Purification of phosphatidylcholine from crude soybean lecithin

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Abstract : Phosphatidylcholine (PC) is widely used in supplements and pharmaceutical applications due to its transmembrane signaling property and its being a source of polyunsaturated fatty acids. It is also applied in the encapsulation of drugs and active ingredients. Crude soybean lecithin (CSL), a by-product of soybean oil refinery, contains only ~18% PC, which is not pure enough for pharmaceutical applications. The other substances are phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA) and neutral oil. In this study, different percentages (85%, 90%, 95% and absolute) of ethanol were used for fractionation of CSL to obtain PC-enriched fractions. Effects of CSL to ethanol ratios (1: 1, 1: 2 and 1: 3 (w/ v)) and temperature (ambient (~ 26°C), 40, 50 and 60°C) on fractionation of PC were studied. Normal phase high performance liquid chromatography (NP-HPLC) with UV detector was used for identification and quantification of the phospholipids. It was found that 1: 1 of CSL to absolute ethanol at 40°C gave the highest purity of PC enriched fraction (64.68% PC; 6.61% PE; 1.21% PI and 21.72% neutral oil). Further purification of PC was performed by adding different ratios of water to PC-enriched fraction (0.1, 0.2, 0.3, 0.4 and 0.5 to 1.0, v/v). Results showed that 84.11% purity with 19.77% yield of PC could be obtained by using 0.2 ml of water to 1 ml of PC-enriched fraction. This method is considered appropriate for industrial PC purification because it used environmental friendly and acceptable solvent for food.

Keywords : Crude Lecithin, Ethanol, Phosphatidylcholine, Purification, Water

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1. Introduction

Lecithin is a group of naturally occurred phospholipids (PL) presents in association with neutral lipids (oil) in egg yolk and vegetable oilseeds such as sunflower and soybean. Commercial lecithin is typically produced from crude lecithin or gum, a by-product of the degumming processing of soybean oil due to its lower production cost. Crude soybean lecithin is a complex mixture. It comprises of PL containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA) triglyceride and free fatty acid. Deoiled soybean lecithin has been widely used as emulsifiers, viscosity regulators, anti-spattering and dispersing agents in food and cosmetic industries [1]. In addition, lecithin is used as a food supplement owing to its major component – PC - which provides the choline, a mediator for transmembrane signaling and a source of polyunsaturated fatty acids (PUFA) [2]. Furthermore, due to the amphiphilic characteristic of PC, it is able to form vesicles or liposomes that can be used in the encapsulation of drugs, protein or peptide antigens [3]. Highly purified PC (≥85% purity) is used in pharmaceutical applications [4].

Various methods have been used for purifying PC, including solvent extraction [1, 3, 5-8], supercritical fluid extraction (SFE) [9-10], and column chromatography [8, 11-12]. Column chromatography and SFE are not only considered to be high efficient separation techniques but also have low solvent residues. However, these techniques have the least industrial possibility due to the high cost of instrument and chromatographic media. Solvent extraction is considered more suitable for industrial application due to its being relatively inexpensive and easy to scale up. Among several solvents including ethanol [5, 6], methanol [13], iso-propanol [8], butanol/chloroform [14], and hexane [3, 15], ethanol is the most preferred for lecithin fractionation. PC is more soluble in ethanol than other phospholipids; thus, ethanol extract is assigned as a PC-enriched fraction [6]. Insoluble fractions contain a high content of PI and are defined as PI-enriched fractions. The remaining substances of both fractions include oil and PE. Fractionation of lecithin with ethanol depends on several parameters such as extraction time, crude lecithin to ethanol ratio and temperature [8]. However, ethanol extraction method reported in the previous studies stopped low purity of PC or needed to combine with other methods to get high purity. The polarity of solvent and co-solvents affects PC extraction. The effect of solvent ratio of ethanol to isopropyl alcohol was studied. The increased yield and purity of PC was achieved by combination of hexane and acetonitrile as the solvent system for partitioning [11]. Thus, the ethanol extraction method could be improved by using co-solvent for polarity adjustment. So, polarities of phospholipids are related to their chemical structures and hydratability [16]. PC is composed of choline or trimethylamine group which shows positive charge and hydrates at all pH. PE consists of ethanolamine group which shows lower polarity than PC. The positive charge of the amine group and the negative charge of the phosphate group can form an internal salt. This salt has no net charge and gives poor hydration. PI is also hydratable due to its five hydroxyl groups of inositol [17]. Polarity of ethanol can be heightened by adding water as co-solvent. Thus, added water in PC-

enriched fraction caused more hydration of PC in this phase. Meanwhile, the non-polar neutral oil tended to be immiscible and separated from the PC-enriched fraction.

In this study, effects of concentration and volume of ethanol and temperature on the crude soybean lecithin fractionation were studied. The suitable condition which gave highest PC in PC-enriched fraction was selected. Further purification of PC was done by adding water as co-solvent to the PC-enriched fraction, and the ratio of water to the PC-enriched fraction was studied.

2. Methodology

2.1 Chemicals

Acetonitrile and methanol were HPLC grade. Ethanol, chloroform, acetone and 85% phosphoric acid were AR grade purchased from RCI Lab-Scan Co., Ltd. (Thailand). PC, PE, PI and lyso-phosphatidylcholine (LPC) standards (approx. 99% purity) were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). Crude soybean lecithin (CSL) was obtained from Industrial Enterprises Co., Ltd. (Thailand).

2.2 Crude lecithin fractionation with various concentrations of ethanol at various ratios of crude lecithin to ethanol

In the first set of experiments, the fractionation was performed by using different concentrations of ethanol (85, 90, 95 and absolute). One gram of CSL was weighted into each 10 ml centrifuge tube, and then 1 ml (concentration previously described) of ethanol was added. The sample was mixed by vortex mixer for 3 min and centrifuged at 2500 × *g* for 10 min. The upper phase (ethanol phase or PC-enriched fraction) and lower phase (PI-enriched fraction) were analyzed by normal phase high performance liquid chromatography (NP-HPLC). The optimum ethanol concentration which gave the highest PC in the PC-enriched fraction was selected for further experiments. The optimum ratio between CSL to selected ethanol concentration was varied from 1: 1 to 1:3 (w/v).

2.3 Effect of temperature on crude lecithin fractionation

One gram of CSL was weighted into each 10 ml centrifuge tube, and then the suitable concentration and ratio of ethanol were added. The sample was mixed by vortex mixer and incubated at ambient temperature (26°C), 40, 50 and 60°C for 3 min. The sample was then further mixed by vortex mixer for 1 min and centrifuged at 2500 × *g* for 10 min. The upper and lower phases were analyzed by NP-HPLC. The optimum temperature which gave highest PC in the PC-enriched fraction was selected for further purification of PC.

2.4 Purification of PC from PC-enriched fraction using different ratios of water

One milliliter of the PC-enriched fraction derived from optimum condition (method 2.2) was added into each 10 ml centrifuge tube, and then five ratios (0.1, 0.2, 0.3, 0.4 and 0.5 ml) of deionized water were added. The samples were mixed by vortex mixer for 3 min and then centrifuged at 2500 × *g* for 10 min. The upper and lower phases were analyzed by NP-HPLC. A flow chart of the crude soybean lecithin fractionation procedure is outlined in Fig. 1.

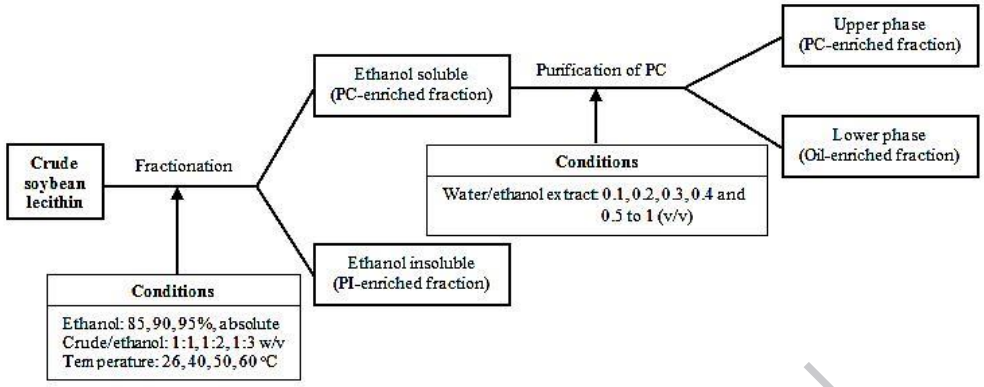

Fig.1 Flow chart of the crude soybean lecithin fractionation procedure

2.5 Determination of phospholipids by NP-HPLC

The HPLC system consisted of a 515-model pump (Waters Associates, Milford, MA, USA), a Rheodyne 7125 valve injector, a 10-µl loop (Cotati, USA) and a SPD-M20A photodiode array detector (Shimadzu, Japan) equipped with a semimicro-cell and operated at a wavelengths of 205 nm. The samples were dissolved in chloroform, filtered (0.45 µm) and analyzed by NP-HPLC according to Loapaiboon and Krisnangkura [18]. The column was µPorasil (300 mm × 3.9 mm.ID., 10 µm; Waters, Ireland) with Mightysil Si60 (4.6 mm × 5 mm; Kanto, Japan) guard column operated at ambient temperature (23-26°C). The isocratic mobile phase was acetonitrile-methanol-water-85% phosphoric acid (100: 1: 1: 1.5, v/v/v/v) and the flow rate was 1.0 ml/min. It was filtered and degassed in an ultrasonic bath for 15 min prior to use. Identification of each PL was done by comparison to the retention time of each standard. The PL was quantified against the standard curve, and the quantity of each PL standards to the total peak area of the UV absorbance was known. The yield of PC was calculated according to equation [1]:

$$\text{Yield of PC} = 100 \times \frac{\text{the amount of PC in the fractionated material}}{\text{the amount of PC in crude lecithin}} \quad [1]$$

3. Results and Discussion

The composition of CSL determined by NP-HPLC was 45.73% neutral oil, 22.85% PC, 21.13% PI and 9.90% PE. There is considerable variation in the composition of CSL, because of the difference in breeds and techniques of the degumming process. Meeren et al. [19] reported that the lipid profile of crude lecithin was 43.1% neutral lipids, 10.2% PE, 10.6% PI and 18.6% PC.

3.1 Effect of ethanol concentration and CSL/ ethanol ratio on fractionation of PC

The purity and yield of PC in PC-enriched fraction were affected by both ethanol concentration and CSL/ethanol ratio. The composition and yield of PC in PC-enriched fraction and PI-enriched fraction are presented in Table 1. At different ethanol concentrations, the yield of PC in PC-enriched fraction ranged from 13.02 to 34.60 %. The composition of PC- and PI-enriched fraction was affected by ethanol concentration. The lowest ethanol concentration (85%) gave the highest percentage of PC in the PC-enriched fraction. Although, high purity of PC was observed by using 85% ethanol, more PI, LPC and the least yield of PC was observed in the PC-enriched fraction. Absolute ethanol provided the highest yield of PC in the PC-enriched fraction and the lowest PC residue in the PI-enriched fraction. However, the purity of PC was decreased due to increasing oil content. This result was similar to the study of Wu and Wang [6] who reported that the highest concentration of ethanol could result in the best fractionation of PC.

Table 1 Composition* and yield of PC when using various concentrations of ethanol to extract PC from CSL.

Compositions (%) of PC-enriched fraction	Ethanol concentration (%, v/v)			
	85	90	95	absolute
Oil	3.50±0.67	12.38±1.09	17.83±1.52	21.72±1.13
PI	2.73±0.93	1.73±0.64	1.02±0.48	1.21±0.56
PE	5.75±0.65	5.84±0.46	6.25±0.00	6.61±0.20
PC	75.31±2.10	71.20±2.66	67.94±1.64	64.68±1.21
LPC	8.71±0.91	5.78±0.55	4.59±0.18	3.43±0.07
Others	3.99±1.04	3.07±1.23	2.37±0.18	2.36±0.36
Yield of PC	13.02±0.53	18.95±0.66	29.58±0.89	34.60±0.69
Compositions (%) of PI-enriched fraction	Ethanol concentration (%, v/v)			
	85	90	95	absolute
Oil	46.37±0.58	39.96±1.05	35.47±1.39	42.77±1.02
PI	22.03±0.95	25.84±0.64	27.08±0.45	26.21±0.59
PE	9.57±0.64	10.80±0.49	12.74±0.03	10.86±0.35
PC	19.53±2.30	20.33±2.93	21.49±1.45	17.16±1.29
LPC	0.54±0.25	0.68±0.35	1.05±0.06	0.63±0.04
Others	1.97±0.80	2.38±1.06	2.17±0.70	2.36±0.50
Yield of PC	64.49±0.99	63.45±0.79	66.71±0.58	52.26±0.74

*mean value and SD (n=3)

The effect of CSL to ethanol ratio is presented in Table 2. The yield of PC in PC-enriched fraction tends to increase with an increase in the ethanol ratio. On the other hand, other impurities such as PI, PE and LPC had little difference. Although 1: 2 and 1: 3 ratios of CSL to ethanol showed the high yield of PC in PC-enriched fraction, the percentage of oil was also high. This higher oil content made lower purity PC in PC-enriched fraction. It is possible that ethanol is able to dissolve PC but it is also trapped by the oil. The partial extraction at the ratio of 1:1 CSL to ethanol presented in the highest purity of PC was selected for further purification.

Table 2 Composition* and yield of PC when using various ratios of crude lecithin to ethanol.

Component	Composition (%) of PC-enriched fraction			Composition (%) of PI-enriched fraction		
	Ratio of CSL to ethanol			Ratio of CSL to ethanol		
	1: 1	1: 2	1: 3	1: 1	2: 1	3: 1
Oil	21.72±1.13	30.40±1.35	35.72±1.49	42.77±1.02	34.74±1.23	33.29±1.45
PI	1.21±0.56	1.37±0.10	1.32±0.23	26.21±0.59	33.56±0.75	35.85±0.36
PE	6.61±0.20	7.50±0.14	7.13±0.62	10.86±0.35	13.93±0.36	14.02±0.21
PC	64.68±1.21	57.13±0.71	52.62±0.32	17.16±1.29	14.47±0.65	13.00±0.28
LPC	3.43±0.07	2.48±0.07	2.33±0.17	0.63±0.04	0.76±0.06	ND
Others	2.36±0.36	1.13±0.33	0.88±0.15	2.36±0.50	2.54±0.44	3.85±0.57
Yield of PC	34.09±1.75	45.17±1.67	42.69±1.58	52.35±1.54	42.54±1.47	38.48±1.08

*mean value and SD (n=3), ND = not detected

3.2 Effect of temperature on CSL fractionation

Fig. 2 shows that the purity of PC was not affected by extraction temperature. Although higher temperature showed higher yield of PC, it was not significantly different. Nevertheless, the viscosity of CSL was decreased by increasing extraction temperature that caused quick homogeneity. The PC-enriched fraction at 40°C of CSL with absolute ethanol at 1: 1 ethanol to CSL ratio were composed of 63.97% PC, 7.5% PE, 1.64% PI, 22.14% oil and 2.54% LPC.

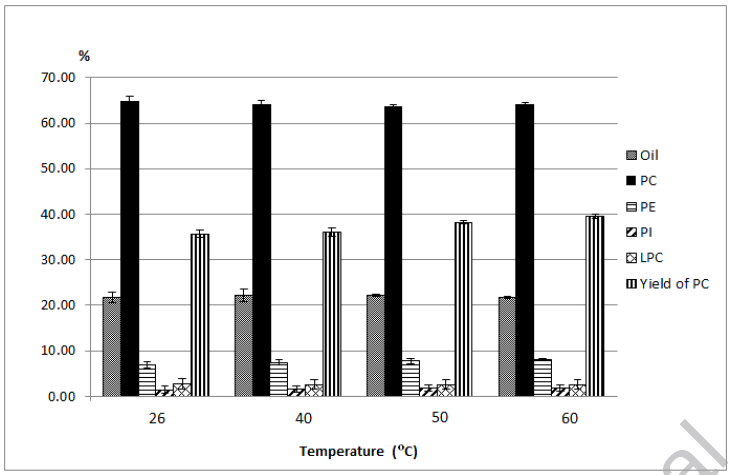

Fig. 2 Compositions and yield of PC-enriched fraction extracted from CSL with absolute ethanol at different temperatures.

It was found that only 64 to 65% PC was obtained by using absolute ethanol as extractor for CSL. In order to obtain higher purity of PC, further purification of PC-enriched fraction was needed.

3.3 Effect of water to PC-enriched fraction ratio on the purity of PC

When different contents of water (0.1-0.5 ml) were added into the PC-enriched fraction (1 ml), the samples were separated into upper (ethanol soluble) and lower (oil) phases as shown in Table 3. The ethanol solution was made to be more polar by adding a small amount of water (0.1-0.5 ml) into the PC-enriched fraction, which caused more hydration of PC in this phase. Meanwhile, the non-polar neutral oil tended to be immiscible in the more polar phase and separated into the lower phase. Addition of 0.2 and 0.3 ml water to 1 ml of PC-enriched fraction shows similar purity of PC and barely detectable oil. Although at 0.3 ml water showed higher yield of PC, higher content of LPC was also observed. More LPC was found in the upper phase than in the lower phase. This result was in agreement with Wu and Wang [6] who suggested that the LPC is more polar, so it is more easily trapped in the PC-enriched fraction. Increasing water content in the PC-enriched fraction also led to the increase of solubilized LPC in the ethanol phase. Subsequently, the polarity of ethanol solution was changed. Thus, some oil (4-10%) was found in the upper phase at higher amounts of water (0.4 and 0.5 ml). Therefore, purification of PC with 0.2 ml of water to 1 ml of PC-enriched fraction was considered to be the optimum condition. The 84.11% purity of PC with 19.77% yield of PC in the upper phase was obtained.

Table 3 Composition* and yield of PC after purifying the PC-enriched fraction with various ratios of water.

Composition (%) of upper phase	Ratio of water to PC-enriched fraction					
	Control	0.1: 1	0.2: 1	0.3: 1	0.4: 1	0.5: 1
Oil	22.14±1.34	5.36±0.47	0.45±0.78	0.00±0.00	4.53±1.00	10.88±1.08
PE	7.51±0.54	7.44±0.63	5.18±0.35	4.31±0.37	3.81±0.37	4.21±0.40
PC	63.97±0.97	76.92±0.83	84.11±1.45	80.94±1.08	74.74±0.62	72.75±0.19
LPC	2.54±1.01	5.04±0.56	6.83±0.52	10.67±0.29	12.15±0.24	11.27±0.73
Others	3.84±0.33	5.24±0.16	3.43±0.48	4.08±0.46	4.77±0.33	0.89±0.39
Yield of PC	36.10±0.90	8.90±0.78	19.77±0.02	32.63±0.47	7.51±0.79	6.23±0.34
Composition (%) of lower phase	Ratio of water to PC-enriched fraction					
		0.1: 1	0.2: 1	0.3: 1	0.4: 1	0.5: 1
Oil		35.30±0.78	24.28±0.97	21.20±0.04	20.44±1.34	20.48±1.17
PI		1.80±0.09	1.12±0.98	0.94±0.17	0.78±0.34	0.93±0.05
PE		11.03±0.25	11.27±0.63	11.40±0.42	10.82±0.34	10.79±0.51
PC		46.36±1.32	56.27±0.94	58.72±1.35	60.58±0.87	60.86±0.45
LPC		1.74±0.04	2.09±0.23	2.35±0.25	2.71±0.17	3.22±0.15
Others		3.78±0.55	4.96±0.32	5.39±0.52	4.66±0.48	3.72±0.44
Yield of PC		18.93±0.56	32.32±0.14	41.55±0.37	65.75±0.89	54.39±1.08

*mean value and SD (n=3)

Some PC can be lost from the upper phase and trapped in the oil because of its hydrophobic part. PE is less polar than PC, allowing it to be more easily trapped by oil in the lower phase. Segers and Sande [16] suggested that because hydratability rate of PE is lower than PC, the hydratability of each phospholipid is related to their chemical structure. PC consists of trimethylamine group, which may cause PC to be well-hydrated. PE is poorly hydrated because it forms an internal salt with a six-atom ring between the dissociated phosphate group and the protonated amino group [17]. So, this salt has no net charge and may therefore cause PE to have low polarity. Purification of PC from PC-enriched fraction with water is an effective method for separating PC from oil and PE. The oil and PE were removed together in a convenient step and a purity of over 84% PC was achieved. In addition, an advantage of this method is to avoid using inflammable toxic acetone and there is no need for low temperature for precipitation.

4. Conclusion

High purity (84.11%) of PC was obtained within 2 simple steps of purification of CSL. The CSL was fractionated by using 1:1 (w/v) of CSL to absolute ethanol at 40 $^{\circ}$C. Then, further purification of the PC-enriched fraction was achieved by addition of 0.2 ml of water to 1 ml PC-enriched fraction to remove the oil and PE. This method is considered appropriate for industrial purification of PC due to its use of environmental friendly and acceptable solvents for food.

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