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Extraction and Purification of Phosphatidic Acid of C_{18} Fatty Acids from Powdered Soybean Phospholipids

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A simple process for efficient extraction of phosphatidic acid from powdered soybean phospholipids is reported. The purity of the extracted phosphatidic acid reached 99 % as analyzed by thin layer chromatography and high-performance liquid chromatography. The fatty acids of the extracted phosphatidic acid were C_{18} fatty acids (stearic and oleic acids, 43.6 and 56.4 %, respectively) as determined by gas chromatography-mass spectrometry. This process may be scaled up for commercial production of high-purity phosphatidic acid.

Key Words: Powered soybean phospholipids, Phosphatidic acid, Extraction, Stearic acid, Oleic acid.

INTRODUCTION

Phosphatidic acid is a small phospholipid commonly found in cell membranes. It also serves as a central intermediate for the synthesis of membrane and storage lipids[1]. Recently, phosphatidic acid has also been implicated in various cellular processes such as signal transduction, membrane trafficking, hormone secretion by endocrine cells and cytoskeletal rearrangement[2-5]. As such, phosphatidic acid is regarded as essential for the survival, proliferation and reproduction of cells or organisms[3]. Because of its important biological functions, it is of great interest to use phosphatidic acid to improve human health. Indeed, when consumed as a supplementation of soy lecithin phosphatidic acid and phosphati-dylserine complex, for instance, phosphatidic acid has been shown to improve pituitary adrenal reactivity and psychological response to mental and emotional stress[6]. It has also been used as a drug[7] or supplement in some cosmetics[8].

To support commercial application of phosphatidic acid, large quantifies of phosphatidic acid of high purity need to be produced from readily available sources. Soybean phospholipids contain relatively high level of phosphatidic acid. Dijksra and Opstal[9] patented one of the early processes to extract phosphatidic acid from soybean oils. However, this process did not support the extraction of pure phosphatidic acid, only producing gums containing high levels of both phosphatidic acid and inorganic salt[9]. Preparation of a

phosphatide mixture using phosphatidase D was also patented[9], but the limited availability of phosphatidase D makes this process not suitable for phosphatidic acid production for commercial applications. Pure phosphatidic acid was obtained using TLC[10], HPLC[11] and extraction with a supercritical solvent[12]. These analytic methods, however, can only produce small quantities of phosphatidic acid for research purposes. One study reported the attempt to chemically synthesize phosphatidic acid[13], but the yield and purity were too low to be applicable for industrial phosphatidic acid production. As such, an efficient process is still needed to prepare large quantifies of phosphatidic acid of high purity. Here we report the development of a new process that permits the effective extraction of phosphatidic acid to high purity (> 99 %) from powdered phospholipids derived soybeans. This process can be readily adapted to industrial phosphatidic acid production at commercial scales.

EXPERIMENTAL

Powered soybean phospholipids (Tsinghua Unisplendour, Weihai China) were washed in five folds (by weight) of 95 % ethanol at 45 ºC for 2 h, with continuous mixing by a stir bar at 100 rpm to remove ethanol-soluble components that can interfere in subsequent phosphatidic acid extraction. After centrifugation at 700 x g for 10 min at room temperature, the supernatant (ethanol fraction) was removed and discarded. This ethanol extraction procedure was repeated four more times.

Following the ethanol extraction, the phospholipid pellets were extracted using methanol (5 folds, by weight) for 12 h at room temperature with gentle mixing by a stir bar. The phosphatidic acid-containing methanol fraction was collected after centrifugation at 700 x g for 10 min at room temperature. The solid residue was extracted using fresh methanol thrice and the methanol extracts were pooled. The pH of the methanol extract was raised to 8-9 using NaOH (0.1 N). Following centrifugation at 700 x g for 10 min at room temperature, the supernatant was collected while the pellets were discarded. The pH of the methanol extract was then lowered to 6 using HCl (0.1 N). One fourth volume (relative to the methanol extract) of hexane was added, mixed well and left to stand still for 2 h to separate the phosphatidic acid-containing hexane phase from methanol phase. After removing the methanol phase, phosphatidic acid was recovered by evaporating off the hexane at 45 ºC in a rotary evaporator. The recovered phosphatidic acid precipitate was redissolved in 60 mL of methanol. One mL of zinc chloride solution (60 %) was added to precipitate the phosphatidic acid. After centrifugation 700 x g at for 10 min at room temperature, the supernatant was discarded and the pellets were retained, washed three times with 200 mL of acetone and dried under a steam of nitrogen gas. At least 0.2 g of crude phosphatidic acid was obtained from 30 g of powdered soybeans phospholipids.

The phosphatidic acid extracted was analyzed by TLC using the TLC plates (10 cm × 20 cm) of Silica gel F_{254}. The mobile phase[14] consisted of chloroformethanol-triethylamine-water (30:35:34:8). A soybean phosphatidic acid (99 % purity, Sigma Chemical Co. Pengyuan, China) was included as the reference. The TLC was allowed to run for 2 h. A bromothymol blue solution (400 mg/L, in 0.01 M sodium carbonate) was used for colour development and visualization as described previously[15]. The R_f of both the extracted phosphatidic acid and the phosphatidic acid reference was determined to be 0.51. As shown in Fig. 1, the extracted soybean phosphatidic acid co-migrated as the phosphatidic acid standard and no other lipid was visible. These results indicate that the extracted phosphatidic acid was free from other lipids.

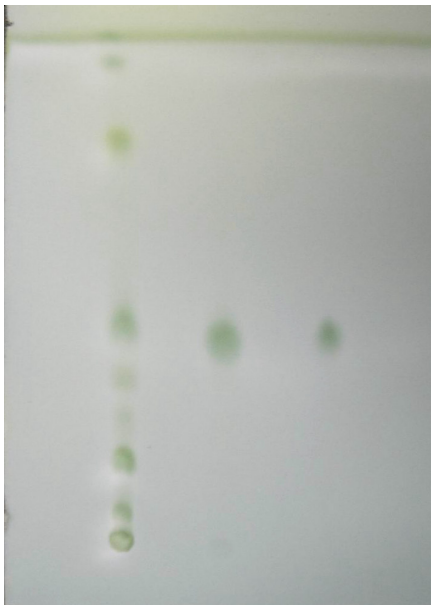
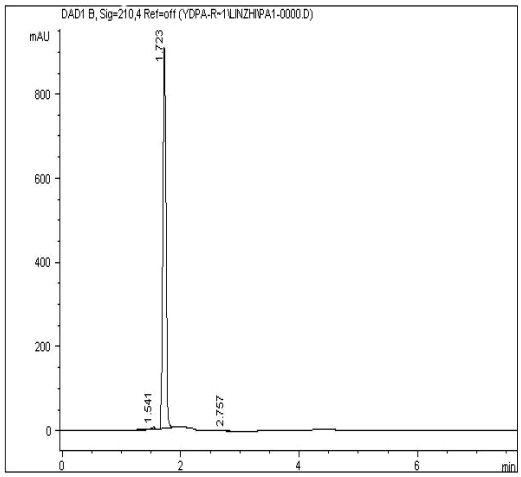


Fig. 1. TLC analysis of the phosphatidic acid extract (1) powered soybean phospholipids (2) phosphatidic acid extract (3) phosphatidic acid reference

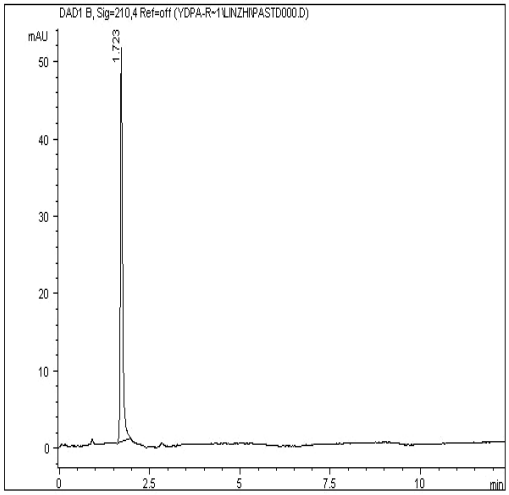
The extracted phosphatidic acid was further confirmed by HPLC analysis using an Agilent HPLC 1100 system (Agilent Technologies, Beijing). The analytical column (4.00 mm × 300 mm, hypersil SiO_2 5 μm) has a silica normal-phase (Dalian Elite Analytical Instruments, China). The detection wavelength was set at 210 nm. Chromatographic separation was carried out using a mobile phase of hexane-isopropanol-H_2O (5:3:0.4) at a constant flow rate of 1.5 mL/min. Exhibiting a single major peak of the same retention time as the phosphatidic acid reference (Fig. 2), the phosphatidic

acid in the phosphatidic acid extract was further confirmed and appeared to be of high purity. Estimated from the peak areas, the purity of the phosphatidic acid extract was *ca.* 99.1 %.



(A)

#	Com Meas. Ret. Time	Main Peak Heiqh	Main Peak Area	Main Peak Area %
1	1.541	4.996	39.456	1.128
2	1.723	912.760	3439.711	98.352
3	2.757	2.078	18.168	0.519



(B)

#	Com Meas. Ret. Time	Main Peak Heiqh	Main Peak Area	Main Peak Area %
1	1.723	51.017	227.572	100.000

Fig. 2. HPLC chromatogram of the phosphatidic acid extract (A) and the phosphatidic acid reference (B)

RESULTS AND DISCUSSION

The fatty acid components of the extracted phosphatidic acid were identified by gas chromatography-mass spectrometry (GC-MS) analysis. Between 20 to 50 mg of dried phosphatidic acid extract was dissolved in 0.75 mL *n*-heptane. Then 0.1 mL of KOH (2 N)-methanol solution was added and mixed for 2 min using a vortexer[13]. Following centrifugation at 1,700 x g for 10 min at room temperature, the upper *n*-heptane phase containing the fatty acid methyl esters was removed and immediately injected into a Finnigan Trace DSQ GC-MS instrument controlled by the Xcalibur software (Thermo Electron Corp.). The methyl esters were separated by a flexible quartz capillary column (length, 15 m; inside diameter, 0.25 mm) using helium as the carrier gas at a flow rate of 1 mL/min (split flow ratio was 1:50). The initial temperature was 110 °C, which was held for 10 min and then the temperature was increased to 220 °C at a rate of 10 °C/min and held for 18 min. The injector temperature was held at 250 °C. Standard electron impact ionization was used at an ion source potential of 70 eV at 230 °C. The methyl esters of the fatty acids were identified by comparing the mass spectra of the sample with those of the library NIST (NIST Mass Spectral Search Program, version 2002). Two fatty acids were identified in the PA extract: stearic acid (C_{18}:0) and oleic acid (C_{18}:1 *cis*-9) (Fig. 3), which account for 43.6 and 56.4 %, respectively.

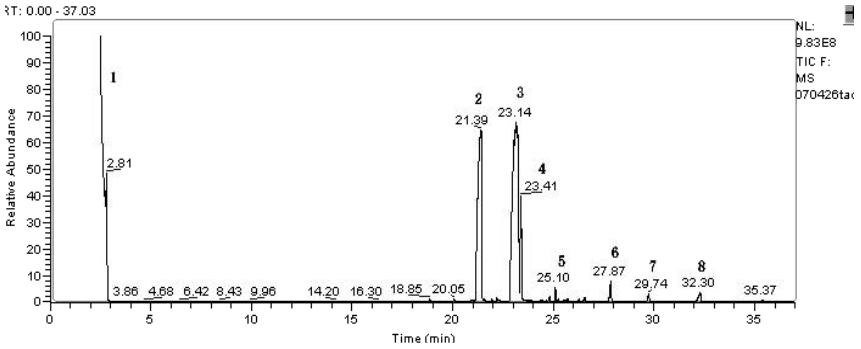


Fig. 3. GC-MS chromatogram of the phosphatidic acid extract. Peak (1) solvent; peak (2) stearic acid (C_{18}:0); peak (3) oleic acid (C_{18}:1 *cis*-9).

The process described here is relatively simple, yet permits efficient extraction of phosphatidic acid to high purity from powdered soybeans phospholipids, a readily available source. This process also improves upon previous processes by increasing yield (0.2 g of phosphatidic acid from 30 g of soybean phospholipids) and purity (> 99 %). This purity is high enough for most applications, including pharmaceutical applications. No special

or sophisticated equipment is needed and all the solvents and chemicals used in the process of phosphatidic acid extraction are inexpensive and commercially available. As such, this process can be readily adapted to industrial production of phosphatidic acid.

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