and radical scavenging properties in both in vivo and in vitro models [7–9]; at the same time, this natural compound is strongly anti-inflammatory [10,11]. In this context, we recently described the antioxidant properties of a natural extract obtained from AFA in protecting human erythrocytes and plasma samples against oxidative damage in vitro [12]. Thus, the characterization of AFA microalga content and the quantitative separation of its components is worthwhile for its increasingly attractive biological and commercial possibilities.

In the case of any of the well-characterized cyanobacteria species like *Spirulina* or *Synechocystis*, the photosynthetic apparatus is known to be composed of a central core containing a chlorophyll binding protein and a peripheral accessory light-harvesting complex called phycobilisome, containing proteins which can constitute up to 60% of the soluble protein content [13] and give these cells the typical blue color. In intact cells, phycobilisomes are composed of α and β subunit

Abbreviations: ACN, acetonitrile; AFA, aphanizomenon flos-aquae; FA, formic acid; PC, phycocyanin; PCB, phycocyanobilin; PFF, peptide fragment fingerprinting; PMF, peptide mass fingerprinting; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid

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polypeptides called phycobiliproteins which associate into (αβ)-monomers. (αβ)-monomers assemble together forming $(\alpha\beta)_3$-trimers, which in turn, associate to form a $(\alpha\beta)_6$-hexamer, the functional unit of the phycobilisome [14].

The brilliant blue color of the phycobiliproteins originates from covalently attached linear tetrapyrrole prosthetic groups, known as phycobilins [15]. Among the five different bilin pigments, having different numbers and arrangements of conjugated double bonds [16], only the blue colored phycocyanobilin (PCB) is present in both allo- and phyco-cyanins of *Synechocystis* PCC 6803 phycobilisomes [17]. Moreover, in this species, the α and β subunits of allophycocyanin, as well as the α subunit of PC, bind one bilin chromophore, while the β subunit of PC binds two bilin chromophores [18]. The spectroscopic properties of individual phycobiliproteins depend largely on the chemical nature of the bilin they carry [19]; however, a strong absorption at 620 nm is commonly observed [20]. Interestingly, the chemical structure of PCB is very close to that of bilirubin, a biliary pigment which acts in vivo as a powerful scavenger of oxygen reactive species [21], supporting the possible role played by bilins in their scavenger action.

In this context, a study has been conducted with the goal of characterizing the main AFA component, PC, and to compare it with those described in literature. Hence, PC from AFA was characterized with respect to its specific light absorbance, subunit make up and molecular weight. For this reason, two methods were used: the well tested sucrose gradient ultracentrifugation [22] suitable for the preparation of purified components, and a more useful method to obtain a large quantity of purified PC, usable for its antioxidant and anti-inflammatory properties without undesired contaminants.

2. Experimental

2.1. Sample preparation in a low ionic strength medium

AFA cells were suspended in 20 mM MES (morpholinoethanesulfonic acid) pH 6.35, 5 mM $CaCl_2 \times 2\ H_2O$, 5 mM $MgCl_2 \times 6\ H_2O$, 1 mM benzamidine, 1 mM aminocaproic acid and disrupted by 15 cycles of 30 s in a Braun Homogenizer. The cell debris was eliminated by centrifugation at $9800 \times g$ for 10 min at room temperature in a JA20 Beckman rotor. The supernatant was spun at $148{,}000 \times g$ in a TFT 50.38 Kontron rotor at 4 °C. The supernatant was collected and used for HPLC and electrophoretic separations without any further purification.

2.2. Ultracentrifuge sample preparation

Phycobilisomes were isolated according to Rajagopal et al. [23] with some modifications. One gram of fresh weight of AFA cells were suspended in 10 ml of 1 M K-phosphate buffer at pH 7.0 containing 1 mM PMSF, 1 mM sodium azide, 2 mM EDTA and 2% 2-mercaptoethanol. Cells were disrupted by vortexing twice for 1 min with half their volume in glass beads (0.17 mm diameter). The vortexed cells were incubated with 2% Triton-X 100 for 35 min and then the crude extract of phycobilisomes was separated from cell debris by centrifugation at $35{,}000 \times g$ for 40 min. The supernatant was collected and layered on a sucrose density step gradient (2.0, 1.0, 0.5 and 0.25 M). Phycobilisomes were obtained after spinning the gradient using swing-out rotors at $150{,}000 \times g$ for 12 h in an ultracentrifuge.

2.3. Purification of PC using a hydroxyapatite column

PC was purified from a dried AFA extract (Nutratec, Urbino, Italy) as follows: 500 mg of extract was suspended in 50 ml of 100 mM Na-phosphate buffer pH 7.0, brought to 50% saturation by the addition of solid ammonium sulfate and allowed to stand for 60 min at 4 °C. The precipitate was recovered by centrifugation at $10{,}000 \times g$ for 30 min at 4 °C; the clear colourless supernatant was discarded and the blue precipitate was dissolved in a small volume of 5 mM Na-phosphate buffer pH 7.0 and dialysed overnight at 4 °C against the same buffer. The dialysed PC was then placed in a 2.5 cm × 25 cm hydroxyapatite column (Bio-Rad Laboratories, CA, USA) and fractions were eluted with Na-phosphate buffer pH 7.0 of increasing ionic strength (from 5 to 150 mM). The fractions showing an absorbance ratio of 620 nm/280 nm greater than four were pooled and brought to saturation with ammonium sulfate. After centrifugation, the blue precipitate was dissolved in 150 mM of Na-phosphate buffer pH 7.0 and dialysed against the same buffer. The purified PC was stored in darkness at 4 °C.

2.4. Spectroscopic measurements

The purity of PC was evaluated according to the absorbance ratio A_{620}/A_{280} [18]. All visible and UV spectra were measured using a Varian Cary 100 spectrophotometer. Fluorescence was measured using a Varian Cary Eclipse spectrofluorimeter. Analyses were conducted at room temperature (20 °C).

2.5. High-performance liquid chromatography

Protein separation by HPLC was performed using a reversed phase Vydac Protein C-4 column (250 mm × 4.6 mm i.d.) packed with 5 μm porous butyl silica particles [24,25]. This column was operated at a flow rate of 1 ml/min for optimum separation efficiency. All solutions were filtered through a Millipore (Milan, Italy) type FH 0.5 μm membrane filter and degassed by bubbling with helium before use. Optimization of chromatographic separations was performed using a Beckman (Fullerton, CA, USA) System Gold system, consisting of a Model 126 solvent delivery pumps and a Model 168 UV diode-array detector. Samples were introduced onto the column by a Model 210A sample injection valve with a 50 μl sample loop.

The Vydac C-4 columns were pre-equilibrated with 20% (v/v) aqueous acetonitrile (ACN) solution containing 0.1% (v/v) trifluoroacetic acid (TFA) and samples were eluted using a linear gradient from 20 to 100% (v/v) ACN in 60 min, followed by a 10 min isocratic elution with the eluent containing 100% ACN.

2.6. SDS-PAGE analysis

To effect separation of α and β subunits, pure PC was dissolved in a cracking buffer containing 2% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol; the mixture was then

boiled for 5 min. SDS-PAGE analysis was conducted on 15% polyacrylamide gel using protein markers with a range of molecular weight from 14,400 to 200,000. Electrophoresis was run at 12 mA at room temperature and the gel was finally stained with colloidal Coomassie or Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, CA, USA). To evaluate the molecular weight of pure PC, native electrophoresis on 10% polyacrylamide gel was performed using protein markers with a range of molecular weight from 31,000 to 140,000. Electrophoresis was run at 12 mA at 4 °C.

2.7. In-gel digestion

Spots were carefully cut out from colloidal Coomassie stained gels and subjected to in-gel trypsin digestion according to Shevchenko et al. [26] with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/μl of trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in an ice bath. After 30 min the supernatant was removed and discarded, 20 μl of 50 mM NH_4HCO_3 were added to the gel pieces and digestion allowed to proceed at 37 °C overnight. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to mass spectrometric analysis, the peptide mixtures were redissolved in 10 μl of 5% formic acid (FA).

2.8. Peptide mass fingerprinting (PMF) by MALDI-TOF-MS

Peptides were desalted and concentrated according to Gobom et al. [27]. Homemade 5 mm nanocolumns were packed with POROS R2 resin (PerSeptive Biosystems, Framingham, MA) in a constricted GELoader tip (Eppendorf, Hamburg, Germany). A syringe was used to force liquid trough the columns by applying gentle air pressure. The columns were equilibrated with 20 μl of 5% FA and the analyte solutions were added. The columns were washed with 20 μl of 5% FA and the bound peptides subsequently eluted directly onto the MALDI target with 0.5 μl of α-cyano-4-hydroxycinammic acid (CHCA) solution (20 μg/μl in ACN, 0.1% TFA, 70:30, v/v).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed using a Biflex III (Bruker Daltonik, Germany). Positively charged ions were analyzed in reflectron mode, using delayed extraction. Typically, 100 shots were averaged to improve the signal-to-noise ratio. Spectra were calibrated using trypsin autolysis products (*m/z* 842.51 and 2211.10) as internal standards. In a few cases this was not possible and external calibration was carried out using peaks from tryptic digest of β-lactoglobulin. Protein identification was performed by searching in a non-redundant protein sequence database (NCBInr) using Mascot program (http://www.matrixscience.com).

2.9. Peptide fragment fingerprinting (PFF) by RP-HPLC-ESI-MS/MS

Peptide mixtures were separated using a LC-Packings (a Dionex company, Italy) autosampler/nano HPLC. A sample volume of 8 μl was loaded by the autosampler and concentrated on a commercial trap column (Zorbax 300 SB-C18, 5 μm, 300 μm i.d. × 5 mm, Agilent Technologies) at a flow rate of 10 μl/min. Separation was performed using a homemade capillary column (Zorbax 300 SB-C18, 3.5 μm, 75 μm i.d. × 10 cm, Agilent Technologies) and a flow rate of 200 nl/min. HPLC solvents contained 0.1% FA and either 2% ACN (solvent A) or 98% ACN (solvent B). The column was pre-equilibrated with 100% solvent A. Elution was performed by multisegment gradient with a first step from 0 to 5% solvent B in 5 min, a second step from 5 to 12% solvent B in 2 min, a third from 12 to 50% solvent B in 30 min and a last from 50 to 100% solvent B in 8 min.

Peptides were eluted directly into an ion trap Esquire 3000 plus (Bruker-Daltonik, Germany). Capillary voltage was 1.5–2 kV and a dry gas flow rate of 3 l/min was used with a temperature of 230 °C. The scan range used was from 400 to 1800 *m/z*. For protein identification, the following parameters were used: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide mass tolerance ± 1.2 Da, fragment mass tolerance ± 0.9 Da, missed cleavages 2.

3. Results and discussion

In a previous paper [24] it was reported that, in the case of *Synechocystis* cyanobacteria the isolation of the light-harvesting apparatus might be obtained by the simple breaking of cells in low ionic strength buffer and removal of thylakoid membranes by centrifugation. The complete resolution of phycobilisome protein components could be obtained by subsequent injection of the total mixture of phycobilisomes into C4 reversed phase HPLC and elution by the H_2O–ACN–TFA gradient system. Applying the same method to AFA and analyzing the supernatant by HPLC and electrophoresis, the presence of contaminant proteins along with phycobilisome components was detected. Fig. 1 shows that the chromatographic runs contain two main peaks, corresponding to phycocyanins and many other proteins; the result was confirmed by SDS-PAGE analysis (right inset of Fig. 1). Tryptic digestion of proteins contained in the SDS-PAGE bands leads to protein identification by PMF using MALDI-TOF and/or by PFF using ESI-MS/MS. Table 1 reports the number of gel bands in the first column. A list of peptides observed by ESI-MS/MS and by simple MALDI-TOF are indicated in the second and third columns, respectively. The fourth column shows the accession number of the identified protein and the database in which the protein was found. If the sequence(s) did not match a protein from AFA, a homologous organism is indicated. Finally, in the last column, the list of identified proteins is given.

The left inset of Fig. 1 shows the absorption spectrum of the supernatant (dashed line) which presented the characteristic peak of PC at 620 nm but also a strong aspecific absorbance below 300 nm (ratio A_{620}/A_{280} of 0.27) corroborating the presence of many other cellular proteins.

At this point, to obtain a more purified phycobilisome, the cellular extract was loaded onto a sucrose gradient ultracentrifuge tube and run at high speed to separate the phycobilisome apparatus by a well consolidated method used previously for *Spirulina platensis* cyanobacteria [23]. The left part of Fig. 2

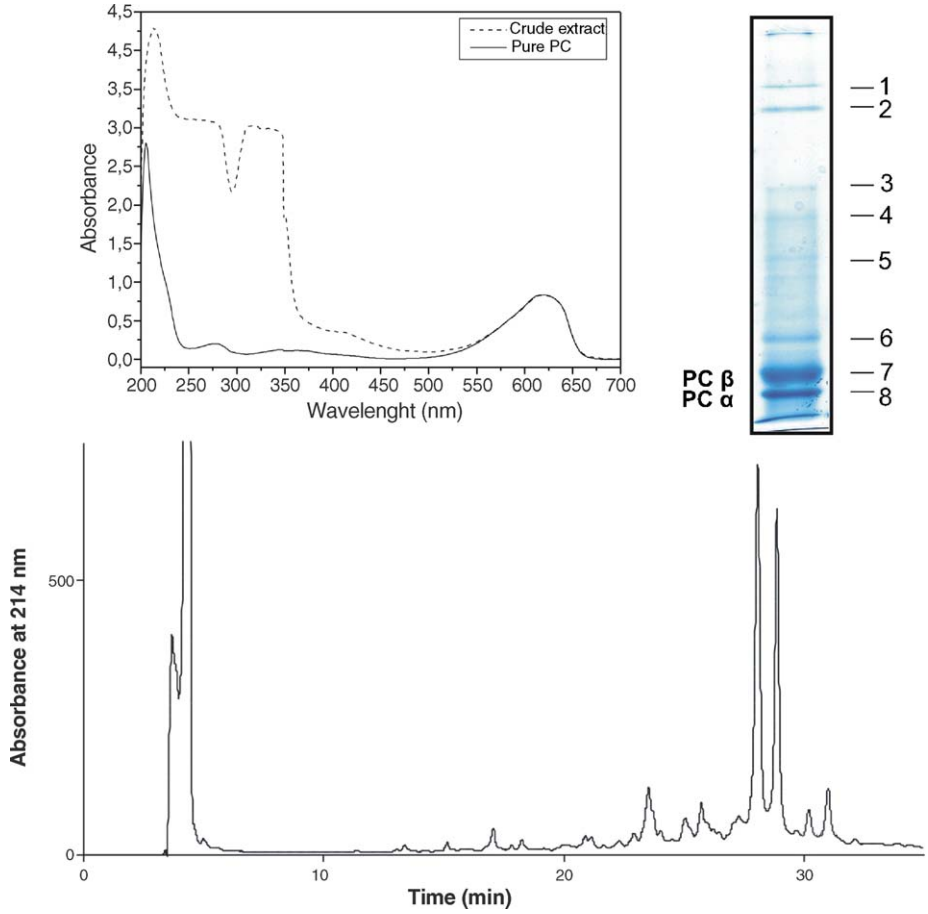

Fig. 1. Chromatographic profile of the phycobilisome extract in a low ionic strength medium; SDS-PAGE analysis (right inset) and UV–vis spectra (left inset) of the crude extract (dashed line) and of pure PC (solid line) in aqueous solution. PC has a characteristic maximum of absorption at 620 nm and is considered pure when the ratio A_{620}/A_{280} is greater than 4.

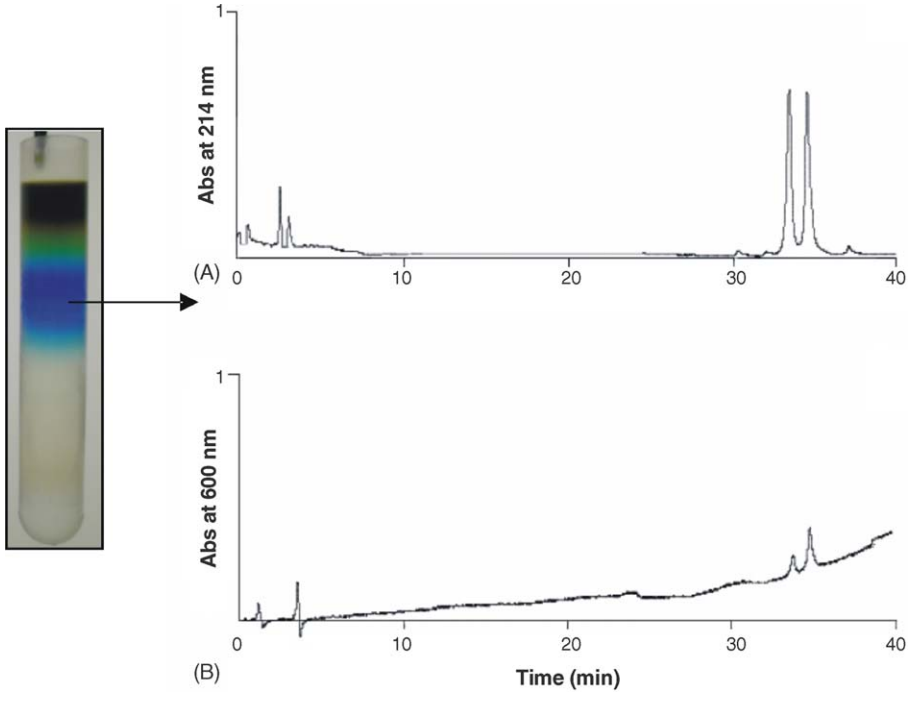

Fig. 2. Ultracentrifugation of cellular AFA extract by sucrose gradient (0.25–2.0 M in phosphate buffer). RP-HPLC chromatogram of the blue band recorded at 214 (panel A) and 600 (panel B) nm.

Table 1
Identified proteins by mass spectrometry on in-gel tryptic digested bands

Number of gel band	Number of peptides identified by PFF	Number of peptides identified by PMF	NCBI accession number [homologous organism]	Theoretical M_r (Da)	Protein
1	3	8	gi|53764980 [Anabaena variabilis ATCC 29413]	45580	S-adenosylmethionine synthetase
2	2	12	gi|46135558 [Anabaena variabilis ATCC 29413]	64478	Uncharacterized conserved protein
	2	8	gi|53764325 [Anabaena variabilis ATCC 29413]	89961	Phosphoketolase
3	3	8	gi|417058 [Fremyella diplosiphon]	53472	Glutamine synthetase
4	3	6	gi|23128142 [Nostoc punctiforme PCC 73102]	45784	Enolase
	2	8	gi|45508985 [Anabaena variabilis ATCC 29413]	50466	ABC-type nitrate/sulfonate/bicarbonate transport systems
5	3	8	gi|45510539 [Anabaena variabilis ATCC 29413]	32200	Hypothetical protein Avar03000794 (rod linker Mw 32000)
6	2	5	gi|48894065 [Trichodesmium erythraeum IMS101]	19302	Inorganic pyrophosphatase
7	2	4	gi|51105738 [Anabaena mendotae]	8307 (fragment)	Phycocyanin β subunit
8	2	4	gi|304586 [Cyanidium caldarium]	17608	Phycocyanin α subunit

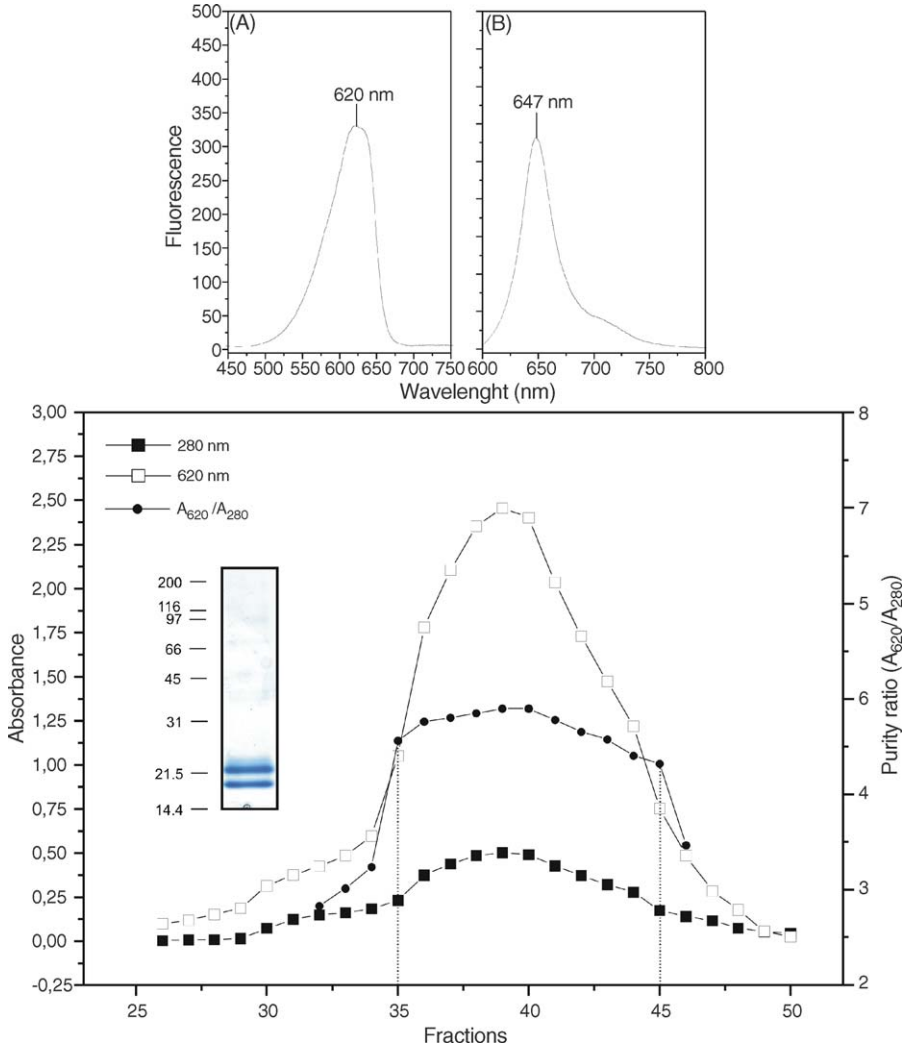

Fig. 3. Chromatographic profile of PC after purification on a hydroxyapatite column. Fractions showing an absorbance ratio A_{620}/A_{280} greater than 4 were pooled and analyzed by SDS-PAGE (left inset). Upper panel: excitation (A) and emission (B) spectra of pure PC in aqueous solution. Upon excitation at 620 nm the protein has a fluorescence emission at 647 nm.

shows the ultracentrifugation result. Three main colored bands can be observed, the darkest blue band (indicated by an arrow) was collected and analyzed with the methods reported below. HPLC analysis of band 3 (Fig. 2, panel A) showed the presence of two main peaks, indicating that the ultracentrifuge sucrose gradient of native cellular extract permits the collection of purified phycobilisome from which the main components may be isolated. For a preliminary characterization of peaks, the HPLC unit was equipped with a photodiode array and fluorescence detectors connected in series, which allow the simultaneous detection of the eluting peaks. The chromatogram of purified proteins recorded by visible light at 600 nm is shown in Fig. 2, panel B. It may be observed that the two main peaks showed an absorption at 600 nm, typically due to the presence of bilin pigments probably still connected with the polypeptide backbone. Interestingly the spectral absorptions recorded by diode-array (data not shown) revealed that the right peak has a higher absorption than the other, particularly at 330 and 620 nm. In fact, integration of peak areas at 600 nm, a measure of PCB content, gives results within experimental error of the 1:2 ratio, as expected from the known bilin content of the β-subunit of PC in *Synechocystis* PCC 6803 [24].

This method, although yielding highly purified PC, is not suitable for the quantitative preparation of PC for therapeutic use. Of the various chromatographic separations tested, the simplest providing high PC recovery resulted passing dried AFA extract through a hydroxyapatite column and eluting the protein with Na-phosphate buffer of increasing ionic strength. The optical absorbance below 300 nm decreases significantly and a predominant peak at 620 was observed (Fig. 1, left inset, solid line) suggesting that most of proteins were removed as they passed through the column and the solution collected between fractions 35 and 45, shown in Fig. 3, contains prevalently PC (final purity ratio A_{620}/A_{280} of 4.78). Moreover, in agreement with literature [17], the fluorescence analysis of pure PC showed that upon excitation at 620 nm, the protein had a fluorescence emission at 647 nm (upper inset of Fig. 3), which is typical of proteins containing bilin as chromophore. SDS-PAGE (left inset of Fig. 3) also confirmed that upon purification, most proteins were removed and two main bands corresponding to PC can be observed.

SDS-PAGE analysis of pure PC (Fig. 4A) revealed that the apparent molecular mass of subunit α was 18,500 whereas the β subunit mass was 21,900. Native electrophoresis (Fig. 4B) showed that PC purified from AFA had a molecular weight of 121,000, indicating that α and β subunits are associated in trimeric form $(\alpha\beta)_3$.

The data reported corresponded to data obtained for pure PC from *Spirulina* spp. [28–32], corroborating that the main constituent of AFA is a phycoprotein.

To better characterize the proteins still contained in the fraction collected from hydroxyapatite column, the solution was injected onto a C4 reversed phase HPLC and eluted by the H_2O–ACN–TFA gradient system, as previously reported.

The chromatogram recorded by UV at 214 nm after passing the mixture through the hydroxyapatite column is similar to that observed in Fig. 2, confirming that the latter method provides

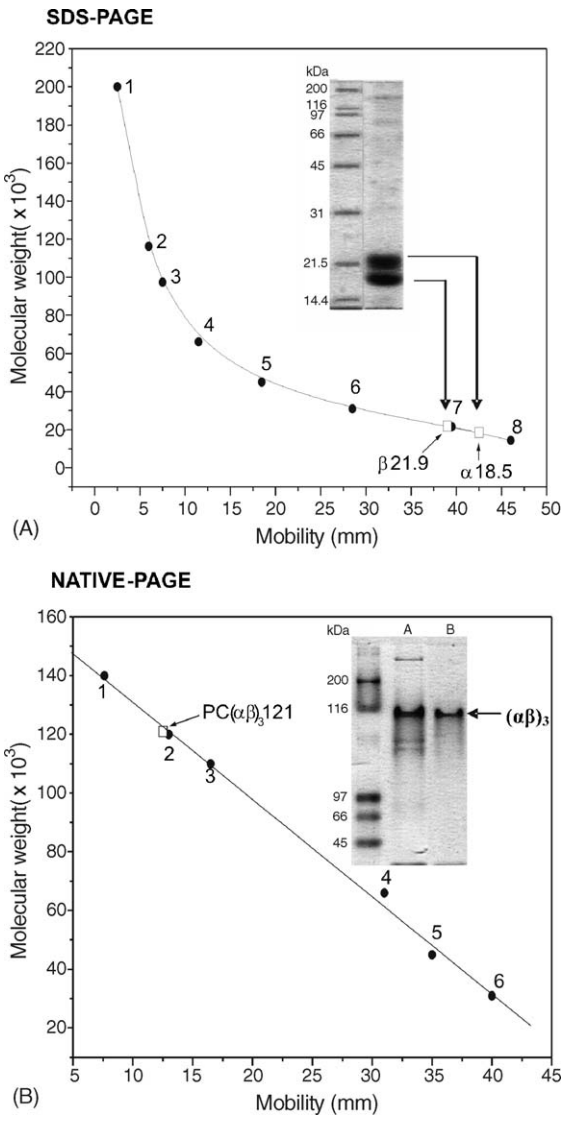

Fig. 4. (Panel A) Electrophoretic mobility and SDS-PAGE analysis of PC subunits on 15% polyacrylamide gel. Molecular standards: (1) myosin, 200,000; (2) β-galactosidase, 116,000; (3) Phosphorylase b, 97,400; (4) serum albumin, 66,200; (5) ovalbumin, 45,000; (6) carbonic anhydrase, 31,000; (7) trypsin inhibitor, 21,500; (8) lysozyme, 14,400. (Panel B) Electrophoretic mobility and native-PAGE analysis of pure PC on 10% polyacrylamide gel. Molecular standards: (1) lactate dehydrogenase, 140,000; (2) glutathione reductase, 120,000; (3) glucose 6-phosphate dehydrogenase, 110,000; (4) serum albumin, 66,200; (5) ovalbumin, 45,000; (6) carbonic anhydrase, 31,000.

a highly purified PC at higher concentrations and in a shorter time. This is an interesting aspect in that the purification method does not remove the chromophore which is involved in the well documented reaction with atmospheric oxygen and in the scavenger properties of PC [9]. This gives credit to the isolation of phycoproteins by hydroxyapatite for therapeutic usage since the prosthetic group of proteins is preserved.

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