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Formation of Complex Interface and Stability of Oil-in-Water (O/W) Emulsion Prepared by Soy Lipophilic Protein Nanoparticles

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ABSTRACT: A lipophilic protein nanoparticle (LPP) was fabricated by ultrasonication of the soy lipophilic protein (LP), which contains hydrophobic proteins and phospholipids. This LPP (R_h = 136 ± 0.8 nm, ζ-potential = −20 mV, pH 7.0) had an improved dispersibility and acted as an emulsifier. The oil/water (O/W) emulsion stabilized by this LPP exhibited superior physical stability over long-term storage (8 weeks), during a stress storage test (200 mM NaCl addition and heating at 90 °C), and in the presence of Tween 20 (1.0−4.0 wt %), in contrast to those emulsions stabilized by β-conglycinin and glycinin. Langmuir−Blodgett method and interface pressure determination revealed that LPP formed rigid and rough granular film at air/water interface. The excellent stability of emulsions stabilized by LPP highlights the synergic effect between hydrophobic proteins and phospholipids. These findings suggest that the complexes of hydrophobic protein aggregates and biosurfactant could form a stable interface which could be developed into a novel strategy to fabricate a stable food emulsion.

KEYWORDS: *soy lipophilic protein, nanoparticles, oil/water emulsion, physical stability, Tween 20, displacement*

INTRODUCTION

Soy proteins are widely used as ingredients in foods, mainly as emulsifiers and interfacial stabilizers in the food industry, due to their amphiphilic structures.[1] Soy glycinin and β-conglycinin, often referred to as 11S and 7S globulins, respectively, according to their sedimentation coefficients, represent two of the most common storage protein components in soybean seed. Their emulsifying properties and related dynamic interfacial adsorption have been studied extensively.[2,3] The β-conglycinin fraction has better emulsifying activity than the glycinin fraction, as it adsorbs readily at the interface due to its flexible structure and its possession of N-linked glycans and extension regions in the α and α′ subunits of β-conglycinin.[3,4] Previous work in our laboratory demonstrated that unlike glycinin, β-conglycinin can form smaller soluble aggregates with less compact conformation during heating.[5] These structural properties of β-conglycinin may contribute to its greater emulsifying activity and higher protein load at the interface compared with glycinin.[4] However, both glycinin- and β-conglycinin-stabilized emulsions exhibited destabilization after being treated with heat; such an effect is likely induced by a depletion flocculation mechanism.[4]

Soy glycinin and β-conglycinin can be sequentially fractionated by precisely adjusting the temperature, pH, and ionic strength.[6,7] Samoto et al.[8] have developed a novel heating method for fractionating soy protein through the modulating nitrogen solubility index (NSI) of defatted soy flour. Soy protein can be fractionated into three components using this method: glycinin, β-conglycinin, and lipophilic protein (LP, which is a group of protein fractions associated with lecithin), at relative abundances of 23, 46, and 31%, respectively.[7] The LP fraction contains approximately 8−10% polar lipids, mainly consisting of phospholipids (PLs), including phosphatidylethanolamine (PE), phospatidylcholine (PC), and phosphatidylinositol (PI).[9] These PLs in soy protein cannot be removed by degreasing treatment using hexane because they can form the lipid−protein complexes with hydrophobic oil bodies binding protein (OBBP).[8] Not surprisingly, LP has a poor water dispersibility. In addition, the lipids in LP can release flavor volatiles upon being exposed to lipoxygenase catalysis and autoxidation; which contribute to the unfavorable bean flavors in soy protein products.[9] Recently, it has been demonstrated that soy LP fraction showed some health benefits, for example, in regulating lipid metabolism, lowering plasma cholesterol and triacylglycerol levels in rats,[10] and decreasing albumin levels in urine for patients with renal disease.[11] However, the potential use of the LP fraction as a soy protein ingredient, based upon its emulsification properties and related interfacial behavior, has not been explored.

The LP fraction comprises a group of proteins, which includes residual fragments of glycinin, β-conglycininm and oil bodies binding proteins, mainly oleosins, caleosins, and steroleosins,[8] indicating its membrane protein origin. Oil bodies or oleosomes have highly robust micelle-like structures with an outer phospholipid monolayer and an interior filled

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with triacylglycerides (TAGs). Oleosins were anchored as hairpin-like structures with their hydrophilic parts remaining outside the oil bodies,[12] providing extreme stability to prevent coalescence. The oleosins in soybean oil bodies are mainly alkaline and hydrophobic proteins with relatively low molecular masses (24, 18, and 17 kDa).[13] The amphiphilic triblock structure of oleosins, which consist of two hydrophilic N- and C-terminal regions as well as a central hydrophobic region, are predicted to irreversibly bind to the lipid/water interface[14] and to improve emulsifying stability of oil bodies against coalescence. Tzen and co-workers have previously reconstituted stable artificial oil bodies (AOB) by sonicating the mixture of TAGs, phospholipids, and oleosins to encapsulate the hydrophobic drugs.[14,15] Apart from oleosins, intact oil bodies can also be used as an emulsifying agent.[16] Our previous work demonstrated that the soybean oil body emulsions coated with ι-carrageenan are digested at a relatively slow rate, which may contributed to satiety.[17] One common feature of all oil bodies is the presence of a phospholipids-protein complex at the surface, which is responsible for maintaining oil body stability.[16]

Generally, commercial food emulsions are usually prepared by homogenizing mixtures of small molecular weight surfactant and protein, which actually have totally different interfacial stabilization mechanisms. The interfacial stability mechanism of small molecular weight surfactants, namely, the Gibbs−Marangoni mechanism, relies on this high degree of mobility. However, proteins are supposed to migrate to the interface, partially unfold, and then link together to form an elastic network, which is beneficial to stabilizing the emulsion.[18] The coexistence of both proteins and small molecular weight surfactant during emulsion processing may result in emulsion destabilization. A small weight molecular emulsifier, such as polyoxyethylene sorbitan monolaurate (Tween 20), coexisted with protein emulsions, resulting in the inevitable competitive displacement of proteins at oil/water interfaces and then the occurrence of unpredictable creaming, flocculation, coalescence, even collapse of the emulsion.[19,20] This is of particular importance to the food industry. Mackie and co-workers[21,22] have proposed an orogenic mechanism of protein displacement from the air/water interface by small molecular weight surfactant and demonstrated the heterogeneity of pure protein film resulting in the adsorption of surfactant onto the protein interface. The surfactants expand their domain and eventually displace the interfacial protein network. Recently, many works have focused on the formation of complex and rigid interface with mixed biopolymers,[23,24] hydrocolloids,[25] and particles,[26,27] with particular emphasis on the competitive adsorption of proteins and other macromolecules at the interface.

In light of the fact that LP is plentiful in OBBP and PLs (they are the main stabilizers of soy oil body), it has potential applications in the food industry. The coexistence of oleosins and phospholipids at the surface of oil bodies suggests support of such an approach. The objective of this study was to explore the possibility of using the LP fraction as an emulsifier and interfacial stabilizer. We have compared the interfacial adsorption behavior, dilatational modulus, and physical stability of emulsion stabilized by LP, and those by soy glycinin, β-conglycinin, with particular emphasis on their ability to resist displacement by low molecular weight surfactant (Tween 20). Our long-term goal is to develop a new strategy to fabricate a more stable food emulsion by forming a complex interface of hydrophobic protein fraction and phospholipids, which actually are thermodynamically competing components.

MATERIALS AND METHODS

Materials. Defatted soy flour with low heat treatment (NSI is 89.5%) was provided by Shandong Yuwang Industrial and Commercial Co., Ltd., China. The phospholipase A_1 (PLA_1), Lecitase Ultra (EC 3.1.1.32) with activity of 10000 LU/mL and Alcalase 2.4 L FG (EC 3.4.21.62) with activity of 2.4 AU/mL were provided from Novozymes AS (Denmark). Polyoxyethylene sorbitan monolaurate (Tween 20) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Corn oil was purchased from a local supermarket and used without further purification. All other chemicals used were of analytical grade.

Preparation of Soy Glycinin, β-Conglycinin, and LP. Soy glycinin, β-conglycinin, and LP were prepared according to the method of Samoto et al.,[8] with modifications as described below. Defatted soy flour with low heat treatment was treated with dry heating at 70 °C for 2 h in the oven, by which the NSI of defatted soy flour decreased to 75%. Fifty grams of heated defatted soy flour was added to 400 mL of water, followed by adjustment to pH 8.0 with NaOH (5 N). The mixture was stirred for 1 h at 20 °C and centrifuged at 3000*g* for 10 min to obtain a supernatant and precipitate. Then, a reducing agent, Na_2SO_3, was added to a final concentration of 10 mM to the water extract, and the pH was adjusted to 5.8 with H_2SO_4 (3.5 N). The fraction precipitated on centrifugation (3000*g*, 10 min) was designated the glycinin fraction. The supernatant was adjusted to pH 5.0 with H_2SO_4 (3.5 N) and then heated for 15 min at 55 °C. To it was added 50 mM NaCl, and the pH was adjusted to 5.5 with NaOH (5 N). The mixture was then centrifuged at 3000*g* for 10 min, with the resulting precipitate being designated the LP fraction. The supernatant was adjusted to pH 4.5 with H_2SO_4 (3.5 N). After centrifugation, the precipitate was designated the β-conglycinin fraction. The protein content and lipid content of the protein products are shown in Table 1. Protein content was determined according to the Dumas combustion method (Elemental Analyzer rapid N cube, Germany) using an N factor of 6.25. For lipid content determination, each fraction was freeze-dried and then added to a 10-fold weight of chloroform/methanol (2:1 by volume), followed by stirring for 2 h at 50 °C. The extract was then dried through evaporation, and the weight of the solidified compounds was determined.

Table 1. Yield and Composition of the Soy Protein Production[a]

sample	yield (%)	protein content (%)	lipid content (%)
β-conglycinin	6.81 ± 0.18 b	91.12 ± 2.26 ab	1.45 ± 0.05 b
glycinin	12.37 ± 0.38 a	95.36 ± 0.34 a	2.36 ± 0.03 c
lipophilic protein	10.81 ± 1.68 ab	85.08 ± 0.48 b	7.48 ± 0.05 a

[a]Different letters (a−c) in a column indicate significant ($p < 0.05$) differences among samples.

Ultrasonic Treatment. LP dispersion (0.5%, w/v) was prepared by dispersing the lyophilized LP in 20 mM phosphate buffer (pH 7.0) and stirred for 1 h at 25 °C with a magnetic stirrer. Then the dispersion was treated in an ice bath for 5 min using a probe ultrasonic homogenizer (Sonic Ruptor 400 Ultrasonic Homogenizer, OMNI, USA) with 30% power.

Particle Size and Zeta-Potential Measurments. The measurements of size distribution, zeta-potential, and polydispersity index (PDI) of protein particles were performed using a Nano ZS Zetasizer instrument (Malvern Instruments, Worcestershire, UK). Samples were diluted to 0.1 wt % with 20 mM phosphate buffer (pH 7.0) before loading into the cuvette (PCS8501). All measurements were carried out at 25 °C in triplicate. A refractive index of 1.450 was used for dispersion (protein) and 1.331 for the continuous phase (20 mM phosphate buffer, pH 7.0). The apparent hydrodynamic radius (R_h) of protein samples was analyzed by means of the "cumulants" method

and Stokes–Einstein equation using Dispersion Technology software (DTS) (V4.20).[23]

Atomic Force Microscopy (AFM). AFM images were recorded in tapping mode at a drive frequency of approximately 320 kHz, and the scan rate was 1.0 Hz using a MultiMode SPM microscope equipped with a Nanoscope IIIa Controller (Digital Instruments, Veeco, Santa Barbara, CA, USA). Point Probe NCHR silicon tips of 125 μm length with a spring constant of 42 N/m were purchased from Nano World (Arrow NC cantilevers, Nano world, Switzerland). Typical resonant frequencies of these tips were about 290 kHz. Aliquots (2 μL) of protein dispersion with or without ultrasonication (diluted to 10 μg/mL with 20 mM phosphate buffer, pH 7.0) were placed on a freshly cleaved mica disk and air-dried for 20 min at ambient temperature (25 °C).

Surface Hydrophobicity. The surface hydrophobicity of protein was determined by titration with ANS according to the method of Liu et al.,[28] with modifications as described below. The aliquots (1 mL) of protein solutions (0.2 mg/mL) were placed in the cell of an F7000 fluorescence spectrophotometer (Hitachi Co., Japan), and aliquots (10 μL) of ANS (5 mM in 5 mM phosphate buffer, pH 7.0) were titrated to reach a final concentration of 50 μM. The molar coefficient (5000 M^{-1} cm^{-1} at 350 nm) was used to calculate ANS concentration. The relative fluorescence intensity (F) was measured at 390 nm (excitation; slit width, 5 nm) and 470 nm (emission; slit width, 5 nm). Data were elaborated using the Lineweaver–Burk equation (eq 1)

$$1/F = 1/F_{max} + (K_d/L_0)(1/F_{max}) \quad (1)$$

where L_0 is the fluorescent probe concentration (μM), F_{max} is the maximum fluorescence intensity (at saturating probe concentration), and K_d is the apparent dissociation constant of a supposedly monomolecular protein/ANS complex. F_{max} and K_d can be calculated by standard linear regression fitting procedures. The ratio F_{max}/K_d, corrected for protein content, represents the protein surface hydrophobicity index (PSH).

Preparation and Stability of Emulsion. The β-conglycinin, glycinin, and LP were dissolved in sodium phosphate buffer solution (20 mM, pH 7.0) and then gently stirred overnight at ambient temperature (25 °C). Ultrasonic treatment was applied to the LP dispersion before emulsification. The pre-emulsions were formed by mixing the corn oil and the protein solutions (finally containing 10% v/v corn oil and 0.5% w/v protein) and homogenized at 10000 rpm for 1 min by using a T25 Ultraturrax homogenizer (IKA, Staufen, Germany). O/W emulsions were prepared by homogenizing the pre-emulsions through an M-110EH-30 microfludizer processor (Microfludics, USA) at 500 bar. Sodium azide (0.02% w/v) was added to the emulsions to prevent microbial growth. A long-term storage test of the emulsions was performed by monitoring the change of the emulsion droplet size at ambient temperature (25 °C) for 8 weeks. Stress storage stability of emulsion was tested by heating the emulsions in the presence of 200 mM NaCl at 90 °C for 30 min. The effect of low molecular weight surfactant Tween 20 on emulsion stability was evaluated by monitoring the mean particle diameter ($d_{3,2}$) and creaming index (CI) in the presence of Tween 20 at concentrations of 1, 2, and 4% (w/v).

Droplet Size Distribution and Microstructure of Emulsions. The mean particle size and size distribution of emulsions were measured by using a Mastersizer 2000 (Malvern Instruments Co. Ltd., Worcestershire, UK) at 25 °C. The refractive indices of corn oil and phosphate buffer were taken as 1.467 and 1.330, respectively; adsorption index = 0.001. The particle sizes measured are reported as the volume-weighted mean diameter $d_{3,2} = \Sigma n_i d_i^{\ 3}/\Sigma n_i d_i^{\ 2}$, where n_i is the number of droplets with diameter d_i. The microstructure of emulsions was studied using a TCS SP5 confocal laser scanning microscope (CLSM, Leica Microsystems Inc., Heidelberg, Germany) with a 100 mm oil immersion objective lens. Aliquots (1 mL) of emulsion were mixed with 40 μL of staining solution containing 0.1% (w/v) Nile Red (fluorescent dye). The stained emulsions (50 μL) were placed on concave confocal microscope slides and examined using an argon krypton laser having an excitation line of 488 nm and a helium neon laser (He/Ne) with excitation at 633 nm.

Creaming Index. The CI was determined according to the method of Moschakis et al.,[29] with modifications. Emulsion samples were put into 5 mL flat-bottom glass tubes immediately after treatment. The tubes were sealed with plastic tops to prevent evaporation. The emulsion samples were stored quiescently at ambient temperature (25 °C) for 10 days. The extent of creaming was characterized by the creaming index (CI, %): CI = $(H_S/H_E) \times 100\%$, where H_S is the height of the serum layer and H_E is the total height of the emulsion.

Microstructure of Protein Network at the Air/Water Interface. Surface pressure measurements were made using a Wilhelmy plate and a Langmuir trough (KSV Minitrough, KSV, Finland). The β-conglycinin, glycinin, and lipophilic protein nanoparticle (LPP) dispersions (containing 100 μg of protein) were spread at the air/water interface (subphase was Milli-Q water with a resistivity of 18.2 MΩ·cm) carefully. The spread protein films were allowed to equilibrate for 30 min, and then the film was compressed at a barrier speed of 10 mm/min until the surface pressure reached 15 mN/m; the interfacial structure was sampled by transferring Langmuir–Blodgett (LB) films onto freshly cleaved mica and imaging the samples by AFM. For the displacement of protein from the air/water interface with Tween 20, Tween 20 was added into the subphase immediately after the compression stopped. As the surface pressure increased to 20 mN/m, the protein film was dipped. The protein coverage area fraction was measured using simple threshold techniques.[30] Surface roughness (R_q) of the protein LB films were analyzed using Digital Nanoscope software (version 5.30.3, Digital Instruments, Veeco).

Modification of Lipophilic Protein Nanoparticle (LPP). Modification of protein particles was modulated by hydrolysis with phospholipase and proteinase. For the lipolysis of LPP, phospholipase A1 (PLA1) was added into the 0.5 wt % LPP dispersion (pH 7.0) at 1000 LU/g protein and incubated at 40 °C under stirring at 200 rpm for different durations. For the proteolysis of LPP, Alcalase 2.4 L was added into the LPP (0.5%, w/v) dispersion (pH 7.0) at 10 mg/g protein and incubated at 55 °C for different durations. The samples were freeze-dried and stored in 4 °C until the subsequent analysis.

Dynamic Surface Properties of Protein at the O/W Interface. The interfacial adsorption kinetics and dilatational rheological parameters of protein at the O/W interface were monitored by the pendant drop technique with an optical contact angle meter (OCA-20, Data-physics Instruments GmbH, Germany) equipped with an oscillating drop accessory (ODG-20). The experiments were carried out at 25 °C. Protein dispersions (0.05%, w/v) were placed in the syringe, and a drop of protein solution was delivered and allowed to stand for 3 h to achieve protein adsorption at the O/W interface. Surface tension measurements were performed to check the absence of surface-active contaminants in the buffer solutions. The surface tension (σ) was calculated according to fundamental Laplace equation. The surface pressure is $\pi = \sigma^0 - \sigma$, where σ^0 is the surface tension of distilled water. During the first step, at relatively low pressure when diffusion is the rate-determining step, a modified form of the Ward and Tordai equation can be used to correlate the change in interfacial pressure with time defined by eq 2[30]

$$\pi = 2C^0KT(D\theta/3.14)^{1/2} \quad (2)$$

where C^0 is the concentration in the bulk phase, K is the Boltzmann constant, T is the absolute temperature, D is the diffusion coefficient, and θ is adsorption time (s). If the diffusion of proteins at the interface controls the adsorption process, a plot of π against $\theta^{1/2}$ will then be linear, and the slope of this plot will be the diffusion rate (k_{diff}). To obtain surface dilatational parameters, sinusoidal interfacial compression and expansion were performed by decreasing and increasing the drop volume at 10% of deformation amplitude ($\Delta A/A$) and 0.1 Hz of frequency. The surface dilatational modulus (E^*) derived from the change in interfacial tension (σ), resulting from a small change in surface area (A), can be described by eq 3.[31]

$$E^* = d\sigma/(dA/A) = -d\pi/d\ln A = E' + iE'' \quad (3)$$

The dilatational modulus (E^*) is a complex quantity and is composed of real and imaginary parts ($E = E' + iE''$). The real part of the dilatational modulus or storage component is dilatational elasticity (E'). The imaginary part of dilatational modulus or loss component is surface dilatational viscosity (E'').

Statistical Analysis. Unless specified otherwise, three independent trials were carried out, each with a new batch of sample preparation. The results are presented as the mean ± standard deviation. Analysis of variance (ANOVA) was performed on the data using the SPSS 13.0 statistical analysis system.

RESULTS AND DISCCUSION

Formation of Lipophilic Protein Particle. Soy glycinin, β-conglycinin, and the LP fraction were fractionated as described above. We compared the yield, protein, and lipid contents of three soy protein components (Table 1); the results indicated that the LP fraction was rich in lipids, accounting for 7.48 ± 0.049 wt % of its weight, higher than those of glycinin (2.36 ± 0.026 wt %) and β-conglycinin (1.45 ± 0.047 wt %). Our previous thin-layer chromatography (TLC) analysis of the lipid composition in the LP fraction indicated the presence of PLs, including PE, PC, and P).[9] The LP fraction was further analyzed by SDS-PAGE (Figure 1), which suggests that LP was

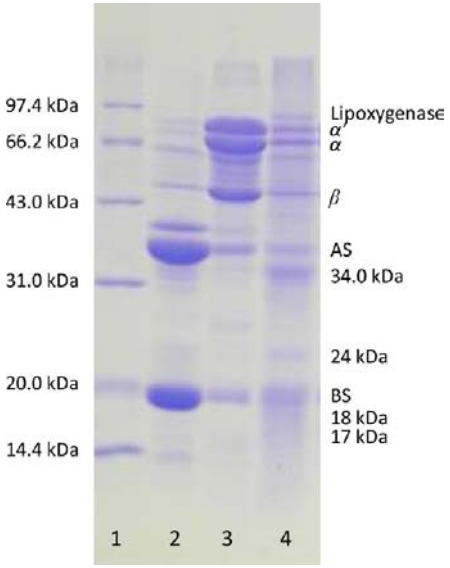

Figure 1. SDS-PAGE of the β-conglycinin, glycinin, and soy lipophilic protein. Lanes: 1, marker; 2, glycinin; 3, β-conglycinin; 4, LP. AS, acidic subunit; BS, basic subunit.

a complex composed of a group of proteins, including residual subunits of glycinin, β-conglycinin, and lipoxygenase (Lx). The 34 kDa protein was initially considered as a soybean oleosin because it could bind to the oil body strongly, but was later recognized as being located in protein storage vacuoles.[13] The 24 and 18 kDa proteins are believed to be soybean oleosins.[8,13] The yield of LP fraction calculated from SDS-PAGE was underestimated as it is lower than those obtained from the nitrogen determination method (Table 1). Such underestimation may due to the high level of PLs present in the LP fraction, which may result in relatively weak Coomassie Brilliant Blue (CBB) staining, as reported by Samoto et al.[8] Another reason may be the PLs contributed the nitrogen content of the determination results.

The soy LP fraction is hydrophobic by nature, which may result in low solubility in water. Surprisingly, we observed that the turbid LP dispersion (pH 7.0) became transparent when it was ultrasonicated for 5 min, suggesting that the water dispersibility of LP was greatly improved upon such treatment (Figure 2). Furthermore, a significant change of the particle size

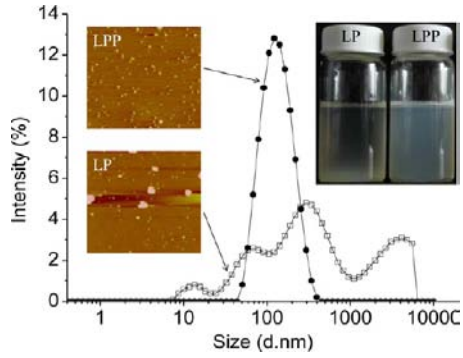

Figure 2. Particle size distribution and AFM image (2 μm × 2 μm) of soy lipophilic protein (LP) and soy lipophilic protein nanoparticle (LPP).

distribution of LP in water was also observed after ultrasonic treatment (Figure 2). The ultrasonicated LP was monodispersed, with a PDI of 0.20. The morphology of the two samples was then analyzed by AFM (Figure 2), which revealed that the LP changed from agglomerates to the spherical shape with uniform size, suggesting the formation of LPP. Dynamic light scattering showed the characteristics of the LPP with R_h of 136.0 ± 0.8 nm and ζ-potential of −20.0 ± 0.3 mV at pH 7.0.

The surface hydrophobicity (H_o) of protein was used to monitor the structural changes of LP before and after sonication. Table 2 compares the surface hydrophobicity of LPP with that of other soy protein fractions. The overall surface hydrophobicity index (PSH) of LPP, defined as the number and affinity of hydrophobic sites, was decreased significantly ($p < 0.05$), in contrast to the original LP, to even less than those of glycinin and β-conglycinin. These results are related to the transformation of hydrophobic patches on the surface to the interior of protein aggregates, indicating the formation of LPP. In view of the high level of PLs of the LP fraction, we hypothesize that the LPP formed by ultrasonication possesses a core−shell structure, in which the hydrophobic surface of LP proteins (mainly OBBP) is stabilized or covered by amphiphilic phospholipids, thus greatly improving the water dispersibility of LP protein.

Physical Stability of Emulsion Stabilized by LPP. We further analyzed the evolution of the mean droplet diameter ($d_{3,2}$) during storage and the physical stability of emulsion stabilized by LPP, β-conglycinin, and glycinin (Figure 3). Among them, the emulsion stabilized by LPP exhibited the best stability with nearly constant droplet size after being stored for 8 weeks, in contrast to the emulsions stabilized by β-conglycinin and glycinin, which exhibited significantly increased ($p < 0.05$) droplet size. Specifically, the $d_{3,2}$ increased from 0.40 ± 0.01 to 2.75 ± 0.16 μm for β-conglycinin and from 0.52 ± 0.04 to 5.53 ± 0.21 μm for glycinin. The stress stability test of emulsions, which were characterized by heating at 90 °C in the presence of 200 mM NaCl, were assessed by droplet size distribution, creaming index, and microstructure with CLSM observations, as shown in Figure 4. After stress treatment, the emulsions stabilized by β-conglycinin (Figure 4A,D) and glycinin (Figure 4B,E) were flocculated with severe creaming;

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Table 2. Surface Hydrophobicity of the β-Conglycinin, Glycinin, and Lipophilic Protein[a]

sample	F_{max}	K_d	F_{max}/K_d
β-conglycinin	619.90 ± 117.16 a	6.01 ± 2.43 a	99.75 ± 22.19 a
glycinin	854.83 ± 143.44 a	4.65 ± 0.90 a	188.89 ± 19.24 b
lipophilic protein	3380.84 ± 305.87 b	13.52 ± 1.22 b	233.33 ± 28.87 c
lipophilic protein nanoparticle	755.50 ± 98.21 a	24.94 ± 3.54 c	34.83 ± 6.79 d

[a]Different letters (a–d) in a column indicate significant ($p < 0.05$) differences among samples. F_{max}, the maximum fluorescence intensity; K_d, the apparent dissociation constant of the proteins–ANS complex; and PSH, protein surface hydrophobicity index.

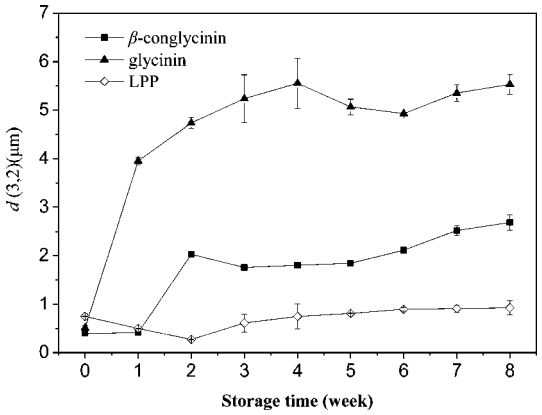

Figure 3. Time evolution of the mean droplet diameter of emulsions (10% v/v corn oil, 0.5% w/v protein, pH 7.0) stabilized by β-conglycinin, glycinin, and soy lipophilic protein nanoparticle (LPP).

the droplet size distribution was shifted from monodispersion to polydispersion. Meanwhile, for the emulsion stabilized by LPP, there was only negligible flocculation with the emulsion remaining stable and homogeneous (Figure 4C,F). The effects of NaCl addition and heating may have weakened the electrostatic repulsion between the emulsion droplets, leading to flocculation and coalescence.[18] The excellent stability of emulsion stabilized by LPP upon stress treatment might result from both electrostatic repulsion and steric hindrance.

The behavior of protein-stabilized emulsion might be altered when the small molecular weight surfactant was added. Generally, nonionic surfactant Tween 20 can pack at the interface effectively by lowering the surface free energy and be able to displace the proteins.[21] Extensive works have revealed that the competitive displacement of milk protein from the droplet surface by Tween 20 could result in the depletion flocculation and creaming of the emulsion.[19,20,32] Interestingly, the improvement in oil body emulsion stability against coalescence and creaming resulting from Tween 80 addition observed recently[33] showed the difference between emulsions stabilized by globular protein and oil body membrane. Figure 5 compares the mean droplet diameter ($d_{3,2}$) and the CI of the emulsions stabilized by three soy proteins in the presence of Tween 20. With increasing Tween 20 concentration from 1 to 4%, a gradual increase of the $d_{3,2}$ (Figure 5A) and CI (Figure 5B) was observed in the emulsions stabilized by β-conglycinin and glycinin, but not with the emulsion stabilized by LPP, further indicating an arrested coalescence. Flocculation of the emulsions stabilized by β-conglycinin and glycinin resulting from Tween 20 could be explained by the weakeden static repulsion between the emulsion droplets. At higher levels of Tween 20, there would be more protein displaced by the uncharged surfactant from the droplet surface, resulting in the weaker static repulsion and more extensive flocculation between emulsion droplets. In addition, the CI of LPP-stabilized emulsion was significantly lower ($p < 0.01$) than that of those stabilized by β-conglycinin and glycinin in the presence of Tween 20 (Figure 5B). These results highlight the unique adsorption behavior and structure of the LPP network at the interface.

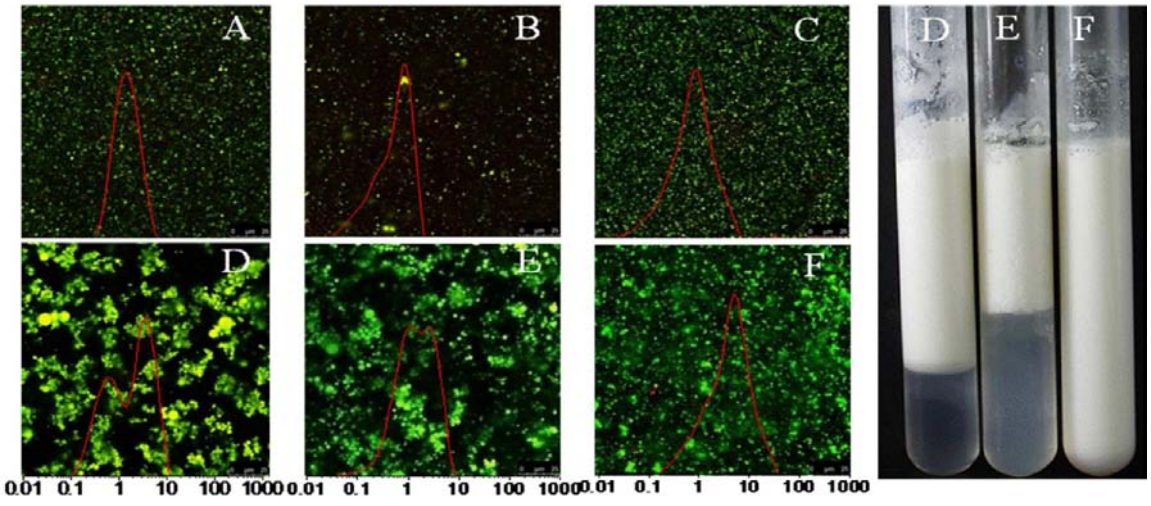

Figure 4. Microstructure and appearance of emulsions stabilized by β-conglycinin (A, D), glycinin (B, E) and soy lipophilic protein nanoparticle (LPP) (C, F): (A–C) without thermal treatment and NaCl addition; (D–F) with 200 mM NaCl addition and thermal treatment at 90 °C for 30 min. Droplet size distributions of the emulsions determined by light scattering (Mastersizer) were superimposed on the micrographs with horizontal scale indicating particle size in micrometers.

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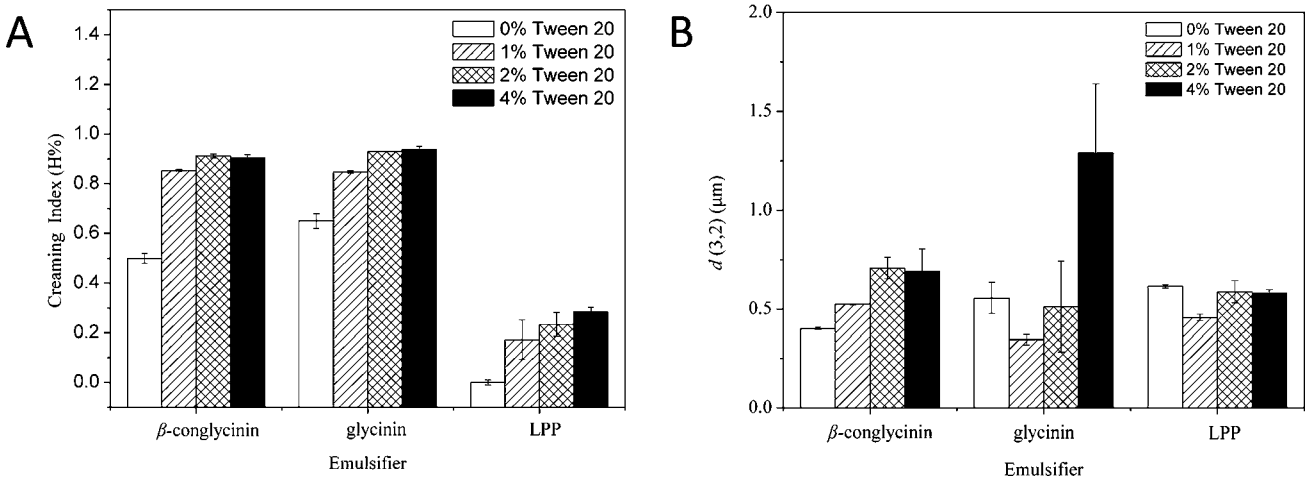

Figure 5. Creaming index (A) and average droplet size (B) of the emulsions (10% v/v corn oil, 0.5% w/v protein, pH 7.0) prepared by β-conglycinin, glycinin, and soy lipophilic protein nanoparticle (LPP) in the presence of Tween 20 (0, 1, 2, and 4% w/v).

Microstructure and Displacement of LPP Interface by Tween 20. Observation of the interfacial microstructures of LB film by AFM provided important information on how the displacement of interfacial protein networks by surfactants occurred.[21,22] We compared the time evolution of surface pressure (π) on the air/water interface of three soy protein fractions when Tween 20 was added into the subphase (Figure 6). The surface pressure of LPP film had a strong growth when

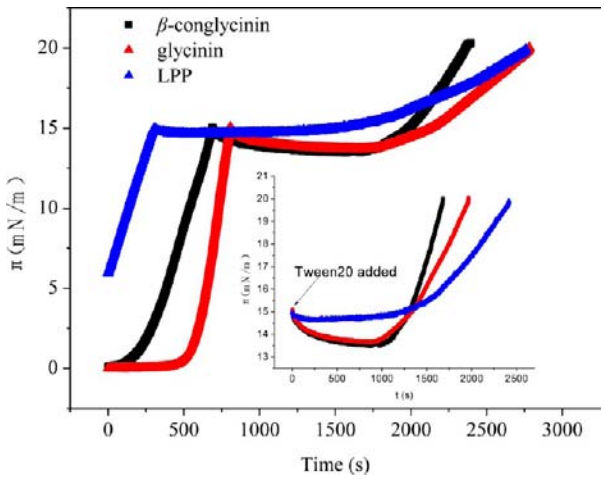

Figure 6. Time−surface pressure evolutions of the LB films comprised with β-conglycinin, glycinin, and soy lipophilic protein nanoparticle (LPP) at the air/water interface during displacement by Tween 20.

it was compressed, but that of glycinin and β-conglycinin showed flat growth first and then increased gradually. These results could be explained as follows: the LPP film at the air/water interface was more rigid and elastic than the other two soy globular protein. In addition, when the compression was stopped and Tween 20 was added into the subphase, the surface pressure of glycinin and β-conglycinin decreased due to the desorption of the emulsifiers (protein) from the interface to bulk phase, in contrast to the stable surface pressure of LPP, suggesting that the LPP interface probably does not experience a desorption process. Furthermore, the surface pressure of LPP film increased more tardily in the presence of Tween 20 when compared to those of the other soy globular proteins. As Mackie et al.[21] noted, Tween 20 adsorption to protein films results in a mixed interfacial layer of protein and surfactant, with the surfactant domain depending on the type of protein. As LP is more hydrophobic than glycinin and β-conglycinin, its expulsion by the surfactant and the development of surfactant-rich domains is expected to be a much more difficult and slower process.

The microstructure of the spread LB film on the air/water interface before (sampled as π = 15 mN/m) and after (sampled as π = 20 mN/m) displacement by Tween 20 was then visualized by AFM (Figure 7A). The three-dimensional images indicated the variation in heights of protein particles, aggregated structure, and protein network at the air/water interface. We observed a significant difference of structural heterogeneity of the LB film, which was characterized by the surface roughness (R_q), among the three soy proteins. Generally, the LPP can form a rougher and more granular interfacial film (R_q = 2.525 ± 0.739 nm) than β-conglycinin (R_q = 0.886 ± 0.06 nm) and glycinin (R_q = 0.971 ± 0.083 nm); the LPP film presented unusual aggregated structures of interfacial networks, in contrast to the fine and compact interfacial structures of β-conglycinin and glycinin. The rough microstructure of LPP film was very similar to the interfacial structure of soybean oil body at the air/water interface described previously by Waschatko et al.[34] In their work, the adsorption and structural modification over time at the air/water interface of oil bodies with and without trypsin digestion by Brewster angle microscopy (BAM) were compared. Their results suggest that oil bodies, when acting as an emulsifier, could migrate to the interface, then rupture and form an aggregated and dense film, with the surface pressure increased with time, presumably due to the formation of a cross-linked film of aggregated oleosins and lipids. However, the decrease of surface pressure and a homogeneous film were observed when subjected to trypsin digestion, probably due to the digested oleosins becoming aggregated and being expelled to the subphase, leaving the interface stabilized by phospholipids.

Figure 7A (bottom panel) shows AFM data for the displacement of soy protein from the air/water interface by Tween 20 at a surface pressure of 20 mN/m. In these images the bright patches show the protein domains and the dark patches show the regions of surfactant Tween 20. It was obvious that the film composed of β-conglycinin and glycinin

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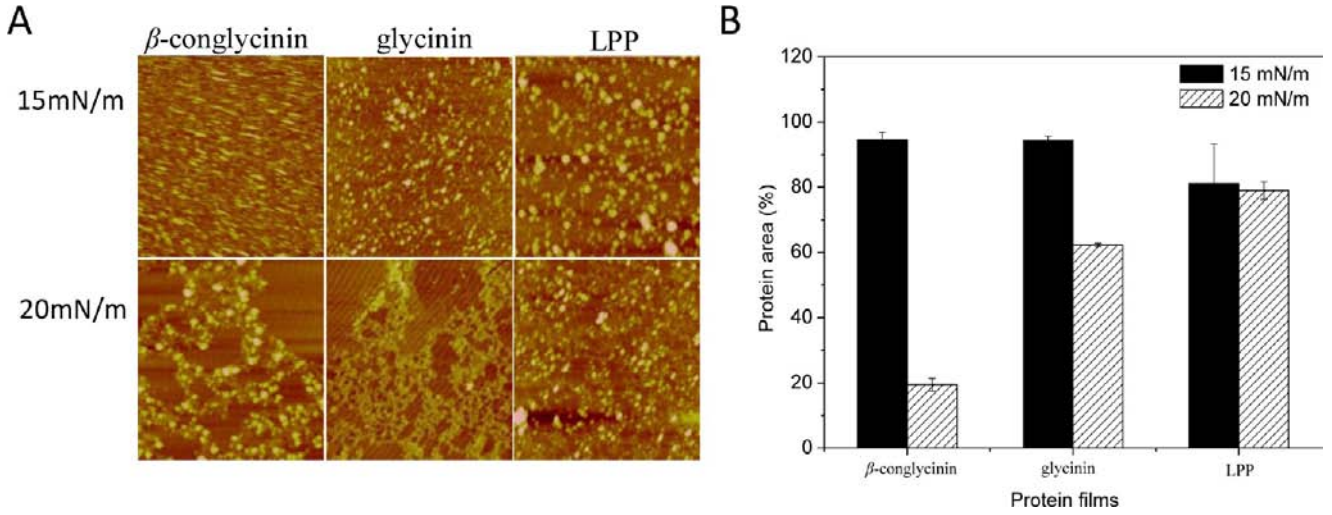

Figure 7. Atomic force microscopy (AFM) images (2 μm × 2 μm) of LB films (A) and the protein area fraction (B) of the β-conglycinin, glycinin, and soy lipophilic protein nanoparticle (LPP) before (15 mN/m) and after (20 mN/m) displacement by Tween 20 at the air/water interface.

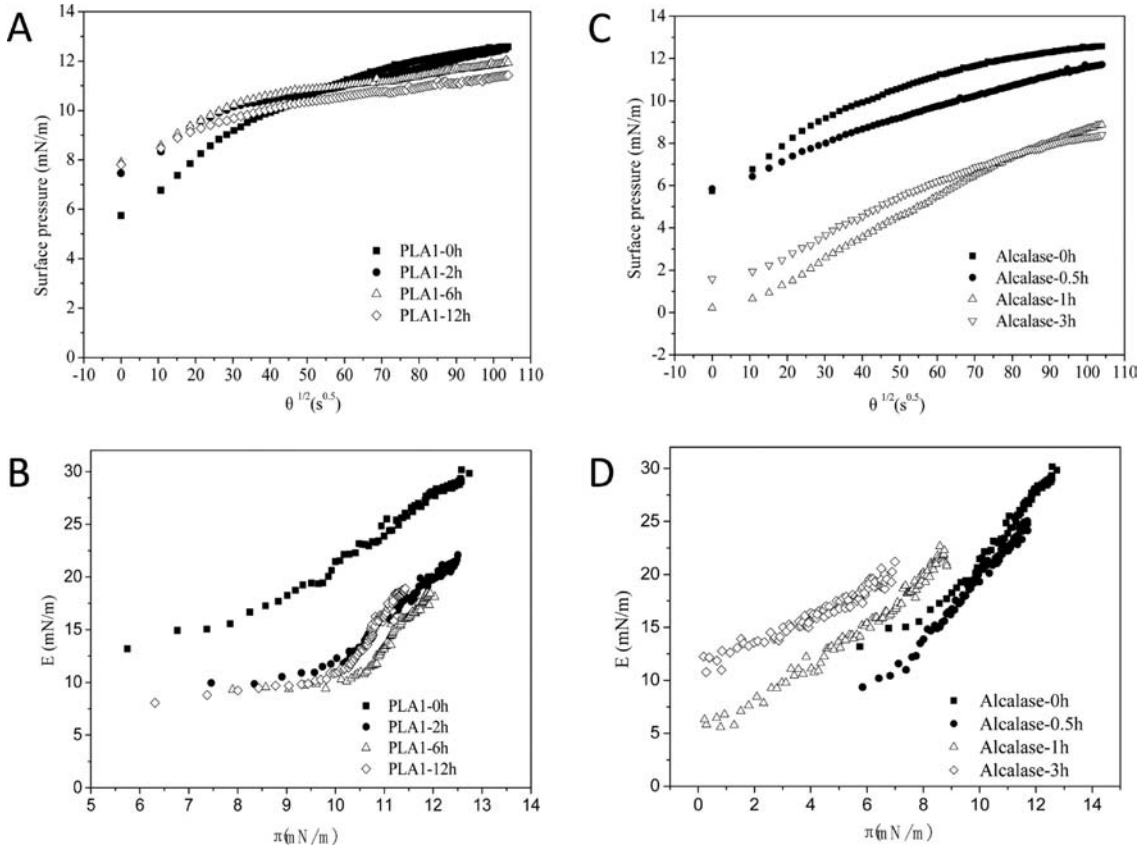

Figure 8. Dynamic surface pressure (A, C) and dynamic dilatational modulus (B, D) of soy lipophilic protein nanoparticle (LPP) (0.05% w/v, pH 7.0) hydrolyzed by PLA1 (A, B) and Alcalase (C, D) at the O/W interface.

showed more dark patches, which were originally occupied by protein but now displaced by surfactant. The protein occupied surface area before and after displacement calculated from AFM images shown in Figure 7B. Interestingly, there was no obvious change on the interfacial protein area of LPP film after displacement, indicating their strong ability to inhibit displacement by Tween 20. The image also exhibited the lace-like interfacial network of β-conglycinin after the Tween 20 displacement (Figure 7A), similar to the phenomenon observed previously for most globular proteins, suggesting the displacement occurred by an orogenic mechanism.[21,22]

In the orogenic model,[21] the displacement of protein by nonionic surfactant derives from defects of the pure protein film in nature. As proteins adsorb at the interface, they become partially unfolded and also interact with each other; the space that is available for future adsorption of proteins is reduced, and eventually holes or patches are increased in the interfacial network. Small molecular weight surfactants can enter these holes, which are too small to allow protein adsorption, and

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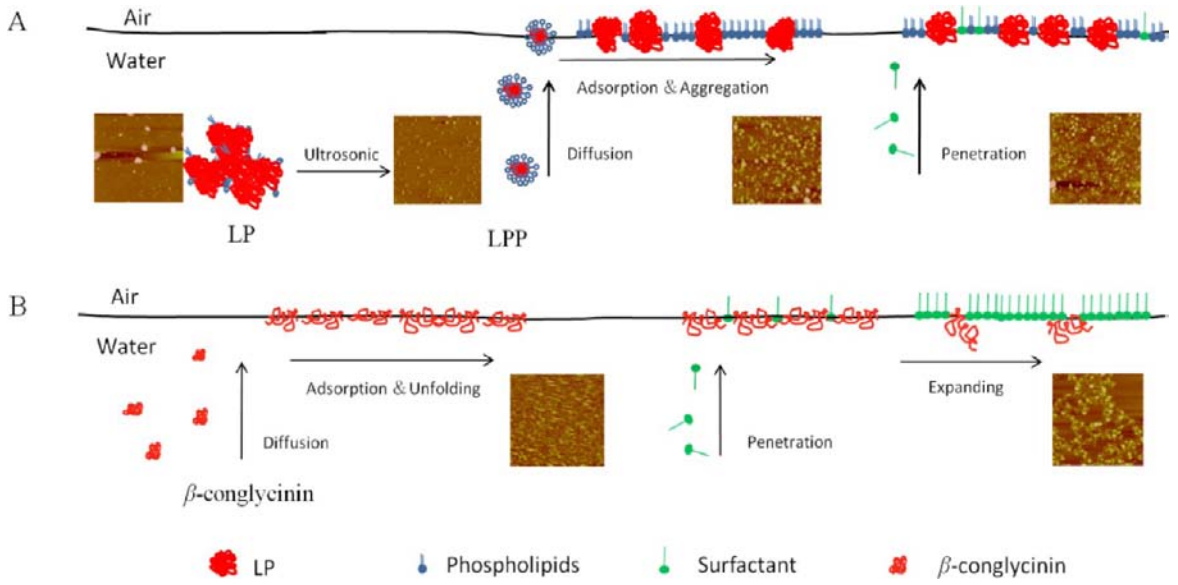

Figure 9. Schematic diagrams showing the formation and stabilizing mechanism of the soy lipophilic protein nanoparticles (LPP) complex interface.

grow and expand their domain on the interface before eventually destroying the protein network. In this experiment, we observed that, unlike β-conglycinin and glycinin, the LPP was able to resist the displacement, mainly due to their unique composition of hydrophobic protein and their abundant phospholipids. It has been demonstrated that most of the oleosins could not be displaced when oil body emuthelsion was incorporated with Tween 80 at high concentration.[33] In consideration of the fact that the LP fraction was associated with lecithin (phospholipids), it is very likely that LPP formed a complex film at the air/water interface, in which the phospholipids covered the holes or defects of the pure protein interface, thus preventing the penetration and adsorption of Tween 20 and, therefore, inhibiting the displacement.

Formation of the Complex Interface of LPP. We then probed the dynamic interfacial adsorption and dilatational modulus of LPP at the O/W interface by the pendant drop technique, in which the LPP was modified by PLA1 and proteinase (Alcalase). The changes of surface pressure (π) with adsorption time ($\theta^{1/2}$) were used to characterize the dynamic adsorption process. Panels A and C of Figure 8 show the time evolution of surface pressure in the O/W interface for LPP after being hydrolyzed by PLA_1 and Alcalase for different times. As shown in Figure 8A, the hydrolysis of phospholipids for different times yields similar surface pressures. Preliminary study indicated the mean particle size of LPP increased greatly after they were treated with PLA1 (data not shown). Phospholipids adopt lamellar and hexagonal structures in water on the basis of their concentration, to build liposomes and micelles.[35] It is likely that the hydrolysis of phospholipids could destroy the micelle structure of LPP and subsequently lead to an aggregation. The hydrolysis of phospholipids yielded more hydrophilic small molecular weight surfactants, including lysophospholipids (lyso-PLs) and free fatty acids, and exhibited high π values over the long adsorption times. After the protein components of LPP were hydrolyzed by Alcalase, the surface pressure (π) decreased visibly (Figure 8C). Such proteolysis may lead to the aggregation of LPP, but the small peptide fragments from the proteolysis of LPP are difficult to diffuse to the interface, ultimately leading to the decline of surface pressure.

Interfacial dilatational rheology of adsorbed layers may serve as an indicator of structural state of proteins adsorbed at the O/W interface and macromolecular interactions. The evolution of surface dilatational modulus (E_d) as a function of surface pressure (π) for LPP, after it was modified with phospholipase and proteinase, was measured by the oscillating drop technique (Figure 8B,D). The high π value (Figure 8A) and low E_d value (Figure 8B) exhibited by the PLA1-hydrolyzed LPP suggest that the interface was dominated by the lyso-PLs and free fatty acids. The hydrolysis of PLs would inhibit the diffusion of LPP onto the interface. The dilatational modulus of LPP at the O/W interface decreased greatly by the hydrolysis of phospholipids and protein, suggestive of a synergic effect between hydrophobic proteins and phospholipids in LPP. Deleu et al.[36] compared the different effects of phospholipids and oleosins on the stability of reconstituted rapeseed oil bodies at various phospholipid/oleosin ratios. Oil bodies rich in oleosins were resistant to coalescence but quickly resulted in flocculation and creaming due to the lower absolute zeta-potential. Addition of phospholipids to oil bodies increases electrostatic repulsive forces of oil droplets and avoids flocculation. These results demonstrated the synergic effect between proteins and phospholipids in the LP fraction, not only during the formation of protein particle but also in maintaining the stability of the interface.

General Discussion. In this work, we employed a simple ultrasonication treatment to prepare LPP (Figure 2), in which the water dispersibility of soy LP was greatly improved. Moreover, the modified LP could serve as a new bulk food functional ingredient because it exhibited great potential as an emulsifier and interface stabilizer (Figures 5 and 6), particularly as the O/W emulsion stabilized by LPP exhibited excellent physical stability after being subjected to long-term storage and a stress storage test and in the presence of Tween 20 (Figures 3 and 4). For better understanding of the stabilization mechanism of LPP, we propose schematic illustrations as shown in Figure 9.

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LP could reconstitute to form nanoparticles (LPP) after it was ultrasonically treated; during the process the hydrophobic surface of LP proteins was stabilized or covered by phospholipids, thus greatly improving its diffusion capacity. As a carrier, LPP could help to deliver hydrophobic protein, which originally had very limited solubility in water, to migrate to the interface. When LPP arrived at the interface, it began to unfold partially until rupture, and the hydrophobic protein fraction was unloaded and aggregated quickly on the interface, forming a cross-linked complex film of hydrophobic aggregates and lipids;[34] thus, a rough and elastic interfacial protein network is formed (Figures 6 and 7). The complex interface formed, with increased dynamic interfacial adsorption and dilatational modulus of the interface simultaneously (Figure 8A,B), provided the oil droplets with the synergic effects: the steric interactions (by hydrophobic protein) and electrostatic repulsive forces (by phospholipids).[36] It is resistant not only to coalescence but also to the flocculation and creaming of emulsion (Figures 3 and 4). However, glycinin and β-conglycinin, which are similar to the majority of globular proteins, form flexible and viscose interfacial structures (Figures 6 and 7) and develop inevitably an interfacial network with holes or patches.[21] Small molecular weight surfactants such as Tween 20 could penetrate the patches, growing and expanding its domain on the interface[21] and displacing the protein network (Figure 5). Meanwhile, LPP formed a complex interface in which the phospholipids covered the holes or defects in the protein film, thus delaying the penetration and adsorption of Tween 20 and consequently preventing the displacement. As a main constructive interface stabilizer, the hydrophobic protein aggregates cannot be displaced by the surfactant,[34] exhibiting integrity of oil droplets and arresting the coalescence of emulsion. Our findings provide evidence that the complexes of hydrophobic protein aggregates and biosurfactant could form a more stable interface, which would be a novel strategy to fabricate a more stable food emulsion.

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Notes

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