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Separation and identification of the light harvesting proteins contained in grana and stroma thylakoid membrane fractions

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Abstract

This paper presents the results of a study performed to develop a rapid and straightforward method to resolve and simultaneously identify the light-harvesting proteins of photosystem I (LHCI) and photosystem II (LHCII) present in the grana and stroma of the thylakoid membranes of higher plants. These hydrophobic proteins are embedded in the phospholipid membrane, and their extraction usually requires detergent and time consuming manipulations that may introduce artifacts. The method presented here makes use of digitonin, a detergent which causes rapid (within less than 3 min) cleavage of the thylakoid membrane into two subfractions: appressed (grana) and non-appressed (stroma) membranes, the former enriched in photosystem II and the latter containing mainly photosystem I. From these two fractions identification of the protein components was performed by separating them by reversed-phase high-performance liquid chromatography (RP-HPLC) and determining the intact molecular mass by electrospray ionization mass spectrometry (ESI-MS). By this strategy the ion suppression during ESI-MS that normally occurs in the presence of membrane phospholipids was avoided, since RP-HPLC removed most phospholipids from the analytes. Consequently, high quality mass spectra were extracted from the reconstructed ion chromatograms. The specific cleavage of thylakoid membranes by digitonin, as well as the rapid identification and quantification of the antenna composition of the two complexes facilitate future studies of the lateral migration of the chlorophyll-protein complexes along thylakoid membranes, which is well known to be induced by high intensity light or other environmental stresses. Such investigations could not be performed by sodium dodecylsulfate–polyacrylamide gel electrophoresis because of insufficient resolution of the proteins having molecular masses between 22 000 and 25 000.

Keywords: Proteomics; Photosynthesis; Grana fraction; Stroma fraction; Proteins; Digitonin

1. Introduction

Higher plants harvest solar energy and operate a light-driven electron transport system through the activities of two main complexes, both residing in the thylakoid membrane: photosystem I and photosystem II (PSI and PSII, respectively). Each photosystem is composed of several chlorophyll-protein complexes, most of which function as antennae that capture visible light [1]. These are called light harvesting chlorophyll *a/b* pigment-proteins (LHCI and LHCII, respectively, or Lhca and Lhcb). The major light-harvesting proteins of PSII have been designated Lhcb1, Lhcb2, and Lhcb3 [1,2] on the basis of the nomenclature for the genes encoding these proteins [3], while other less abundant proteins have been called minor antenna proteins and designated Lhcb4, Lhcb5 and Lhcb6. The antenna proteins of PSI are composed of four polypeptides, which are the gene products of *Lhca1, Lhca2, Lhca3*, and *Lhca4* [4]. Both antenna proteins of PSI and PSII are hydrophobic and embedded in the thylakoid membrane. Their amino acid sequence is strongly conserved and consequently their molecular masses are very similar, commonly between 22 000 and 29 000.

Within each chloroplast, the thylakoids form a continuous network which contains two distinct types of membrane domains, the stacked grana thylakoids and the unstacked stroma thylakoids (see Fig. 1). The lateral heterogeneity of grana and stroma lamellae in chloroplasts is generally

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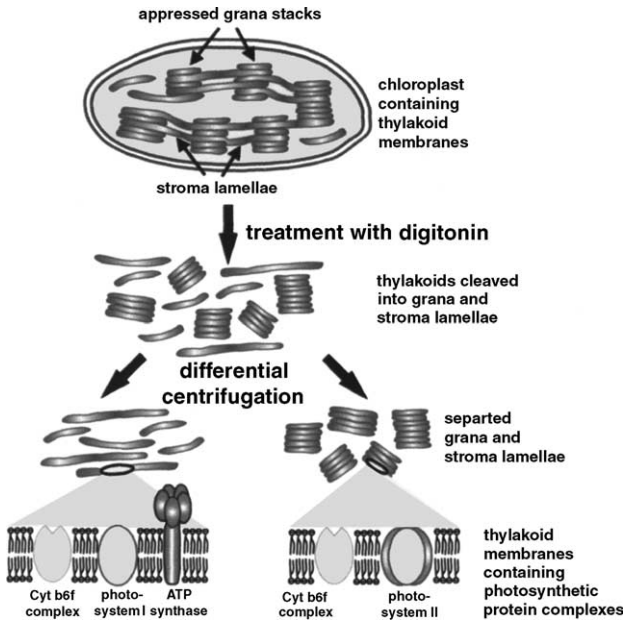

Fig. 1. Schematic illustration of fractionation of stroma and grana lamellae from thylakoid membranes using digitonin treatment and differential ultracentrifugation.

accepted, which means that PSI is predominantly localized in stroma lamellae and peripheral membranes of the grana, while PSII is localized mainly in grana appressions [5]. However, despite the lateral heterogeneity of the photosynthetic membrane, the two photosystems need to operate in series and they often exchange components. It is well documented that under light stress or other environmental stresses, the light harvesting proteins of PSII migrate to PSI. This migration between appressed (grana) and non-appressed (stroma) membranes has had a profound impact on current models for both the distribution of excitation energy and photosynthetic electron transport between the two photosystems [6–9]. Thus, it is of general interest to identify and quantify the proteins involved in the migration, particularly if handling of the sample and processing time can be significantly reduced.

The pioneering work by Michel and Michel–Wolwertz [10] and Sane et al. [11] using a mechanical press treatment or by Jacobi and Lehmann using sonication [12], demonstrated that PSI and PSII could be partially separated. Both methods are time consuming, while the same results can be obtained by solubilization of thylakoid membrane with digitonin [13,14]. Once PSI has been separated from PSII, the method commonly used for identification their relative components has been sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In this paper we present an alternative method using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled on-line with electrospray ionization mass spectrometry (ESI-MS) that has been adapted for separation and identification of antenna proteins from isolated and purified PSI and PSII [15–17]. In this study, we utilized this analytical strategy for characterizing the components present in the two photosystems obtained upon cleavage of the thylakoid membranes with digitonin and subsequent fractionation by ultracentrifugation.

2. Experimental

2.1. Chemicals

Reagent-grade sodium chloride, magnesium chloride, sorbitol, *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), tris-hydroxymethylaminomethane (Tris), sodium fluoride, trifluoroacetic acid (TFA), methanol, ethanol, formamide, as well as HPLC-grade water and acetonitrile, were obtained from Carlo Erba (Milan, Italy). Digitonin was obtained from Sigma (Milan, Italy).

2.2. Separation of grana/stroma lamellae

Spinach (*Spinacia oleracia* L.) was grown at 20 °C with a light period of 12 h and incident light intensity of 300 μE m^{-2} s^{-1}. The leaves were dark-adapted for 24 h before harvesting. Thylakoid membranes were prepared from spinach leaves according to Kyle et al. [18] and resuspended to a concentration of 200 μg Chl/ml in 15 mM Tricine (pH 7.8), 0.1 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM NaF (buffer A). Recrystallized digitonin (1% in water) was added to the stirred membranes to give a final concentration of 0.4%. The 2 min detergent treatment was terminated by a 10-fold dilution of the sample with resuspension buffer A at 0 °C. Differential centrifugation according to Anderson and Boardman [19] yielded pellets following 1000 g for 10 min, 10 000 g for 30 min, 40 000 g for 30 min and 144 000 g for 60 min. In some cases the thylakoid membranes were sheared using a French presser cell (27.6×10^6 Pa) according to the method of Arntzen et al. [20] before differential centrifugation [20]. The pellets were resuspended in buffer A and injected directly onto the column without any further sample pretreatment.

2.3. High-performance liquid chromatography and electrospray mass spectrometry

Chromatographic separations were performed using a System Gold HPLC system, consisting of two solvent delivery pumps (Model 126, Beckman, Fullerton, CA, USA), a UV detector (Model 168, Beckman), and a fluorescence detector (Model LC 240, Perkin-Elmer). The UV absorbance was monitored at 214 nm, while fluorescence emission was monitored at 330 nm after excitation at 280 nm. Samples were introduced onto the column by a sample injection valve (Model 210A, Beckman) with either a 20 μl or a 50 μl sample loop. The proteins were separated in a reversed-phase microbore column packed with 5-μm porous butyl silica particles (Vydac Protein C_4, 250 mM × 1 mM i.d., Group, Hesperia, CA, USA). The column was operated

at a flow rate of 0.05 ml/min and room temperature. All solutions were filtered through a membrane filter (type FH 0.5-μm, Millipore, Milan, Italy) and degassed by sparging with helium before use. The Vydac C_4 columns were pre-equilibrated with 10% (v/v) aqueous acetonitrile solution containing 0.05% TFA and samples were eluted using a gradient consisting of a first linear gradient from 10 to 40% (v/v) acetonitrile in 15 min, followed by a second gradient segment from 40 to 90% (v/v) acetonitrile in 60 min. At the end of the run, the column was flushed with 100% acetonitrile for 3 min. This post run gradient was used in order to ensure that hydrophobic contaminants of LHCII were eluted from the column by the second gradient segment up to 100% acetonitrile.

The system used for HPLC–ESI-MS experiments consisted of a low-pressure gradient micropump (model Rheos 2000, Flux Instruments, Basel, Switzerland) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), and an injector (model 7125, Rheodyne, Cotati, CA, USA) with a 20 μl sample loop. ESI-MS was performed on ion trap mass spectrometer (Esquire 3000 plus, Bruker Daltonik, Bremen, Germany). The 0.05 ml/min flow through the microbore column was conducted directly into the mass spectrometer. For analysis with pneumatically assisted ESI, an electrospray voltage of 3–4 kV and a nitrogen sheath gas flow were employed. The temperature of dry gas was set to 300 °C. The scan range was 500–2000, fine tuning for ESI-MS of proteins was accomplished by infusion of 3.0 μl/min of a 0.4 pmol/μl solution of cytochrome *c* or a 6.9 pmol/μl solution of carbonic anhydrase in 0.050% aqueous TFA-solution containing 20% (v/v) acetonitrile.

3. Results and discussion

The relative distributions of the chlorophyll–protein complexes in spinach thylakoid membrane fractions, derived from appressed and non appressed regions, were determined following their resolution and identification by RP-HPLC coupled on line with ESI-MS. The separation of thylakoid membranes into two grana and stroma lamellae fractions was optimized upon incubation with digitonin at different times and ratios of chlorophyll to digitonin. In order to minimize any seasonal effects, leaves were collected at different periods of the year. Moreover, they were harvested at night in the dark before extraction with the aim to eliminate any light effects. Fig. 1 reports a schematic illustration of fractionation of stroma and grana lamellae upon digitonin cleavage. The digitonin was added to thylakoid membranes at 0 °C in order to slow down its effect. After incubation for different time periods, the digitonin effect was stopped by diluting the sample by a factor of 70 with buffer and separating the membrane fractions by ultracentrifugation (Fig. 1). In this way the heavier grana membranes were easily and quickly separated from the lighter stroma membranes, which are recovered from the supernatant. The antenna proteins in both fractions were solubilized using buffer A and directly injected onto the HPLC column.

3.1. On-line RP-HPLC–ESI-MS studies

The components of intact thylakoid membranes and those of the two membrane fractions were chromatographed on a reversed-phase C_4 microbore column using a linear water–acetonitrile elution gradient in trifluoroacetic acid. Fig. 2A reports the chromatogram obtained upon injection of proteins from intact spinach thylakoid membranes. The chromatogram shows that most of the peaks, probably corresponding to the more abundant proteins, such as light harvesting proteins, were completely resolved within retention times ranging from 25 to 40 min, corresponding to a relatively narrow range of acetonitrile concentration of only 11.2% (v/v). This is in accordance with the expectation that all antenna proteins of PSI and PSII are similarly

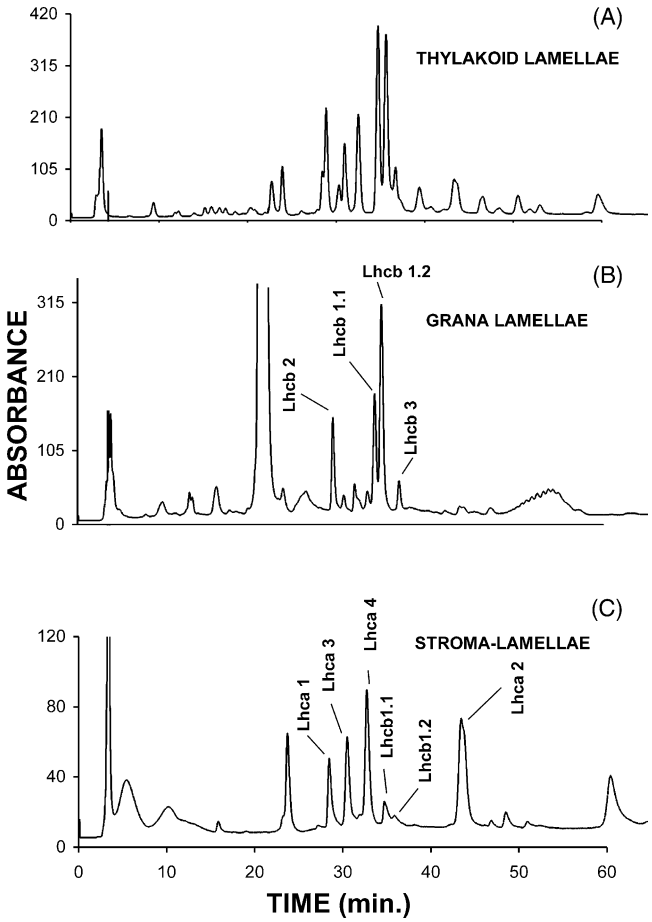

Fig. 2. Chromatographic fingerprints of the antenna proteins contained in intact spinach thylakoid membrane (A) in subfraction grana (B) and in stroma lamellae fraction (C). Column, Vydac Protein C_4 (250 mm × 1 mm i.d.) packed with 5 μm butyl silica; mobile phase, 15 min linear gradient from 10 to 40% (v/v) acetonitrile in water containing 0.05% (v/v) TFA followed by a second gradient from 40 to 90% (v/v) in 60 min; flow rate 0.05 ml/min; detection, UV, 214 nm; injection volume, 50 μl, sample size, approximately 20 μg total protein.

hydrophobic due to their highly similar amino acid sequence [4,21] with three membrane-spanning helices [22].

3.2. Proteins comprising the grana thylakoid membrane fractions

Fig. 2B and C report the chromatograms obtained upon injection of grana and stroma membrane fractions, respectively. The runs shown refer to a membrane fragmentation obtained upon incubation of spinach thylakoids with digitonin at a digitonin-to-chlorophyll ratio of 1:5 for 2–4 min at $T = 0\,^{\circ}C$. Interestingly, the sum of both chromatograms gives approximately the total protein pattern obtained upon injection of intact thylakoid membranes (Fig. 2A), indicating that the entire membrane was cut into two main fractions under the experimental conditions used.

In the grana fraction (Fig. 2B), three main peaks are well resolved and two minor peaks with retention times ranging from 28 to 37 min are observed. These peaks show different elution times (first column of Table 1) and different peak areas. The identification of the eluting proteins was performed by ESI-MS. The reconstructed ion current chromatogram (RICC) is shown in Fig. 3. It was recorded simultaneously with the UV trace upon injection of the grana membrane fraction. Most of the UV peaks showed a corresponding peak in the reconstructed ion chromatograms. Thus, the identification of proteins contained in each RICC peak gave the identity of protein(s) present in each UV trace. The deconvolution analysis (showed in the insets of Fig. 3) indicated the presence of several proteins with molecular masses in the range of 24 000–25 000, summarized in Table 1.

Chromatographic retention times may serve as a first indication of the identity of a protein, although coincidental elution of other compounds at the same position in the chromatogram may result in false positive identification. Thus, an unequivocal protein identification was performed by comparison of the measured molecular masses with the mass range predicted from the DNA sequences obtained from the literature and from the SWISS-PROT database at http//:www.ExPAsy.ch. As reported in a recent papers, species where the genome is known show an excellent correspondence between measured and expected molecular masses for most of the proteins, with mass deviations typically less than 0.02% [23], which is characteristic for intact molecular mass measurements using quadrupole ion trap mass spectrometers. In the particular case of spinach, however, most of the antenna genes are not known, with the exception of Lhcb1.2 and Lhcb3 [24]. Nevertheless it has been demonstrated that each antenna protein type showed molecular masses that fell within a relatively narrow range of mass values, and hence, it is reasonable to suggest an assignment of an experimental molecular mass to one of the six types of antenna proteins, if it fits into these narrow ranges. Moreover, values reported were calculated assuming that a conserved Arg, not a Met, was the N-terminal residue of the mature polypeptide, as indicated by tandem mass spectroscopy [25]. The calculation also took into account the fact that an acetyl group blocks this Arg both in Lhcb1 and Lhcb2 [24]. Thus, through this comparison with sequence data for tomato [26], each protein was identified and consequently each peak reported in the chromatogram of Fig. 2B was labeled with the corresponding antenna protein.

It is worth remarking that both grana and stroma fractions contain the proteins together with a considerable amount of phospholipids. It is well known that membrane proteins are not easy to analyze by ESI-MS [27], because detergents such as Triton or dodecylmaltoside [17] have to be added to the sample solutions in order to keep the proteins in solution. In our case, the use of detergent, apart the small amount of digitonin, was avoided, however at the cost of the presence of phospholipids. Phospholipids, however, are known to inhibit ionization during the electrospray process as well [28], and thus, they have to be removed for successful ESI-MS analysis. The data reported here reveals that the on-line hyphenation of RP-HPLC to ESI-MS circumvented the problem of ion suppression. Consequently, RP-HPLC–ESI-MS not only efficiently removed the phospholipids from the samples but also fractionated

Table 1
Identification of protein components of grana lamellae (PSII), and stroma lamellae (PSI), by RP-HPLC–ESI-MS

Thylakoid membrane fraction	HPLC elution times	M_r measured ± (S.D.)[a]	M_r expected	Accession no.	Protein identification
Grana lamellae	27.89	24 761 ± 0.9	24 788 [37]	P14279	Lhcb2
	32.41	24 936 ± 1.4	24 879 [37]	P07369	Lhcb1.1
	33.39	25 014 ± 0.5	25 015 [38]	P12333	Lhcb1.2
	35.06	24 321 ± 1.3	24 308 [37]	P27489	Lhcb3
Stroma lamellae	28.79	24 321 ± 1.3	24 308 [37]	P27489	Lhcb3
	30.38	25 298 ± 1.0	25 340 [26]	P27522	Lhca3
	32.15	22 353 ± 0.5	22 336 [26]	S14305	Lhca4
	34.19	24 936 ± 0.5	24 943 [26]	P07369	Lhcb1.1
	35.08	25 014 ± 0.2	25 015 [38]	P12333	Lhcb1.2
	43.08	23 241 ± 1.6	23 079 [26]	P10708	Lhca2

[a] The mean ± S.D. of three experiments is presented.

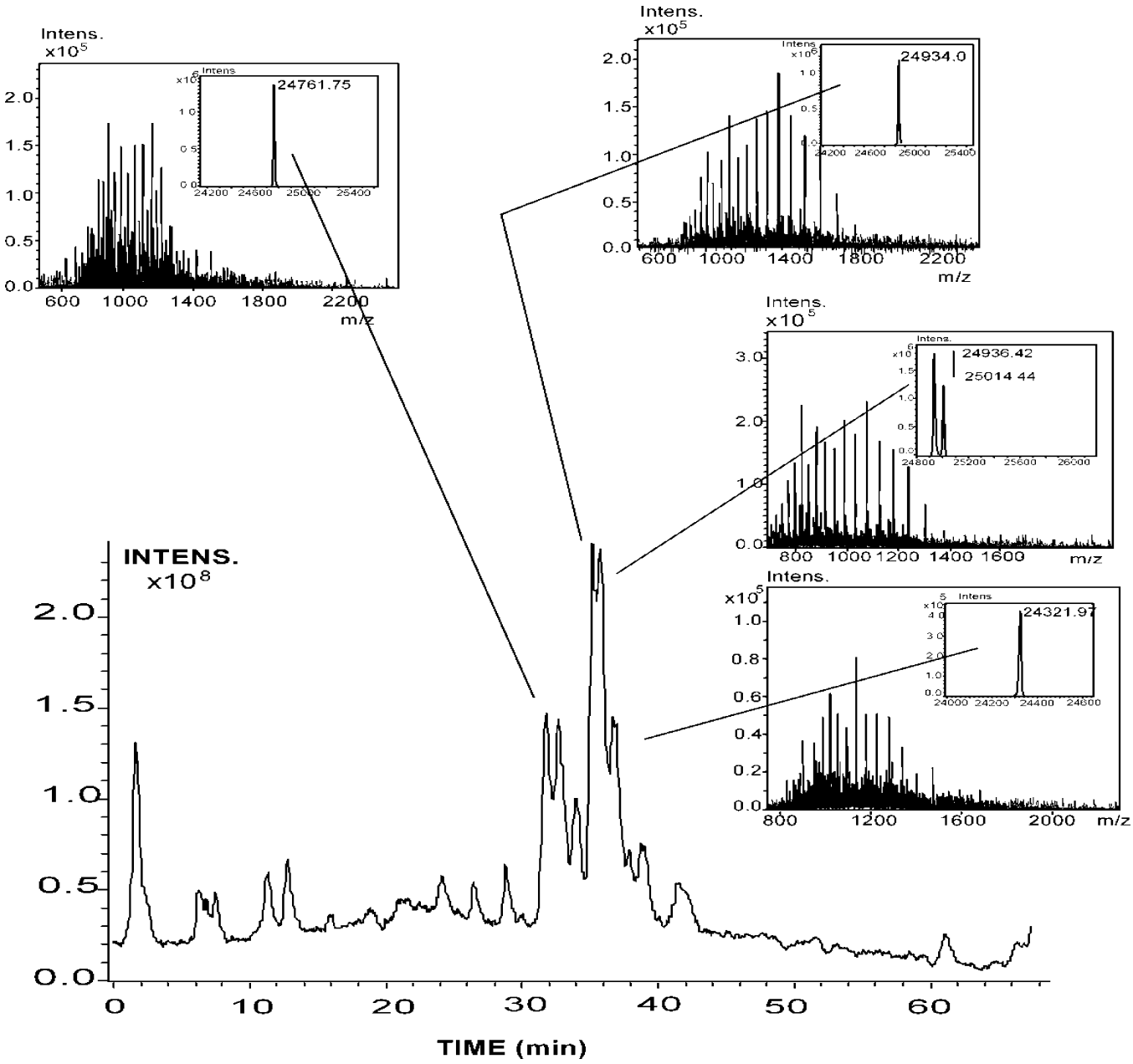

Fig. 3. Identification of the protein components of the antenna proteins in grana fraction by RP-HPLC–ESI-MS. Column, Vydac Protein C_4 (250 mm × 1 mm i.d.) packed with 5 μm butyl silica; mobile phase, 15 min linear gradient from 10 to 40% (v/v) acetonitrile in water containing 0.05% (v/v) TFA followed by a second gradient from 40 to 90% (v/v) in 60 min; flow rate 0.05 ml/min, flow of column effluent entering the mass spectrometer, 50 μl/min; detection, ESI-MS, 500–200; injection volume, 100 μl.

the different protein components prior to their ESI-MS investigation. In fact, mass spectra of high quality, showing no adduction with phospholipids, were extracted from the reconstructed ion current chromatograms. The calculated masses were in excellent agreement to those previously reported [29,30] and demonstrate that RP-HPLC–ESI-MS is highly suited to the separation and characterization of proteins in complex and difficult matrices.

From a physiological point of view, the grana thylakoid fraction contains prevalently the antenna proteins of PSII. Interestingly, however, the absence of components from PSI is observed only upon incubation of thylakoid membranes with digitonin at a chlorophyll-to-digitonin ratio of 1:5 and incubation time of 2–4 min. Shorter incubation times or lower chlorophyll-to-digitonin ratio gave rise to more peaks in the HPLC chromatograms. These were more similar to those recorded upon injection of intact membrane (see Fig. 1A), indicating that no significant cutting of the membrane had occurred. In contrast, longer incubation times or higher digitonin concentrations gave rise to a

more extensive solubilization of the membrane and consequently similar HPLC chromatograms for grana and stroma fractions, due to the complete solubilization of the entire membrane. On this note the absence of PSI contamination in grana fractions has been used as parameter for a good separation of thylakoid membrane into grana and stroma fractions.

In order to examine the repeatability of the chromatographic profiles and resolution of the grana proteins, the standard deviations (S.D.) and the relative standard deviations (R.S.D.) of the retention times of the resolved proteins were calculated from the chromatograms obtained by 10 repeated injections of the grana lamellae. This experiment was repeated on other samples extracted from spinach leaves by a second and a third preparation. The results are reported in Table 2 and show that for all preparations the R.S.D. were better than 0.88% for elution time for all proteins. Furthermore, in order to gain some insights from the comparison of different chromatographic profiles displayed from grana, we calculated the peak area ratios as the quotients of the peak area of Lhcb2 and Lhcb3 divided by the peak area of the Lhcb1.1 protein, obtained by multiple injections ($n = 6$) of the grana preparation. The results are reported in the right part of Table 2 and show that the R.S.D. of the calculated peak area ratios are better than 7.8%. Thus, the method used, besides being rapid, simple and reproducible, has proven to be effective for separating and identifying each component of the grana fraction, despite the presence of a significant amount of phospholipids.

3.3. Proteins comprising the stroma thylakoid membrane fractions

The identification of stroma proteins was performed in an analogous fashion to that reported for grana proteins. Fig. 4 shows the RICC that was recorded simultaneously with the UV trace upon injection of the stroma fraction. The deconvolution analysis (showed in the insets of Fig. 4) indicated the presence of several proteins with molecular masses different from those determined for PSII and fitting the range expected for the antenna proteins of PSI. Table 1 summarizes the measured molecular masses with the mass range predicted from the PSI DNA sequences obtained from the literature and from the SWISS-PROT database at http//:www.ExPAsy.ch. Through this comparison, most proteins were identified as antenna proteins of PSI (Lhca1-Lhca4). However, some proteins previously found in the grana were also found in stroma lamellae, although in small amounts. Thus, once each protein was identified, all peaks in the Fig. 1C chromatogram were labeled for the corresponding antenna protein.

The repeatability of the chromatographic profiles was also examined for the stroma fraction. For this purpose the S.D. and the R.S.D. of the retention times of the resolved proteins were calculated from the chromatograms obtained by 10 repeated injections and a second and a third set of repeat sample preparations. The results are reported in the left part of Table 3. The R.S.D. were better than 3.2% for all the preparations. Also the peak area ratios were calculated as for grana and the results are reported in Table 3 which show

Table 2
Repeatability of retention times and peak areas of protein components of grana (PSII) isolated from spinach leaves by three different preparations

Antenna protein	No. of preparations	Repeatability of retention times				Repeatability of area (V s)			
		Mean retention time (min), $n = 10$	R.S.D. (%)	Mean of three preparations	Mean R.S.D. (%)	Peak area ratio	R.S.D. (%)	Mean of three preparations	Mean R.S.D. (%)
						Lhcb2/1.1	*Lhcb2/1.1*	*Lhcb2/1.1*	*Lhcb2/1.1*
Lhcb2	1	27.90	0.87			0.4421	0.24		
	2	27.88	0.91	27.89	0.88	0.4409	1.06	0.441	0.90
	3	27.90	0.87			0.4418	1.40		
Lhcb1.1	1	32.41	0.10						
	2	32.42	0.24	32.41	0.14	1		1	
	3	32.41	0.10						
						Lhcb1.2/1.1	*Lhcb1.2/1.1*	*Lhcb1.2/1.1*	*Lhcb1.2/1.1*
Lhcb1.2	1	33.34	0.10						
	2	33.34	0.10	33.39	0.13	0.5768	7.70	0.573	
	3	33.35	0.19			0.5721	7.82		7.77
						0.5720	7.81		
						Lhcb3/1.1	*Lhcb3/1.1*	*Lhcb3/1.1*	*Lhcb3/1.1*
Lhcb3	1	35.07	0.87			0.086	1.62		
	2	35.07	0.87	35.06	0.82	0.087	1.90	0.087	1.98
	3	35.05	0.73			0.088	2.44		

Repeatability of the peak area was calculated as the quotients of the peak area of Lhcb2, Lhcb3 and Lhcb1.2 divided by the peak area of Lhcb1.1 protein. The data were obtained using UV detection.

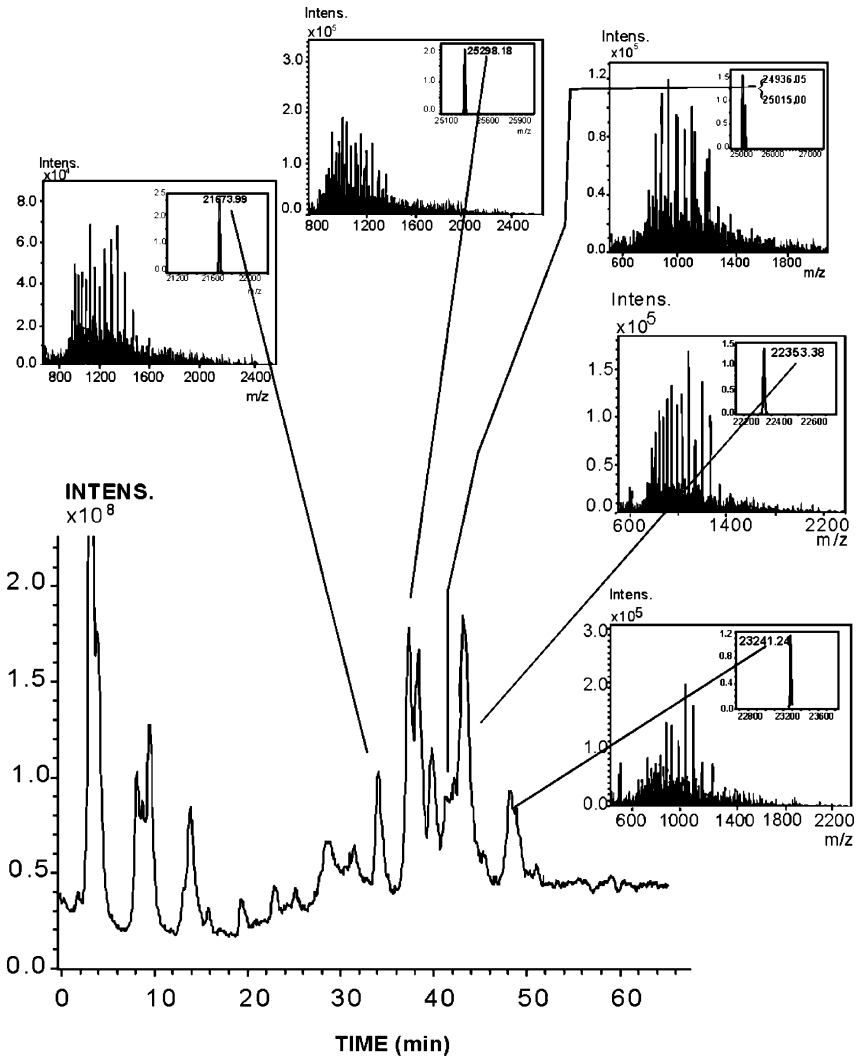

Fig. 4. Identification of the protein components of the antenna proteins in stroma lamellae fraction by RP-HPLC–ESI-MS. Column, Vydac Protein C_4 (250 mm × 1 mm i.d.) packed with 5 μm butyl silica; mobile phase, 45 min linear gradient from 27.5 to 63.5% (v/v) acetonitrile in water containing 0.05% (v/v) TFA followed by a second gradient from 40 to 90% (v/v) in 60 min; flow rate 0.05 ml/min, flow of column effluent entering the mass spectrometer, 50 μl/min; detection, ESI-MS, 500–200; injection volume, 100 μl.

that the R.S.D. of the calculated peak area ratios are better than 6.4%.

From the data reported it may be ascertained that both the chromatographic separation and the retention times of the proteins resolved from grana and stroma, either from the same or from different preparations are highly reproducible, prompting us to conclude that RP-HPLC separation is useful in detecting proteins still embedded in the membrane. In particular, digitonin seems to cleave the thylakoid membrane at preferential sites during short incubations, giving reproducible subfractions and providing a reliable method to elicit rapidly and simultaneously the relative distributions of the chlorophyll–protein complexes in the plant thylakoid membrane. In fact, the relative areas underlying the HPLC peaks represent the relative protein concentration with excellent accuracy, since the antenna proteins of both PSI and PSII are strongly conserved and it may be assumed that all have similar optical extinction coefficients. Thus, it may be inferred that the physiological distribution of antenna proteins in leaves, once maintained overnight at dark, is the following: grana contains essentially the PSII photosystem, while the stroma lamellae contain mainly the PSI complex and a approximately 10% of Lhcb1 and Lhcb2. The last evidence is in agreement with a recent report from Zhang and Scheller [14], although they were not able to determine the relative amounts by SDS-PAGE.

Consequently, another great advantage of chromatographic analysis is the possibility to rapidly and quantitatively determine the protein migration between grana and stroma, which is known to be related to different environmental conditions to which the plants might be exposed during growing, such as light intensity [31], temperature [32] and senescence [33]. This is possible with this method, since extraction of thylakoid membranes from leaves,

Table 3
Repeatability of retention times and peak areas of protein components of stroma (PSI) isolated from spinach leaves by three different preparations

Antenna protein	No. of preparations	Repeatability of retention times				Repeatability of area (V s)			
		Mean retention time (min), $n = 10$	R.S.D. (%)	Mean of three preparations	Mean R.S.D. (%)	Peak area ratio	R.S.D. (%)	Mean of three preparations	Mean R.S.D. (%)
						Lhca1/4	*Lhca1/4*	*Lhca1/4*	*Lhca1/4*
Lhca1	1	28.77	0.69			0.2302	7.15		
	2	28.80	0.49	28.79	0.55	0.2291	6.46	0.2299	6.39
	3	28.80	0.49			0.2304	5.56		
						Lhca3/4	*Lhca3/4*	*Lhca3/4*	*Lhca3/4*
Lhca3	1	30.48	1.32			0.1502	5.39		
	2	30.36	1.27	30.38	1.34	0.1504	5.31	0.1503	5.28
	3	30.32	1.44			0.1505	5.15		
						Lhca4/4	*Lhca4/4*	*Lhca4/4*	*Lhca4/4*
Lhca4	1	32.31	1.44			1		1	
	2	32.03	2.10	32.15	1.89				
	3	32.12	2.14						
						Lhcb1.1/4	*Lhcb1.1/4*	*Lhcb1.1/4*	*Lhcb1.1/4*
Lhcb1.1	1	34.23	3.20			0.0790	5.48		
	2	34.23	3.20	34.19	3.22	0.0795	6.43	0.0794	6.07
	3	34.11	3.27			0.0797	6.31		
						Lhcb1.2/4	*Lhcb1.2/4*	*Lhcb1.2/4*	*Lhcb1.2/4*
Lhcb1.2	1	35.06	2.39			0.0398	6.31		
	2	35.12	2.58	35.08	2.44	0.0399	6.42	0.0398	6.34
	3	35.06	2.39			0.0398	6.31		
						Lhca2/4	*Lhca2/4*	*Lhca2/4*	*Lhca2/4*
Lhca2	1	43.08	0.73			0.5773	5.41		
	2	43.13	1.03	43.08	0.83	0.5777	5.36	0.5776	5.36
	3	43.03	0.75			0.5780	5.32		

Repeatability of the peak area was calculated as the quotients of the peak area of Lhca1, Lhca3, Lhcb1.1, Lhcb1.2 and Lhca2 divided by the peak area of Lhca4 protein. The data were obtained using UV detection.

fragmentation and separation as well as the separation and identification of protein components requires very little time. Thus, the reduced manipulation of the sample helps to eliminate artifacts and makes the interpretation of results more reliable. In this respect, the different elution times of PSII components with respect to that of PSI (see Table 1) allow us to reveal and quantify which proteins of PSII have migrated into the stroma. Clearly, such an investigation could be performed with the usage of RP-HPLC with UV detection alone, since now the proteins contained in each UV peak have been identified by on-line hyphenation with ESI-MS.

Since it has been demonstrated by radiolabeling and immunoblotting methods that phosphorylation of some PSII proteins is the driving force to dissociate the antenna protein from PSII and diffuse them laterally to the stroma lamellae [34,35], using HPLC–ESI-MS it is possible to measure the molecular masses of the migrated antenna proteins and recognise which protein(s) is (are) really phosphorylated. Finally, tandem MS of trypsin-digested antenna proteins will provide partial sequence information which is suitable not only to allow identification of the amino acid involved in the phosphorylation process, but to gather insights into the molecular mechanism by which the chloroplast modulates the adaptation of the photosynthetic apparatus to environmental changes. This is particularly interesting in plants where the entire genome is known, such as *Arabidopsis thaliana*. The method may be further adapted by miniaturization of the analytical steps, a concept which can be successfully realized by the use of monolithic capillary columns [36].

4. Conclusion

Our study has shown that digitonin causes specific and rapid (less than 3 min) cleavage of the thylakoid membrane into two subfractions: appressed and non-appressed membranes, avoiding time consuming sucrose-gradient ultracentrifugations for the separation of the protein components of PSI and PSII. Moreover, direct analysis of these two subfractions by RP-HPLC–ESI-MS allows a rapid separation and unequivocal identification of proteins contained in

each photosystems. All phospholipids are separated from the analytes and high quality mass spectra were extracted from the reconstructed ion current chromatograms. The good agreement with the molecular masses of the individual antenna proteins calculated on the basis of their nucleotide derived amino acid sequences facilitates better study of the lateral distribution of the chlorophyll–protein complexes along thylakoid membranes, which is well known to be affected by high intensity light or other environmental stresses.

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