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High-performance liquid chromatography coupled on-line with electrospray ionization mass spectrometry for the simultaneous separation and identification of the Synechocystis PCC 6803 phycobilisome proteins

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Abstract

The complete resolution of the protein components of phycobilisome from cyanobacterium Synechocystis 6803, together with their detection and determination of molecular mass, has successfully been obtained by the combined use of HPLC coupled on-line with electrospray ionization mass spectrometry. The method proposed consists of the isolation of the light-harvesting apparatus of cyanobacterium, by simply breaking cells in low-ionic-strength buffer, and subsequent injection of the total mixture of phycobilisomes into a C_4 reversed-phase column. Identification of proteins was performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the samples collected from HPLC or by measuring the protein molecular mass coupling HPLC with mass spectrometry. The latter method allows the simultaneous separation of the phycobiliproteins, phycocyanin and allophycocyanin, from linker proteins and their identification, which due to their similar amino acid sequence and their similar hydrophobicity, might not be detected by denaturing SDS–PAGE. Under the experimental conditions used, the pigment phycobilin is not removed from the polypeptide backbone, determining the hydrophobicity of the phycoproteins and hence their interaction with the reversed-phase column as well as in determining the protein–protein interaction into the phycobilisome aggregation. Removal of the pigment, in fact, abolishes HPLC separation, emphasizing the essential role that the pigments play in maintaining the unusual tertiary structure of these proteins.

Keywords: Bacteria; Synechocystis PCC 6803; Proteins; Phycobilisome proteins

1. Introduction

Cyanobacteria are the oldest known oxygenic organisms, which by their photosynthetic activity probably made a fundamental contribution to the development of the present oxygen-enriched atmosphere. The cyanobacterial photosynthetic apparatus consists of more than one protein complex: photosystems I (PSI) and II (PSII) as well as the cytochrome b_6f complex and ATP synthase [1]. Both PSI and PSII are composed of a central core, containing a chlorophyll binding protein, also conserved in higher plants and a peripheral accessory light-harvesting complex containing proteins not

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found in plants, called phycobiliproteins [2]. The latter belong to more than one family of water-soluble proteins, organized into macromolecular aggregates known as phycobilisomes [1]. This macrostructure consists of allophycocyanin cores surrounded by phycocyanin and phycoerithrin (when present) on the periphery [2]: all these proteins are assembled through specific interactions with polypeptides called linkers, whose molecular masses range from 7800 to 100 000 [3]. Phycocyanin is the major constituent of the phycobilisome, while allophycocyanin functions as the bridging pigment between phycobilisomes and the photosynthetic lamellae [2]. Both phycocyanin (PC) and allophycocyanin (APC) are each composed of two dissimilar polypeptide chains, a and b, of approximately M_r 17 000 and 18 000 [2]. Cell breakage in a low concentration buffer leads to the dissociation of the phycobilisome into phycobiliproteins from their associated linker polypeptides [4], as water-soluble complexes varying in composition depending on the particular protein and source organism [2].

The brilliant blue colors of the phycobiliproteins originates from covalently attached, linear tetrapyrrole prosthetic groups, known as phycobilins [5]. Among the five different bile pigments, with different numbers and arrangements of conjugated double bonds [6], only the blue colored phycocyanobilin (PCB) is present in the APC and PC of Synechocystis PCC 6803 phycobilisomes [4], the specie investigated in this study. In this specie, the α and β subunit of allophycocyanin (PHAA and PHAB, respectively) as well as the α subunit of a PC (PHCA) bind 1 bilin chromophore, while the β subunit of PC (PHCB) binds two bilin chromophores [2]. Phycobilin chromophores are bound to the polypeptide chain at conserved positions either by one cysteinyl thioether linkage through the vinyl constituent of the pyrrole ring A, or on both the A and D, of the tetrapyrrole [7,8]. The spectroscopic properties of individual phycobiliproteins depend in large measure on the chemical nature of the bilin they carry [9]. A strong absorption at 615 nm is observed [10].

In these light harvesting proteins, pigments collect or harvest the light energy, trap the excitation energy at the special pair, and finally transduce the light energy into stably separated charges. Pigments alone, however, are unable to perform the primary steps in the present form of photosynthesis. Thus, proteins are essential elements that orient the pigments, give them the appropriate conformation and physical separation, and modulate the absorption properties needed for the special steps in the light reactions [11]. It is obvious that analysis of protein structure is essential for an understanding of photosynthesis. On the other hand, characterization of phycobiliproteins with respect to subunit composition, bilin type and content, as well as the amino acid sequence requires separation of each subunit [12]. This is not an easy task due to the close similarity of the isoelectric points, the molecular mass and the hydrophobicity index of the phycobilin proteins. Several methods have been employed in the past, all sharing the following disadvantages: the separation is time consuming and requires numerous purification steps, some of them involving extreme experimental conditions [13,14]. As a consequence bilin prosthetic groups sometime undergo oxidative degradation. Swanson and Glazer [15] proposed a procedure for separating the α and β subunits of phycobiliproteins derived from different species by reversed-phase high-performance liquid chromatography (HPLC), starting from phycocyanin and allophycocyanin complexes obtained by standard chromatographic protocols [16].

The method reported in this paper is based on the direct injection of the whole phycobilisome assembly of the cyanobacterium Synechocystis 6803 into a reversed-phase high-performance chromatography column. The prior dissociation of the assembly from the core complex is simply performed by solubilization of cell breakage products into low concentration of buffer. The identification of proteins contained in each peak was performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and by molecular mass determination coupling the HPLC on-line with triple quadrupole mass spectrometry (MS) and an electrospray ion source device (ESI). The developed HPLC–ESI-MS procedure allows the resolution of all phycobiliproteins. Their molecular mass values are in good agreement with the computed molecular masses of these protein based on their DNA sequences, including the presence of chromophore tightly bound to the polypeptide.

2. Experimental

2.1. Chemicals

Reagent-grade phosphoric acid, magnesium chloride, sodium chloride, silver nitrate, sodium carbonate, trifluoroacetic acid, methanol, ethanol, formamide, as well as HPLC-grade water and acetonitrile, were obtained from Carlo Erba (Milan, Italy). Sucrose, tricine, trishydroxymethylaminomethane (Tris), 2-(*N*-morpholino)ethanesulfonic acid (MES) were obtained from Sigma (Milan, Italy).

2.2. Sample preparation

Synechocystis PCC 6803 was grown at 37°C at 5 μE m^2 s^{-1} in BG11 medium [17] supplemented with 5 m*M* glucose. The cells were harvested by centrifugation at 9000 rpm for 10 min at room temperature in a JA20 Beckman rotor and disrupted by 15 cycles of 30 s in a Braun homogenizer. The cell debris were eliminated by centrifugation as before and the supernatant was spun at 35 000 rpm in a TFT 70 Kontron system at 4°C. Thus the supernatant containing phycobilisomes [18] was collected and used for HPLC separation without any further purification. The bilin chromophore was partially removed by overnight treatment of phycobilisomes with 5 *M* urea, 2 m*M* dithiothreitol (DTT) and heating at 50°C. The spectrophotometer used was a Varian 4 equipped with a thermostatic bath.

2.3. SDS–PAGE

SDS–PAGE analysis was carried out by a Protean II Bio-Rad apparatus (180 mm×160 mm, 1.5 mm thick), using the method described by Schagger and von Jagow [19]: 16.5% T, 5.4% C in the separating gel and 10% T, 3% C in the spacer gel; a constant voltage of 100 V was applied over night at room temperature[1].

Gels were stained with Comassie brilliant blue R-250 dissolved in acetic acid–methanol–water (10:40:50, v/v).

The bilin carrying proteins in the gel were highlighted in a Bio-Rad UV transilluminator after soaking the gel in $ZnCl_2$ solution [20].

2.4. High-performance liquid chromatography

Protein separation by HPLC was performed using a reversed-phase Vydac Protein C_4 column (250×1.0 mm I.D.) packed with 5-μm porous butylsilica particles [21]. 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 166 UV detector or a fluorescence detector LC40 [22]. Samples were introduced onto the column by a Model 210A sample injection valve with either a 20-μl or a 50-μl sample loop.

The Vydac C_4 columns were pre-equilibrated with 20% aqueous acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid (TFA) and samples were eluted using a gradient consisting of a first linear gradient from 20 to 55.4% (v/v) acetonitrile in 40 min, followed by a gradient segment from 55.4% to 95% acetonitrile in 1 min.

2.5. Electrospray mass spectrometry

The HPLC–ESI-MS experiments were carried out by splitting the outlet of the HPLC system and coupling it with a Perkin-Elmer API 2000 or API 365 triple quadrupole mass spectrometer equipped with the electrospray ion source [23]. For HPLC–MS analysis, with pneumatically assisted electrospray, a spray voltage of 5 kV and a sheath gas pressure of 500 kPa were employed. Protein mass spectra were recorded by scanning the first quadrupole; scan range was 500–1800 u in 2 s. A typical positive ion spectrum of a single protein consists of a series of peaks, each of which represents a multiply charged ion of the intact protein having a specific number of protons attached to the basic sites of the amino acid sequence. The m/z values for the ions have the general form $[M+zH]/z$, where z equals the number of protons attached. It follows that the

[1] T=[g acrylamide+g *N*,*N*′-methylenebisacrylamide (Bis)]/100 ml solution; C=g Bis/% T.

molecular mass can be readily calculated from two measured, adjacent m/z values, given the additional information that two adjacent multiply charged ions differ by one charge. Once M and z are determined for one pair of peaks, all other m/z signals can be deconvoluted into one peak on a real mass scale, which has a typical peak width at half height of 10–20 u. The mass spectrometer was tuned for chromatographic conditions with a 2 μg/μl solution of cytochrome *c* (Sigma) added at a flow-rate of 1 ml/min to the column effluent (50 μl/min, 50% acetonitrile in 0.05% TFA) by means of a T-piece before entering the ESI source, resulting in a flow-rate of 50 μl/min into the mass spectrometer. The separations were performed using a 250 mm Vydac Protein C_4 column of either 4.6 mm or 1.0 mm I.D. (Separation Group, Hesperia, CA, USA), packed with 5-μm porous butylsilica particles. All solutions were filtered through a Millipore type FH 0.5-μm membrane filter and degassed by bubbling with helium before use.

3. Results and discussion

The complete resolution of the protein components of phycobilisome from cyanobacterium Synechocystis 6803, together with their detection and determination of molecular mass, has successfully been achieved by the combined use of on-line HPLC–ESI-MS. The method proposed consists in the isolation of light-harvesting apparatus of cyanobacterium, by simple breaking of cells in low-ionic-strength buffer, and subsequent injection of the total mixture of phycobilisomes into a C_4 reversed-phase HPLC system and elution by the water–acetonitrile–TFA gradient system. The optimal separation of the protein components of phycobilisome is obtained by a multisegment gradient, as reported in the Experimental section. In order to examine the reproducibility of the chromatographic profile and resolution of the phycobilin proteins, the standard deviations (SDs) and the relative standard deviation (RSDs) of the retention times of the resolved proteins were calculated from the chromatograms obtained by six repeated injections of the phycobilisome sample. This experiment was repeated on other samples of phycobilisome isolated from cyanobacteria by a second and a third preparation. The RSDs were better than 0.4% for the main peaks (data not shown). Thus, by using the best experimental conditions reported, the phycobilisome complexes may be resolved into four main peaks and many smaller peaks (Fig. 1). 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 recorded by visible light at 600 nm is shown in Fig. 1A while the one recorded by UV at 214 nm is shown in Fig. 1B. Reported in Fig. 1C is the chromatogram detected by fluorescence emission at 330 nm upon excitation at 280 nm, typically used to identify proteins. It may be observed that the UV chromatogram in Fig. 1B is comparable to that of Fig. 1C recorded in fluorescence, with the exception of peak 7, which is strongly reduced in panel C. This correspondence indicates that most of the peaks, labeled 1–9, could be proteins. However, only peaks 6, 7, 8 and 9 show an absorption at 600 nm (Fig. 1A), typically due to the presence of bilin pigments probably still connected with the polypeptide backbone. Thus, it may be inferred that these peaks represent the allo- and phycocyanoproteins whereas the peaks eluting at short times, labeled 1, 2, 3, 4, 5 may contain the linker proteins. They do not in fact show any absorption at 600 nm and elute at a shorter time in agreement with their lower hydrophobicity deducted from DNA sequence (see Table 1). However, as seen in Fig. 1, the two types of allo- and phycyanoproteins elute from the reversed-phase column with retention times ranging from 28.1 to 31.05 min, which corresponds to a narrow range of acetonitrile concentration of only 3.92% (v/v), as expected by their close similarity in amino acid sequence, molecular mass and hydrophobicity.

In the inset of Fig. 1A are the spectral absorptions, recorded by diode-array detection, of peaks 6, 7, 8 and 9. It may be observed that peak 7 has a higher absorption than the other peaks, particularly at 330 nm and 660 nm. In fact integration of peak areas at 660 nm, a measure of phycocyanobilin (PCB) content, gives results within experimental error of the 1:2 ratio, as expected from the known bilin content of the β-subunits of phycocyanin in Synechocystis 6803 [4]. Thus, it may be deduced that the stronger

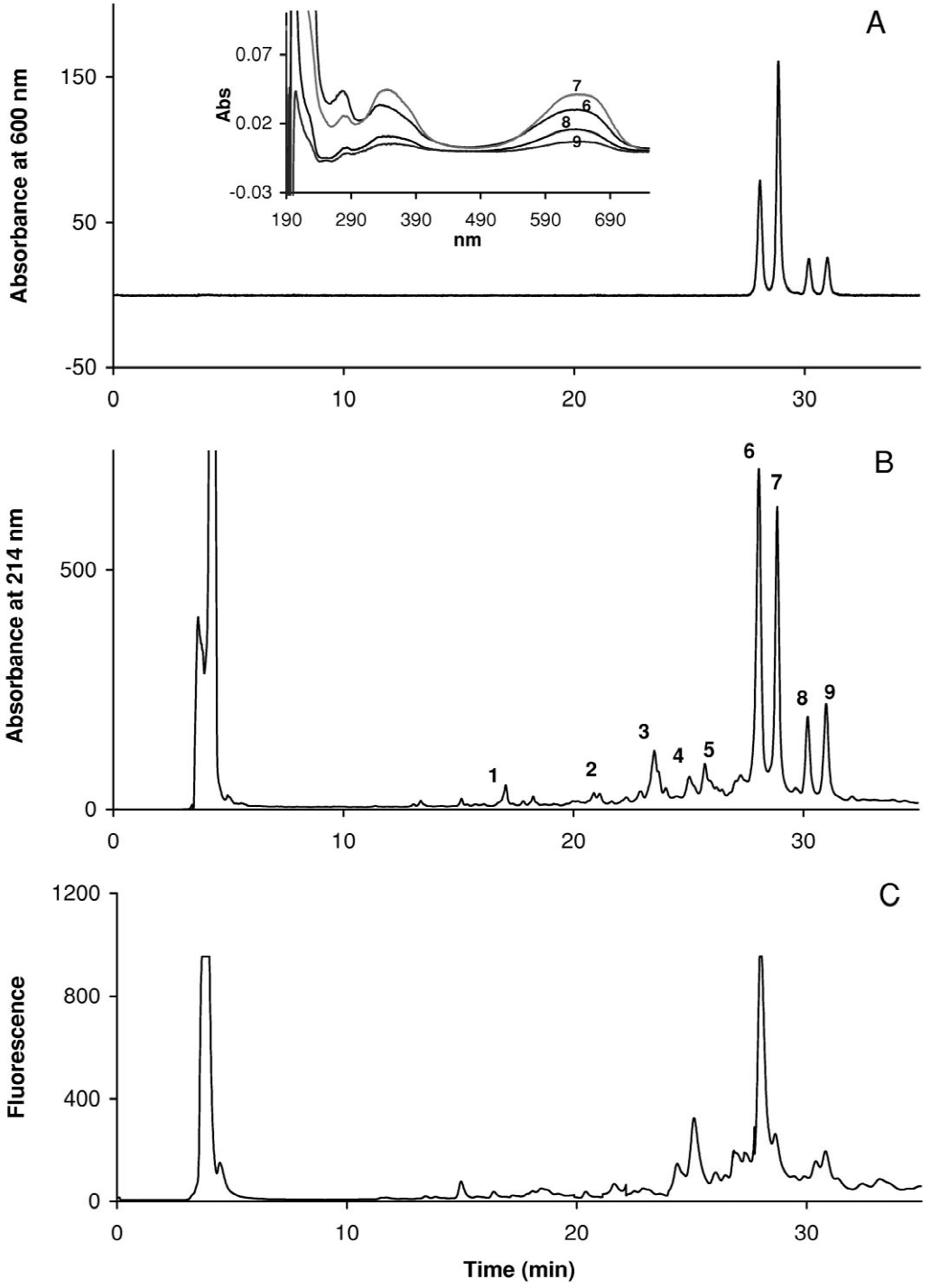

Fig. 1. Chromatographic profile of Synechocystis PCC 6803 phycobilisome proteins separated by reversed-phase HPLC. (A) Chromatogram recorded at 600 nm. The inset shows the spectra of the four main peaks obtained by the diode array detector. Labeling refers to peak numbers reported in panel B. (B) Chromatogram recorded at 214 nm. (C) Chromatogram recorded by fluorescence detection: excitation wavelength at 280 nm and emission at 330 nm.

Table 1
Identification of the Synechocystis 6803 phycobilisome components by measuring the protein molecular mass

Protein	Molecular mass from DNA sequence	Molecular mass by SDS–PAGE	Molecular mass by HPLC–ESI-MS	Aliphatic index	HPLC	
					Elution time (min)	Peak number
PHAA	17 280	18 000	17 860	89.0	30.2	8
PHAB	17 215.6	17 000	17 820	98.9	31.0	9
PHCA	17 586.6	17 500	18 180	82.7	28.0	6
PHCB	18 126.4	18 000	19 320	92.6	28.9	7
PYC1	7706		7542	69.8	17.0	1
PYS1	9322		9242	73.98	21.1	2
PYR1	32 389.4	<36 000	31 980	82.7	23.5	3
PYR2	30 797.3	>29 000	30 123	79.3	25.1	5

The molecular masses were determined by the deconvolution of the ESI-MS spectra recorded during the chromatographic run into a C_4 reversed-phase column coupled on-line with by a mass spectrometer equipped with an electrospray ion source. The values of molecular mass deduced from DNA sequence as well as the aliphatic index have been calculated by using the Prot Parameter tools Expasy program. The SDS–PAGE molecular masses were deduced by the marker used in the electrophoresis. HPLC elution time and peak numbers refer to Fig. 1.

reduction in fluorescence of peak 7 is related to its higher absorption at 330 nm.

By this preliminary characterization, it may be inferred that the four main peaks in the chromatograms displayed in Fig. 1 represent the two components phycocyanin and allophycocyanin, respectively, and on the basis of the abundance expected from examination of the literature on phycocyanin and allophycocyanin, it is possible that peaks 6 and 7 represent the phycocyanines, in particular peak 7 contains β-subunit due to the presence of two bilins, and peaks 8 and 9 the allophycocyanines, respectively.

In order to better identify the proteins contained in each HPLC peak, we performed both SDS–PAGE electrophoresis on the protein collected from each peak and determined the protein molecular mass by ESI-MS.

For the first purpose, we searched for the best conditions to resolve the protein components of the phycobilisomes on a semi-preparative (250×10 mm I.D.) column, with the aim of collecting an amount of purified polypeptide sufficient for peak identification by SDS–PAGE. To achieve this, the best resolution obtained on the analytical column was retained by keeping the ratio of the gradient volume constant in order to maintain the same gradient shape. Then the fractions of eluate collected throughout the chromatographic run were lyophilized, dissolved in 50% (v/v) glycerol, 62.5 m*M* Tris–HCl buffer (pH 6.8), containing 0.1 *M* dithiothreitol and 5% SDS, and analyzed by denaturing SDS–PAGE. The SDS–PAGE gel displayed in Fig. 2 shows that lines 1, 2 and 4 corresponding to HPLC peaks 1, 2 and 4 do not show any protein, probably due to either insufficient material collected in the fractions or to the absence of protein material. However, peaks 6, 7, 8 and 9 contained phycocyanin and allophycocyanin as predicted, their apparent molecular masses being in the range 17 000–18 000. This identification is supported by the correspondence of the mobilities of the proteins collected throughout the chromatographic

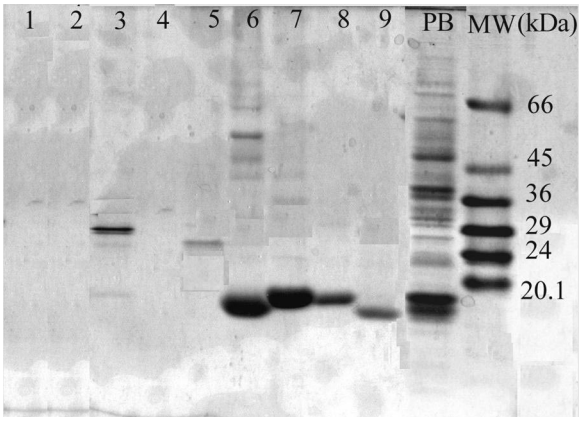

Fig. 2. SDS–PAGE characterization of peaks collected from the reversed-phase HPLC column. The numbers at the top of the lines correspond to peaks labeled in Fig. 1B. PB indicates the whole phycobilisome apparatus while M_r indicates the molecular mass of the marker ($\cdot 10^3$).

run in peaks 6–9, and those observed when running the total proteins contained in phycobilisome (line PB). Moreover, SDS–PAGE gel staining confirms that the abundance of the four main proteins separated from the phycobilisome sample is in the order phycocyanin>allophycocyanin, as observed in the chromatogram reported in Fig. 1. It is worth noting that from the different order in which the phycobilin proteins are resolved by RP-HPLC and by denaturing SDS–PAGE it can be inferred that the hydrophobic character of these proteins is not directly related to their molecular masses, which range between 17 000 and 18 000.

Concerning the minor peaks 3 and 5 present in Fig. 1B, the SDS–PAGE shows two faint bands at about 30 000–32 000 which may be tentatively attributed to the linker polypeptides PYR 1 and PYR 2 whose expected molecular masses are 30 797 and 33 389, respectively.

Finally, for an unequivocal and complete identification of the protein contained in each HPLC peak, the molecular mass of each protein was determined by coupling HPLC on-line with ESI-MS. For this purpose the outlet of the HPLC column was split in two directions: one towards the electrospray source of the mass spectrometer and the other one to the UV detector.

Fig. 3A reports the reconstructed ionic current (RIC) recorded during the chromatographic run with the mass spectrometer. Most of the RIC peaks show a concomitant UV and fluorescence detection (Fig. 1B and C), suggesting that each peak could contain ionizable chemical material, such as proteins. Fig. $3B_1$–B_4 shows the reconstructed ion chromatogram, based on the individual ESI-MS spectra (B_1–B_4) collected for the main peaks eluting from the RP-HPLC column at 31.14 (peak 6), 33.18 (peak 7), 35.9 (peak 8) and 36.4 (peak 9) min, respectively as well as the mass spectra in absolute mass unit after deconvolution (Fig. $3C_1$–C_4). It is important to remark that the retention times of RIC do not correspond exactly with the chromatogram reported in Fig. 1 due to the adapted experimental condition used for HPLC on line with the spectrometer. However, the analysis confirms that the peak eluting from the column at 22.9 min (peak 4) was a non-protein impurity related to the detergent used, while all the other peaks show a typical Gaussian distribution of multicharged species, as expected for proteins. The deconvolution analysis, in fact, indicated the presence of proteins with molecular masses in the range 17 000 to 19 000 for peaks 6, 7, 8 and 9, many proteins with molecular masses below 10 000 in peaks 1 and 2, and over 30 000 for peaks 3 and 5. In Table 1 the experimental molecular masses, obtained by the deconvolution of the ESI-MS spectra, have been correlated to the molecular masses expected for phycobilisomes protein components deduced from their DNA sequences [24], as well as with the hydrophobicity calculated for each protein from the SwissProt database, in an effort to assign a protein to each peak. Using this comparison, each main peak (numbered 6, 7, 8 and 9) on the UV chromatogram can be assigned to the corresponding PHCA, PHCB, PHAA and PHAB proteins, respectively, in agreement with the above spectroscopic analysis and SDS–PAGE electrophoresis. The molecular mass values obtained, in fact, are very close to those expected from the gene sequence, taking into account that differences observed (ranging from 580 to 604.4) are due to the presence of residual bilin pigment still bound to the proteins, whose molecular mass is 587. Moreover, the discrepancies of 580 and 593.4 observed for PHAA and PHCA, respectively, are into the experimental error, whereas the difference of 604.4 as observed for PHAB could indicate the presence of a methylation on the apoprotein [16]. In the case of PHCB, the difference is 1194, confirming the presence of two bilin pigments in this protein. Moreover, the assignment reported reveals that in the electrophoretic migration order PHCB >PHAA>PHCA>PHAB observed in SDS–PAGE, the PHAA shows a higher apparent molecular mass than measured. This may create confusion for the identification of proteins by SDS–PAGE, whereas the close agreement between the molecular mass determined by ESI-MS and the DNA sequence permits confidence in the method used here. Moreover, this indicates that post-translational modifications are absent in these proteins, especially the phosphorylation which should add at least 79 to the protein, according with the evidence that phosphorylation has never been documented unequivocally in cyanobacteria [25].

Concerning the other minor peaks, labeled 1, 2, 3 and 5, although their ESI spectra are less than phyco-

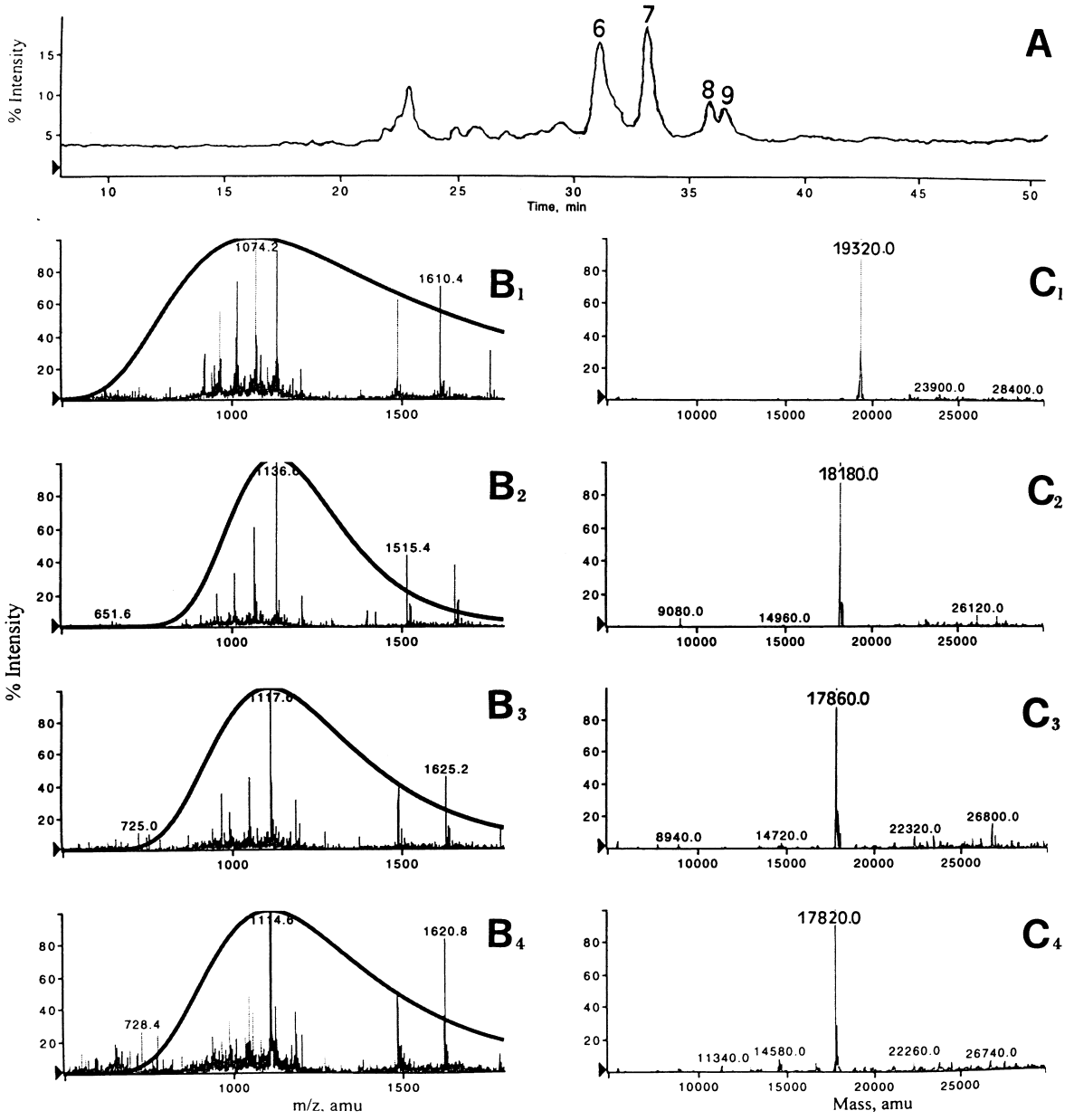

Fig. 3. Determination of the molecular mass of proteins eluting from HPLC by mass spectrometry coupled on-line. (A) Reconstructed ionic current (RIC) during the chromatographic run recorded by the mass spectrometer. (B_1–B_4) ESI-MS spectra collected for the peaks eluting the RP-HPLC column at 28.1 (peak 6), 28.9 (peak 7), 30.2 (peak 8) and 31.0 (peak 9) min, respectively. The ESI spectra were measured with a scan range of 500–1800 u in 2 s. The dark line indicates the theoretical charge state distributions. (C_1–C_4) Mass spectra in absolute mass after deconvolution of the relative ESI-MS spectra showed in (B_1-B_4).

and allocyanins, as there is less material present, deconvolution analysis was able to provide mass values of 30 123 and 31 980 for peaks 3 and 5, respectively and 7342 and 8822 for peaks 1 and 2, allowing PYR 1, PYR 2, PYC 1 and PYS 1 to be assigned to the peaks in that order. In this case the

mass accuracy is lower than phycoproteins due to their lower protein concentration. This assignment agrees with that previously observed by SDS–PAGE as well as with their shorter retention time related to their low hydrophobicity index (see Table 1). Thus, by simple extraction of phycobilisome and subsequent liquid chromatography, all proteins may be simultaneously separated and identified.

As expected, elution by acetonitrile does not remove the phycobilin from the polypeptide backbone, in contrast to that previous observed for light harvesting protein in higher plants [21] where the chlorophyll pigments were completely removed from proteins. However, the two different kind of pigments are differently bound to the polypeptide chain. Nevertheless, the phycobiliproteins when running on SDS–PAGE show a faint blue band, indicating the presence of bilin pigment on the proteins and confirming that the denaturing and reductant conditions of solubilization are not sufficient to remove the bilin from the polypeptide backbone. However, in order to confirm the presence of bilin pigment in the polypeptide chain after HPLC separation, we took advantage of the known affinity of phycobilin pigments for Zn ions which increases the natural fluorescence of bilin [20]. Fig. 4 compares the SDS–PAGE of peaks 6, 7, 8 and 9 when stained by Comassie (Fig. 4A) with the same proteins treated with $ZnCl_2$ and illuminated by a transilluminator at 320 nm. The appearance of a visible fluorescence following the addition of $ZnC1_2$ (Fig. 4B) in the phycobilin protein containing band, confirms that molecules present in the peak collected from the preparative column still retain their chromophore(s), in agreement with indications from ESI-MS measurements. The faint fluorescence observed at higher molecular mass may be related to the aggregation complexes. On the other hand, using more drastic conditions (overnight treatment with 5 *M* urea, high temperature and ditiothreitol) we are able to partially remove the chromophore from the protein, as confirmed by the disappearance of absorption at 620 mn (data not shown). In this case, the phycobilisome analysis by SDS–PAGE shows a polypeptide pattern similar to that found under control conditions (inset of Fig. 5), but the HPLC separation is strongly reduced (Fig. 5). This result suggests that the presence of chromophore inside the protein gives rise to a peculiar conformational structure of the polypeptide chain, as reported elsewhere [11], which

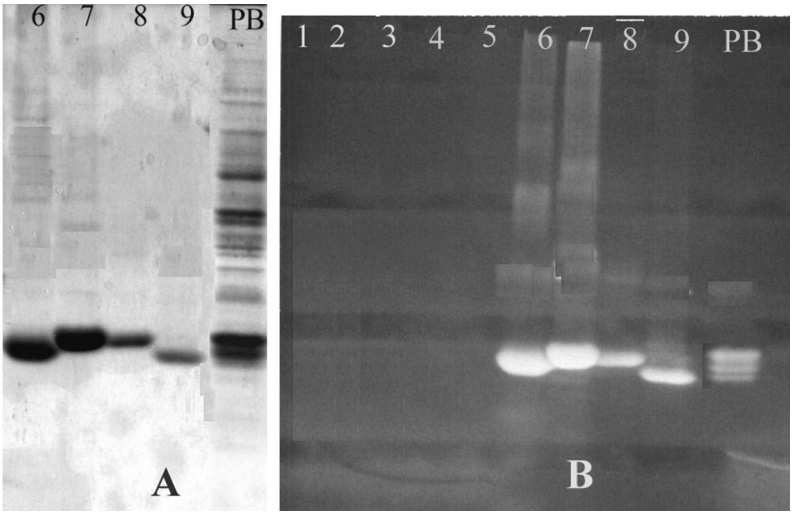

Fig. 4. Fluorescence of bilin chromophores present in the allophycocyanin and phycocyanin upon addition of Zn ions. The presence of bilin pigment with the polypeptide was ascertained by taking advantage of the known affinity of phycobilin pigments for Zn ions which increases the natural fluorescence of bilin [20]. Proteins were collected from RP-HPLC semipreparative columns and subjected to SDS–PAGE using the experimental conditions reported in Fig. 2. (A) Comassie blue-stained polypeptide bands; (B) fluorescence of polypeptides resulting from excitation at 320 nm. The numbers at the top of the lines correspond to peak labels reported in Fig. 1.

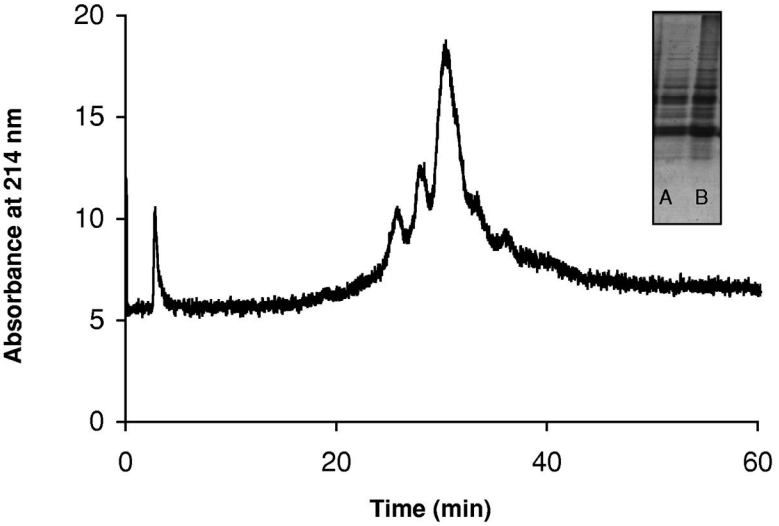

Fig. 5. Chromatographic profile of Synechocystis PCC 6803 phycobilisome proteins after removal of the bilin pigments. Pigments were removed by overnight treatment of phycobilisome with 5 *M* urea and ditiothreitol (DTT) at 50°C. The HPLC separation was performed using the experimental conditions reported in the legend for Fig. 1. The inset reports the SDS–PAGE separation of the untreated (line A) and treated sample (line B).

confers different hydrophobicity on the phycoproteins and consequently different interactions with the reversed-phase column as well as among proteins–proteins in vivo. This observation highlights the main role that the pigments may play in the tertiary structure of this kind of proteins and consequently in vivo in the protein–protein interactions necessary to form functional supramolecular organization of phycobilisomes. It is well documented, in fact, that both in higher plants and in cyanobacteria the aggregation of light harvesting proteins into supracomplex occurs only after the pigment(s) has been inserted into the apoproteins [26,27]. To this regard, treatment of phycobilisome, or intact cyanobacteria, with UVB for 2 h, gives rise to a strong decrease of the HPLC peak corresponding to the β-phycocyanin without degradation of the polypeptide chain, suggesting that the decreased absorption at 214 nm is probably due to alteration of the pigments of β-phycocyanin, which is known to contain two bilins (data not shown). This preliminary investigation, which is progress in our lab, is an example of the potentiality of the combined use of HPLC–ESI-MS method for studying and characterizing each protein into a supramolecular structure.

4. Conclusions

In conclusion, the reversed-phase HPLC method reported in this paper, besides being rapid, simple and precise, has proven to be effective in detecting differences in the protein components of phycobilisome, such as allocyanins and phycocyanins, which due to their similar amino acid sequences and their similar molecular masses, might be not identified by denaturing SDS–PAGE. In particular, our data have shown that PHAA may be impossible to identify only by the apparent molecular mass in SDS–PAGE, as well as the linker proteins, due to their small amounts.

Moreover, unlike the chromatographic method proposed by Swanson and Glazer [15], which is limited to the separation of the α and β subunits of phycocyanin and allocyanin, the method presented here allows the simultaneous separation of allo- and phycocyanins and linker polypeptides without sample preparation. The reduced manipulation of the sample helps to eliminate artifacts and makes the interpretation of results more reliable, especially when screening phycobiliproteins for interesting structural features or characterization of mutant

phycobiliproteins lacking one or more bilin peptides as well as in detecting differences in the protein components of light-harvesting complexes isolated from different cyanobacteria. This knowledge is expected to shed light on the composition and supramolecular organization of phycobilisomes and may increase the understanding of the molecular mechanisms underlying their physiological adaptations to environmental conditions [28,29], such as the response to UV irradiation and adaptation [30–32] to the nutrient deprivation.

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