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ORIGINAL PAPER

β-Cyclodextrin-Mediated Removal of Soy Phospholipids from the Air–Water Interface

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Abstract Binding of soy phosphatidylcholine (PC) and phosphatidylinositol (PI) with β-cyclodextrin (βCD) was studied using a spread monolayer technique at the air–water interface. First, surface pressure (π) versus surface concentration (Γ) isotherms of both PC and PI were characterized by forming spread monolayers on an aqueous subphase. PC and PI monolayers reached saturation at Γ of 1.98 and 3.24 μmol/m^2, respectively, at 25 °C. Subsequently, desorption of PC or PI from the spread monolayer in the presence of 2–14 mM βCD in the subphase was studied by measuring changes in π of monolayer. This desorption was indicative of a complex formation between βCD and PC or PI. The amount of PC or PI bound to βCD was determined by converting the net change in π to Γ by using π–Γ isotherms. From the saturated monolayers at the air water-interface, approximately 30% of PC and 50% of PI could be removed by 14 mM βCD. It was calculated that the free energy change required to transfer a PL from the monolayer at air–water interface to the aqueous phase in presence of βCD was decreased by 6–7 kcal/mol. Hydrolysis of PC in the monolayer by phospholipase A_2 (PLA_2) improved extraction efficiency of βCD. By incorporating 2.29 μM PLA_2 and 10 mM βCD in subphase, up to 80% of PC monolayer could be desorbed from the air–water interface. These results are discussed in terms of the potential use of βCD to remove PLs bound to soy protein.

Keywords Soy phospholipids · β-Cyclodextrin · Soy protein · Off-flavor · Spread monolayer

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Introduction

In soy protein (SP), residual phospholipids (PLs) are one of the main precursors of off-flavors. Several studies have demonstrated that oxidative deterioration of ester-bound polyunsaturated fatty acids (PUFA) in soy PLs generates volatile and non-volatile compounds that cause objectionable beany odor and bitter taste [1–5]. Not surprisingly, the majority of treatments developed to overcome off-flavor in freshly isolated SP have focused either on reducing or masking end-products of PL oxidation [6]. Moderate to modest gains in improving SP flavor have been achieved via such approaches. However, autoxidation of PUFA of residual PLs during storage or processing of treated SP yields additional off-flavor compounds. This makes it extremely difficult to obtain bland SP for food applications. Therefore, any comprehensive solution for flavor improvement of SP must address removal of both phospholipids as well as by-products of PL oxidation.

Association of PLs with soy protein, oleosin, presents a major hurdle to their removal. It has been shown that oleosin–PL complexes remain intact during SP extraction from soybean and are isolated along with purified SP [7]. Previously, removal of PLs from SP has been attempted using solvents like aqueous alcohols (78–97%, v/v) [8]; hexane/alcohol azeotropes [9, 10]; methylene chloride, and aqueous acetone [11]. While these treatments proved very effective in reducing PL levels and concomitantly improving SP flavor, exposure to solvent resulted in severe protein denaturation and subsequent loss of solubility [9, 10, 12]. Thus, solvent treatment is not particularly suited to prepare food-grade SP. Mixtures of supercritical carbon dioxide (SC-CO_2) and ethanol (EtOH) have also been described to extract PLs from soybean flakes [13, 14]. PL extraction by this method has proved very selective.

While phosphatidylcholine (PC) and phosphatidylethanolamine (PE) could be readily solubilized using SC-CO_2/EtOH, phosphatidylinositol (PI) and phosphatidic acid (PA) showed limited solubility in the same solvent. Recently, salting out of PL-containing SP fraction, oleosins, has been described [7, 15]. However, use of strong kosmotropic salts (ammonium sulfate or sodium sulfate) also caused a significant loss of storage proteins as they salted out along with oleosins. Therefore, it is quite evident that a milder treatment that could effectively remove SP-bound PL without alteration of protein structure or functionality would be beneficial in addressing the off-flavor problem in SP.

In a previous report, we demonstrated that β-cyclodextrin (βCD) forms an inclusion complex with 2-nonanone and suggested that this approach could be equally effective in removing SP bound off-flavor volatiles with an alkyl chain of medium length (less than nine carbons) [16]. Here, we extend the same argument by hypothesizing that βCD could form inclusion complexes with SP-bound PLs and effectively extract them into the aqueous phase.

Soy PLs, such as PC, PE or PI, etc., consist of a polar head group and two fatty acids (FA) attached to a glycerol backbone. FA acyl chains are non-polar and have a cross-sectional diameter of $\sim$4.0 Å [17], which would likely facilitate their inclusion in the hydrophobic cavity (diameter = 6–6.5 Å) of βCD. This assertion is supported by several experimental studies, which have shown that βCD can form complexes with lipids containing single or double alkyl chains of variable length [18–25].

To test our hypothesis, a model surface monolayer study was conducted wherein desorption of soy PLs from the air–water (a/w) interface was studied in the presence of βCD in the aqueous subphase. The rationale behind this approach was based on assuming energetic equivalence between transferring PLs from the air (hydrophobic phase) into the aqueous phase and removing PLs from the local hydrophobic environment of SP (oleosin)–PL complex. Therefore, if βCD could extract soy PLs from the a/w interface into water, it can be argued that βCD can overcome a similar energy barrier to remove SP–bound PLs via formation of an inclusion complex.

Materials and Methods

Food grade βCD (Cavitron 82800) was donated by Cargill Inc. (Minneapolis, MN). Lyophilized soybean phospholipids L-α-phosphatidylcholine (PC) and L-α-phosphatidylinositol sodium salt (PI) with a purity of approximately 99% were purchased from Sigma-Aldrich Corp. (St. Louis, MO). According to the supplier, the fatty acid composition of the soy PC was 14% palmitic acid (16:0), 4% stearic acid (18:0), 12% oleic acid (18:1), 65% linoleic acid (18:2), and 5% linolenic acid (18:3). The soy PI contained primarily linoleic and palmitic acids. Based on these compositional data, the average molecular weights of soy PC and PI were calculated as 776 and 857 g/mol, respectively. These values are in close agreement with those reported in the literature for soy PC and PI [26]. Stock solutions of PC (25 mg/mL) and PI (5 mg/mL) were prepared in $CHCl_3$ (99.9%, HPLC Grade; Sigma-Aldrich Corp.) by vortexing and storing at −20 °C and warming at ambient temperature before use. Phospholipase A_2 (PLA_2) from porcine pancreas, with a stated activity of >10,000 U/mL, was obtained from Sigma-Aldrich Corp. (St. Louis, MO). Deionized water from a Milli-Q ultrapure water system (Millipore Corp., Bedford, MA) with a resistivity of 18.2 mΩ cm was used in all experiments. All other chemicals used were of reagent grade.

π–Γ Isotherms of Phospholipids

In a typical experiment, 959 mL of 10 mM Tris–HCl buffer (pH 7.4) containing 15 mM NaCl was used as the subphase and placed in a thermostated Teflon trough ($35 \times 7 \times 3.7$ cm^3) maintained at 25 °C. Aliquots of PC or PI stock solution (0.5 mg/mL in $CHCl_3$) were spread in increments of 10 μL on the subphase until a saturated monolayer was reached. Surface concentration, Γ (μmol/m^2), of PLs was calculated using the amount spread at the interface, the total interfacial area of the trough (0.0245 m^2) and the average molecular weights of 776 and 857 g/mol for PC and PI, respectively. The surface tension (γ) at the air–water interface was monitored using a Wilhelmy plate hanging from an electrobalance (Cahn Instruments Co., Escondido, CA). The change in γ in response to an incremental increase in Γ (μmol/m^2) of PLs was reported as surface pressure (mN/m), and calculated as follows:

$$\pi = \gamma_0 - \gamma \quad (1)$$

where γ_0 and γ are surface tension readings at the a/w interface of pure buffer and buffer with spread PLs, respectively. All measurements were reported for equilibrium conditions as determined by a change in π by no more than 0.2 mN/m over a period of 30 min. The data thus obtained were plotted as π–Γ isotherm and fitted with mathematical models using the Sigma Plot software (version 9.0, Systat Software Inc., San Jose, CA). A minimum of three independent measurements were carried out for each experiment. All experiments were performed in a glove box (Cole-Palmer Instruments Co., Vernon Hills, IL) to prevent surface contamination and air drafts and to maintain a relative humidity of at least 90%.

Spread Monolayer Study of Soy PLs in the Presence of βCD

Saturated monolayers of PLs were formed by spreading 74 μL of 0.5 mg/mL PC ($\Gamma = 1.98$ μmol/m^2) or 130 μL of 0.5 mg/mL PI ($\Gamma = 3.24$ μmol/m^2) on the air–buffer interface. These Γ values correspond to the inflection points, i.e., the beginning of a saturated monolayer, in the π–Γ isotherms. βCD (0–14 mM) was included in the subphase prior to formation of monolayers. A βCD solution with a 14-mM concentration was prepared by heating the solution to 50 °C and then cooling it to 25 °C. The solution thus prepared remained clear with no sign of aggregation of βCD during the course of the experiment. The subphase was stirred gently and the change in π of these monolayers was monitored continuously until a new equilibrium was achieved. It should be noted that βCD itself is not surface active even at higher concentrations [27], and therefore, cannot influence π of a pure interface. We have confirmed this fact in our experiments as well. New equilibrium values of π in the presence of βCD were then converted to surface concentration (Γ) values for PC and PI using the π–Γ isotherms. Any change in Γ of PC or PI observed during the period of equilibration with βCD was taken as the amount of PL desorbed into the bulk phase in the form of βCD–PL complex.

Spread Monolayer Study of Soy PC in the Presence of βCD and PLA_2

The effect of enzymatic hydrolysis of PL on desorption efficiency of βCD was probed by including PLA_2 in the bulk phase. Experimental conditions were chosen to optimize the performance of PLA_2. Briefly, a saturated monolayer of soy PC was formed by spreading 120 μL of 0.5 mg/mL PC stock solution on an aqueous surface (pH 7.0, 10 mM $CaCl_2$) at 37 °C. This corresponded to monolayer coverage of 3.25 μmol/m^2 or 51.08 Å^2 interfacial area per molecule. A higher coverage was used in these experiments than in the βCD only experiments (see above) in order to observe the full effect of PLA_2 on desorption of PL by βCD. After forming the saturated monolayer, 100 μL of PLA_2 (10,000 U/mL) was injected into the bulk phase through a side port, without penetrating the monolayer. The final concentration of PLA_2 in the bulk phase was 2.29 μM (0.104 U/mL). PC hydrolysis at the a/w interface was followed by monitoring changes in surface pressure (π) [28]. The experiment was repeated by spreading a saturated monolayer of soy PC ($\Gamma = 3.25$ μmol/m^2) in the presence of 10 mM βCD in the subphase and the system was allowed to reach an equilibrium π. Thereafter, 100 μL of PLA_2 (10,000 U/mL) was injected into the bulk phase. Surface pressure was monitored continuously until it reached a new equilibrium value. The equilibrium π values were converted to Γ values using the π–Γ isotherm as before.

Analysis of Binding Data

The interaction of phospholipids with βCD cannot be analyzed using the traditional solution-phase complexation process. The complication arises from the fact that PLs have poor water solubility and βCD lacks the ability to penetrate the PL monolayer/bilayer assembly [23, 29]. Debouzy et al. [30] described α-cyclodextrin (αCD)-mediated extraction of soy PI from the bilayer to the aqueous phase as a two-step process. First, αCD pulls the polar heads of PI into the aqueous phase and partially exposes the *sn*-2 acyl chains of PI, which are subsequently expelled from the bilayer due to the thermodynamic need of the bilayer to maintain its continuity. In the second step, the *sn*-2 acyl chains of the PI molecules are incorporated into the cavity of αCD. Regardless of the actual mechanism by which cyclodextrins extract PL from a bilayer or monolayer, the process can be generally depicted as a two-step process. First, PL from the monolayer ($PL_{Monolayer}$) is desorbed into the solution phase, followed by inclusion of solution phase PL ($PL_{Solution}$) into the βCD cavity to form a water soluble complex (PL•βCD). This two-step process can be represented as follows:

$$[PL]_{Monolayer} \overset{K_1}{\longleftrightarrow} [PL]_{Solution} \overset{K_2}{\longleftrightarrow} [PL \cdot \beta CD]_{Solution} \quad (K_3) \tag{2}$$

where, K_1 and K_2 are the step-wise equilibrium constants, and K_3 is the overall equilibrium constant. K_1, K_2 and K_3 can be represented as:

$$K_1 = \frac{[PL]_{Solution}}{[PL]_{Monolayer}} \tag{3}$$

$$K_2 = \frac{[PL \cdot \beta CD]_{Solution}}{[PL]_{Solution}[\beta CD]} \tag{4}$$

$$K_3 = \frac{[PL \cdot \beta CD]_{Solution}}{[PL]_{Monolayer}[\beta CD]} \tag{5}$$

In Eqs. 4–5, [βCD] represents the free concentration of βCD remaining in the bulk phase after complex formation. Substituting the values of $[PL]_{Monolayer}$ and $[PL]_{Solution}$ in Eq. 5,

$$K_3 = K_1 K_2 \tag{6}$$

Since, PL in the monolayer is practically insoluble in the aqueous phase, it can be considered as an independent homogenous phase that acts a reservoir of PL. In such a phase, the activity of PL, i.e., $a_{PL\text{-}monolayer}$, would be unity regardless of the amount of PL at the surface [31]. For long chain PL, $[PL]_{Solution}$ at equilibrium with a PL monolayer

or bilayer is equal to its critical bilayer (or monolayer) concentration (CBC), which is of the order of 10^{-10} M for PL [17]. Substituting, these values of $[PL]_{Monolayer}$ and $[PL]_{Solution}$ in Eq. 3, K_1 is essentially CBC, which is of the order of 10^{-10} M.

For estimating K_2 by Eq. 4, [PL·βCD] and [βCD] need to be determined. Assuming that the inclusion complex [PL·βCD] is completely solubilized in the subphase, [PL·βCD] can be calculated from the amount of PL depleted from the monolayer as follows [32]:

$$[PL \cdot \beta CD] = (\Gamma_I - \Gamma_F)\frac{A}{V} \quad (7)$$

where, A is the area of the a/w interface (m^2), V is the volume of the aqueous bulk phase (equal to 0.959 L in this case), Γ_I and Γ_F are the initial and final surface concentration, i.e., in the absence and in the presence of βCD in the subphase, respectively, of PL at the a/w interface. For a 1:1 complex formed between PL and βCD, the [βCD] can be estimated from $[\beta CD]_{Total}$ and [PL·βCD]. That is,

$$[\beta CD] = [\beta CD]_{Total} - [PL \cdot \beta CD] \quad (8a)$$

If a 1:2 complex formation between PL and βCD is assumed, then Eq. 8a can be modified as

$$[\beta CD] = [\beta CD]_{Total} - 2[PL \cdot \beta CD] \quad (8b)$$

However, since experimental [PL·βCD] was at least five orders of magnitude lower than $[\beta CD]_{Total}$ (see Tables 1, 2), for all practical purposes $[\beta CD] \approx [\beta CD]_{Total}$. Substituting values of [PL·βCD], $[PL]_{Solution}$ (which is same as CBC), and [βCD] in Eq. 4, K_2 can be estimated. Since $[PL]_{Solution}$ is equal to CBC, it remains a constant during the extraction process so long as a PL monolayer exists at the a/w interface. Finally, the free energy change (ΔG_3) for the transfer of PL from the monolayer to βCD can be calculated as:

$$\Delta G_3 = -RT\ln K_3 = -RT\ln K_1 K_2 \quad (9)$$

Results and Discussion

π–Γ Isotherms of Soy Phospholipids

The π–Γ isotherms of PC and PI on pure buffer are shown in Fig. 1. The data for soy PI are in agreement with those obtained previously under identical conditions [33]. Similar validation for soy PC could not be done as there is no report in the literature on the spread monolayer of soy PC. Some notable differences were observed in π–Γ isotherms

Table 1 Binding parameters for βCD complexation with soy PC

$[\beta CD]_{Total}$ (mM)	$[\Gamma]_{PC,\ Initial}$ (mol/m^2)	$[\Gamma]_{PC,\ Final}$ (mol/m^2)	$[PC{\cdot}\beta CD]_{Solution}$ (mol/L)	$[\beta CD]_{Free} = [\beta CD]_{Total} - [PC{\cdot}\beta CD]$ (mol/L)	K_1 (M)	K_2 (M^{-1})	K_3	ΔG_3 (kcal/mol)	Ratio $[PC{\cdot}\beta CD]_{solution}/[\beta CD]_{Free}$
3	3.25E−06	1.53E−06	4.39E−08	0.003	1.00E−10	1.46E+05	1.46E−05	6.57	1.46E−05
6	3.25E−06	1.47E−06	4.55E−08	0.006	1.00E−10	7.59E+04	7.59E−06	6.96	7.59E−06
8	3.25E−06	1.43E−06	4.63E−08	0.006	1.00E−10	7.72E+04	7.72E−06	6.95	5.79E−06
10	3.25E−06	1.39E−06	4.73E−08	0.010	1.00E−10	5.92E+04	5.92E−06	7.10	4.73E−06
12	3.25E−06	1.40E−06	4.72E−08	0.012	1.00E−10	4.72E+04	4.72E−06	7.24	3.93E−06
14	3.25E−06	1.40E−06	4.72E−08	0.014	1.00E−10	3.93E+04	3.93E−06	7.34	3.37E−06

Table 2 Binding parameters for βCD complexation with soy PI

$[\beta CD]_{Total}$ (mM)	$[\Gamma]_{PI,\ Initial}$ (mol/m^2)	$[\Gamma]_{PI,\ Final}$ (mol/m^2)	$[PI{\cdot}\beta CD]$ (mol/L)	$[\beta CD]_{Free} = [\beta CD]_{Total} - [PI{\cdot}\beta CD]$ (mol/L)	K_1 (M)	K_2 (M^{-1})	K_3	ΔG_3 (kcal/mol)	Ratio $[PI{\cdot}\beta CD]/[\beta CD]_{Free}$
2	3.25E−06	2.06E−06	3.03E−08	2.00E−03	1.00E−10	1.51E+05	1.51E−05	6.55	1.51E−05
4	3.25E−06	1.97E−06	3.24E−08	4.00E−03	1.00E−10	8.10E+04	8.10E−06	6.92	8.10E−06
6	3.25E−06	1.87E−06	3.52E−08	6.00E−03	1.00E−10	5.86E+04	5.86E−06	7.11	5.86E−06
8	3.25E−06	1.71E−06	3.92E−08	8.00E−03	1.00E−10	4.90E+04	4.90E−06	7.21	4.90E−06
10	3.25E−06	1.68E−06	4.00E−08	1.00E−02	1.00E−10	4.00E+04	4.00E−06	7.33	4.00E−06
12	3.25E−06	1.72E−06	3.88E−08	1.20E−02	1.00E−10	3.23E+04	3.23E−06	7.46	3.23E−06
14	3.25E−06	1.64E−06	4.09E−08	1.40E−02	1.00E−10	2.92E+04	2.92E−06	7.52	2.92E−06

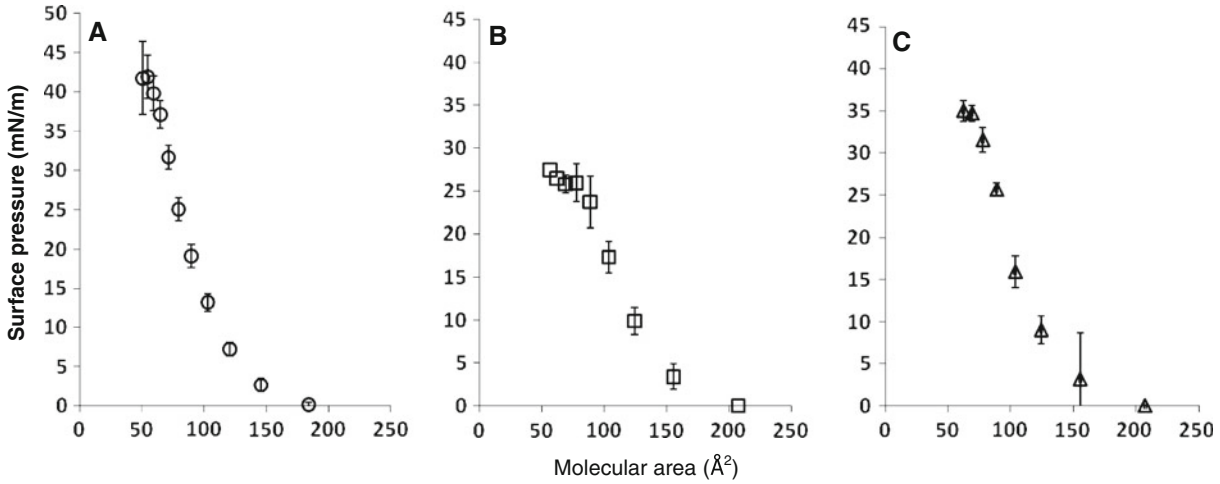

Fig. 1 Surface pressure (π)–molecular area (Å^2) isotherms of soy phospholipids: **a** PI at 25 °C; **b** PC at 25 °C; **c** PC at 37 °C on aqueous subphase. The subphase was 10 mM Tris–HCl buffer, pH 7.4, containing 15 mM NaCl

for both soy PLs. At 25 °C, the evolution of π was similar for both soy PC and PI up to $\Gamma = 1.06$ μmol/m^2. However, for Γ in the range of 1.06–2.40 μmol/m^2, π for soy PC increased more steeply than soy PI and reached a constant value at around $\pi = 24.87$ mN/m. At this value of π, the soy PC monolayer was deemed saturated. Soy PI monolayer, on the other hand, reached saturation at $\pi = 41.77$ mN/m corresponding to $\Gamma = 3.30$ μmol/m^2 at 25 °C. These differences between soy PC and soy PI could be attributed to differences in the physical nature of their polar head groups at the a/w interface. It has been shown that in PC monolayers, the phosphorylcholine group is submerged in the aqueous subphase and is oriented parallel to the interface [34]. When a PC monolayer approaches saturation, the phosphorylcholine group is shifted upwards towards the interface due to expulsion of solvated water molecules [35]. This movement of the phosphorylcholine group from the subphase to interface exerts a lateral pressure on the acyl chains aligned in the gas (air) phase and prohibits further packing of the PC monolayer. On the other hand, the anionic phosphorylinositol moiety in a PI monolayer is oriented perpendicularly to the interface and remains submerged in the aqueous subphase due to six hydrogen bonds formed between its hydroxyl groups and subphase water molecules [36]. This allows for maximal packing of PI alkyl chains in a monolayer, which eventually manifests in a higher surface pressure as compared to PC.

Temperature also seemed to have an effect on the evolution of the soy PC isotherm. For the soy PC monolayer at 37 °C, evolution of π was less steep than at 25 °C and saturation was achieved at $\pi = 35$ mN/m compared to 24.87 mN/m at 25 °C. These differences could be attributed to increased hydrophobic interaction between PC acyl chains at 37 °C which might effectively increase the packing efficiency in the monolayer [37].

The surface pressure isotherms were fitted with sigmoidal models for soy PC at 25 °C and 37 °C (Eqs. 10, 11) and for soy PI at 25 °C (Eq. 12).

$$\pi = \frac{26.72}{1 + e^{-\left(\frac{\Gamma - 1.461\times10^{-6}}{2.026\times10^{-7}}\right)}} \tag{10}$$

$$\pi = \frac{37.54}{1 + e^{-\left(\frac{\Gamma - 2.001\times10^{-6}}{4.225\times10^{-7}}\right)}} \tag{11}$$

$$\pi = \frac{42.92}{1 + e^{-\left(\frac{\Gamma - 1.461\times10^{-6}}{3.379\times10^{-7}}\right)}} \tag{12}$$

All models exhibited excellent fit as illustrated by a regression coefficient (R^2) of more than 0.99 in all three cases. These equations were used to transform changes in π values into Γ values at the a/w interface in the presence of βCD in the subphase.

βCD: Soy PL Binding

When saturated monolayers of soy PLs were formed on a subphase containing βCD, a time-dependent decrease in π was observed (Figs. 2a, 3a). As π of a monolayer depends on Γ [38], the time-dependent decrease in π indicated depletion of soy PL molecules from the a/w interface. This depletion occurred only when βCD was present in the subphase; in the absence of βCD, π remained steady for an extended period of time. The Γ values derived from experimentally determined values of π and Eqs. 10 and 12 for soy PC and soy PI, respectively, confirmed this trend. As shown in Fig. 2b, the Γ of soy PC monolayer formed on a βCD-free subphase (control) remained constant during the duration of the experiment. However, when the same

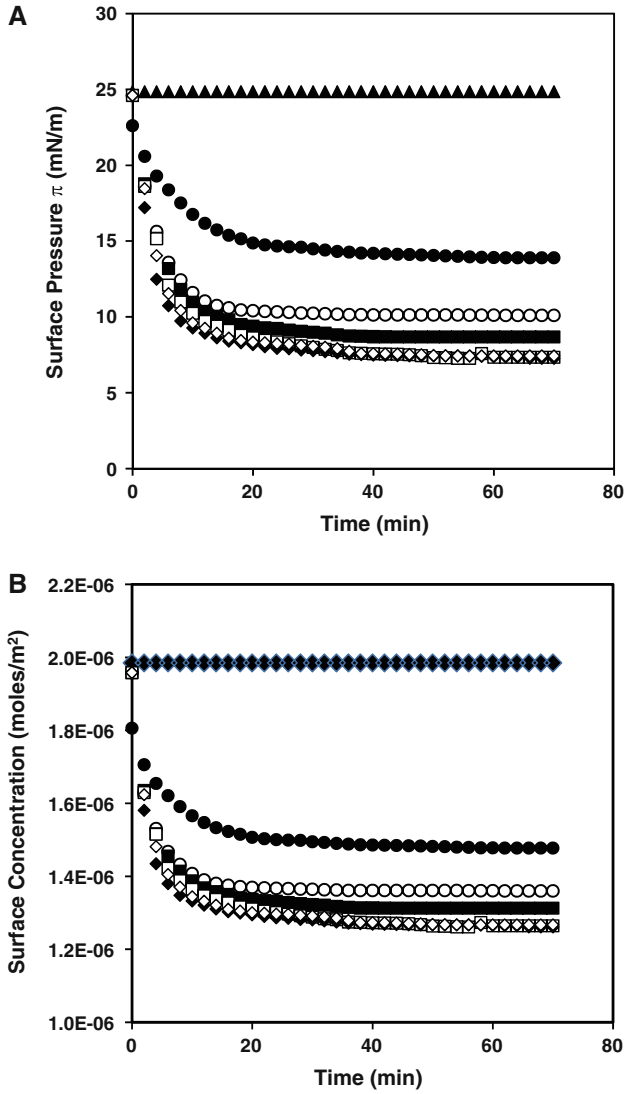

Fig. 2 Change in surface pressure (**a**) and surface concentration (**b**) of soy PC spread monolayer at air–buffer (10 mM, pH 7.4, 15 mM NaCl) interface in the presence of 0 mM (*triangles*), 3 mM (*filled circles*), 6 M (*open circles*), 8 mM (*filled squares*), 10 mM (open squares), 12 mM (*filled diamonds*) and 14 mM (*open diamonds*) βCD in the subphase

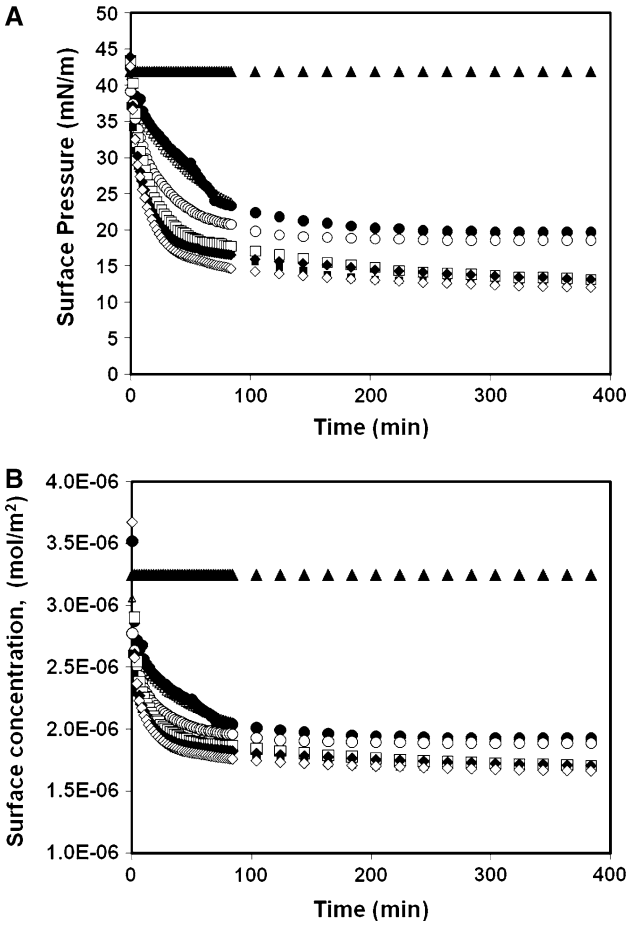

Fig. 3 Change in surface pressure (**a**) and surface concentration (**b**) of soy PI spread at air–buffer (10 mM, pH 7.4, 15 mM NaCl) interface in the presence of 0 mM (*filled triangles*), 2 mM (*open triangles*), 4 mM (*filled circles*), 6 M (*open circles*), 8 mM (*filled squares*), 10 mM (*open squares*), 12 mM (*filled diamonds*) and 14 mM (*open diamonds*) βCD in the subphase

monolayer was formed on a subphase containing 2–14 mM βCD, Γ decreased with time and reached a constant value after about 30 min as a result of desorption of soy PC from the interface. The extent of net decrease in Γ after an extended period (80 min) increased with increase of subphase βCD concentration. It should be pointed out that although desorption of soy PC began immediately after spreading, the initial decrease in Γ of soy PC did not necessarily represent true values. This is because the solvent used to spread soy PC takes 5–10 min to evaporate [39]. Therefore soy PC monolayer evolution at the interface could be uneven during this time period. For this study, those initial Γ values were discarded, as we were only interested in determining equilibrium values of Γ representing maximum desorption of soy PC in the presence of subphase βCD. Soy PI also exhibited βCD-dependent reduction of π and Γ (Fig. 3a, b).

Since an energy barrier (∼13.65 kcal/mol) precludes spontaneous dissolution of PLs into the aqueous phase [40], PL depletion from the a/w interface was taken as an evidence of soy PL complexation and solubilization by subphase βCD. The total amount of soy PL desorbed by βCD was calculated by determining the net change in Γ of soy PL spread monolayer in the presence of the latter. Statistically, the reduction in Γ of both PLs by βCD was significant ($p < 0.05$).

Previously, it has been reported that while βCD could sequester fatty acids and single acyl chain lipids, such as monoglycerides and lysophospholipids spread at the a/w interface [41–43], βCD-mediated extraction of phospholipids containing two long acyl chains, such as 1,

2-dihexadecanoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphatidylcholine (DOPC) and egg PC, from the spread monolayer at the a/w interface was not possible [21, 43]. On the other hand, βCD was able to extract almost all of cholesterol and about 25% of phospholipids from human erythrocytes [29]. These conflicting results could be attributed to structural differences among phospholipids tested. The spread monolayers of both DPPC and DOPC are known to be in a liquid condensed phase owing to the lack of or limited number of *cis*-double bonds. On the other hand, about 60% of fatty acids in soy PLs are polyunsaturated (PUFA) [44] and this high degree of PUFA may render spread monolayers of soy PL to exist in a liquid expanded phase, making them more accessible for complexation with subphase βCD.

Two key observations were made during desorption of soy PC and PI by βCD. First, at all βCD concentrations, the amount of soy PI removed was almost 1.5 times that of soy PC. Overall, about 30% of soy PC and 49.3% of soy PI were removed from the saturated monolayer at the a/w interface by 14 mM βCD. Incidentally, this compares with extraction rate of about 25–30% of the total PL from erythrocytes by βCD [29]. Polarity difference between the head groups of PC and PI might play a role in the efficiency of their removal from the a/w interface. For example, in the case of αCD, it has been shown that the transfer of interfacial PL followed a two step process, wherein the polar head group of PL was first extracted via interaction with hydroxyl groups of αCD followed by the inclusion of the acyl chain into the αCD cavity [30]. Accordingly, PLs with highly polar groups, such as PI (dipole moment of 26.6 D), tend to exhibit stronger attraction than the ones with relatively less polar head groups, such as PC (dipole moment of 16.7 D) or PE [30, 45], towards cyclodextrins in the subphase. Second, desorption reached a plateau at a βCD concentration range of 10–14 mM (Fig. 4). This may be attributed to structural restrictions for desorption at low surface pressures [21, 41]. In this study, soy PC and PI desorption by 2–14 mM βCD occurred in the π range of 25–7.2 and 41–12 mN/m, respectively. At high surface pressures (25 mN/m for soy PC and 41 mN/m for soy PI), the acyl chains are tightly packed and exhibit strong lateral pressure. Once a PL molecule is partially pulled down by βCD, the lateral force of the monolayer expels the partially exposed molecule fully into the bulk phase in order to maintain its continuity [46]. However, at well below saturated monolayer coverage (e.g., $\pi = 7$ mN/m for soy PC and 12 mN/m for soy PI), acyl chain interactions (the crowding effect) might be weak and therefore expulsion of PL was no longer favored.

The transfer of a PL molecule from the a/w interface to the aqueous subphase is an energy intensive process. For

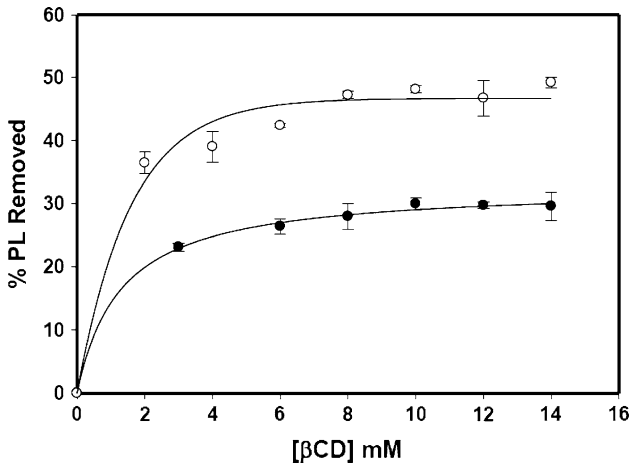

Fig. 4 Reduction in surface concentration of PC (*filled circles*) and PI (*open circles*) in the presence of 0–14 mM βCD in the subphase

instance, the free energy change (ΔG) involved in the transfer of a PL monomer from an aqueous phase into a lipid bilayer or a micelle can be calculated using Eq. 13,

$$\Delta G_1 = -RT \ln \frac{1}{\mathrm{CBC}} \tag{13}$$

where R is the gas constant, T is the temperature, and CBC is the critical bilayer concentration of phospholipid in mole fraction units. Equation 13 is also true for the formation of a micelle or a monolayer in which case CBC would represent critical micelle or critical monolayer concentration (CMC). The CBC or CMC value for a diacyl PL with 18 carbons is typically about 10^{-10} M [17, 47]. Thus, from Eq. 13, ΔG_1 for the transfer of PL from the monolayer to the subphase is about 13.65 kcal/mol. Using Eq. 9, it was calculated that the free energy change (ΔG_3) for transferring 1 mol of soy PC or soy PI from the monolayer to subphase containing 2–10 mM βCD ranged from 6.55 to 7.52 kcal/mol (Tables 1, 2). This indicated that the presence of βCD in the subphase and consequent inclusion of PL into the βCD cavity effectively lowered the overall free energy change ($\Delta\Delta G = \Delta G_3 - \Delta G_1$) for the transfer of PL into the aqueous subphase by −6.13 to −7.10 kcal/mol.

From the known dimensions of βCD and PL acyl chains, it has been predicted that about 4.7 methylene units of an acyl chain can fit in the 7.9-Å long cavity of βCD [48]. Stoichiometric ratios of 1:3–1:4 (acyl chain: βCD) have been reported for various PL–βCD inclusion complexes [31, 49]. But in the case of soy PL, since the acyl chains are highly unsaturated, the kinks introduced by *cis*-double bonds may sterically restrict inclusion of more than one βCD molecule per acyl chain. Therefore, we can tentatively assume that for soy PLs the acyl chain: βCD ratio might be restricted to 1:1.

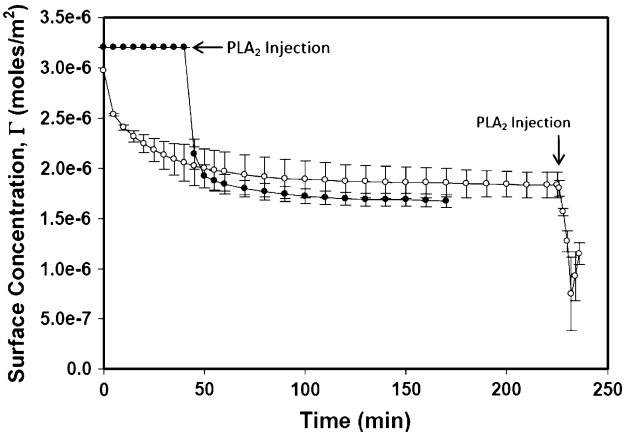

Fig. 5 Extent of desorption of soy PC monolayer in the presence of 2.29 μM PLA_2 only (*filled circles*) and a combination of 10 mM *β*CD and 2.29 μM PLA_2 (*open circles*) in the subphase

As discussed earlier, desorption of PL from the a/w interface was incomplete and reached a plateau at all *β*CD concentration (Figs. 2, 3, 4). Although this was suggested to be due to low surface pressure which disfavored expulsion of PL from the a/w interface [21, 42], an alternative explanation could be that, for steric reasons, phospholipids with two acyl chains might not be good ligands for forming inclusion complexes with *β*CD. In other words, desorption of soy PC from the a/w interface by subphase *β*CD could be more effective if the soy PC at the monolayer was first converted into fatty acid (FA) and lyso-phosphatidylcholine (lyso-PC) using phospholipase A_2 (PLA_2). These hydrolysis products may more easily form complexes with subphase *β*CD. This was tested and the results are shown in Fig. 5. As shown, the spread monolayer of soy PC was very stable for an extended period when both PLA_2 and *β*CD were not present in the subphase. However, even in the absence of *β*CD in the subphase, when PLA_2 was injected into the bulk phase, the surface concentration decreased in a time-dependent manner and reached a constant value. This hydrolysis-induced desorption of soy PC is likely due to the higher solubility of FA and lyso-PC than PC in the subphase. For instance, the solubility (or CMC) of DPPC in water is about 5×10^{-10} M, whereas that of lyso-phosphatidylcholine (C16) is about 7 μM [17]. As the solubility of lyso-PC decreases 3.2-fold per CH_2 increment in chain length, the solubility (CMC) of an 18 carbon lyso-PC would be about 0.7 μM. Taking into account the surface area of the PL monolayer and the volume of the bulk phase in the Langmuir trough used in this study, the net amount of soy PC desorbed ($\sim$1.63 μmol/m^2) into the subphase in the presence of PLA_2 (Fig. 5) translates to about 0.83 μM concentration of lyso-PC plus FA in the subphase. This is approximately close to the solubility limit of C18 lyso-PC and FA, i.e., 0.7 μM. Thus, although the soy-PC monolayer might have been completely hydrolyzed by PLA_2, only a portion of the liberated FA and lyso-PC was desorbed into the subphase and a significant amount remained at the interface due to solubility limitations. However, when 10 mM *β*CD also was included in the subphase, a greater amount of soy PC and its hydrolysis products was desorbed into the subphase (Fig. 5). Since *β*CD was allowed to equilibrate with the soy PC monolayer prior to injection of PLA_2 into the subphase, the initial reduction in Γ reflected sequestration of intact soy PC, followed by binding of hydrolysis products FA and lyso-phosphatidylcholine from the interface. As shown in Fig. 5, about 80% of PC monolayer was desorbed and sequestered during a combination of *β*CD and PLA_2 treatments as opposed to desorption of only about 30% of PC monolayer with *β*CD treatment only (Fig. 4).

Two main facts emerge from this study. First, *β*CD alone can induce a moderate removal of soy PLs from the a/w interface in a manner that is dependent on monolayer saturation as well as PL polarity. Secondly, hydrolysis of soy PLs at the a/w interface improves the efficiency of binding of PL and its hydrolysis products by *β*CD. Since a significant energy barrier for desorption from the interface to the subphase is overcome by soy PLs by binding to *β*CD's hydrophobic cavity, *β*CD could prove useful in extracting protein-bound PLs that reside in a similar hydrophobic environment. Thus, *β*CD could be potentially used in addressing off-flavor caused by soy PLs in soy protein. However, the removal of soy protein-bound PLs entails that the interaction energy between PL and soy protein (SP) be overcome by *β*CD–soy PL interaction. In soy protein, residual PLs exist as oleosin particles (protein–PL complexes) having a diameter of 100–200 nm [7]. Although the energetics of protein–PL interactions in these oleosin particles is not known, a generalization can be made that the energy needed to desorb the protein-bound PL might be comparable in magnitude to that of desorption of PL from the air–water interface into the aqueous subphase. On an average, the ΔG of interaction of PCs with apo-lipoproteins is in the range of -7 to -10 kcal/mol [50], which is lower than the ΔG of adsorption of PL at the a/w interface, i.e., -13.65 kcal/mol. Therefore, an energetic parity does exist for removing PL from soy protein oleosin. This, however, remains to be investigated.

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