Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 837 (2006) 101–107
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Isolation of lipids from photosystem I complex and its characterization with high performance liquid chromatography/electrospray ionization mass spectrometry

Hongjun Yao [a], Yujie Shi [b], Rongfu Gao [a], Guifeng Zhang [c], Rumin Zhang [a], Caixia Zheng [a], Bingjiu Xu [d,*]

[a] *Key Laboratory of Genetics and Breeding of Forest Trees and Ornamental Plants, Ministry of Education, Beijing Forestry University, Beijing 100083, PR China*
[b] *School of Pharmaceutical Sciences, Peking University, Beijing 100083, PR China*
[c] *Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100080, PR China*
[d] *Department of Analytical Chemistry, School of Chemical Biology and Pharmaceutical Sciences, Capital University of Medical Sciences, Beijing 100054, PR China*

Received 11 October 2005; accepted 6 April 2006

Abstract

A method for simultaneous analysis of lipids extracted from photosystem I complex was developed with high performance liquid chromatography/electrospray ionization mass spectrometry. The photosystem I complex was firstly solubilized and separated using deoxycholate polyacrylamide gel electrophoresis method after ultrasonic treatment of the sample (leaves of pea, *Pisum sativum* L.). The Photosystem I complexes were electrophoretically eluted from the deoxycholate polyacrylamide gel electrophoresis bands containing them, and the electron transport activity of the eluent measured as confirmation. Lipids, which were isolated from the complex having photosystem I activity, were separated and characterized with high performance liquid chromatography/electrospray ionization mass spectrometry. Five lipids, monogalactosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidylglycerol, sulphoquinovosyldiacylglycerol and phosphaditylcholine were found combining with photosystem I complex. Different species of these lipids were found in the ESI mass spectra and the compositions of the acyl groups in them were determined.

Keywords: Photosystem I; Lipids; Reversed-phase high performance liquid chromatography; Electrospray ionization mass spectrometry

1. Introduction

Photosynthesis is a biological process taking place in photosynthetic organisms, by which the light energy irradiated on these organisms is transformed into chemical energy [1]. The energy-converting process is carried through on the photosynthetic membranes (the thylakoid membranes) of chloroplast of the photosynthetic organisms [2]. The multiple functions carried out by thylakoid membranes are supported by a very complex array of proteins, lipids and pigments. Photosystem I (PSI) is one of the two pigment-containing reaction centers of oxygenic photosynthesis. It catalyzes the transfer of electrons from lumenal plastocyanin to stromal ferredoxin, using the energy of absorbed photons. In this process the interactions among protein molecules and among protein and lipid molecules play a significant role [3].

In comparison with other biological membranes, the thylakoid membrane is unique in its composition. It is well established that it is composed of only five categories of lipids: nonpolar monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG); as well as the polar phosphatidylglycerol (PG), sulphoquinovosyldiacylglycerol (SQDG) and phosphaditylcholine (PC) [4]. The structures of the thylakoid lipids are shown in Fig. 1.

Besides being the structural components of the thylakoid membrane and PSI complex, thylakoid lipids are commonly

* Corresponding author. Tel.: +8610 82802014.
E-mail address: xbjhfl@263.net (B. Xu).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.04.011

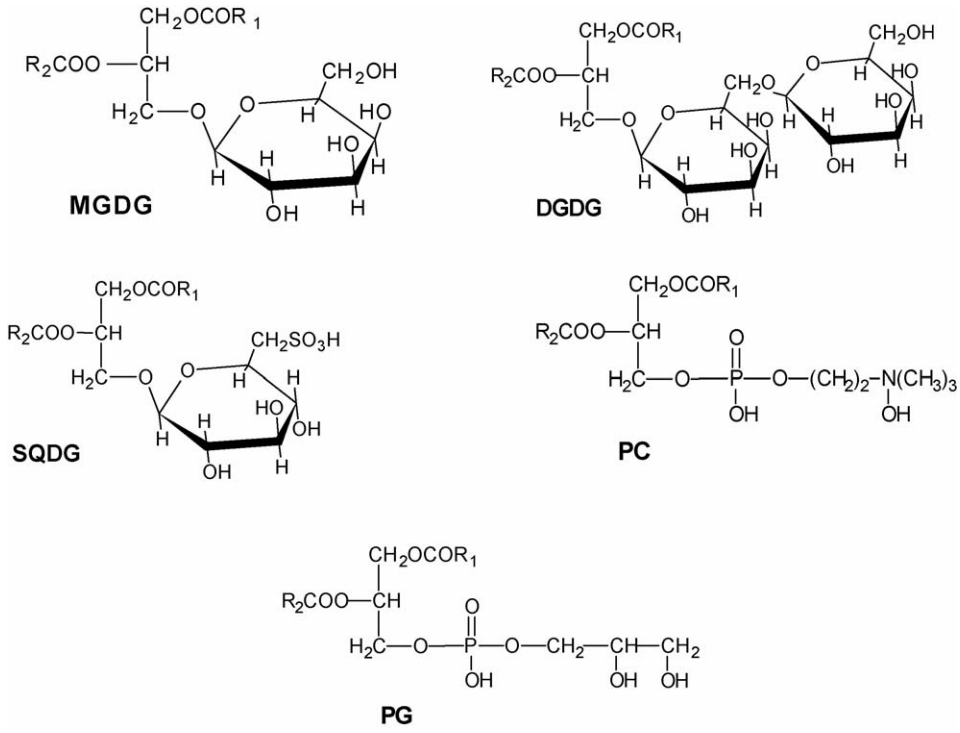

Fig. 1. The structures of lipids in thylakoid membrane.

involved in the photochemical reaction and the maintenance of the structural integrity and stability of PSI complex. While there is an in-depth understanding on the role of thylakoid proteins and pigments in the assembly and functioning of the photosynthetic apparatus, the compositions and functions of the thylakoid lipids combined in PSI complex remain to be thoroughly investigated [5].

At present, researchers pay increasing attention to the lipid composition of the thylakoid membrane. While a variety of TLC and HPLC methods have been established for lipid analysis [6], RP-HPLC is mainly used for phospholipids analysis [7,8], however, the studies on the glycolipids using the same technique are not as abundant [9]. HPLC–MS technique is in rapid progress, with which lipids of unknown composition can be separated, quantified as well as identified on-line [10–12]. It is the development of soft-ionization techniques (such as electrospray ionization, ESI) in mass spectrometry that make possible the analysis of macromolecules extracted from plants, such as lipids [13]. As a result, a number of ESI-MS protocols for lipid analysis have been worked out [14,15]. Phospholipids have been successfully analyzed by HPLC–MS backed by powerful data-processing software [16–18]. Nevertheless, there are fewer reports on MS analysis of glycolipid after reversed-phase separation [19,20].

In order to identify the lipids in PSI complex, we establish a method for the separation of PSI complex using deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) and subsequent analysis of lipids extracted from the complex using on-line coupled RP-HPLC–MS (ESI).

2. Experimental

2.1. Reagents and materials

The reagents for DOC-PAGE were obtained from Amresco (Solon, Ohio USA); 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), dichlorophenolindophenol (DCPIP) and 1,1′-dimethyl-4,4-bipyridinium dichloride (methyl viologen, Mv) from Sigma(St. Louis, MO, USA). HPLC reagents were purchased from Fisher (Fair Lawn, NJ, USA). Digitonin was purchased from China National Pharmaceutical Industry Corporation.

Mature pea leaves (*Pisum sativum* L.) were collected after hydro-cultured for 20 days in greenhouse. The freshly picked leaves were stored at 4 °C after rinsing and removing the surface water, until further treatment.

2.2. Preparation of the PSI complex

PSI complex was prepared essentially as described by Oku et al. [21–23], with modifications. Pre-cooled leaves were ground in the extract buffer into slurry (for ca. 1 min). After centrifugation, the precipitation was re-suspended in suspension buffer. After measurement of chlorophyll content of the suspension, digitonin (the amount of which depended on the content of the chlorophyll in the mixture, generally 100 times that of the latter) was added, thoroughly mixed up and treated in ice bath with ARTEK300 Ultrasonic cell homogenizer for 2 min (instead of a 24 h solubilization on the shaking table at 4 °C [23]). After standing still at 4 °C for 30 min, the mixture was subject to

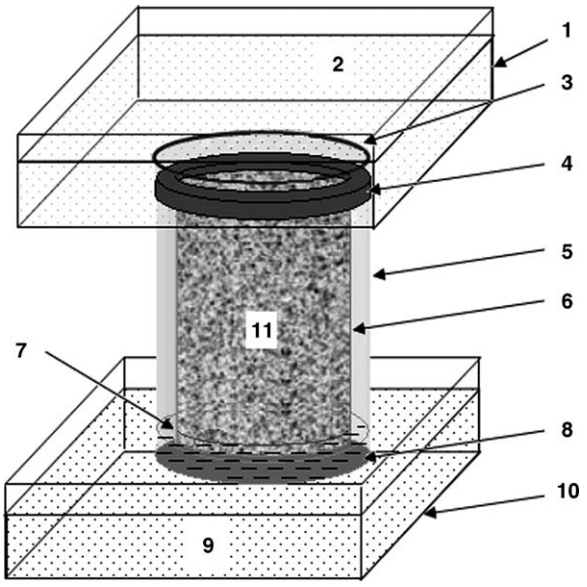

Fig. 2. A homemade elution device for PAGE gel particles. 1, 10: Electrode slot; 2, 9: electrode buffer; 5: outer-tube; 6: inner-tuber; 3: seam between outer tube, 4: seal between the outer and inner tube; 7: collection solution at the bottom of the outer tube; 8: dialysis membrane; 11: DOC-PAGE gel particles + 0.5% (w/v) agarose.

centrifugation (20,000 × g) at 0 °C for 20 min. The supernatant was separated with DOC-PAGE immediately. The concentration of gel and its corresponding buffer systems were prepared as described by Picaud [23]. Electrophoresis was carried out at 0–5 °C.

2.3. Measurement of PSI complex activity

After DOC-PAGE separation, the solutes contained in the bands were individually eluted and their activities measured to ensure the correct selection of the PSI. However, it was found that it took too much time to elute the PSI with the instruments available. Moreover, the PSI solutions thereby derived were severely diluted owing to too large a volume of the collection liquid. Therefore we built up a new device for our purpose (Fig. 2). The electron transport activity of PSI (as measured by PSI-mediated electron transfer from reduced DCPIP to Mv) was measured at 25 °C with an oxygen electrode unit (Hansatech Ltd., England) and a light source. The reaction medium was prepared according to Hsu and Chien [24].

2.4. Lipid extraction

Lipids were extracted according to Folch et al. [25], with modifications. The first green DOC-PAGE band was carefully cut off, shredded, and put into a mixture of 4 ml water, 5 ml chloroform and 10 ml methanol. After full mixing and standing for 30 min, another 5 ml of chloroform was added into the mixture and shaken up. After extraction (1 min), 5 ml more water was added into the mixture, shaken up (1 min), and filtered. The filtrate was then subject to centrifugation. The lower phase was collected and filtered with 0.45 μm filter membrane, the solvent was evaporated under nitrogen stream, and the residue was stored at −20 °C before analysis.

2.5. Analysis of the lipids with RP-HPLC

The HPLC Series 1100 system used for the analysis of the lipids was composed of a binary pump, a column temperature stabilizer and a diode array detector (Agilent, Waldbronn, Germany). Agilent ChemStation software was used for instrument control and data processing. The UV detection was set at 205 nm. A Waters 3.9 mm × 300 mm column packed with μ-Bondapak C_{18} 125 Å 10 μm packing (Waters, Milford, MA USA) was used for separation with the mobile phase flow rate set at 1 ml/min. Several mobile phase systems, including acetonitrile–water, methanol–water, acetonitrile–methanol–water and *n*-hexane–*iso*-propanol–water were tested. Finally, water and acetonitrile were respectively chosen as solvent A and B. The gradient used was as follows: before injection, the column was rinsed with 95% A- and 5% B solvents for 5 min. After injection, B increased linearly to 65% in 8 min, and to 95% in 7 min, and remained at that for 10 min.

2.6. Liquid chromatography/electrospray mass spectrometry

The on-line HPLC separation was performed using Agilent 1100 system. The mobile phase gradient (apart from a few drop of ammonium acetate added into the solvent A to enhance the ionization), the flow rate and the reversed-phase column were the same as above. A part of the eluent from a split valve, 100 μl/min of flow-rate, was introduced into the ion source of the mass spectrometer. The mass spectra were obtained using a Finnigan LCQ ion trap mass spectrometer (Thermo electron, San Jose, CA, USA) equipped with an electrospray ionization source (Spectronex AG, Basel, Switzerland). The spray voltage was 3.5 kV. The heated capillary was kept at 275 °C. The scan range was set from *m/z* 300 to 2000. After electrospray ionization, tandem MS (MS/MS) function was performed in data dependent mode, by collisional activating of the solute molecules colliding with argon gas [26]. The collision energy value was set at 35–45%.

3. Results and discussion

3.1. DOC-PAGE separation of photosystem I

The preparation procedure of thylakoid membrane was first put forward by Oku and Tomita [21] We slightly modified the method by applying ultrasonic homogenization, in order to increase the PSI complex yields in much less time (40 min rather than 24 h) and to obtain thylakoid membrane samples of fairly high purity.

The result of the DOC-PAGE separation of the PSI complex is shown in Fig. 3. The complex was eluted with agarose electrophoretic method using the homemade device shown in Fig. 2. Due to the shorter electrophoretic pathway of this device, the PSI complex elution can be finished rapidly. The PSI derived is also

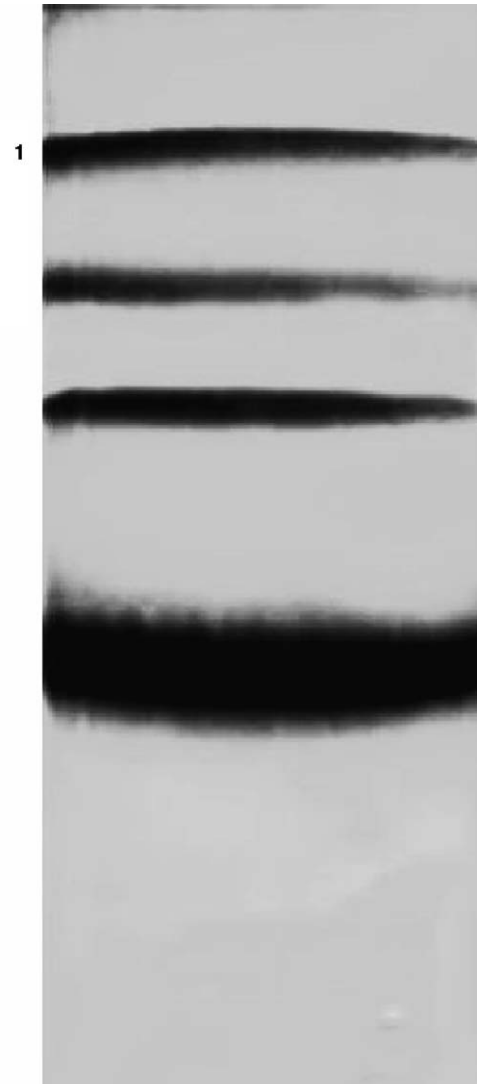

Fig. 3. DOC-PAGE separation of PSI complex. For DOC-PAGE conditions see separation of PSI complexes with DOC-PAGE, experimental section 1 is the band cut for extracting lipids.

more concentrated since only small amount of collection solution is needed. The PSI activity of the eluent was measured to be 3.52 μmol mg(chl)$^{-1}$ min^{-1} of oxygen absorption, this proved that the material extracted was indeed PSI complex.

3.2. RP-HPLC separation of lipids

Given that the lipids extracted from PSI complex were mixtures composed of substances of a very wide range of polarity, it would be difficult to separate all its ingredients using isocratic elution system. By carefully selecting the appropriate packing material and optimizing the LC parameters for the separation, satisfactory results were achieved on a reversed phase column (Waters, μ-Bondapak C_{18} 125 Å 10 μm). Among the different mobile phases studied, an acetonitrile/water gradient elution turned out to be the most effective. The result obtained from the optimized chromatographic system is illustrated in Fig. 4. Analysis of the UV spectra of five major peaks (RT = 9.48, 12.56, 16.50, 19.67, 21.13) suggested that they might all be lipids. Coincidently there are five categories of lipids in thylakoids membrane [4], as suggested by the authors. Further identification of the compounds using HPLC–MS(ESI) was subsequently carried out.

3.3. HPLC–ESI-MS

Phospholipids analysis by HPLC–MS(ESI) is rapidly becoming an established technique [27,28], yet up to now few reports have been published on the identification of individual lipids of plant chloroplasts [29]. Plant chloroplasts have glycolipids (i.e. MGDG, DGDG and SQDG) as well as phospholipids (i.e. PG and PC) as their ingredients. MGDG and DGDG are uncharged species, whereas SQDG is negatively charged at neutral pH. It was reported that MGDG and DGDG were positively charged during MS analysis. Molecular ions, sodium adducts and protonated molecular ions were easily observed with strong respective signals [29]. SQDG and all the phospholipids could also be analyzed in positive-ion mode [16–18].

The total ion current (TIC) of lipids extracted from PSI protein complex is shown in Fig. 5. MGDG, DGDG, SQDG, PG and PC were the unique lipids in thylakoids membrane [4], the extracted ion chromatogram (EIC) of $[M+Na]^+$or $[M+H]^+$ ion of these lipids was used to characterize their structure. Fig. 6 shows EIC of MGDG, DGDG, SQDG, PG and PC from PSI complex extracted with Folch technique [25], using the reversed phase HPLC coupled with ESI-MS in the positive-ion mode. It can be seen in Fig. 6 (which show the intensities of the strongest ion currents that can be extracted from the respec-

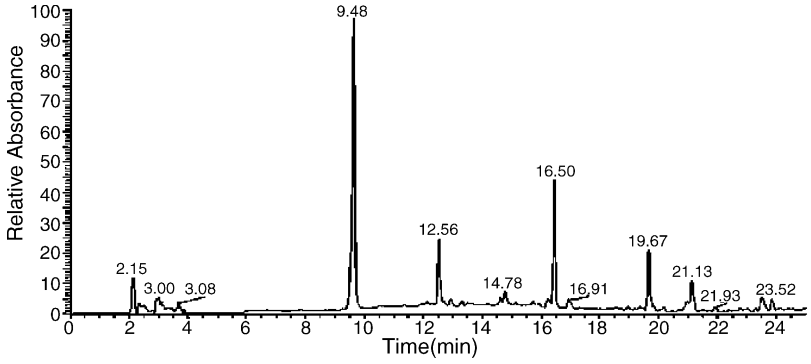

Fig. 4. RP-HPLC chromatogram of lipids extract from PSI complex. HPLC conditions and gradient of mobile phase of acetonitrile–water, see Section 2.5.

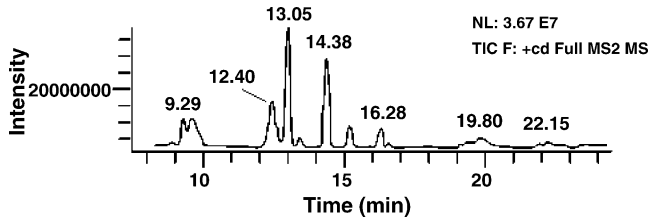

Fig. 5. Positive ion mode TIC of lipids extract from PSI protein complex separated by RP-HPLC–ESI-MS. For HPLC–MS conditions, see Section 2.6; gradient of mobile phase of acetonitrile–water, see Section 2.5.

tive lipid peaks), that the strengths of extracted ion currents was in the following order: those from MGDG, DGDG and SQDG (very strong, as shown in Fig. 6a–c), that from PG (rather strong, Fig. 6d), and PC (very weak, Fig. 6e). The last one barely exceeded the background noise. Judging from the weak signal of PC, it appeared that PC did exist in our samples, as evidenced by the relevant peak in the chromatogram as well as the mass spectrogram.

ESI mass spectra of MGDG, DGDG, SQDG, PG and PC are shown in Fig. 7. The major peaks identified in the spectra were assigned to the protonated molecular ions $[M+H]^+$ $[M+Na]^+$ or $[M+2Na]^{++}$ of individual MGDG, DGDG, SQDG, PG, PC molecular species. Ion *m/z* with 777.3 in Fig. 7a shows $[M+Na]^+$ of MGDG with acyl composition of C34:2. $[M+Na]^+$ of DGDG and PC were shown in Fig. 7b and f with acyl composition of C34:3 and C34:4, respectively. $[M+Na]^+$ and protonated molecular ion of SQDG were found in one ESI mass spectra in Fig. 7c and d. *m/z* 863.2 and 837.4 were $[M+Na]^+$ of SQDG with acyl composition of C34:2 and C32:1 with their protonated molecular ion *m/z* 819 and 793, respectively. $[M+2Na]^+$ and $[M+Na]^+$ of PG with acyl composition of C34:3 also found in the same ESI mass spectra in Fig. 7e.

Table 1
Molecular species and fatty acid compositions of lipids isolated from PSI protein complex

Lipid class	Species	MW	$[M+H]^+$	$[M+Na]^+$ or $[M+2Na]^+$	Composition of fatty acid
MGDG	M1	752.6		775.5	C34:3
	M2	754.6		777.3	C34:2
	M3	756.6		779.5	C34:1
DGDG	M1	914.6		937.5	C34:3
SQDG	M1	792.5	793.0	837.4	C32:1
	M2	818.5	819.5	863.2	C34:2
PG	M1	750.5		796.7	C34:2
	M2	791.5		817.6	C36:3
PC	M1	781.6		804.6	C36:4
	M2	783.6		806.6	C36:3
	M3	787.6		810.3	C36:1

Singly charged ions of MGDG, DGDG, SQDG, PG and PC were found in the ESI mass spectra of individual components. Even the same type of lipids extracted from plants may contain many different species because of the different sizes of fatty acyl groups in their molecules and the different numbers of double bonds (as well as their position) in the acyl groups [30]. For instance, in our work MGDG ions (*m/z* 773, 775 and 779) were found in the mass spectra, indicating that MGDG species might have the following acyl groups: C34:3, C34:2 and C34:1, respectively. The same results were found in other lipid components (see Table 1).

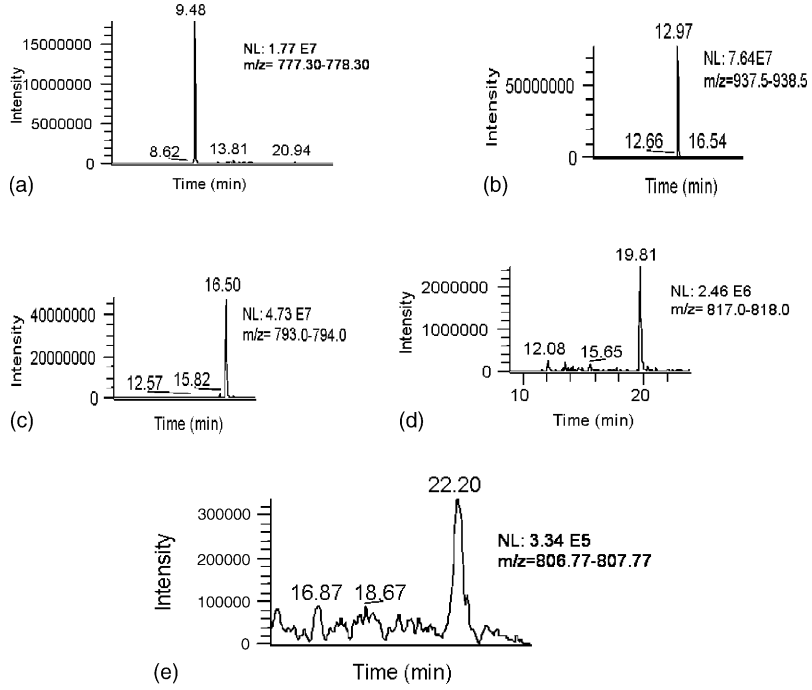

Fig. 6. Positive ion mode EIC of lipids. (a) EIC of *m/z* = 777.3 (MGDG); (b) EIC of *m/z* = 937.6 (DGDG); (c) EIC of *m/z* = 793.0 (SQDG); (d) EIC of *m/z* = 817.5 (PG); (e) EIC of *m/z* = 806.5 (PC). For HPLC–MS conditions, see Section 2.6; gradient of mobile phase of acetonitrile–water, see Section 2.5.

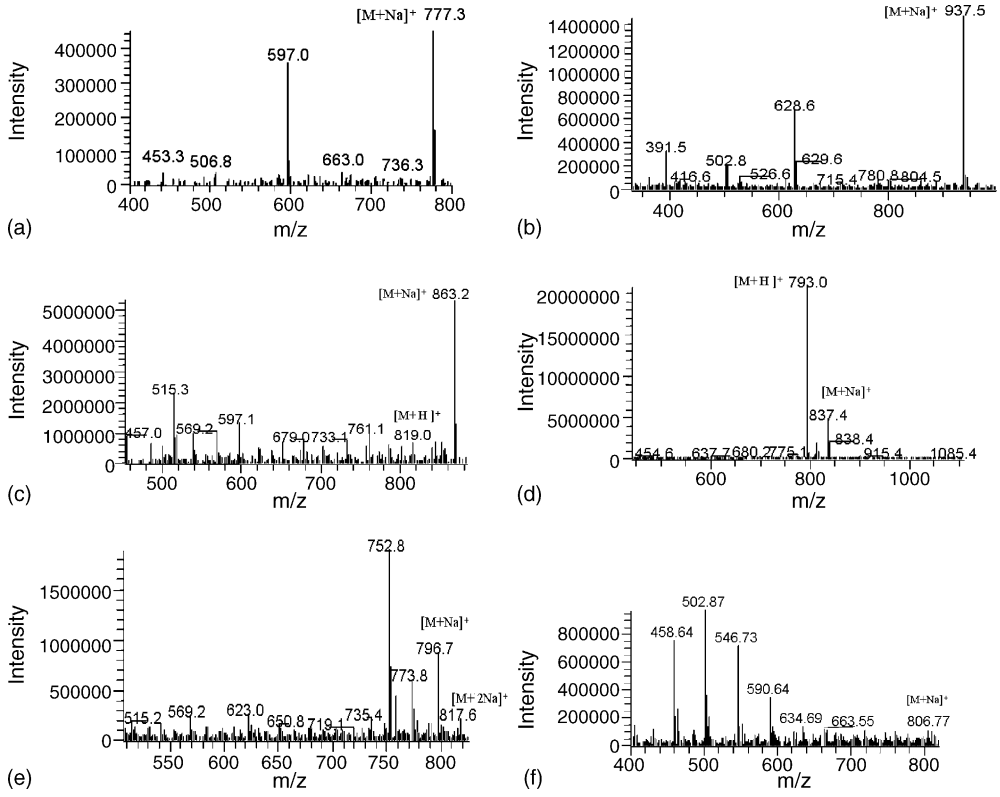

Fig. 7. Positive ion mode ESI-MS spectrum of lipids extracted from PSI. (a) MS spectrum at RT = 9.48 (MGDG, *m/z* 777.3); (b) MS spectrum at RT = 12.95 (DGDG, *m/z* 937.5); (c) MS spectrum at RT = 16.37(SQDG, *m/z* 819.0,863.2); (d) MS spectrum at RT = 16.53 (SQDG, *m/z* 793.0, 837.4); (e) MS spectrum at RT = 19.22 (PG, *m/z* 817.6); (f) MS spectrum at RT = 22.22 (PC, *m/z* 806.9). For HPLC-MS conditions, see Section 2.6; gradient of mobile phase of acetonitrile–water, see Section 2.5.

Palsdottir and Hunte [32] pointed out that "the acyl chains of lipid are usually refined as fully saturated, although it is often evident from the density that one or more double bonds would be energetically more feasible to produce the extreme bending angles that are frequently observed". The existence of acyl groups with one or more of the double bonds was supported by our mass spectrometric evidence obtained.

We tried to study further the collision-induced dissociation MS/MS spectra of lipid molecular ions. MS/MS spectrum of sodium-adducted molecular ion of MGDG was shown in Fig. 8. Product ions at *m/z* 243, the characteristic MS/MS ion of head group of MGDG, from MGDG + Na precursors had an *m/z* value consistent with the chemical formula $C_9H_{16}O_6Na$, which is consistent with the fragment structure proposed by Kim et al. [19,30]. However, MS/MS spectrum of MGDG was the only case in which a rational formula could be derived; for other lipids, DGDG, SQDG, PG and PC, although their characteristic primary ions reported by Ruth et al. [19,29,30] were confirmed by our observations, it was difficult to further derive the structures of the acyl groups from MS/MS spectra. It should be noticed that Ruth et al. used FAB to study the standard lipid samples, whereas in our case ions from the first stage of LC–MS/MS were further dissociated in a collision-induced process.

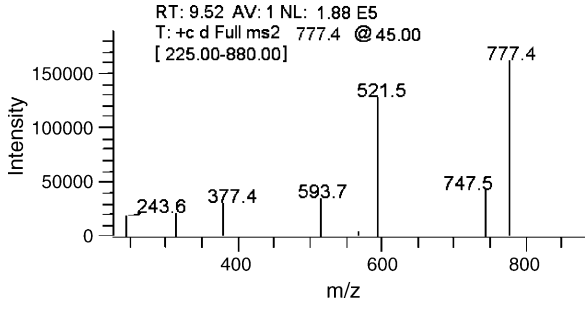

Fig. 8. MS/MS spectrum of MGDG. For MS/MS conditions, see Section 3.3.

4. Conclusions

A more efficient method of thylakoid membranes protein complex solubilization and a useful homemade elution device for DOC-PAGE separation of photosystem I were introduced in this research. Further analysis showed that MS technique makes possible quick and simple fragmentation of lipid head groups, thereby determining the *m/z* values of the most important acyl substitution groups in each type of lipids. This clearly demonstrates the advantage of ESI/MS over traditional analytical methods for the study of the constituents and distribution of lipids of thylakoid [11,31].

In our work, three glycolipids (MGDG, DGDG, SQDG) and two phospholipids (PG, PC) were found in the PSI complex, and the acyl groups they contained determined. The method developed in this work could be used for the separation of individual lipids in the thylakoid and other related phospholipids and glycolipids compounds, as well as for the identification of

the constituent acyl groups contained in these lipids. On the basis of the above-mentioned information, the distribution and functioning of lipids in thylakoid membrane, and further, the photosynthesis process, might be better understood.

Acknowledgement

The financial support of the Natural Science Foundation of China (Grant No. 20175001) and Education Reinvigoration Plan of Beijing Forestry University (Grant No. 200302014) are gratefully acknowledged.

References

[1] H. Miki, G. Zoltán, V. Zsuzsanna, K. Masamoto, N. Sato, M. Tsuzuki, H. Wada, Plant Physiol. 124 (2000) 795.
[2] W. Yang, S. Liu, F. Feng, H. Haitong, J. Guizhen, X. Yinong, K. Tingyun, Plant Sci. 166 (2004) 1575.
[3] M.S. Webb, B.R. Green, Biochim. Biophys. Acta 1060 (1991) 133.
[4] B. Brugger, G. Erben, R. Sandhoff, F.T. Wieland, W.D. Lehmann, Proc. Natl. Acad. Sci. USA 94 (1997) 2339.
[5] Govindjee, D. Krogmann, Photosynth. Res. 80 (2004) 15.
[6] T. White, S. Bursten, D. Federighi, R.A. Lewis, E. Nudelman, Anal. Biochem. 258 (1998) 109.
[7] G.M. Patton, S.J. Robins, Methods Mol. Biol. 110 (1998) 193.
[8] G. Mal, J. Ska, L. Dariusz, K. Strzalka, Chem. Phys. Lipids 110 (2001) 85.
[9] F.D. Conforti, C.H. Harris, J.T. Rinehart, J. Chromatogr. 645 (1993) 83.
[10] B. Barroso, R. Bischoff, J. Chromatogr 814 (2005) 21.
[11] E. Robert, C. Jochimsa, W. Lehmannb, G. Erben, S. Staffer, C. Reininger, W. Stremmel, Biochim. Biophys. Acta 1682 (2004) 63.
[12] J. Adachi, N. Yoshioka, R. Funae, J. Adachi, S. Matsushita, N. Yoshioka, R. Funae, T. Fujita, S. Higuchi, Y. Ueno, J. Chromatogr 806 (2004) 41.
[13] D.N. Heller, R.J. Cotter, Anal. Chem. 59 (1987) 2806.
[14] J.J. Li, W.P. Randy, C.R. James, Anal. Chem. 76 (2004) 4676.
[15] S. Peter, L. Jukka, B. Johansson, Anal. Chem. 76 (2004) 4271.
[16] A. Carrier, J. Parent, S. Dupuis, J. Chromatogr 876 (2000) 97.
[17] A.A. Demmers Jeroen, D. Annemieke, A.J.R. Heck, J.A. Killian, FEBS Lett. 541 (2003) 28.
[18] L. Gerhard, L. Bernd, R. Jan, W. Drobnik, G. Schmitz, Biochim. Biophys. Acta 1686 (2004) 108.
[19] Y.H. Kim, J.S. Choi, J.S. Yoo, Y.M. Park, M.S. Kim, Anal. Biochem. 267 (1999) 260.
[20] W.G. Chai, C. Leteux, A.M. Lawson, Anal. Chem. 75 (2003) 118.
[21] T. Oku, G. Tomita, Physiol. Plant 38 (1976) 181.
[22] C.X. Zheng, R.F. Gao, Chin. Sci. Bull. 43 (1998) 1973.
[23] A. Picaud, S. Bycker, J. Duranron, Photosynth. Res. 3 (1982) 203.
[24] B.D. Hsu, R.F. Chien, Physiol. Plant 73 (1988) 65.
[25] J. Folch, M. Lees, G.S. Stanley, J. Biol. Chem. 226 (1957) 497.
[26] X. Han, W. Richard, Anal. Biochem. 295 (2001) 88.
[27] G. Isaac, D. Bylund, J.E. Månsson, J. Neurosci. Methods 128 (2003) 111.
[28] B.H. Nathalie, B. Laurent, V.A. Fay, Free Radical Biol. Med. 7 (2004) 1604.
[29] J.H. Gil, J.K. Hong, J.C. Choe, Y.H. Kim, Bull. Korean Chem. Soc. 24 (2003) 1163.
[30] R. Welti, X.M. Wang, T.D. Williams, Anal. Biochem. 314 (2003) 149.
[31] F. Giuffrida, F. Destaillats, F. Robert, L.H. Skibsted, F. Dionisi, Free Radical Biol. Med. 37 (2004) 104.
[32] H. Palsdottir, C. Hunte, Biochim. Biophys. Acta 1666 (2004) 2.