Separation of Phosphatidylcholine and Its Molecular Species by High-Performance Liquid Chromatography

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Isocratic separation of phosphatidylcholine (PC) and PC molecular species from a soybean leaf lipidic extract using high-performance liquid chromatography (HPLC) is described. PC separation was achieved within 20 min on a semipreparative silica normal phase column using isooctane/2-propanol/water (40:53:7 v/v/v). As much as 1.25 mg of PC was obtained after a single injection. Individual molecular species of PC were separated on an analytical C18 reversed-phase column using methanol/0.1 M ammonium acetate (95:5 v/v, pH 7.4). Spectrophotometric detection of PC and molecular species was made at 205 nm.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been used often to analyze animal lipids (Hamilton and Comai, 1988; Patton, et al., 1982) but less frequently used to analyze plant lipids (Rezanka and Podojil, 1989). In recent studies on plant phospholipids, several investigators demonstrated that phospholipid molecular species could be separated by reversed-phase HPLC on the basis of the degree of unsaturation of the fatty acid constituents (Bishop, 1987; Norman and St. John, 1986; Demandre et al., 1985; Kesselmeier and Heinz, 1985; Lynch et al., 1983). Much attention has been given to this subject since the discovery that significant changes occur in the composition of molecular species in plants during acclimation to low temperature (Thompson, 1984).

The conventional procedure for analyzing molecular species of plant phospholipids consists of separating the lipid extract into the various phospholipid classes by preparative column or thin-layer chromatography and then isolating the molecular species by reversed-phase HPLC. Since the conventional preparative techniques are tedious and time-consuming, a preparative HPLC method has been sought for the separation of plant phospholipids.

Rezanka and Podojil (1989) reported recently on the development of a preparative HPLC technique for separating phospholipid classes in milligram quantities from a green freshwater alga lipidic extract. The molecular species of the phospholipids were separated also on a preparative scale, which was the first report of this development. The present paper describes a semipreparative HPLC method for separating phosphatidylcholine (PC) from a soybean leaf extract that is simple, rapid, and isocratic. PC molecular species were separated isocratically on an analytical HPLC column. Although the separation of PC was the primary goal in this study, this method should be applicable to the separation of other plant phospholipids.

EXPERIMENTAL PROCEDURES

Materials. Leaves of soybean (*Glycine max*) were harvested from plants grown in the field. An egg PC standard was purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade solvents were obtained from EM Science (Cherry Hill, NJ). All other chemicals were of reagent grade and were used without further purification.

Extraction of Plant Lipids. The procedure for extracting lipids from the leaves was similar to that described by St. John (1986). Soybean leaves (30–40 g, fresh weight) were homogenized with 150 mL of chloroform/methanol (2:1 v/v) in a Waring-type blender. The lipid extract was filtered through cheesecloth and was combined with 150 mL of water in a 500-mL separatory funnel. The mixture was shaken vigorously a few times and then allowed to stand for 4 h at room temperature before the lower $CHCl_3$ phase containing the total leaf lipids was removed. The lipid extract was collected in a flask and subsequently poured over sodium sulfate in a chromatographic column to remove traces of water. The extract was then taken to dryness on a rotary evaporator. The residue was dissolved in 40 mL of chloroform and stored at 5 °C for ca. 1 h before fractionation.

Separation of Lipid Classes. Sep-Pak silica cartridges (Waters Associates) were used to fractionate the lipid extract into neutral lipids, glycolipids, and phospholipids. Two cartridges that were connected in series were first washed with 15 mL of chloroform. Solvents and samples were applied with a 50-mL syringe (Hamilton) containing a Luer tip. A 10-mL aliquot of the extract was applied to the cartridges followed by the addition of 20 mL of chloroform, which eluted the neutral lipids and plant pigments. A 20-mL volume of chloroform containing 15% methanol was applied next to remove the glycolipids. Finally, 20 mL methanol were added to elute the phospholipids. The phospholipid column eluate was reduced to approximately 5 mL on a rotary evaporator.

HPLC Separation of PC. PC in the phospholipid fraction was isolated by a normal phase HPLC method recently described by Moreau et al. (1989), who used an analytical silica column and a flame ionization detector to identify many of the neutral and polar lipids that were extracted from the leaves of several different plants. The mobile phase used in the present study was adopted from the earlier investigation. The isocratic separation of PC was performed here on a semipreparative Excello silica column (R. E. Gourley Co., Laurel, MD), which was 10 × 150 mm and contained 5-μm particles. The mobile phase was isooctane/2-propanol/water (40:53:7 v/v/v) delivered at 4.0 mL/min with a ternary pump (Spectra-Physics Model SP 8800). Samples were injected on the HPLC with a syringe loading injector (Rheodyne Model 7125) fitted with a 1000-μL loop. PC was detected with a variable-wavelength detector (Spectra-Physics Model SP 8440) set at 205 nm and 0.08 AUFS. The elution profiles were recorded and integrated on a Beckman Model 740 integrator. The PC eluate was collected manually after it passed through the detector and was stored in screw-cap vials under N_2 at 5 °C.

An analytical Excello silica column (4.6 × 150 mm) contain-

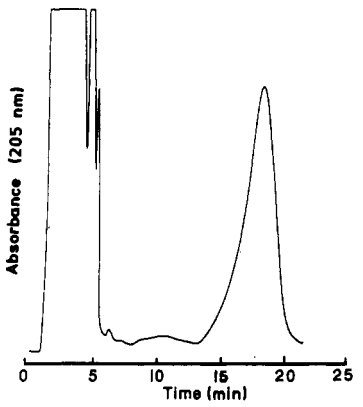

Figure 1. HPLC separation of soybean PC on a semipreparative Excello silica column (10 × 150 mm). Peak corresponds to 1.25 mg of PC. The mobile phase was isooctane/2-propanol/water (40:53:7 v/v/v) delivered isocratically at 4.0 mL/min. Detection was made at 205 nm.

Table I. Effect of Mobile Phase Composition on the Retention Time of Soybean PC on Semipreparative Column[a]

composition of mobile phase			
isooctane, %	2-propanol, %	H_2O, %	retention time, min
40	55	5	33.3
40	53	7	19.3
40	51	9	8.8

[a] Flow rate was 4.0 mL/min.

ing 5-μm particles was also used to test the purity of PC isolated from the semipreparative column. The equipment and conditions were the same as described above with the exception that the flow rate was 2.0 mL/min.

HPLC Separation of Molecular Species. Separation of molecular species of PC was performed on a C18 reversed-phase analytical column (Excello Ultra Pac ODS, Gourley), which was 4.6 × 150 mm and contained 5-μm particles. The mobile phase was methanol/0.1 M ammonium acetate (95:5 v/v, pH 7.4), which was delivered at 2.0 mL/min. The sample loop was 100-mL capacity. The molecular species were collected manually after passing through the UV detector, which was set at 205 nm.

Chemical Analyses. The fatty acid compositions of the purified PC and of the individual PC molecular species were determined on a Hewlett-Packard Model 5880 gas chromatograph (GC) equipped with a SP 2330 capillary column and a flame ionization detector. The fatty acids were transesterified with boron trifluoride/methanol (Supelco). Lipid phosphorus was determined by the method of Bartlett (1959) as modified by Marinetti (1962).

RESULTS AND DISCUSSION

HPLC Separation of PC. A typical chromatogram for the separation of PC from soybean leaf extract is shown in Figure 1. The retention time for PC was ca. 19 min at the flow rate of 4.0 mL/min. Table I shows the effect of the mobile phase composition on the retention time of PC. Higher percentages of water reduced the retention time. The PC peak in the chromatogram corresponds to 1.25 mg, as determined by lipid phosphorus analysis. A total of 4.0 mg of polar lipids in 1000 μL was injected on the column. As much as 10 mg/injection of an egg PC standard was satisfactorily resolved by this semipreparative column (chromatogram not shown).

Aliquots of the isolated soybean PC and an egg PC standard are compared in the analytical chromatograms shown in Figure 2. The peaks of soybean PC and egg PC correspond to 25 and 80 μg, respectively. The absorbance of soybean PC at 205 nm was over 3-fold greater

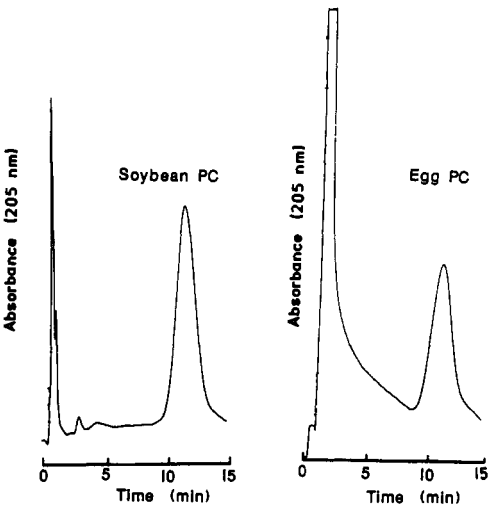

Figure 2. Chromatograms of soybean PC (25 μg) and egg PC (80 μg) separated on an analytical Excello silica column (4.6 × 150 mm). The mobile phase was isooctane/2-propanol/water (40:53:7 v/v/v) delivered isocractically at 2.0 mL/min. Detection was made at 205 nm.

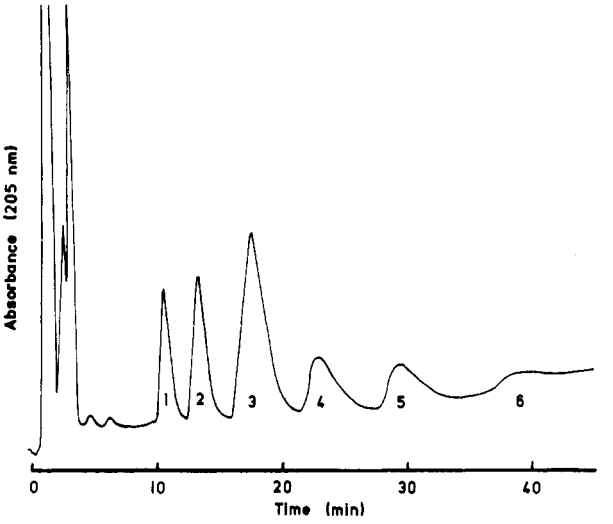

Figure 3. Separation of molecular species of soybean PC on an analytical Excello Ultra Pac ODS column (4.6 × 150 mm). The mobile phase was methanol/0.1 M ammonium acetate (95:5 v/v, pH 7.4) delivered isocractically at 2.0 mL/min. Detection was made at 205 nm.

Table II. Fatty Acid Composition of Soybean PC[a] and Egg PC[b]

	% fatty acid by wt				
	16:0	18:0	18:1	18:2	18:3
soybean PC	18.0	9.0	2.1	40.3	30.6
egg PC[c]	30	14	30	14	0

[a] Data were obtained from the lipid phosphorus analysis in the present study. [b] Data were reported by Smith and Jungalwala (1981). [c] Other fatty acids include 20:4 (4%), 22:5 (1.5%), and 22:6 (2%).

than that of egg PC per unit mass. It is known from other lipid studies that UV absorbance is related to the degree of unsaturation of the fatty acids in acyl lipids (Bishop, 1987). The data in Table II show the difference in the fatty acid composition of the soybean PC isolated in this study and egg PC reported by Smith and Jungalwala (1981). From these data, it was calculated that the number of double bonds in fatty acids of the purified soybean PC was 3-fold greater than in fatty acids of the egg PC.

HPLC Separation of Molecular Species. Figure 3 shows the analytical chromatograms for the separation of

Table III. Molecular Species Found in Soybean PC Isolated by Semipreparative HPLC[a]

peak no., Figure 3	retention time, min	molecular species
1	11.1	18:3/18:3
2	13.8	18:2/18:3
3	18.1	18:2/18:2
		16:0/18:3
4	23.4	18:1/18:2
		16:0/18:2
5	30.4	ND[b]
6	39.5	ND[b]

[a] Flow rate was 2.0 mL/min. [b] ND, not determined.

soybean PC into molecular species. The complete analysis time was about 40 min at a flow rate of 2.0 mL/min. Table III shows the assignment for the individual molecular species of soybean PC in Figure 3, which was based on the results of the fatty acid analyses of the present study (data not shown) and the results of related studies (Demandre et al., 1985; Norman and St. John, 1986).

Peaks 1 and 2 were assigned as 18:3/18:3 and 18:2/18:3, respectively. Peak 3 was identified as a mixture consisting of 18:2/18:2 and 16:0/18:3. Peak 4, which is also a mixture, is assigned as 18:1/18:2 and 16:0/18:2. Assignments were not made to peaks 5 and 6 because of the results of the fatty acid analyses of the respectve molecular species were not reproducible. Assignments of the molecular species to the major peaks (1–4) were in good agreement with that reported for PC from tobacco leaves (Demandre et al., 1985) and PC from spinach (Norman and St. John, 1986). There are plans to conduct a detailed examination of the molecular species of soybean PC with an advanced technique using a combination of liquid chromatography (LC) and mass spectrometry (MS).

In conclusion, the method described here appears to be sensitive enough to monitor qualitative and quantitative changes in the composition of PC and molecular species in higher plants during development.

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Registry No. 16:0, 57-10-3; 18:0, 57-11-4; 18:1, 112-80-1; 18:2, 60-33-3; 18:3, 463-40-1; 18:3/18:3PC, 2701-19-1; 18:2/18:3PC, 128134-52-1; 18:2/18:2PC, 998-06-1; 16:0/18:3PC, 128115-09-3; 18:1/18:2PC, 75803-87-1; 16:0/18:2PC, 75813-28-4.