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Resolution and identification of the protein components of the photosystem II antenna system of higher plants by reversed-phase liquid chromatography with electrospray-mass spectrometric detection

Danilo Corradini[a,*], Christian G. Huber[b], Anna Maria Timperio[c], Lello Zolla[c]

[a]*Istituto di Cromatografia del CNR, P.O. Box 10, Via Salaria Km 29300, I-00016 Monterotondo Stazione (Rome), Italy*
[b]*Institut für Analytische Chemie und Radiochemie, Leopold-Franzens-Universität, Innrain 52a, A-6020 Innsbruck, Austria*
[c]*Dipartimento di Scienze Ambientali, Università della Tuscia, Via S. Camillo de Lellis, Blocco D, I-01100 Viterbo, Italy*

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Abstract

Reversed-phase liquid chromatography (RPLC) was interfaced to mass spectrometry (MS) with an electrospray ion (ESI) source for the separation and accurate molecular mass determination of the individual intrinsic membrane proteins that comprise the photosystem II (PS II) major light-harvesting complex (LHC II) and minor (CP24, CP26 and CP29) antenna system, whose molecular masses range between 22 000 and 29 000. PS II is a supramolecular complex intrinsic of the thylacoid membrane, which plays the important role in photosynthesis of capturing solar energy, and transferring it to photochemical reaction centers where energy conversion occurs. The protein components of the PS II major and minor antenna systems were extracted from spinach thylacoid membranes and separated using a butyl-silica column eluted by an acetonitrile gradient in 0.05% (v/v) aqueous trifluoroacetic acid. On-line electrospray MS allowed accurate molecular mass determination and identification of the protein components of PS II major and minor antenna system. The proposed RPLC–ESI-MS method holds several advantages over sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the conventional technique for studying membrane proteins, including a better protein separation, mass accuracy, speed and efficiency.

Keywords: Proteins; Photosystem II; Light-harvesting complex

1. Introduction

The molecular mass of a protein is a fundamental parameter in the biochemical characterization of both natural and recombinant proteins. The determination of the molecular mass of proteins is traditionally performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). This technique is based on the assumption that fully denatured proteins bind through hydrophobic interaction a constant amount of SDS (1.4 g of SDS per 1 g of protein) [1], resulting in complexes of approximately constant charge-to-mass ratios and, consequently, identical electrophoretic mobilities. There-

*Corresponding author. Tel.: +39-6-9067-2254; fax: +39-6-9067-2269.

E-mail address: danilo.corradini@mlib.cnr.it (D. Corradini)

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fore, in a sieving medium protein–SDS complexes migrate proportionally to their effective molecular radius and thus to the protein molecular mass [2]. The result is that SDS–PAGE determines an apparent molecular mass, the accuracy of which range from a few per cent for a well behaving globular protein to about 30% for a heavily glycosilated protein.

The advent of electrospray ionization (ESI) for the interfacing of mass spectrometry (MS) and liquid chromatography (LC) has greatly enhanced the utility of mass spectrometry in structural biochemistry [3]. However, LC–ESI-MS of integral membrane proteins is complicated by the strong hydrophobic nature of these proteins. Mostly they are solubilized and maintained in solution by using either high concentrations of solubilizing agents, such as urea, or detergents, which are not compatible with ESI-MS. Nevertheless, an increasing number of applications of LC–ESI-MS for the investigation of membrane proteins have been recently reported [4–7].

This study has been performed to develop a rapid and straightforward LC–ESI-MS procedure for the resolution and molecular mass determination of the individual intrinsic membrane proteins that comprise the photosystem II (PS II) major and minor antenna system. This light harvesting complex (LHC) is located in the peripheral region of photosystem II, which is the supramolecular complex intrinsic of the thylacoid membrane responsible for splitting water to form molecular oxygen, electrons and protons [8].

The major light harvesting complex of photosystem II (PS II major antenna system) is composed of the protein binding chlorophylls type I, type II and type III (also denominated Lhcb1, Lhcb2 and Lhcb3 or just LHC II), which are encoded by the nuclear genes *Lhcb1*, *Lhcb2* and *Lhcb3*. The LHC II complex is traditionally separated from the minor antenna system and from the reaction center complexes by sucrose gradient ultracentrifugation [9]. Up to 65% of the PS II chlorophyll is bound to this complex [10]. Components of the PS II minor antenna system, binding about 10% of the PS II chlorophyll, are the proteins CP29, CP26 and CP24 encoded by the nuclear genes *Lhcb4*, *Lhcb5* and *Lhcb6*, respectively [11].

The protein components of the PS II major and minor antenna system are traditionally resolved by SDS–PAGE into several closely related proteins with apparent molecular masses in the range 20 000–31 000 [11]. However, most of these values diverge from the molecular masses calculated for the individual LHC proteins on the bases of their nucleotide-derived amino acid sequences [12–18].

This paper reports the results of a study performed to obtain the resolution and the precise molecular mass determination of the protein components of the PS II major and minor antenna system using a reversed-phase (RP) LC column coupled to a triple quadrupole mass spectrometer with an electrospray ion source. The developed LC–ESI-MS procedure allows the resolution of all PS II antenna proteins, whose molecular mass values are in good agreement with the computed molecular masses of these proteins based on their DNA sequences.

2. Experimental

2.1. Chemicals

Reagent-grade phosphoric acid, magnesium chloride, sodium chloride, silver nitrate, sodium carbonate, trifluoroacetic acid (TFA), formic acid, acetic acid, methanol, ethanol, formamide, as well as high-performance liquid chromatography (HPLC)-grade water and acetonitrile, were obtained from Carlo Erba (Milan, Italy) or Merck (Darmstadt, Germany). Acrylamide, *N,N′*-methylene-bis-acrylamide, and all other reagents for SDS–PAGE were purchased from Bio-Rad (Segrate, Italy). Sucrose, tricine, tris-hydroxymethylaminomethane (Tris), *n*-octyl β-D-glucopyranoside, *n*-dodecyl β-D-maltoside, chlorophyll a and chlorophyll b, as well as 2-(*N*-morpholino)ethanesulfonic acid (MES) were obtained from Sigma (Milan, Italy). Triton X-100 and *n*-octylsucrose were purchased from Calbiochem (San Diego, CA, USA).

2.2. Liquid chromatography and electrospray mass spectrometry

Optimization of chromatographic separations was performed using a Beckman (Fullerton, CA, USA)

Model 342 liquid chromatograph, equipped with two Model 114 M solvent delivery pumps, a Model 420 system controller, a Model 340 dynamically stirred high-pressure mixer, a Merck–Hitachi (Darmstadt, Germany) Model F-1050 fluorescence spectrophotometer equipped with a 2-μl flow cell, and a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 5-μl sample loop. The LC–ESI-MS experiments were carried out with a Model Rheos 4000 low-pressure gradient pump (Flux Instruments, Karlskoga, Sweden), a Model S4100 column oven (Sykam, Gilching, Germany), a Rheodyne Model 8125 microinjector equipped with a 20-μl sample loop, a Model Linear UV–Vis 200 variable-wavelength detector (Linear Instruments, Fremont, CA, USA) equipped with a 3 mm/1.2 μl flow cell, and a personal computer-based data system from Gynkotek (Germering, Germany). ESI-MS was performed on a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with the electrospray ion source. For LC–MS analysis with pneumatically assisted ESI an electrospray voltage of 4.5 kV and a sheath gas pressure of 410 kPa were employed.

Protein mass spectra were recorded in the positive ion mode by scanning the third quadrupole; scan range and scan time are given in the figure captions. The mass spectrometer was calibrated using a solution of L-methionyl-arginyl-phenylalanyl-alanine (20 pmol/μl, Finnigan) and apomyoglobin from horse skeletal muscle (5 pmol/μl, Sigma) in water–methanol (50:50, v/v) to give average molecular masses of the observed signals. 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 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 [19]. 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 μl/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. (The Separation Group, Hesperia, CA, USA), packed with 5-μm porous butyl silica particles. All solutions were filtered through a Millipore (Milan, Italy) type FH 0.5-μm membrane filter and degassed by bubbling with helium before use.

2.3. Isolation of chloroplast thylakoid and PS II membranes

Chloroplast thylakoid membranes (PS II membranes) were isolated from spinach leaves according to the method of Berthold et al. [20] with the following modifications. Leaves were powdered in liquid nitrogen and subsequently homogenized in an ice-cold 20 m*M* tricine buffer, pH 7.8 containing 0.3 *M* sucrose and 5.0 m*M* magnesium chloride (B1 buffer). The homogenization was followed by filtration through one layer of Miracloth (Calbiochem) and centrifugation at 4000 *g* for 10 min at 4°C. Pellets were suspended in B1 buffer and centrifuged as above. This second pellet was resuspended in 20 m*M* tricine buffer, pH 7.8 containing 70 m*M* sucrose and 5.0 m*M* magnesium chloride (B2 buffer) and centrifuged at 4500 *g* for 10 min. Pellets containing the thylakoid membranes were then resuspended in 50 m*M* MES buffer, pH 6.3 containing 15 m*M* sodium chloride and 5 m*M* magnesium chloride (B3 buffer) at 2.0 mg chlorophyll/ml for 15 min after adding Triton X-100 at a final ratio of 20 mg/mg chlorophyll. The concentration of chlorophyll was determined according to the method described by Porra et al. [21]. The incubation was terminated by centrifugation at 40 000 *g* for 30 min at 4°C. This pellet containing the PS II complex and corresponds to the PS II enriched membrane preparation described by Berthold et al. [20], which is referred to as BBY, from the initials of the author's names. The BBY preparation was resuspended in B3 buffer containing 20% (v/v) glycerol and stored at −80°C.

2.4. Isolation of the PS II major and minor antenna system by sucrose gradient ultracentrifugation

The light-harvesting complex was isolated from the PS II membranes as described by Dainese and Bassi [10] with the following modifications. PS II membranes were pelleted by centrifugation at 10 000 *g* for 5.0 min at 4°C, suspended in B3 buffer at 1.0 mg/mg chlorophyll and then solubilized by adding 1% (w/v) *n*-dodecyl β-D-maltoside. Unsolubilized material was removed by centrifugation at 10 000 *g* for 10 min. The supernatant was rapidly loaded onto a 0.1–1.0 *M* sucrose gradient containing B3 buffer and 5.0 m*M* *n*-dodecyl β-D-maltoside. The gradient was then spun on a Kontron Model Centricon T-1080 ultracentrifuge equipped with a Model TST 41.14 rotor at 39 000 rpm for 18 h at 4°C. Green bands were harvested with a syringe. The SDS–PAGE analysis of these green bands revealed that band 2 contained a mixture of the protein components of the PS II major and minor antenna system, whereas band 3 contained essentially the protein components of the PS II major antenna, as was previously reported [9]. The material harvested from the second (band 2) and the third (band 3) band of the sucrose gradient ultracentrifugation were subjected to RPLC–ESI-MS analysis.

3. Results and discussion

The analytical strategy employed in this study was focused on the combined use of RPLC coupled on-line with a mass spectrometer equipped with an electrospray ion source in order to obtain the resolution of the protein components of the PS II major and minor antenna system together with their detection and molecular mass determination. Protein separation by RPLC was performed using a reversed-phase column of either 4.6 or 1.0 mm internal diameter packed with a porous butyl-silica stationary phase.

3.1. Optimization of the protein resolution

Initial phases of this investigation were centered on the optimization of the resolution of the protein components of the major and minor antenna system of the photosystem II complex by RPLC. During these initial phases of the study, either UV detection at 214 nm or fluorescence detection with excitation and emission wavelengths set at 280 and 330 nm, respectively, were utilized to expedite the optimization procedure avoiding the complexity of on-line ESI-MS detection. The experiments were carried out subjecting to chromatography the proteins isolated by sucrose gradient ultracentrifugation as described in the Experimental section. The sucrose gradient ultracentrifugation produced five different green bands where the second one (band 2) contained a mixture of the protein components of the PS II major and minor antenna system, whereas the third green band (band 3) contained mainly the protein components of the PS II major antenna system.

In RPLC–ESI-MS, the chemical composition of the mobile phase plays an important role both on the chromatographic separation and on spray stability and signal sensitivity [22,23]. In addition, sample detection by ESI-MS restricts the use of buffers and other mobile phase modifiers to volatile compounds. Thus, a number of factors such as gradient shape, flow-rate, type and concentration of the acidic additive of the water–acetonitrile mobile phase were optimized before testing the utility of on-line RPLC–ESI-MS analysis of the PS II antenna system proteins. The three volatile acidic mobile phase additives TFA, formic acid and acetic acid were evaluated for their capability in resolving the protein components of the PS II major and minor antenna system. These additives are the most commonly used in eluents containing acetonitrile for the RPLC of peptides and proteins. TFA is known to enhance the solubility of most peptides and proteins and to reduce their electrostatic interactions with the residual silanol groups at the chromatographic surface [24–27]. It has low UV transparency and anionic ion-pairing properties. However, TFA is also known to inhibit the ion signal by increasing the conductivity of the electrosprayed solution and ion-pair formation with the proteins, resulting in low sensitivity of electrospray ionization [22,28]. For this reason, mobile phases containing the relatively weak acids formic acid and acetic acid, which are popular alternatives [29,30], were also evaluated for the

separation of the protein components of the PS II major and the minor antenna system.

The chromatographic performance and the resolution of the protein components of the PS II major and minor antenna system were greatly affected by the mobile phase additives, as well as by gradient shape and flow-rate. With mobile phases containing either formic acid or acetic acid at different concentrations ranging from 0.2 to 5% (v/v), peak resolution was rather poor with any of the water–acetonitrile gradient elution profiles and flow-rates employed (data not shown).

Optimum peak resolution and chromatographic performance with the analytical size column (4.6 mm I.D.) were obtained using a 45 min linear gradient elution from 38.0 to 69.5% (v/v) acetonitrile in the aqueous mobile phase containing 0.1% (v/v) TFA. However, in order to avoid low ionization efficiency of the electrospray ion source and analyte signal reduction, in the subsequent experiments with ESI-MS detection the content of TFA in both the starting eluent and the gradient former was reduced to 0.05% (v/v) at the expense of a little decrease in chromatographic performance and resolution.

3.2. On-line RPLC–ESI-MS studies

The optimum chromatographic conditions resulting from the study described in the first part of this discussion were employed for the RPLC–ESI-MS analysis of the protein components of the photosystem II complex isolated by sucrose gradient ultracentrifugation. The material harvested from the second band of the sucrose gradient fractionation, containing both the minor and the major antenna proteins, was resolved into the eight main peaks displayed in the chromatograms reported in Fig. 1, where panel (a) displays the UV trace and panel (b) shows the separation monitored by ESI-MS. Figs. 2 and 3 show the ESI-MS spectra collected during the chromatographic run and, as inserts, the mass spectra in absolute mass units after deconvolution [19].

The deconvolution of the ESI-MS spectra revealed that the peak eluting from the column at 15.3 min (Fig. 1) was a non-protein compound whereas the following peaks were detected as proteins of molecular masses in the range 22 823 to 28 099. It is important to note that complete separation of all components of a protein mixture is not necessary in RPLC–ESI-MS analysis provided their molecular masses are different.

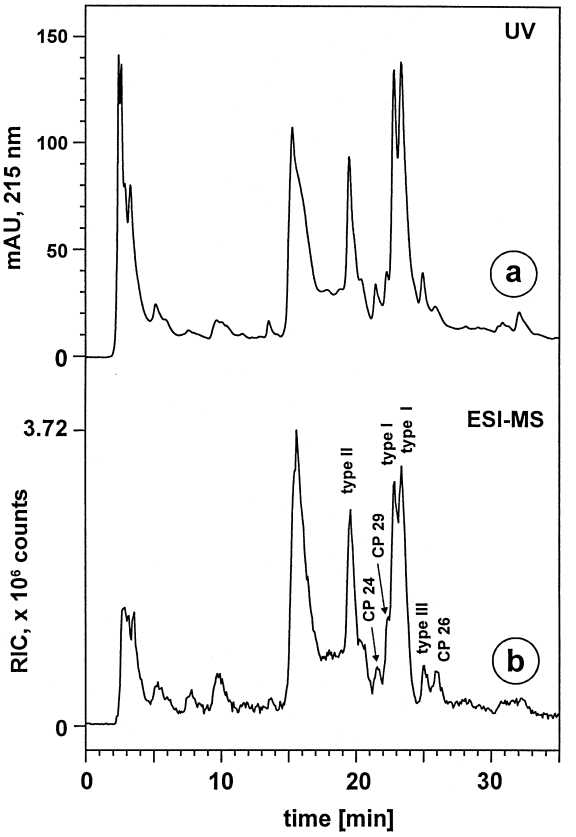

Fig. 1. RPLC–ESI-MS analysis of the protein components of the PS II major and minor antenna system in spinach leave extract. Column, Vydac Protein C_4 (250×4.6 mm I.D.) packed with 5 μm butyl silica; elution, 45 min linear gradient from 38.0 to 69.5% (v/v) acetonitrile in water containing 0.05% (v/v) TFA; flow-rate 1.0 ml/min; detection, UV at 215 nm (a), MS, scan range 500–2500 amu in 5 s (b).

Gene sequences encoding for the protein components of the PS II major and minor antenna system have been reported and used to deduce their complete primary sequence [12–18]. These studies have revealed that the amino acid sequences of the protein components of the PS II major and minor antenna system are highly conserved among different species, and they all seem to belong to a gene group which has several different gene family members [11]. Therefore, the molecular masses calculated for the same proteins in different higher plant species are

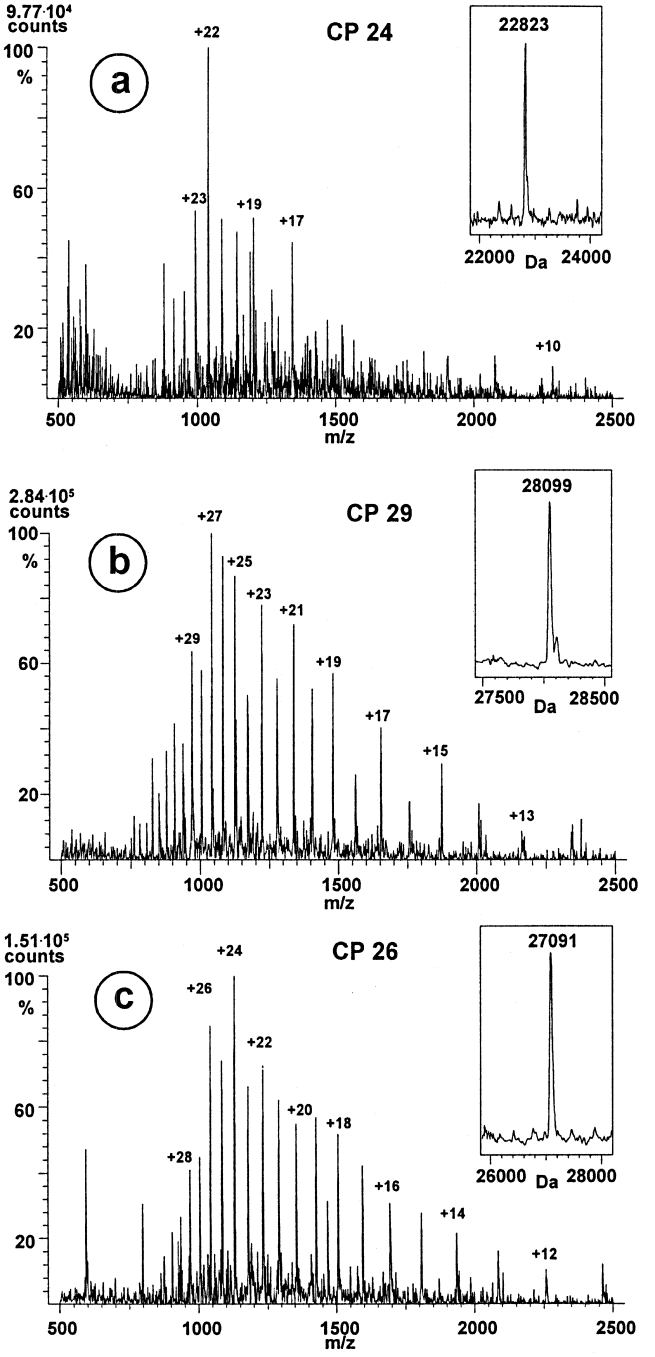

Fig. 2. Electrospray mass spectra of the protein components of the PS II major and minor antenna system eluting the RP-HPLC column at 20.3 (a), 22.3 (b) and 25.8 (c) min, respectively. Scan range 500–2500 u in 5 s; deconvoluted spectra are shown as inserts.

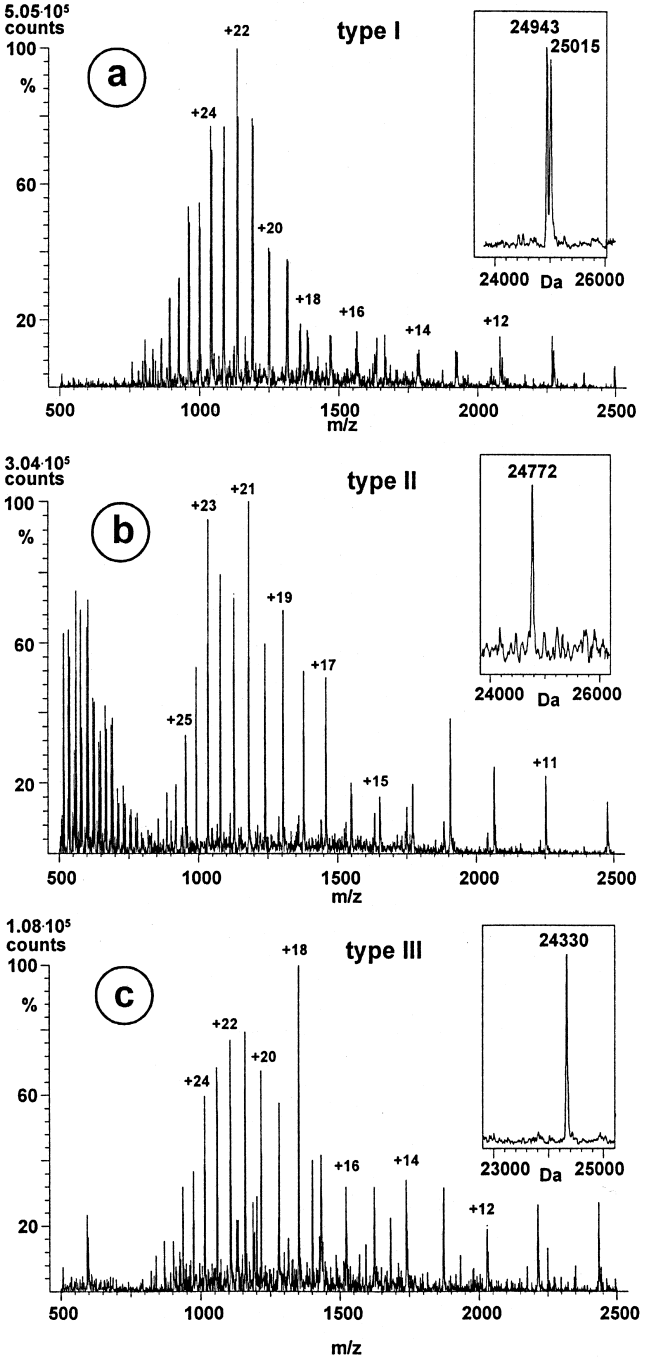

Fig. 3. Electrospray mass spectra of the protein components of the PS II major and minor antenna system eluting the RP-HPLC column at 22.8 (a), 23.1 (a), 24.9 (b), and 19.3 min (c), respectively. Scan range 500–2500 u in 5 s; deconvoluted spectra are shown as inserts.

very close to each other. The isolation of multiple copy numbers of the same gene from several higher plant species [31] accounts for the resolution of different variants of the same proteins reported in literature [32,33] as well as in this paper.

The molecular mass values of the protein components of the PS II major and minor antenna system computed from the protein sequence derived from the genes isolated from several higher plant species are listed in Table 1, which also reports the range of apparent molecular masses in polyacrylamide gel electrophoresis as well as the number of amino acids and the number of genes coding for each protein. The computed molecular mass values of the PS II mature proteins reported in Table 1 were obtained from literature and from SWISS-PROT [34], an annotated protein sequence database available from the World-Wide Web (WWW) molecular biology server ExPAsy [35]. Comparison of the molecular mass values calculated from the protein sequence derived from the isolated genes with those determined by RPLC–ESI-MS and the following considerations have permitted to deduce the peak assignment reported in Fig. 1 and Table 1.

3.3. Proteins comprising the PS II minor antenna system

The PS II minor antenna system comprises three distinct protein binding chlorophyll a/b, denoted CP24, CP26 and CP29 on the basis of their apparent molecular mass in non-denaturing SDS–PAGE [36,37]. The molecular mass of the spinach CP24 protein calculated from its nucleotide-derived protein sequence reported in SWISS-PROT [34] is 22 813. This value is in excellent agreement with the molecular mass of 22 823 determined by ESI-MS (Fig. 2a) for the protein eluting the reversed-phase column at

Table 1
Comparison between experimental and computed values of the molecular mass (M_r) of the protein constituents of the PS II major and minor antenna system

Protein	M_r by RPLC–ESI-MS	M_r calculated from nucleotide-derived sequence			Apparent M_r [11]	No. of amino acids [11]	No. of genes [11]
		Spinach	Other species[a,b]	Accession number[c]			
		22 813		P36494	20 000–22 000	210–211	2
CP24	22 823		22 897[T] [12]	P27525			
			23 022[M]	Q41748			
					26 000–29 000	246–251	1
CP26	27 091		26 624[M]	Q41746			
					29 000–31 000	257–258	–
CP29	28 099		28 558[M] [38]	O24561			
		25 035 [13]			27 000–28 000	230–233	3–16
1	24 943		25 041[P] [14]	P04782			
Type I			25 026[M] [15]	Q00827			
2	25 015		24 969[M] [16]	P27497			
			24 969[M] [17]	P06671			
			24 845[C] [42]	P27518	25 000–27 000	228	1–4
Type II	24 772						
			24 285[B] [18]	P27523	24 000–25 000	223	1–4
Type III	24 330		24 308[T] [43]	P27489			

[a] Plant species: P, petunia; M, maize; T, tomato; C, cotton; B, barley.
[b] Bibliographical references reported in the SWISS-PROT protein sequence database [34].
[c] SWISS-PROT accession number.

retention time 20.3 min (Fig. 1). The computed molecular mass values of the CP24 protein from spinach, tomato and maize reported in Table 1 are quite close to each other. This is in agreement with the high sequence homology displayed by CP24 and the other proteins comprising the PS II antenna system from different species [11].

As far as we know, the primary amino acid sequences of the spinach CP26 and CP29 proteins have not been reported. However, because the primary sequence of the protein constituents of the PS II minor and major antenna system is highly conserved among different species, the experimental molecular masses have been correlated to the molecular masses expected for the CP26 and CP29 proteins deduced from their DNA sequences from other higher plant species. The CP29 protein is the largest of the proteins comprising the PS II major and minor antenna system. The molecular mass calculated from the nucleotide-derived protein sequence deduced from the gene encoding this protein in maize [38] is 28 558. This value is in good correlation with the molecular mass of 28 099 (Fig. 2b) determined for the protein eluting the column at 22.3 min (Fig. 1), which is the greatest value of molecular mass determined in the sample by RPLC–ESI-MS.

The computed value of the expected molecular mass of CP26 from maize reported in SWISS-PROT [34] is 26 624. This value is consistent with the molecular mass of 27 091 (Fig. 2c) experimentally determined for the protein eluting the HPLC column at retention time of 25.8 min (Fig. 1).

3.4. Proteins comprising the PS II major antenna system

The LHC II complex or PS II major antenna system consists mainly of three closely related proteins designated type I, type II and type III, or Lhcb1, Lhcb2 and Lhcb3 on the basis of the nomenclature for the genes encoding these proteins [39]. These proteins consist of a similar number of amino acids, ranging from 223 in type III to 230–233 in type I, and all are related by extended sequence homologies. As a consequence, the molecular masses expected for these proteins on the basis of their nucleotide-derived protein sequences reported in literature are very close to each other, ranging from 24 285 for type III from barley [18] to 25 035 for type I from spinach [13] (see Table 1).

The type I protein is the major constituent of the whole PS II antenna system [40] and is encoded by multiple gene copies in most species, with copy numbers up to 16 in *Petunia* [14]. According to both UV and reconstructed ion current (RIC) traces (see Fig. 1), two partially resolved proteins eluting the column at 22.8 and 23.1 min appear to be the most abundant separated proteins in the sample, whose molecular masses of 24 943 and 25 015 determined by ESI-MS (Fig. 3a) are in good agreement with the computed molecular mass for type I protein from spinach (25 035) [13] and from other higher plant species (see Table 1). The resolution of two variants of type I proteins is in agreement with previous findings reporting the resolution of more than one type I protein by both high-resolution PAGE [32,41] and HPLC [33] and is consistent with the high copy numbers of *Lhcb1* genes isolated in higher plants [14,39].

The type II protein is slightly smaller than type I and is found in amounts equivalent to roughly one third of type I [31]. The experimental molecular mass of the protein eluting the column as the second most detectable protein peak is 24 772 (Fig. 3b), which is in good agreement with the molecular mass of 24 845 calculated for the type II protein from cotton on the basis of its nucleotide-derived protein sequence [42].

The type III protein is the smallest constituent of the LHC II complex and its abundance in the PS II antenna system is between one-tenth to one-twentieth of that of type I [11]. The molecular masses calculated from the protein sequence derived from barley and tomato genes are 24 285 [18] and 24 308 [43], respectively, which are in excellent agreement with the molecular mass of 24 330 (Fig. 3c) experimentally determined for the low UV protein absorbing peak eluting the column at 24.9 min (Fig. 1).

The elution order of the protein constituents of the PS II major antenna system determined from the above assignation is in excellent agreement with that observed with the same silica-based butyl column in a previous study where peak identification was accomplished by a combination of various SDS–PAGE systems and by amino acid microsequence analysis [33]. The SDS–PAGE systems employed

either Coomassie or silver staining or immunological detection using polyclonal antibodies raised against LHC II proteins and against CP24, CP26 and CP29 proteins. Equivalent protein resolution and molecular mass determinations were obtained subjecting to RPLC–ESI-MS analysis the sample corresponding to the BBY preparation [20], containing the PS II major and minor antenna systems together with free pigments and the reaction center complexes.

3.5. RPLC–ESI-MS analysis with the column of microbore format

We have attempted to improve the resolution of the protein components of the PSII major and minor antenna system by reducing the steepness of the acetonitrile gradient in 0.05% (v/v) aqueous TFA. This investigation was performed using a microbore column (250×1.0 mm I.D.) packed with the same 5 μm spherical Vydac C_4 stationary phase that was operated at the same mobile phase velocity employed with the analytical size column. The microbore format of the column was selected for these experiments in order to reduce solvent consumption, which results to be excessive when performing RPLC in analytical size columns at low rate of gradient development and conventional flow-rate.

The use of the microbore column in combination with a shallower acetonitrile gradient allowed the improved resolution of the CP29, CP26 and CP24 proteins at the expense of longer analysis time and of the resolution of the less abundant type III protein (see Fig. 4). The separation of the protein components of the major and minor antenna system, harvested with the second band of the sucrose gradient ultracentrifugation, was obtained with the microbore column using a 120 min linear gradient elution from 42.75 to 71.25% (v/v) acetonitrile in the aqueous mobile phase containing 0.05% (v/v) TFA. This separation yielded essentially the same mass spectral information as reported in Figs. 2 and 3. The RPLC–ESI-MS analysis of the proteins in the sample harvested from the third band of the sucrose gradient ultracentrifugation, containing essentially the protein components of the PS II major antenna system, evidenced the absence of the peaks for proteins of molecular masses of 28 099, 27 091 and 22 823 (data not shown), which further confirmed the assignment of these molecular masses to CP29, CP26 and CP24 proteins, respectively.

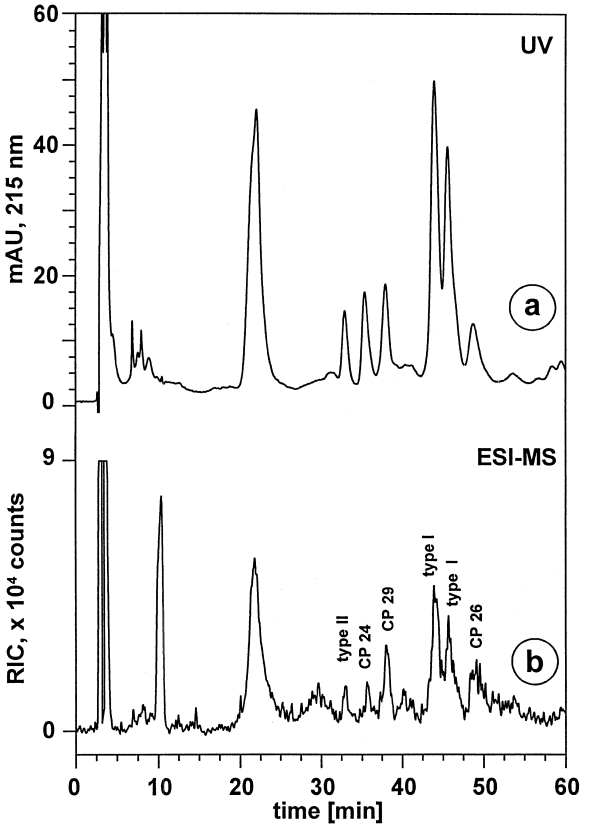

Fig. 4. Microbore RPLC–ESI-MS analysis of the protein components of the PS II major and minor antenna system in spinach leave extract. Column, Vydac Protein C_4 (250×1.0 mm I.D.) packed with 5 μm butyl silica; elution, 120 min linear gradient from 42.75 to 71.25% (v/v) acetonitrile in water containing 0.05% (v/v) TFA; flow-rate 0.05 ml/min; detection, UV at 215 nm (a), MS, scan range 500–2500 u in 5 s (b).

It is worth noting that the apparent molecular masses determined by SDS–PAGE (see Table 1) are neither in good agreement with the molecular masses calculated from the nucleotide-derived protein sequence nor with the experimental data reported here. This can be attributed to the fact that the apparent molecular mass determined by SDS–PAGE depends on the gel system and can be affected by several factors, including sample loading, composition of the electrolyte solution, presence of pigments or/and formation of multimeric complexes.

4. Conclusions

Our study has shown that RPLC–ESI-MS is an effective method for resolving and characterizing the integral membrane proteins comprising the PS II major and minor antenna system, both as isolated complexes by sucrose gradient ultracentrifugation and as the BBY grana membrane preparation directly. In accordance with molecular genetic data reported in literature, showing that higher plants have several *Lhcb1* genes encoding different type I proteins for each specie, two type I proteins of similar molecular mass have been resolved in spinach leaves. The experimental data are in good agreement with the molecular masses of the individual antenna proteins calculated on the basis of their nucleotide-derived amino acid sequences. In addition, the RPLC–ESI-MS method allows the separation of protein constituents of the major and minor antenna system, which are not resolved by conventional SDS–PAGE methods. Other advantages of RPLC–ESI-MS over SDS–PAGE include the accuracy in determining the molecular mass and the higher speed and efficiency.

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