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Rapid resolution by reversed-phase high-performance liquid chromatography of the thylakoid membrane proteins of the photosystem II light-harvesting complex

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Abstract

This paper presents the results of a study performed to develop a rapid and straightforward HPLC method to resolve and identify the protein components of the photosystem II light-harvesting complex (LHC II), which is the major protein–chlorophyll *a/b* complex in higher plants. The method employs a reversed-phase Vydac Protein C_4 column containing 5-μm porous butyl silica which is eluted by a 13.5-min linear water–acetonitrile gradient in the presence of 0.1% (v/v) trifluoroacetic acid. Peak identification was performed by denaturing SDS-PAGE analysis of the fractions of the eluate collected throughout the chromatographic run. This procedure allowed the identification of Lhcb2, Lhcb1 and Lhcb3 protein components of LHC II, which eluted as well-resolved peaks with highly repeatable retention times, in samples isolated from spinach leaves both by the same and by different preparations. The reversed-phase HPLC method has proven to be suitable for detecting dissimilarities in the protein components of LHC II isolated from different plants. © 1997 Elsevier Science B.V.

Keywords: Thylakoid membrane; Light-harvesting complex; Proteins; Photosystem II; Membrane proteins

1. Introduction

The photosystem II (PS II) complex is the portion of the photosynthetic apparatus that is responsible for splitting water to form molecular oxygen, electrons and protons. It is embedded in the thylakoid membrane and contains a reaction center (core) surrounded by a specific light-harvesting system (LHC II), which is the major protein–chlorophyll *a/b* complex in higher plants [1–3]. The LHC II proteins are of great interest since they are expected to play different roles in the PS II antenna system. In fact, they are involved in the distribution of light energy between photosystem I and photosystem II [3], and are capable of adapting to different environmental conditions, including low and high light intensity [4] and heat stress [5].

The proteins associated to chlorophyll in the LHC II have been traditionally resolved by SDS-PAGE into several closely related hydrophobic membrane proteins, typically in the range 23–28 kDa [6–8]. The uncertainness of the number of proteins in the

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complex is mainly due to the different procedures and detergents used to solubilize LHC II from the thylakoid membranes and to the different methods employed in isolating and characterizing the individual membrane proteins in the complex [3]. LHC II isolated in spinach leaves contains two major closely related hydrophobic proteins in the range 28–27 kDa, and a minor intrinsic membrane protein of about 25 kDa [3,9].

We are interested in the development of high-performance separation techniques for the resolution of thylakoid membrane proteins, which may contribute to improve the information on the composition and supramolecular organization of the LHC II, on the differences among species, and on the molecular mechanisms underlying the physiological adaptation of higher plants to environmental conditions.

Traditional approaches by SDS-PAGE are not only cumbersome but also rather ineffective for evaluating differences in the relative quantity of each LHC II component. Recently, we have demonstrated that capillary zone electrophoresis can successfully be applied for the complete resolution of the LHC II thylakoid membrane proteins by using the neutral detergent *n*-octyl-β-D-glucopyranoside at a concentration lower than its critical micelle concentration in the electrolyte solution [10].

In this paper we report the results of a study performed to obtain the rapid resolution of the protein components of LHC II by reversed-phase HPLC, which is more suitable than CZE for the micropreparative scale separation that may be of interest for protein sequencing or peptide mapping of the isolated LHC II proteins. So far, the only attempt to separate the protein components of LHC II by reversed-phase chromatography (RPC) has been recently reported by Damm and Green who have partially separated these proteins using a polymeric-based RPC column eluted by a 90-min three-segment linear water-acetonitrile gradient in 0.1% trifluoroacetic acid [11].

The method reported in this paper allows the complete resolution of the protein components of LHC II extracted from spinach leaves by a 13.5-min linear water–acetonitrile gradient, in the presence of 0.1% (v/v) TFA, using a porous butyl silica-based HPLC column. The application of this method to the resolution of the protein components of LHC II extracted from different plants is described.

2. Experimental

2.1. Instrumentation

The liquid chromatograph was a Beckman (Fullerton, CA, USA) System Gold unit consisting of two Model 126 solvent delivery pumps and a Model 166 UV detector, interfaced with a Kbyte Informatica (Viterbo, Italy) computer utilizing Version 6.0 System Gold software for instrument control, data acquisition and analysis. Samples were introduced onto the column by a Model 210A sample injection valve with a 20-μl sample loop. Fractions of the eluate were collected by a Gilson (Villiers le Bel, France) Model 201 sample collector.

The experiments were performed on a Vydac (The Separation Group, Hesperia, CA, USA) Protein C_4 column (150×4.6 mm I.D.) containing 5-μm porous butyl silica. The eluents were prepared by mixing in a beaker the quantity of acetonitrile and TFA required for the concentration stated with 90% (v/v) of the total water volume estimated. Thereafter, the solution was transferred to a volumetric flask and its final volume was adjusted with water. All solutions were filtered through a Millipore (Milan, Italy) type FH 0.5-μm membrane filter and degassed by sparging with helium before use.

2.2. 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). Acrylamide, *N,N'*-methylen-bis-acrylamide, and all other reagents for SDS-PAGE were purchased from Bio-Rad (Segrate, Italy). Sucrose, tricine, Tris-hydroxymethylaminomethane (Tris), *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, USA).

2.3. Isolation of chloroplast thylakoid and PS II membranes

Chloroplast thylakoid membranes (PS II mem-

branes) were isolated from spinach, pea and ailanthus leaves according to the method of Berthold [12] 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, San Diego, USA) and centrifuged at 4000 *g* for 10 min at 4°C. Pellets were suspended in B1 buffer and centrifuged as above. These second pellets were 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. [13]. The incubation was terminated by centrifugation at 40 000 *g* for 30 min at 4°C. This pellet containing the PS II complex and corresponding to the BBY preparation described by Berthold et al. [12], was resuspended in B3 buffer containing 20% (v/v) glycerol and stored at −80°C.

2.4. Isolation of LHC II by sucrose-gradient ultracentrifugation

The light-harvesting complex was separated from the core complex as described by Bassi and Dainese [14] 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 (Milan, Italy) 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 3 contained essentially LHC II proteins as previously reported by Bassi and Dainese [14]. Consequently, this was the purified starting material used for the HPLC study.

2.5. Polyacrylamide gel electrophoresis

Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12–17% acrylamide gradient gels (15 cm×1.5 mm) containing 7 *M* urea, using a BRL (Bethesda Research Laboratories, Gaithersburg, USA) Model V16 vertical gel-electrophoresis system. Gels were run at room temperature (20±2°C) for 15 h at constant current of 20 mA using 25 m*M* Tris/192 m*M* glycine buffer, pH 8.8, containing 3.5 m*M* SDS. Gels were fixed and stained for 2 h in a 5:1:4 (v/v) methanol–glacial acetic acid–water mixture, containing 0.1% (w/v) Coomassie Blue. For silver-staining, gels were fixed in 50% (v/v) methanol–water and 10% (v/v) ethanol–water solutions, stained with 0.1% (w/v) silver nitrate–water solution and developed in 3.5% (w/v) aqueous sodium carbonate containing 0.05% (v/v) formamide.

3. Results and discussion

The material harvested from band 3 of the sucrose-gradient ultracentrifugation (see insert Fig. 1), containing essentially LHC II [14], was precipitated in 80% (v/v) aqueous acetone at room temperature and collected by centrifugation in order to take away the chlorophyll. The pellet was dried under a stream of nitrogen and dissolved in 2:1 (v/v) acetonitrile–formic acid prior to being chromatographed on a Vydac C_4 column using linear water–acetonitrile elution gradient in trifluoroacetic acid. In a typical chromatographic run, the Vydac C_4 column was pre-equilibrated with 38% (v/v) aqueous acetonitrile solution containing 0.1% (v/v) TFA, and the LHC II proteins were eluted by linear gradient from 38 to 61.75% (v/v) acetonitrile, preceded by 1.0 min isocratic elution with the starting eluent, followed by a second gradient segment from 61.75 to 95.0% (v/v) acetonitrile in 3 min. This post-run gradient was used in order to ensure that eventual hydrophobic contaminants of LHC II were eluted from the column by the second gradient segment up to 95% acetonitrile.

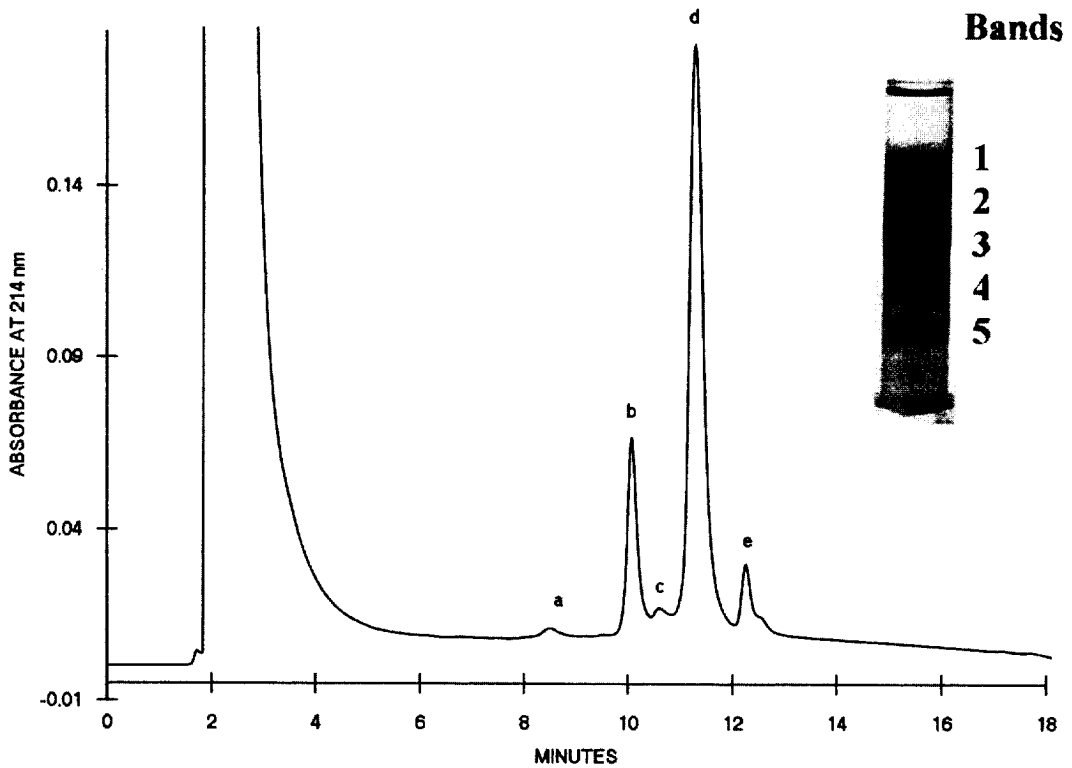

Fig. 1. Reversed-phase HPLC separation of spinach chlorophyll *a*/*b*-binding proteins contained in band 3 of BBY proteins fractionated by sucrose-gradient ultracentrifugation. Column, Vydac Protein C_4 (150×4.6 mm I.D.) containing 5-μm porous butyl silica; linear gradient from 38 to 61.75% (v/v) acetonitrile in water containing 0.1% TFA, preceded by 1.0 min isocratic elution with the starting eluent and followed by 3.0 min linear gradient to 95% acetonitrile; flow-rate, 1.0 ml/min; detection, 214 nm; temperature, ambient. Insert, sucrose-gradient fractionation of BBY proteins from spinach leaves, band 3 contains essentially LHC II proteins.

The chromatogram displayed in Fig. 1 shows that the LHC II proteins were completely resolved in three main and two minor peaks with retention times ranging from 8.3 to 12.2 min. The retention times of these five peaks were not appreciably affected by injecting samples that were not precipitated by acetone. However, with these samples the chromatograms exhibited an additional peak which coeluted with the peak labelled with letter *a*.

The same sample was subjected to denaturing SDS-PAGE, following the procedure reported in the Section 2, and was separated in three bands of molecular mass ranging from 25 to 28 kDa, which were identified as the Lhcb1, Lhcb2, and Lhcb3 [15,16] of the LHC II proteins according to their decreasing molecular mass [3] (see Fig. 2). 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 then analyzed by denaturing SDS-PAGE. The SDS-PAGE gel displayed in Fig. 2 shows that the three main peaks labelled in the chromatogram of Fig. 1 with the letters *b*, *d*, and *e* contain the Lhcb2, Lhcb1, and Lhcb3 proteins, respectively. This identification is supported by the complete correlation between the mobilities of the proteins collected throughout the chromatographic run (corresponding to peaks *b*, *d*, and *e*) and those of the LHC II proteins isolated by the sucrose-gradient fractionation. Moreover, both the SDS-PAGE gel (Fig. 2) and the HPLC chromatogram (Fig. 1) show that the abundance of the three main proteins separated from the LHC II sample is in the order Lhcb1>Lhcb2>Lhcb3, as also has been reported by Machold [17], who observed that the LHC II preparations contain the three proteins,

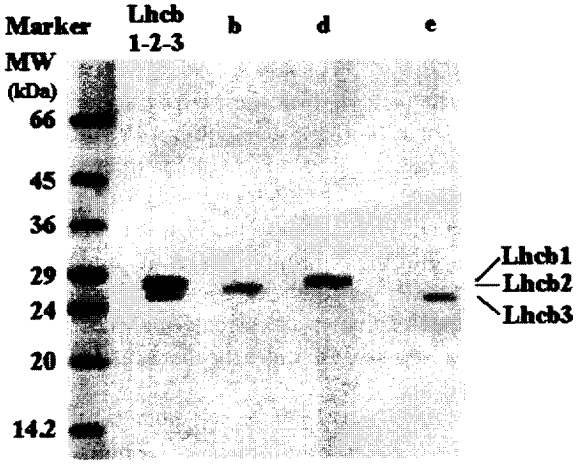

Fig. 2. Denaturing SDS-PAGE of LHC II proteins isolated from spinach leaves by sucrose-gradient ultracentrifugation (LHC II) and of fractions *b*, *d*, and *e* collected throughout the chromatographic run. Slab gel, 12–17% acrylamide gradient gel containing 7 *M* urea; electrolyte solution, 25 m*M* Tris/192 m*M* glycine (pH 8.8) containing 3.5 m*M* SDS; silver-staining, $AgNO_3$ 0.1% (w/v) in water.

Lhcb1, Lhcb2, and Lhcb3, in ratios that vary from 10:3:1 to 20:3:1. This further corroborates the identification of peaks *a*, *b*, and *e* as the separated Lhcb2, Lhcb1, and Lhcb3 proteins. Due to the very low amount of material collected in the fractions corresponding to peaks *a* and *c*, the denaturing SDS-PAGE analysis did not allow any identification for these peaks, although preliminary studies indicate that peak *c* represents a contamination of the minor antennas.

As seen in Fig. 1, the three types of the LHC II proteins elute from the reversed-phase column with retention times ranging from 10.12 to 12.2 min, which corresponds to a narrow range of acetonitrile concentration of only 3.9% (v/v). From this it can be inferred that the three LHC II proteins are hydrophobic to a similar extent. This is in accordance with the expectation that they are hydrophobic intrinsic membrane proteins very similar in amino acid sequence [7,8], all of which have three membrane-spanning helices [18]. From the different order in which the LHC II proteins are resolved by RPC and by denaturing SDS-PAGE, it can be inferred that the hydrophobic character of these membrane proteins is not directly related to their molecular mass. LHC II proteins extracted from thylakoid membranes exposed to illumination at 1000 $\mu E/m^2s$ (white light; μE=microEinstein), in the presence of the phosphatase inhibitor NaF, displayed the same chromatographic behavior of LHC II proteins extracted from thylakoid membranes treated with alkaline phosphatase. This indicates that the phosphorylated LHC II proteins, obtained by illuminating the sample, and the enzymatically dephosphorylated LHC II proteins, cannot be resolved by the RPC column under these experimental conditions.

In order to examine the repeatability of the chromatographic profile and resolution of the LHC II proteins, the standard deviations (S.D.) and the relative standard deviation (R.S.D.) of the retention times of the resolved proteins were calculated from the chromatograms obtained by six repeated injections of the LHC II sample isolated from spinach leaves. This experiment was repeated on other samples of LHC II isolated from spinach leaves by a second and a third preparation. The results are reported in Table 1, and show that for all preparations the R.S.D.s were better than 0.39% for the three proteins.

The high repeatability of both the chromatographic

Table 1
Repeatability of retention times of the protein components of LHC II isolated from spinach leaves by three different preparations

Thylakoid membrane proteins	First preparation		Second preparation		Third separation		Mean of three separations	
	Mean retention time (n=6)	R.S.D. (%)	Mean retention time (n=6)	R.S.D. (%)	Mean retention time (n=6)	R.S.D. (%)	Mean	R.S.D. (%)
Lhcb2	10.1	0.2	10.1	0.2	10.1	0.2	10.1	0.2
Lhcb1	11.3	0.1	11.3	0.4	11.2	0.1	11.3	0.2
Lhcb3	12.2	0.1	12.2	0.1	12.1	0.1	12.2	0.1

Data were calculated for each preparation and for the three different preparations.

separation and the retention times of the proteins resolved from LHC II isolated from spinach leaves, either by the same or by different preparations, prompted us to examine if the reversed-phase HPLC method might be useful in detecting differences in the protein components of LHC II isolated from different plants. For this reason, we isolated LHC II from ailanthus and pea leaves using the same procedure reported in the Section 2 for spinach. These samples were subjected both to denaturing SDS-PAGE and to reversed-phase HPLC using the same column and gradient conditions employed to separate the proteins in the samples isolated from spinach leaves. Each sample of LHC II isolated from each plant was analyzed in triplicate by reversed-phase HPLC in order to evaluate the repeatabilities of the retention times which were comparable to those calculated for the samples isolated from spinach leaves and reported in Table 1.

The separations of proteins in samples of LHC II isolated from ailanthus and pea, obtained by reversed-phase HPLC and by denaturing SDS-PAGE, are reported in Fig. 3 (panels A and B, respectively). It is observed that, besides the high repeatability of chromatographic profile and retention times obtained for the separations performed with samples isolated from each plant, the chromatographic profiles are to some extent different from plant to plant. This is in accordance with the dissimilarities in the amino acid sequence reported in literature for other plants [15], and with the results of the denaturing SDS-PAGE analysis that shows different band patterns for ailanthus and pea (see Fig. 4).

On the other hand, spinach and ailanthus, which display quite similar electrophoretic patterns, have chromatographic profiles that are significantly different in terms of the areas of the separated peaks which, however, elute with very comparable retention times. In order to gain some insights from the comparison of the two different chromatographic

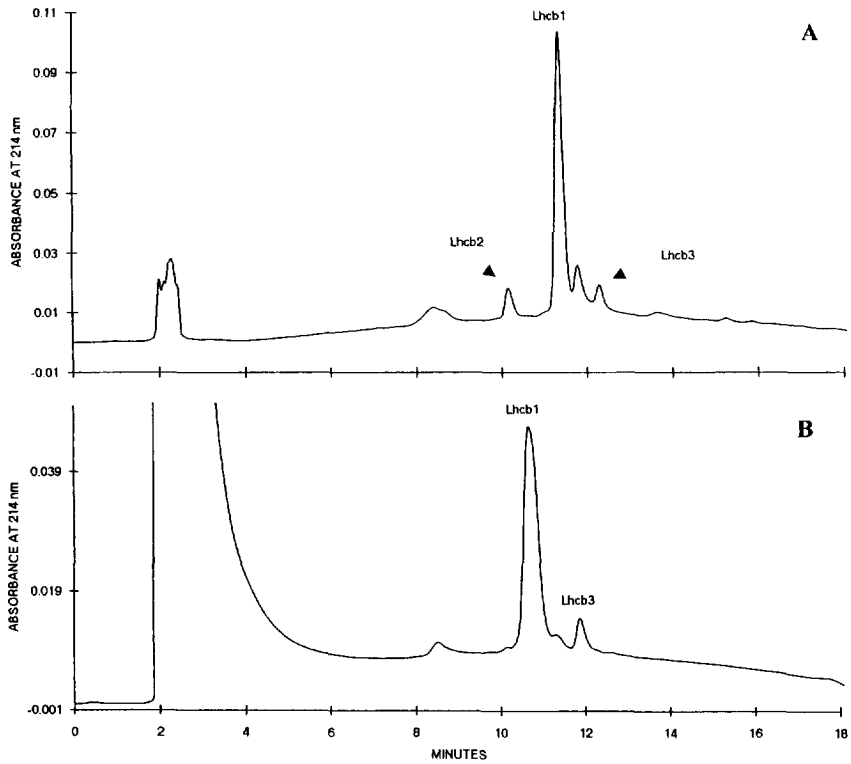

Fig. 3. Reversed-phase HPLC separation of the protein components of LHC II isolated from ailanthus (panel A) and from pea (panel B) leaves. All conditions as in Fig. 1.

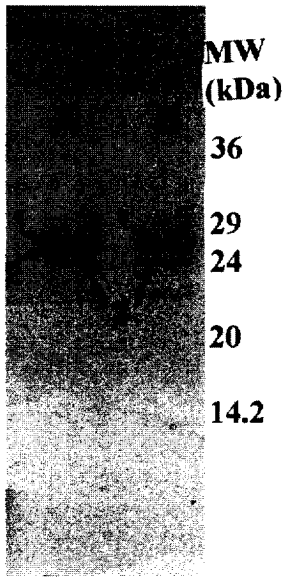

Fig. 4. Denaturing SDS-PAGE gel of protein components of LHC II isolated from ailanthus (lane A) and from pea (lane B) leaves. Symbols: 1, Lhcb1; 2, Lhcb2; 3, Lhcb3; protein components of LHC II. Experimental condition as in Fig. 2, except staining, 0.1% (w/v) Coomassie Brilliant Blue in 5:1:4 methanol–glacial acetic acid–water.

profiles displayed by ailanthus and spinach, 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 protein, obtained by multiple-injection analysis ($n=3$) of the LHC II isolated from the two plants. The results are reported in Table 2 and show that the R.S.D.s of the calculated peak area ratios are better than 9.14%.

The analysis of the values of the peak area ratios calculated for the two plants shows that ailanthus displays a lower Lhcb2/Lhcb1 peak area ratio than spinach which, on the other hand, has a lower Lhcb3/Lhcb1 peak area ratio than ailanthus. It can be inferred from these data that the HPLC method is suitable for the investigation of the organization of the different protein components of photosystem II which might be related to several factors, including different environmental conditions to which the plants were exposed during growing or to structural differences naturally occurring in the LHC II antenna system of different plants.

4. Conclusions

The reversed-phase HPLC method reported in this paper, besides being rapid, simple and precise, has proven to be effective at detecting differences in the protein components of LHC II isolated from different plants that might be not evidenced by denaturing SDS-PAGE, as in the case of spinach and ailanthus reported here. This knowledge is expected to shed light on the composition and supramolecular organization of LHC II, and may increase the understanding of the molecular mechanisms underlying the physiological adaptations of higher plants to environmental conditions.

Furthermore, the use of the volatile mobile-phase additive, trifluoroacetic acid, makes this method suitable to be applied on micropreparative or preparative scale (using wider bore columns) for amino acid sequencing of the isolated proteins or/and of their tryptic peptides.

Acknowledgments

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Table 2
Repeatability of the peak area ratio for ailanthus and spinach calculated as the quotients of the peak area of Lhcb2 and Lhcb3 divided by the peak area of the Lhcb1 protein for triplicate HPLC analysis of LHC II isolated from the two different plants

Peak area ratio	Spinach				Ailanthus			
	Ratio	Mean	S.D.	R.S.D (%)	Ratio	Mean	S.D.	R.S.D. (%)
Lhcb2/1	a 0.304:0.249:0.305	0.3	0.026	9.1	b 0.098:0.097:0.086	0.1	0.005	5.8
Lhcb3/1	c 0.092:0.088:0.108	0.1	0.008	9.0	d 0.105:0.111:0.106	0.1	0.003	2.4

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