JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Emulsifying and Interfacial Properties of Vicilins: Role of Conformational Flexibility at Quaternary and/or Tertiary Levels

Han-Ni Liang[†] and Chuan-He Tang*[,†,§]

[†]Department of Food Science and Technology, South China University of Technology, Guangzhou 510640, People's Republic of China

[§]State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, People's Republic of China

ABSTRACT: Although the functionality of plant proteins (and soy proteins in particular) has been widely investigated in the last decades, the importance of conformational characteristics to their functionalities is still far away from being understood. The aim of the present work was to unravel the role of conformational flexibility at the quaternary and/or tertiary levels in the emulsifying and interfacial properties of phaseolin, an ideal vicilin (or 7S globulin) from red kidney bean. The conformational flexibility at quaternary and tertiary levels of phaseolin was modulated by urea with increasing concentrations from 0 to 8 M, as characterized by using dynamic light scattering (DLS), intrinsic fluorescence and derivative UV spectroscopy, and differential scanning calorimetry (DSC). The emulsifying and interfacial properties, including emulsifying ability, flocculated state of oil droplets (in fresh emulsions), emulsion stability against creaming, and adsorption dynamics at the oil−water interface, were characterized at a specific protein concentration of 0.5% (w/v). The results indicated that increasing the urea concentration resulted in a progressive dissociation of trimeric phaseolin molecules into monomeric subunits, and even a structural unfolding of dissociated subunits; the urea-induced conformational changes at quaternary and/or tertiary levels were reversible, and the molecules at high urea concentrations shared similar structural features to the "molten globule state". On the other hand, increasing the urea concentration progressively improved the emulsifying ability of the protein, and flocculated extent of oil droplets in the fresh emulsions, but led to a progressive decrease in interfacial protein concentration. The improvement of the emulsifying ability was not related to diffusion (during initial adsorption) and penetration at the interface, but highly dependent on ease of structural rearrangement of the adsorbed proteins. These observations clearly confirmed that the flexibility of phaseolin at quaternary and/or tertiary levels plays a vital role in its emulsifying ability, mainly through the way of affecting the ease of structural rearrangement of adsorbed proteins at the interface. The findings could provide an in-depth understanding of the importance of conformational flexibility for the emulsifying properties of oligomeric storage globulins, and thus are of great help to guide the modifications of the proteins for better emulsifying properties.

KEYWORDS: *phaseolin, vicilin (7S globulins), emulsifying ability, conformational flexibility, interfacial property, urea denaturation*

INTRODUCTION

To date, the structure−functionality relationship as well as the molecular bases of the functionalities of globular proteins is still far away from being well understood.[1] This situation becomes more distinct in the research field of plant proteins (and soy proteins in particular) with complex structures. Vicilins, also often denoted as 7S globulins, are one of the major storage proteins for many legume or oilseed proteins of economic importance. The 7S vicilin is a trimer of 50-kDa subunits, associated through noncovalent interactions. These oligomeric globulins have been confirmed to exhibit good emulsifying and foaming properties,[2,3] with the emulsifying properties even better than legumin (also denoted as 11S globulins), due to their lower molecular weight and higher flexibility in tertiary structure.[4] However, despite an analogous subunit arrangement and a rather high similarity in the secondary structure, vicilins from different species exhibit considerable differences in surface properties and conformational characteristics.[2,5] As a consequence, considerable differences in emulsifying properties may occur for these proteins, which have been confirmed in the literature.[5,6] The emulsifying properties of vicilins are usually associated with their surface structural characteristics, e.g., surface hydrophobicity (and/or hydrophobicity/hydrophilicity balance),[2,7] as well as molecular conformational characteristics, e.g., conformational flexibility at the quaternary and tertiary levels. To date, very few studies have addressed the importance of these conformational characteristics to the emulsifying properties of vicilins.

There are several research strategies to investigate the structure−function relationships of vicilins. One strategy is to formulate recombinant proteins with different structural characteristics, through protein engineering. Comparing the native and recombinant samples, Utsumi's group[8−11] widely investigated the structure and physicochemical functions of soybean or other legume vicilins, and found many relationships between structural characteristics (e.g., surface hydrophobicity and thermal stability) and some physicochemical properties (including solubility and emulsifying ability), as well as the role of N-linked glycans in the subunits. In these works, the role of

Received: August 29, 2013
Revised: October 23, 2013
Accepted: October 23, 2013
Published: October 24, 2013

conformational characteristics (e.g., conformational flexibility at quaternary and/or tertiary levels) in the physicochemical functions is little addressed. Another strategy is to compare the physicochemical functions of vicilins with different polypeptide constituents or conformational characteristics from the same vicilin species, or from individual vicilin species. In this regard, we successfully isolated individual vicilins with different polypeptide constituents (or conformational characteristics) from mungbean, red bean, or kidney bean, and compared their physicochemical functions including emulsifying properties, and interestingly found that the emulsifying ability of these vicilins seems to be closely dependent on the flexibility in their quaternary conformation, while the emulsion stability was related to the flexibility of their tertiary conformation.[5,12] However, the relationship between the conformational flexibility and emulsifying properties of vicilins still needs to be further confirmed.

The structure−functionality of a specific vicilin also can be investigated by modulating its conformation and functionality through physical, chemical, and enzymatic modifications. Pedrosa and others[13] indicated that a mild, selective glycation with various carbohydrates at low modification levels can modulate the stability and subunit interactions of vicilin from pea seeds. In another work,[14] they observed that this kind of glycation can efficiently improve the emulsifying capacity and emulsion stability of this vicilin. These observations suggest the presence of the relationships between the tertiary conformational stability and emulsifying properties of the vicilin. Using the same glycation technique with glucose, we studied the influence of glycation at different modification levels on the conformation characteristics and some physicochemical functions (including emulsifying properties) of phaseolin, a vicilin from kidney bean, and confirmed that many conformational characteristics including solubility and/or surface hydrophobicity, and conformational flexibility, contribute to the emulsifying properties of glycated vicilins.[12] Other chemical modifications, e.g., succinylation or acetylation, can also change the conformation and surface-related functionalities, as well as surface properties of vicilins or legumins.[2] In general, the changes in conformations or surface properties induced by those chemical modifications are complex, thus causing a difficulty in understanding the role of conformational flexibility in the functionality. By comparison, some mild modifications of conformations by some protein perturbants (e.g., urea or GdnHCl) seem to be a better choice in this aspect. Urea has been widely applied to modulate unfolding and/or denaturation of globular proteins in many biochemical research fields,[15,16] and even emulsifying activity of proteins.[17] In fact, a high concentration of 6 M urea can act as a subunit dissociation agent in the previous works to isolate individual subunits of soybean β-conglycinin.[18,19] This implies that the intersubunit interactions, e.g., hydrogen bonding and hydrophobic interactions, or intrasubunit interactions of vicilins, can be modulated by urea with increasing concentrations, thus suggesting a good strategy to investigate the structure−functionality relationship of these proteins.

Thus, the present work aimed to provide an insightful knowledge about the importance of conformational flexibility (at quaternary and/or tertiary levels) to the emulsifying properties of vicilins. To achieve this goal, a unique vicilin (phaseolin) that exhibits excellent emulsifying properties (as compared to other vicilins[5,6,11]) was applied as the model vicilin, and its conformations and emulsifying properties were modulated by urea with varying concentrations. In the first part, we mainly investigated the influence of urea concentration (0−8 M) on the conformational characteristics of this vicilin, and found that its conformations at quaternary and/or tertiary levels could be delicately modulated by changing urea concentration. The conformations at tertiary and/or quaternary levels were characterized by using dynamic light scattering (DLS), intrinsic fluorescence, and second-derivative ultraviolet (UV) spectroscopy, whereas the emulsifying ability and emulsion stability were characterized in terms of emulsion size and creaming index. To better understand the structure−function relationship, we also characterized the adsorption dynamics, penetration, and structural rearrangement of vicilin at the oil−water interface. According to the authors' knowledge, this is the first work to indicate the role of conformational flexibility at quaternary and/or tertiary levels in the emulsifying and interfacial properties of oligomeric globulins.

MATERIALS AND METHODS

Materials. Red kidney bean (*Phaseolus vulgaris* L.), cultivated in Shandong province or North-East area of China, was purchased from a local supermarket (Guangzhou, China). The seeds were soaked in deionized water for 12 h at 4 °C and dehulled manually, and the dehulled seeds were freeze-dried and ground to produce the flour. The 1,8-anilinonaphthalenesulfonate (ANS^-), Nile Blue A, and Nile Red were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Fitzgerald Industries International Inc. (Concord, MA, USA). Soy oil (relative density of 0.92 g/mL at 25 °C; insoluble material content, < 0.05%), produced by the pressing technique, was purchased from a local supermarket in Guangzhou (China). The purified water, produced after reverse osmosis and ion exchange treatments, was applied in all the experiments. All other chemicals used were of analytical grade.

Preparation of Phaseolin. The phaseolin (7S globulins) from the kidney bean flour was prepared according to the process of Hall and others,[20] as described in our previous work.[5] In brief, the flour (10%, w/v) was dispersed in 0.025 M HCl (pH 3.5) with 0.5 M NaCl. After being stirred at 25 °C for 2 h, the dispersion was centrifuged (9000 *g*, 20 min) at 4 °C with a centrifuge (CR22G, Hitachi Co., Japan). The obtained supernatant was mixed with 4-fold volumes of deionized water (∼4 °C), and left at 4 °C for at least 2 h for the globulin precipitation. The precipitate was collected by centrifugation at 9000 *g* for 20 min at 4 °C. The resultant pellet was dispersed in 0.5 M NaCl solution, and reprecipitated twice as above. The last obtained precipitate was finally dissolved in 0.5 M NaCl solution and dialyzed against deionized water at 4 °C for 48 h, and then lyophilized to produce the phaseolin sample. The protein content was calculated to be above 95% (dry weight), using the Dumas method with a nitrogen conversion factor of 6.25. The electrophoresis pattern of the protein was similar to that described in our previous work,[5] indicating effectiveness of isolation for the phaseolin.

Urea-Induced Denaturation by Phaseolin. The denaturation experiments in urea with varying concentrations of 0−8 M were done in 50 mM phosphate buffer (pH 7.0). After fully being dissolved in urea solutions with varying concentrations, the protein solutions at protein concentrations (*c*) of 0.01−1.0% (w/v) were stirred for 2 h, and then left overnight at 4 °C for full hydration and action of urea with the protein molecules.

DLS. The particle size distribution of phaseolin molecules in solutions with varying urea concentrations (0−8 M) at a specific *c* value (0.1%, 0.25%, 0.5%, or 1.0%, w/v) was evaluated by using the DLS technique, as described in our previous work.[1] Each sample was determined in duplicate.

Intrinsic Emission Fluorescence Spectroscopy. Intrinsic (or Trp) emission fluorescence spectra of phaseolin solutions in 50 mM phosphate buffer (pH 7.0) at a *c* value of 0.01% but with varying concentrations (0−8 M) of urea were determined in a F4500 fluorescence-spectrophotometer (Hitachi Co., Japan). Protein sol-

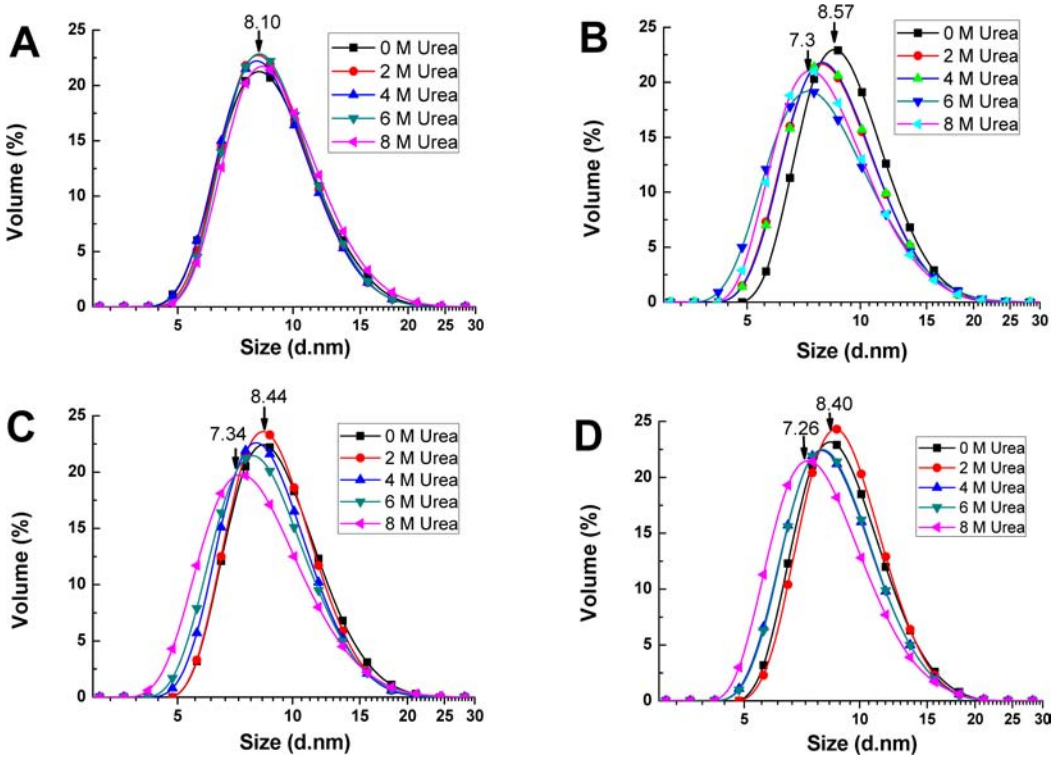

Figure 1. Typical particle size distribution profiles of native and urea-denatured phaseolin at various c values of 0.1% (A), 0.25% (B), 0.5% (C), and 1.0% (D), in the presence of 0−8.0 M urea, respectively.

utions were excited at 290 nm, and emission spectra were recorded from 300 to 400 nm at a constant slit of 5 nm for both excitation and emission.

Second-Derivative Ultraviolet (UV) Spectroscopy. The phaseolin solutions at c = 0.1% (w/v) with varying concentrations of urea (0−8M) were used for the spectral determination. Baseline corrected UV spectra were recorded between 250 and 300 nm at medium speed in the double beam mode with use of a UV2300 spectrophotometer (Techcomp Co., Chnia). The data interval was 0.2 nm. The second-derivative treatment of UV spectra was performed with Origin 8.0 software (Origin-Lab Corp.,Northampton, MA).

Differential Scanning Calorimetry (DSC). The thermal denaturation of various protein samples was examined by using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, Delaware, USA). Each protein sample (approximately 2.0 mg) was weighed into an aluminum liquid pan (Dupont), and 10 μL of 0−8 M urea in 50 mM phosphate buffer (pH 7.0) was added. The pan was hermetically sealed and heated from 25 to 120 °C at a rate of 5 deg/min. A sealed empty pan was used as a reference. Denaturation temperature (T_d), enthalpy change of the endotherm (ΔH), and cooperativity were computed from the thermograms by the Universal Analysis 2000, Version 4.1D (TA Instruments-Waters LLC). For each sample two consecutive measurements were performed (with the means used for statistics analysis), and each sample was replicated 3 times. The sealed pans containing protein samples and buffers were equilibrated at 25 °C for at least 6 h.

To testify the reversibility of urea-induced dissociation and/or denaturation of phaseolin, an experiment for protein refolding was designed as follows: A phaseolin solution at c = 20% (w/v) in 50 mM phosphate buffer (pH 7.0) with 8 M urea was prepared as above, and left for overnight at room temperature; then, the protein solution was dialyzed against the same buffer but without urea at 4 °C for 48; last, the thermal transition of the urea-treated and dialyzed sample (10 μL; in pans) was evaluated as above. The phaseolin solution at c = 20% (w/v) in 50 mM phosphate buffer (pH 7.0) without urea was treated following the same process, and applied as the control.

Preparation of Phaseolin-Stabilized Emulsions. The emulsions stabilized by phaseolin in 50 mM phosphate buffer at pH 7.0 (containing 0.02% sodium azide) at c = 0.5% (w/v), with various concentrations (0−8 M) of urea, were prepared as follows. In brief, 10 mL of soy oil and 40 mL of each phaseolin solution were first prehomogenized with use of a high-speed dispersing unit (model IKA-ULTRATURRAX T25 basic, IKA 190 Works, Inc., Wilmington, NC) at 10 000 rpm for 2 min. Then, the resultant dispersions were further homogenized though a Microfluidizer (M110EH model, Microfluidics International Corporation, Newton, MA), one pass with an overall pressure at 40 MPa at room temperature. Unless stated otherwise, all the preparations were performed at room temperature.

Evaluation of Emulsion Characteristics. *Droplet Size Distribution and Volume or Surface Average Size ($d_{4,3}$ or $d_{3,2}$).* The droplet size distribution and volume average size ($d_{4,3}$) of emulsions, diluted with distilled water or 1.0% (w/v) SDS, were determined by using a laser diffraction technique with a Malvern Mastersizer 2000 (Malvern Instrument Ltd., Worcestershire, UK). The refractive indices of soy oil and the continuous phase were 1.456 and 1.33, respectively, resulting in a relative refractive index of 1.095 for the emulsion. The results are reported as the volume-average diameter $d_{4,3}$ $(=\sum n_i d_i^4/\sum n_i d_i^3)$ and surface-average diameter $d_{3,2}$ $(=\sum n_i d_i^3/\sum n_i d_i^2$, where n_i is the number of droplets with diameter d_i). For each emulsion two consecutive measurements were performed (with the means used for statistics analysis), and each sample was replicated at least 2 times.

Percentage of Flocculation Index (FI%). The FI% of the fresh emulsions (at 0 h) was evaluated according to the method of Castellani and others.[21] The FI% was calculated as the following eq 1:

$$\text{FI\%} = [(d_{4,3} \text{ in water})/(d_{4,3} \text{ in 1\% SDS}) - 1.0] \times 100 \quad (1)$$

Percentage of Adsorbed Proteins (AP%) and Interfacial Protein Concentration (Γ). The AP % and Γ of the fresh emulsions were determined according to the process of Puppo and others,[22] with a few modifications as described in our previous work.[23]

Percentage of Creaming Index. The creaming index (%) of the emulsions was evaluated according to the same method as described in our previous work.[1]

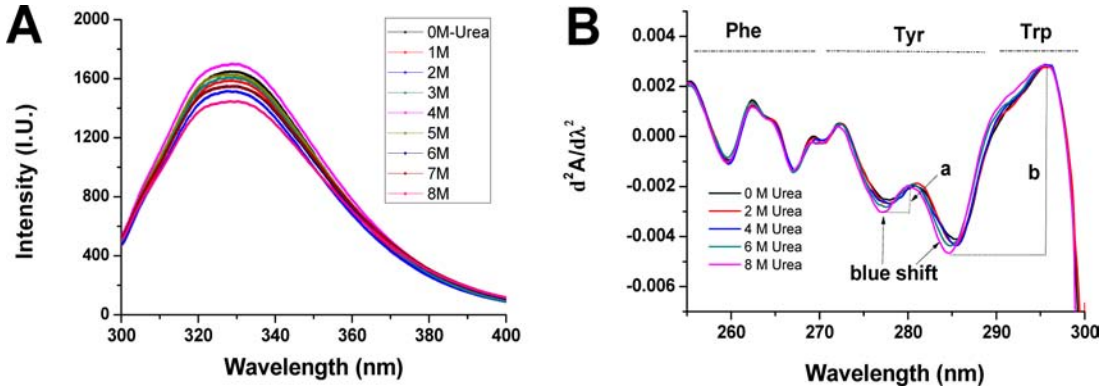

Figure 2. (A) Typical intrinsic emission fluorescence spectra of phaseolin at c = 0.01% (w/v), in the presence of 0−8 M urea. (B) Typical second-derivative UV profiles of phaseolin at c = 0.1% (w/v), in the presence of 0−8.0 M urea. The letters *a* and *b* represent the peak-to-trough amplitude. The assignment of Phe, Tyr, and Trp UV adsorption bands refers to the literature (ref 29).

Confocal Laser Scanning Microscopy (CLSM). The microstructure of the fresh emulsions was determined by using CLSM technique as described in our previous work,[1] with a few modifications. The emulsion samples were stained with an appropriate amount of the mixture of Nile Blue A (0.1%, w/v) and Nile Red (0.1%, w/v) in 1, 2-propanediol (containing distilled water, 20 mL/g).

Dynamic Surface Pressure (π) Measurement. An optical contact angle meter (OGA-20) with oscillating drop accessory ODG-20 (Dataphysics Instruments GmbH, Germany) was used to measure the adsorption kinetics of phaseolin at c = 0.5% (w/v) in 50 mM phosphate buffer (pH 7.0) with varying concentrations (0−8 M) of urea. The oil−water interface was formed for an oil drop at the inverted tip of a syringe, which was submerged into a cuvette with the protein solution. The interfacial tension (γ) was calculated from the drop shape by using the Young−Laplace equation of capillarity, as described by Güzey et al.[24] Surface pressure (π) is defined as the decrease in surface tension of a pure solvent caused by the addition of the protein ($\pi = \gamma_s - \gamma_p$), where γ_s is the surface tension of the aqueous buffer solution (at any test time) and γ_p is the surface tension of the corresponding protein solution (at the comparable test time). All the measurements were performed in duplicate at a room temperature of 25 °C. A period time up to 3 h was needed to achieve protein adsorption at the oil−water interface. For each sample two consecutive measurements were performed (with the average used for statistics analysis), and each sample was replicated at least 2 times.

Statistics. ANOVA of the data was performed, and a least significant difference (LSD) at the $p < 0.05$ level was used to compare the means.

RESULTS

Conformational Characterization: Influence of Urea Concentration on the Conformations. The size and number of particles in the protein solution can be detected by using the DLS technique.[25] We determined the size distribution profiles of phaseolin particles at various c values of 0.1−1.0%, in the presence of 0−8.0 M urea, as displayed in Figure 1. As expected, a similarly homogeneous Gaussian profile of size distribution was observed for the particles in all the solutions, with a distribution peak centered at around 7.2−8.6 nm depending on the c and urea concentration (Figure 1). In the absence of urea, the distribution peak diameter (D_p) of the solution at c = 0.1% (8.1 nm) was distinctly lower than that (8.4−8.6 nm) at higher c values (0.25−1.0%), possibly reflecting enhanced intermolecular attractions at higher c values. This is an indication that the conformation state of phaseolin in the solutions varied with the applied c, with a compacted conformation observed at c = 0.1% rather than at c = 0.25−1.0%. However, we can still observe that in this case at c values of 0.25−1.0%, the D_p progressively decreased with increasing the c (Figure 1). The progressive decreasing in D_p might reflect an increasing contribution of intermolecular repulsion to the conformation state of the proteins in the system. A similar c dependence of the hydrodynamic diameter within a comparable c range (0.25−1.0%, w/v) has been observed for bovine serum albumin (BSA),[1] where it was mainly attributed to increased Coulomb repulsion interaction of proteins. The D_p of phaseolin particles at c = 0.1% agrees well with the diameter (~8.5 nm) of the phaseolin flat-disc structure based on electron microscopy observation.[26] The data are also similar to the hydrodynamic diameter (10.8 nm) observed for the 7S form of soy glycinin with use of the same DLS.[27]

The particle size of phaseolin was changed by urea, but the changes varied with both the applied c and urea concentration. At c = 0.1%, the particle size of phaseolin was nearly unaffected by urea, even at a concentration up to 8 M, while at higher c values, increasing urea concentration resulted in a peak shift toward smaller sizes, e.g., the D_p at c = 0.25% decreased from 8.57 to 7.3 nm with the urea concentration increasing from 0 to 8 M (Figure 1). The differences might to a certain extent confirm the differences in conformational state between at c = 0.1% and at c = 0.25−1.0%. The three subunits or polypeptides in phaseolin are usually associated through noncovalent interactions, e.g., hydrophobic interactions and hydrogen bonding, to form the 7S structure. Urea can disrupt hydrogen bonding and hydrophobic interactions, thus causing dissociation of the phaseolin trimers into monomers. Besides structural dissociation at the quaternary level, the presence of urea might also cause unfolding of dissociated subunits (or polypeptides). Thus, the urea-induced changes in particle size are usually a net result of the contributions from the structural dissociation at the quaternary level (negative) and the subunit structural unfolding at the tertiary level (positive). In the case of c = 0.1%, the slight changes in particle size are thus possibly because the positive contribution of subunit dissociation (at the quaternary level) is compensated by the positive contribution of structural unfolding of dissociated subunits (at the tertiary level). In contrast, the urea-induced changes in particle size at c = 0.25−1.0% might be mainly dominated by the contribution of subunit dissociation at the quaternary level. On the other hand, in the case of c values above 0.1%, we can still observe that a higher urea concentration was needed to initiate the subunit dissociation at higher c values (Figure 1). For example, at c =

0.25% the presence of 2 M urea resulted in a considerable shift of the distribution peak, while at c = 0.5% or 1.0%, it needed a urea concentration of 4 M. This is evidence showing the presence of interparticle Coulomb repulsion, since the latter can drive the protein molecules to form a more compacted conformation.

To further unravel the urea-induced changes in tertiary conformation of phaseolin, we evaluated the influence of urea concentration (0−8 M) on the intrinsic fluorescence and second-derivative UV spectra of phaseolin. Due to the c limitation for the spectral determination, the experiments were performed at c = 0.01% and 0.1% for intrinsic fluorescence and UV spectral determination, respectively. We observe that at c = 0.01%, the Trp chromophores did not suffer a peak shift, with the peak centered at around 330 nm, which is a characteristic of Trp chromophores in a relatively hydrophobic microenvironment,[28] but the quantum yield of fluorescence changed upon increasing the urea concentration (Figure 2A). The quantum yield of fluorescence decreased first as the urea concentration increased from 0 to 2 M, and then on the contrary increased up to a maximal value at 4 M urea (even higher than that at 0 M urea); last, it progressively decreased upon increasing urea concentration from 4 to 8 M. The changing pattern of fluorescence quantum yield might reflect the contribution of both quaternary and tertiary conformational changes (induced by urea; negative) and variation in solvent polarity (positive). The initial decrease in quantum yield can be largely attributed to urea-induced disassociation of the trimeric form at the quaternary level, while the progressive decrease in quantum yield at high urea concentrations seems to be chiefly contributed by urea-induced unfolding of the structure at the tertiary conformational level. In contrast, the abnormal increase in quantum yield upon urea concentration increasing from 2 to 4 M could be largely associated with the decrease in solvent polarity (at higher urea concentrations). Besides the Trp chromophores, the microenvironment of the Tyr chromophores also can be affected by the presence of urea with increasing concentrations (0−8 M). Figure 2B shows the second-derivative UV spectra of phaseolin at c = 0.1% and varying urea concentrations of 0−8 M. We can interestingly see that at this c, the Tyr chromophores suffered a blue shift of the spectral peaks, with much higher extents of changes in magnitude than the Trp chromophores, while the spectra of the Phe chromophores were nearly unaffected by the presence of urea even at 8 M (Figure 2B). This is generally concurrent with the fact that the Phe chromophores are usually located in a very hydrophobic microenvironment, while the Trp chromophores are associated with a relatively hydrophilic microenvironment. The amplitude ratio ($r = a/b$) of the two peak-to-trough values, as marked in Figure 2B, has been well used to indicate the change in solvent polarity of the Tyr chromophores.[29] Upon the increase in urea concentration (0−8.0 M), the r progressively increased from 0.084 to 0.138, suggesting that the solvent polarity of the Tyr chromophores considerably increased. The urea-induced changes in microenvironment polarity of the Tyr chromophores in phaseolin are similar to those induced by thermal treatment.[30] These observations confirmed that the tertiary conformation of phaseolin could be to a certain extent affected by urea with increasing concentration up to 8 M.

However, it is still uncertain whether the above observations about the urea-induced changes in tertiary conformations of phaseolin at low c values (~0.1%) can be applicable to the situation at high c values, e.g., >1.0%. To confirm this, we further investigated the influence of urea concentration on the thermal transition of phaseolin at a high c value of approximately 20% using DSC. The peak transition or denaturation temperature (T_d) and enthalpy change (ΔH) are two important DSC characteristics, reflecting thermal or even conformational stability, and proportion of undenatured protein in a globulin sample (or content of ordered structure), respectively.[31] In the DSC profiles of native phaseolin, a prominent endothermic peak with T_d of 92.5 °C was observed (data not shown), which is consistent with the previous literature,[5,12,32] reflecting the thermal denaturation of phaseolin polypeptides or subunits with uniform polypeptide homogeneity. Upon increasing the urea concentration from 0 to 8 M, we observe that both the T_d and ΔH of the phaseolin component progressively and significantly decreased from 92.5 to about 82−83 °C and from 16.6 to 4.8 J g^{-1}, respectively (Table 1). Similar changes in T_d and ΔH of phaseolin upon increasing urea concentration have been observed in our previous work.[32] Meng and Ma[33] also reported that increasing urea concentration from 0 to 8 M resulted in a progressive decrease in T_d and ΔH of 7S globulin from red bean. In general, urea can effectively disrupt the hydrogen bonding of water and facilitate protein unfolding by weakening hydrophobic interactions.[34] Urea can also cause loss of protein structural stability, by means of enhanced "permittivity" of water for nonpolar residues.[35] The observations thus indicate that the presence of urea with increasing concentrations led to a progressive disassociation of trimeric phaseolin molecules, largely by means of urea-induced disruption of intersubunit noncovalent interactions, e.g., hydrogen bonding and hydrophobic interactions. The observation that the T_d did not further decrease as the urea concentration reached above 6 M (Table 1) suggests that the disruption of phaseolin molecules at the quaternary conformational level might be basically complete at 6 M urea concentration (Table 1). In contrast, the further increase in urea concentration from 6 to 8 M still led to a significant decline in ΔH (Table 1), reflecting a loss of protein structural stability at the tertiary conformational level, possibly due to favored "permittivity" of nonpolar residues for water.

Table 1. DSC Characteristics of Native and Urea-Denatured Phaseolin, with Increasing Concentrations (0−8 M) of Urea[a]

phaseolin samples	T_d (°C)[d]	ΔH (J·g^{-1})[d]
0 M urea	92.52 ± 0.76[a]	16.60 ± 2.11[a]
2 M urea	88.19 ± 1.25[b,c]	10.54 ± 1.62[b,c]
4 M urea	85.76 ± 0.75[c,d]	8.85 ± 2.36[b,c]
6 M urea	82.19 ± 0.41[d]	6.32 ± 1.11[b,c]
8 M urea	83.54 ± 2.44[d]	4.76 ± 0.33[c]
refolded[b]	90.05 ± 0.24[a,b]	10.6 ± 1.37[b,c]
control[c]	90.32 ± 0.01[a,b]	12.09 ± 1.96[a,b]

[a]Each data entry is the mean and standard deviation of 3 replicates. [b]Refolded: the proteins (approximately 20%, w/v) were solubilized in pH 7.0 phosphate buffer containing 8 M urea, and hydrated overnight; the solutions were then dialyzed against the same buffer but without urea for 48 h (to remove the urea). [c]Control: the proteins (approximately 20%, w/v) were solubilized in the same buffer without urea, and then treated as the same process as for the refolded sample (above). [d]Different superscripts (a−d) represent significant difference at the $p < 0.05$ level among the same column.

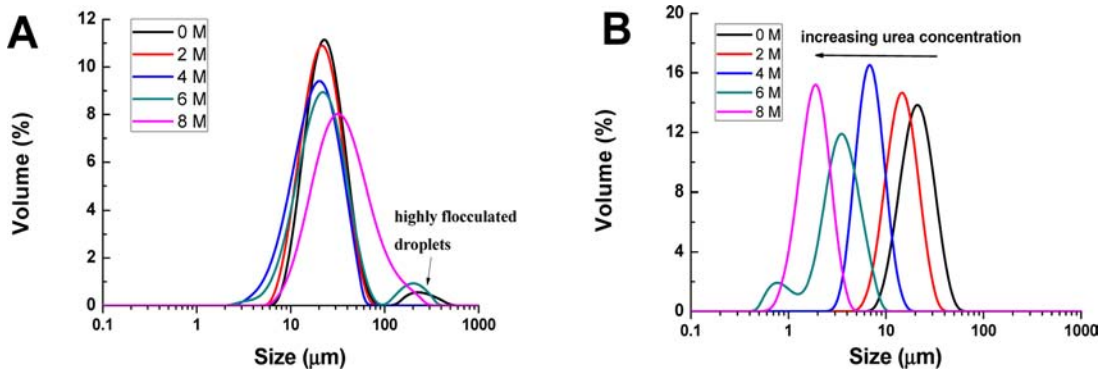

Figure 3. Typical droplet size distribution profiles of emulsions stabilized by phaseolin at c = 0.5%, in the presence of increasing concentrations (0−8 M) of urea: (A) diluted in water and (B) diluted in 1% SDS.

Table 2. Emulsion Characteristics, Including Mean Size Droplet ($d_{4,3}$ and $d_{3,2}$), Percentage of Flocculation Index at 0 h (FI%), Interfacial Protein Concentration (Γ) of Phaseolin-Stabilized Emulsions at c = 0.5%, in the Presence of Increasing Concentrations (0−8 M) of Urea[a]

	$d_{3,2}$ (μm)		$d_{4,3}$ (μm)				
urea conn (M)	water	1% SDS	water	1% SDS	FI (at 0 h)	AP%	Γ (mg/m^2)
0 M	23.4^c	19.9^d	34.7^c	25.3^d	37.0^a	19.2^a	15.9^d
2 M	20.3^b	14.9^c	25.6^b	18.2^c	41.1^b	21.7^a	13.2^c
4 M	16.4^a	7.31^b	22.4^a	9.37^b	139.4^c	25.3^b	7.63^b
6 M	19.2^b	2.53^a	36.1^c	3.43^a	951.9^d	35.4^c	3.69^a
8 M	31.2^d	1.84^a	46.9^d	2.07^a	2169.6^e	39.4^d	2.98^a

[a]Each data entry is the average of at least 2 replications.

The above DSC data indicated that the flexibility of the quaternary conformation for phaseolin progressively increased up to a maximum, but the tertiary conformation of the protein seemed to be slightly affected, as the urea concentration increased from 0 to around 6 M, while the urea concentration was further increased up to 8 M, the structural unfolding of the disassociated phaseolin subunits (or polypeptides) might occur. The high stability of phaseolin against urea-induced denaturation has been previously reported,[32] where it was attributed to its unique structural peculiarity and compactness, largely associated with its amino acid composition.[5]

Using the DSC technique, we also evaluated the reversibility of the urea-induced changes in conformations of phaseolin. To achieve this, we removed the urea in the phaseolin solution at 8 M urea by dialysis, and then evaluated the DSC characteristics of the phaseolin component in the resultant sample. Interestingly, we found that after the removal of urea, the phaseolin exhibited almost the same DSC transition (with the same T_d and ΔH) to the control (dialyzed by the same process, but in the absence of urea; Table 1). This phenomenon indicates that urea-induced structural unfolding of phaseolin molecules at the quaternary or the tertiary conformational level is completely reversible, and the urea-denatured molecules can completely refold to form a structure similar to that of the control (without urea denaturation), thus implying that the conformational state of the dissociated phaseolin subunits at 8 M urea is like that described for the "molten globule state".[36] The reversibility of urea-induced denaturation has also been observed for a protein (with no disulfide bonds; named as β1) from the protein G B1 domain[15] and concanavalin A (an oligermeric globulin).[37]

Emulsifying Properties and Emulsion Characteristics. The emulsifying properties of phaseolin at a specific c value of 0.5%, as affected by variation with urea concentration, were evaluated by using the droplet size of the freshly prepared emulsions. The choice of the concentration is based on the consideration that under the conditions, the amount of proteins (phaseolin) was not enough to fully cover the interface of oil droplets, and thus the importance of conformational flexibility to its emulsifying properties could be better reflected (than at higher c values). Figure 3 shows typical droplet size distribution profiles of various fresh emulsions stabilized by phaseolin at c = 0.05% and varying concentrations (0−8 M) of urea, with water or 1% SDS as the dispersant. The mean droplet sizes of all the emulsions, including volume-averaged diameter, $d_{4,3}$, and surface-averaged diameter, $d_{3,2}$, are summarized in Table 2. The droplet size of a fresh emulsion, with 1% SDS as the dispersant, can reflect the emulsifying ability of the protein, with smaller sizes implying greater ability. With 1% SDS as the dispersant, we can observe that all the emulsions basically exhibited a monodal droplet size distribution profile, reflecting the homogeneity in size of the formed droplets; increasing urea concentration resulted in a progressive shift of the distribution peak toward smaller sizes, and concomitantly, the droplet size ($d_{4,3}$ or $d_{3,2}$; in 1% SDS) progressively and considerably decreased with increasing urea concentration (Figure 3B and Table 2). For example, the $d_{3,2}$ decreased by about 10-fold (from 19.9 to 1.84 μm) as the urea concentration increased from 0 to 8 M. This agrees well with the emulsifying activity index (EAI) data where the EAI progressively increased from 57 to 266 m^2/g (data not shown). The observations suggested a progressive improvement of emulsifying ability of phaseolin by increasing urea concentration.

As compared with the situation with 1% SDS, the droplet size profiles and mean droplet sizes of these emulsions without 1% SDS were much less dependent on the applied urea concentration (Figure 3 and Table 2), reflecting occurrence of droplet flocculation. The flocculated state of droplets in these

fresh emulsions can be reflected by using the percentage of flocculation index (FI%) as the indicator, as included in Table 2. Interestingly, the FI% for these emulsions progressively increased from 37 to 2170 as the urea concentration increased from 0 to 8 M (Table 2), indicating a progressive increase in extent of droplet flocculation. This can be further corroborated by the CLSM observations of these emulsions (Figure 4),

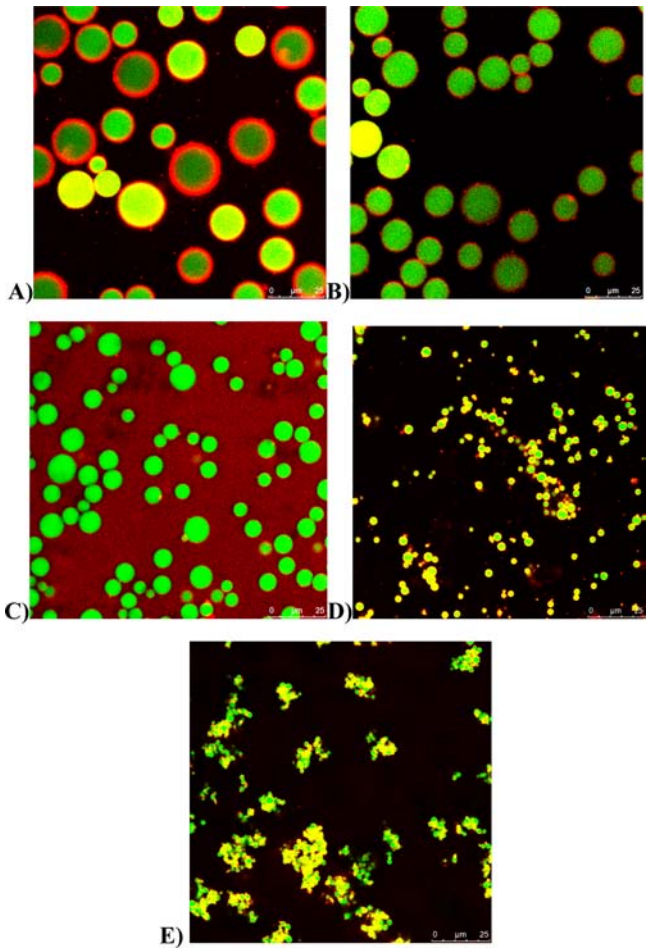

Figure 4. Typical CLSM images of the emulsions stabilized by phaseolin at c = 0.5% in the presence of 0 (A), 2 (B), 4 (C), 6 (D), and 8 M (E) urea, respectively. The scale bars within figures represent 25 μm in length.

where the microstructural morphology of oil droplets changed from a separate to aggregated or clustered state. Especially at 8 M urea, almost all the droplets participated in droplet flocculation, and the formed flocs looked like "grape clusters" (Figure 4E), indicating strong interdroplet attractive interactions.

We also evaluated the percentage of adsorbed proteins (AP %) and interfacial protein concentration (Γ) of these fresh emulsions in the presence of urea (0–8 M). As expected, the AP% progressively increased with the urea concentration (Table 2), which is consistent with the progressive increase in interfacial area of droplets. However, we can see that the increase in AP% was much less than that for the interfacial area (as evidenced by the $d_{3,2}$ data; Table 2). The differences are well in accordance with the Γ data, where it progressively decreased from 15.9 to 3.0 mg/m^2 upon the increase in urea concentration (Table 2). The observations indicated that the concentration of interfacial proteins or the thickness of interfacial films progressively decreased with increasing urea concentration.

The progressive increase in flocculation extent of oil droplets upon increasing urea concentration seems to be contrasting from the general viewpoint that urea can disrupt hydrogen bonding and hydrophobic interactions between the proteins.[34,35] Herein, it should be noted that the urea-induced disruption of intermolecular interactions between the proteins might depend on the magnitude of these interactions, and if the magnitude of interactions (e.g., hydrophobic interactions within the interior of globular proteins) is high enough, they will not be affected by the presence of urea, even at high concentrations. The present work confirmed that in the phaseolin solutions, urea could disrupt its intersubunit noncovalent interactions, but not its intrasubunit hydrophobic interactions, which implies that once the phaseolin molecules unfold at the tertiary conformational level, the unfolded phaseolins might interact through intermolecular hydrophobic interactions that cannot be affected by the presence of urea. Thus, the higher droplet flocculation of droplets at higher urea concentrations might be largely attributed to enhanced interdroplet hydrophobic interactions between adsorbed and unfolded phaseolin subunits. This is supported by the fact that unfolding and structural rearrangement of globular proteins occur once adsorbed at the interface.[36,38]

The stability of these formed emulsions against creaming, upon storage up to 12 days, was also investigated, as illustrated in Figure 5. All the tested emulsions fast developed into two

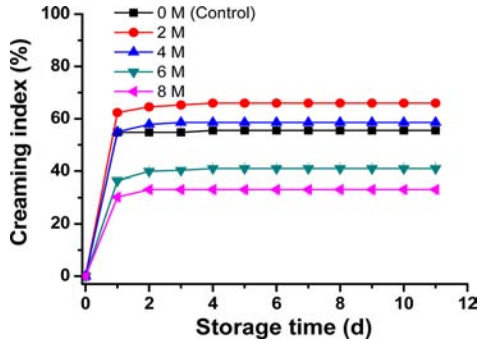

Figure 5. Time evolution of percentage of creaming index on storage time for phaseolin-stabilized emulsions at c = 0.5%, with increasing concentrations (0–8 M) of urea. Each data point is the mean and standard deviation of 3 replicates.

layers (cream and serum layers) within a short period of 1 day, but the creaming index percentage basically remained constant upon further storage up to 12 days (Figure 5). For the emulsion without urea, the creaming index percentage at prolonged periods was about 55%, indicating relative emulsion stability of the emulsion (since the applied oil fraction was 0.2). We can interestingly observe that at prolonged periods of storage, the presence of urea up to 2 M increased the creaming index percentage, but it progressively decreased as the urea concentration further increased from 2 to 8 M (Figure 5). At 8 M urea, the creaming index percentage (about 24%) was considerably lower than that without urea, indicating considerable improvement of creaming stability by increasing urea concentration. The improvement of emulsion stability might be related to the progressive increase in extent of droplet flocculation (Table 2). Droplet flocculation and subsequent

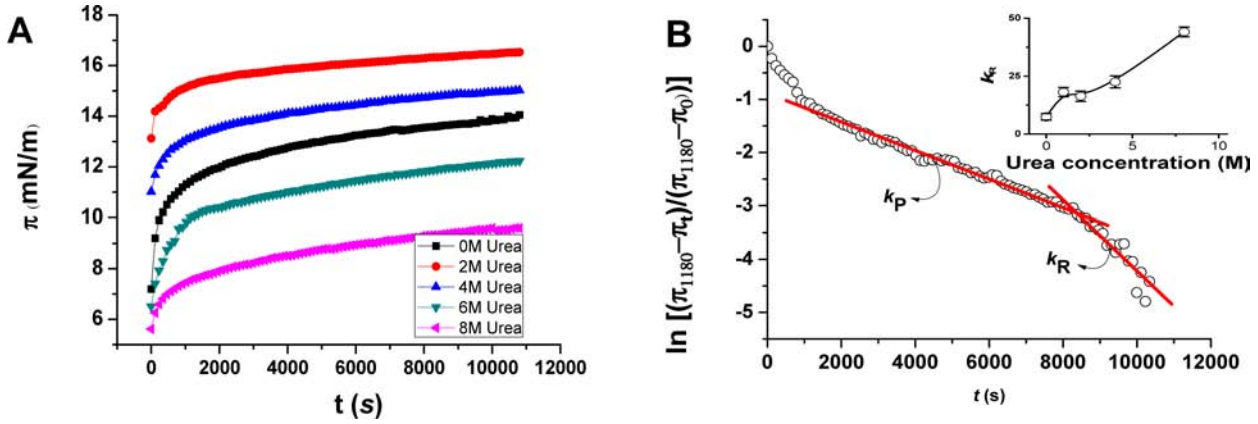

Figure 6. (A) Time evolution of surface pressure for the adsorption of phaseolin at the oil−water interface: phaseolin concentration in solution, 0.5%, w/v; urea concentrations, 0−8 M; temperature, 20 °C. (B) A typical profile of molecular penetration and configurational rearrangement step at the interface for phaseolin (without urea). k_P and k_R represent first-order rate constants of penetration and rearrangement, respectively. The influence of urea concentration on the k_R is displayed within the figure. Each datum is the mean and standard deviation of at least 2 replications.

formation of a gel-like network in the emulsions have been considered to be the underlying mechanism for the extraordinary creaming stability of soy protein-stabilized emulsions.[39]

Interfacial Properties: Adsorption Kinetics and Structural Rearrangements at the Interface. Although the interfacial protein films of droplets produced during emulsification are impossible to characterize, the evaluation of adsorption of the proteins at the quiescent interface may provide valuable information to help understand the emulsifying properties. Figure 6A shows the time evolution of surface pressure (π) for the adsorbed phaseolin films at the oil−water interface, in the presence of 0−8 M urea, reflecting the formation dynamics of the films at the quiescent interface. As expected, the π values in all the cases increased with adsorption time, with the increasing rate progressively decreasing, which is a typical characteristic of protein adsorption at the interface.[24,40] The changing pattern of π and the magnitude of π at the end of measurement (1180 *s*) considerably varied with the urea concentration (Figure 6A). The presence of 2 M urea not only increased the adsorption rate during the initial period (e.g., less than 60 s), but also resulted in a much higher π value at prolonged adsorption period, indicating improvement of adsorption at the interface. However, a further increase in urea concentration from 2 to 8 M contrarily led to progressive reductions in the adsorption rate (during the initial period of adsorption) and the π value at the end of the adsorption process (Figure 6A). We also determined the time evolution of interfacial π without the presence of the protein, at varying urea concentrations of 0−8 M, and found that the time dependence of π was insignificantly changed by variation in urea concentration (data not shown). This confirmed that the differences in adsorption behavior, observed between the different urea concentrations, were not caused by the differences in solvent polarity or dielectric constant, and instead, largely related to the urea-induced changes of molecular characteristics, e.g. molecular size, surface hydrophobicity, and conformational flexibility (at quaternary and/or tertiary levels). The possibility that surface hydrophobicity affected the adsorption of the protein could also be excluded, since we had preliminarily observed that at c = 0.1%, increasing urea concentration from 0 to 8 M progressively decreased the maximal fluorescence quantum yield of ANS^- (a fluorescence probe; data not shown), suggesting that urea might weaken the hydrophobic interactions between the exposed hydrophobic clusters (on the surface of the protein) and the hydrophobic probe. Thus, in the present work, the urea-induced changes in quaternary and/or tertiary conformations of the protein would be the crucial parameter determining its adsorption behavior.

Besides the diffusion process (initial adsorption), the unfolding, penetration, and subsequent structural rearrangement of previously diffused and adsorbed protein at the interface are also highly dependent on the conformational characteristics of adsorbed proteins, e.g., conformational flexibility. To monitor these processes, a first-order phenomenological equation (eq 2)[41,42] can be applied:

$$\ln[(\pi_{11800} - \pi_t)/(\pi_{11800} - \pi_0)] = -k_i t \quad (2)$$

where π_{11800}, π_t, and π_0 are the π at the final time (11800 s) of each step, at any time (t), and at the initial time (t_0), respectively; and k_i is the first-order rate constant. In practice, a plot of eq 2 usually yields two or more linear regions.[41] Figure 6B shows a typical application of eq 2 to the adsorption of the protein at the interface. The first slope is taken as a first-order rate constant of penetration (k_P), while the next slope refers to as a first-order rate constant of molecular rearrangement (k_R) for the adsorbed protein. The results showed that the k_P for the adsorbed protein was slightly affected by variation in urea concentration (increasing from -2.444×10^{-4} to -2.891×10^{-4} s^{-1}; data not shown), but increasing urea concentration from 0 to 8 M resulted in a progressive and significant increase from -7.428×10^{-4} to -33.05×10^{-4} s^{-1} in k_R (Figure 6B, inset). At any test urea concentration, the k_R for the adsorbed protein was considerably higher than the k_P, indicating much more importance of structural unfolding and subsequent rearrangement of adsorbed proteins at the interface for the film formation (than their penetration at the interface). The observations suggest that after initial adsorption, the structural rearrangement of adsorbed proteins could be greatly improved by increasing concentrations of urea.

DISCUSSION

Modulation of Quaternary and/or Tertiary Conformations of Phaseolin by Urea with Increasing Concentrations. In general, native phaseolin molecules are present in the trimeric or 7S form, with three individual subunits or

polypeptides associated together, mainly through intersubunit hydrogen bonding and hydrophobic interactions. The magnitude of these intersubunit interactions that is an indication reflecting quaternary conformational flexibility of vicilins has been confirmed to produce a vital influence on their emulsifying ability.[5] For 7S globulins (or vicilins), the conformation state of their subunits or polypeptides, also referred to as tertiary conformational flexibility of vicilins, is also important for their emulsifying properties (and emulsion stability in particular). However, the importance of quaternary and/or tertiary conformational characteristics on emulsifying properties of these oligomeric globulins is to date still very little understood.

The conformational state of these globulins at quaternary and tertiary levels can be modulated through weakening or strengthening inter- or intrasubunit interactions, by means of variation in environmental conditions or the presence of protein structure-perturbing agents, e.g., urea or GdnHCl. In the present work, we convincingly showed that at c = 0.5%, the presence of increasing concentrations (0−8 M) of urea resulted in a progressive disassociation of trimeric phaseolin molecules into monomeric ones (as evidenced by a progressive decrease in hydrodynamic diameter upon increasing urea concentration; Figure 1). DSC evidence (Table 1) suggested that a urea concentration of 6 M seemed to be enough for the complete disassociation at the quaternary conformational level. This agrees well with the previous findings that the presence of 6 M urea could be effective to dissociate the trimeric form of soybean β-conglycinin into monomeric subunits.[19,43] In contrast, the conformational state of phaseolin at the tertiary level (or conformational state of phaseolin subunits) was much less influenced by the presence of urea. Although the experiments were performed at low c values (e.g., 0.1% or below), the spectroscopic and DSC results clearly revealed that the tertiary conformation of phaseolin becomes more unfolded at higher concentrations of urea (Figure 2 and Table 1). In this case, a urea concentration of approximately 2 M seemed to be a critical concentration above which the conformation of phaseolin subunits became susceptible to urea-induced unfolding. One point is noteworthy to be strengthened that the urea-induced structural unfolding of phaseolin at quaternary and/or tertiary levels is completely reversible (as evidenced by the DSC data; Table 1). This suggests that the urea-dissociated and denatured phaseolin subunits in the solution are present in the state more like the "molten globule state", a concept that has been introduced to describe the conformational state of a globular protein adsorbed at an interface.[36] It has been confirmed that even for monomeric globular proteins (e.g., α-lactalbumin), the native-to-molten-globule-state transformation improves the adsorption of these proteins at the interface; α-lactalbumin in the molten globule state is more easily adsorbed at the interface of more hydrophilic silica particles.[44]

Role of Conformational Flexibility in the Emulsifying and Interfacial Properties of Phaseolin. The emulsification process of a monomeric globular protein (e.g., BSA) in general includes at least three steps: (I) diffusion and adsorption at the droplet interface; (II) penetration and orientation, and structural unfolding at the interface (to form a "molten globule state"); and (III) structural rearrangement and lateral interactions, thus forming a viscoelastic interfacial layer or multilayer.[1] The emulsifying ability of the proteins is mainly related to the above steps I and II, while the last step is vital for the stabilization of oil droplets, through coverage of their interface by proteins. The adsorption of a protein at the interface is usually dependent on the protein concentration in the bulk phase, the diffusion coefficient, the electrical potential set up at the interface by the adsorbed protein (given that the protein is charged), as well as attractive interactions (hydrophobic interactions in particular) between the adsorbed proteins.[45,46] After adsorption, the adsorbed proteins will orientate at the interface and their structure unfold, which is determined by their conformational flexibility and the crowding degree at the interface.[47] For monomeric globular proteins, the elucidation of the role of conformational flexibility in their emulsifying or interfacial properties is specifically based on the tertiary level. For the 7S-form vicilins, the situation is much more complex, since it also involves the contribution of conformational characteristics at the quaternary level. Our previous work[5] had suggested that at a low protein concentration (e.g., 0.5%) the emulsifying ability of vicilins in the trimeric form is highly dependent on their quaternary conformational flexibility. As compared with other vicilins, e.g., from mung and red beans, the phaseolin exhibits highest flexibility in quaternary conformation, which largely accounts for the highest emulsifying ability among the test vicilins,[5,6] implying that the phaseolin is the most appropriate vicilin to investigate the structure−function relationship of these oligomeric globulins.

In the present work, a relatively low c of 0.5% was applied for the characterization of emulsifying properties, at which the protein molecules might be adsorbed at the interface occupying a maximum interface area. This can be corroborated by much higher droplet sizes (in 1% SDS) of the formed emulsions relative to BSA at a comparable c value,[1] wherein the amount of the proteins at a c value of 0.5% was enough to fully cover the droplet interface. The present work indicated that at this c value, increasing urea concentration from 0 to 8 M progressively improved the emulsifying ability of phaseolin (as evidenced by a progressive decrease in droplet size in 1% SDS; Table 2). This observation is well in accordance with the progressive increase in flexibility of quaternary and/or tertiary conformation upon increasing the urea concentration, confirming the importance of conformational flexibility at quaternary and/or tertiary levels for the emulsifying ability of phaseolin.

However, the relative contribution of quaternary and tertiary conformational characteristics to the improvement of the emulsifying ability is dependent on the applied urea concentration. Based on the knowledge about the influence of urea concentration on the conformations of phaseolin (see the above), we can reasonably argue that at urea concentrations less than 6 M, the improvement of emulsifying ability is mainly contributed by the enhanced flexibility of the quaternary conformation, while at high urea concentrations, e.g., 6−8 M, the improvement of emulsifying ability is more related to increased flexibility in the tertiary conformation of phaseolin. The importance of tertiary conformational flexibility of a monomeric globular protein to the emulsifying ability has been well reflected by using BSA as the model.[1]

On the other hand, the adsorption experiments at the quiescent interface showed that only partial structural unfolding at the quaternary level (e.g., induced by 2 M urea) increased the diffusion of the protein to the interface, and the surface pressure at prolonged periods, while a further increase in urea concentration on the contrary impaired the adsorption (Figure 6A). Since the possibility that changes in surface hydrophobicity of phaseolin has been excluded (data not shown), the

improvement of adsorption by the presence of 2 M urea can thus be largely attributed to enhanced flexibility in the quaternary conformation, or dissociation of trimeric 7S-form phaseolin into monomeric subunits. The improvement of adsorption can also be explained by the increased diffusion coefficient, and effective increase in contact sites of the proteins with the interface, as a result of dissociation of trimeric phaseolin. In contrast, the progressive impairment of adsorption at higher urea concentrations might be associated with increased flexibility of dissociated subunits (at the tertiary level). A similar phenomenon for the impairment of adsorption has been confirmed in BSA conformers,[48] where the BSA at acidic pH with more elongated and flexible F form exhibited a poorer adsorption behavior than the normal or basic-form BSA with more compacted conformation. Pereira et al.[48] attributed this observation to formation of a more packed interface with a smaller molecular projected area for the more compacted BSA, thus giving rise to higher surface pressures. In fact, the impairment of adsorption at high urea concentrations is well in agreement with the formation of thinner interfacial films at higher urea concentrations (Table 2). This seemly abnormal observation indirectly supports the above argument that enhanced flexibility in conformation greatly improved the emulsifying ability of the protein, since at higher urea concentrations, less amount of proteins are needed to fully cover the interface of oil droplets.

Besides the initial adsorption, the emulsifying ability of the protein is also affected by conformation flexibility or ease of conformational changes of adsorbed protein at the interface. The present work has indicated that increasing the urea concentration progressively improved the ease of structural rearrangement of adsorbed proteins (as evidenced by the progressive increase in k_R; Figure 6B, inset), confirming the previous argument that the improvement of conformational flexibility of the protein in solution also favors the formation of a more viscoelastic interfacial film.[1] In this aspect, we can interestingly observe a close relationship between the droplet size (in 1% SDS) and the k_R data (Table 1), implying that the emulsifying ability of the protein might be to a large extent related to the ease of structural rearrangement of its "adsorbed proteins" at the interface. In the present work, the enhanced droplet flocculation at higher urea concentrations (Figure 4 and Table 2) is also an indirect evidence for the increased ease of structural rearrangement of adsorbed proteins. Thus, the improvement of the emulsifying ability of phaseolin by urea is contributed by increased flexibility in both quaternary and tertiary conformations.

In summary, urea can delicately modulate the flexibility of quaternary and/or tertiary conformations of phaseolin, depending on the applied concentration. Increasing urea concentration up to 8 M results in a progressive dissociation of trimeric phaseolin molecules into monomeric subunits, and concomitantly, the conformation of dissociated subunits becomes unfolded. The urea-induced changes in conformations are reversible, and seem to be independent of c (at c values above 0.1%). On the other hand, increasing urea concentration (from 0 to 8 M) progressively increases the emulsifying ability of phaseolin at c = 0.5%, and the flocculated extent of oil droplets in the freshly prepared emulsions, but leads to a gradual decrease in Γ. The progressive improvement of the emulsifying ability is not related to the initial adsorption rate and amount of adsorbed proteins, but highly dependent on the ease of structural rearrangement of adsorbed proteins at the interface. The present work clearly confirmed that the flexibility of quaternary and/or tertiary conformation plays a vital role in the emulsifying ability of 7S globulins, mainly by affecting the structural rearrangement of adsorbed proteins at the interface. The findings will have important implications for understanding the molecular mechanism for the emulsifying properties of plant oligomeric globulins, thus providing an effective strategy for improving the functional properties of plant proteins.

AUTHOR INFORMATION

Corresponding Author

*Fax: (086)20-87114263. E-mail: chtang@scut.edu.cn.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by the National Natural Science Foundation of China (serial no. 31171632) and Program for New Century Excellent Talents in University (NCET-10-0398).

REFERENCES

(1) Tang, C. H.; Shen, L. Role of conformational flexibility in the emulsifying properties of bovine serum albumin. *J. Agric. Food Chem.* **2013**, *61*, 3097−3100.

(2) Schwenke, K. D. Reflections about the functional potential of legume proteins. A review. *Nahrung/Food* **2001**, *45*, 377−381.

(3) Moure, A.; Sineiro, J.; Domínguez, H.; Parajó, J. C. Functionality of oilseed protein products: A review. *Food Res. Int.* **2006**, *39*, 945−963.

(4) Dagorn-Scaviner, C.; Gueguen, J.; Lefebvre, J. Emulsifying properties of pea globulins as related to their adsorption behaviors. *J. Food Sci.* **1987**, *52*, 335−341.

(5) Tang, C. H.; Sun, X. A comparative study of physicochemical and conformational properties in three vicilins from *Phaseolus* legumes: Implications for the structure-function relationship. *Food Hydrocolloids* **2011**, *25*, 315−324.

(6) Kimura, A.; Fukuda, T.; Zhang, M.; Motoyama, S.; Maruyama, N.; Utsumi, S. Comparison of physicochemical properties of 7S and 11S globulins from pea, Fava bean, cowpea, French bean with those of soybean − French bean 7S globulin exhibits excellent properties. *J. Agric. Food Chem.* **2008**, *56*, 10273−10279.

(7) Damodaran, S. Functional properties. In *Food Proteins − Properties and characterization*; Nakai, S., Modler, H. W., Eds.; VCH Publishers: New York, NY, 1996; pp 167−234.

(8) Maruyama, N.; Sato, R.; Wada, Y.; Matsumura, Y.; Goto, H.; Okuda, E.; Nakagawa, S.; Utsumi, S. Structure-physicochemical function relationships of soybean β-conglycinin constituent subunits. *J. Agric. Food Chem.* **1999**, *47*, 5278−5284.

(9) Maruyama, N.; Salleh, M.; Takahashi, K.; Yagasaki, K.; Goto, H.; Hontani, N.; Nakagawa, S.; Utsumi, S. Structure-physicochemical function relationships of soybean β-conglycinin heterotrimers. *J. Agric. Food Chem.* **2002**, *50*, 4323−4326.

(10) Maruyama, N.; Salleh, M.; Takahashi, K.; Yagasaki, K.; Goto, H.; Hontani, N.; Nakagawa, S.; Utsumi, S. The effect of the N-linked glycans on structural features and physicochemical functions of soybean β-conglycinin homotrimers. *JAOCS, J. Am. Oil Chem. Soc.* **2002**, *79*, 139−144.

(11) Kimura, A.; Tandang-Silvas, M. R.; Fukuda, T.; Cabanos, C.; Takegawa, Y.; Amano, M.; Nishimura, S.-I.; Matsumura, Y.; Utsumi, S.; Maruyama, N. Carbohydrate moieties contribute significantly to the physicochemical properties of French bean 7S globulin phaseolin. *J. Agric. Food Chem.* **2010**, *58*, 2923−2930.

(12) Tang, C. H.; Sun, X.; Foegeding, E. A. Modulation of physicochemical and structural properties of kidney bean vicilin

(phaseolin) by glycation with glucose: Implications for the structure-function relationship. *J. Agric. Food Chem.* **2011**, *59*, 10114−10123.
(13) Pedrosa, C.; De Felice, F.; Trisciuzzi, C.; Ferreira, S. T. Selective neoglycosylation increases the structural stability of vicilin, the 7S storage globulin from pea seeds. *Arch. Biochem. Biophys.* **2000**, *382*, 203−210.
(14) Pedrosa, C.; Trisciuzzi, C.; Ferreira, S. Effects of glycosylation on functional properties of vicilin, the 7S storage globulin from pea (*Pisum sativum*). *J. Agric. Food Chem.* **1997**, *45*, 2025−2030.
(15) Ferreon, A. C.; Bolen, D. W. Thermodynamics of denaturant-induced unfolding of a protein that exhibits variable two-state denaturation. *Biochemistry* **2004**, *43*, 13357−13369.
(16) Adel, A.; Nadia, M.; Mohamed, O.; Abdelhafidh, G. Study of thermally and chemically unfolded conformations of bovine serum albumin by means of dynamic light scattering. *Mater. Sci. Eng., C* **2008**, *28*, 594−600.
(17) Poon, S.; Clarke, A. E.; Schultz, C. J. Effect of denaturants on the emulsifying activity of proteins. *J. Agric. Food Chem.* **2001**, *49*, 281−286.
(18) Thanh, V. H.; Shibasaki, K. Major proteins of soybean seeds. Reconstitution of β-conglycinin from its subunits. *J. Agric. Food Chem.* **1978**, *26*, 695−698.
(19) Yuan, D. B.; Min, W.; Yang, X. Q.; Tang, C. H.; Zheng, Z. X.; Wang, J. M.; Guo, J.; Zheng, H. G.; Qi, J. R. An improved isolation method of soy β-conglycinin subunits and their characterization. *JAOCS, J. Am. Oil Chem. Soc.* **2010**, *87*, 997−1004.
(20) Hall, T. C.; McLeester, R. C.; Bliss, F. A. Equal expression of the maternal and paternal alleles for the polypeptide subunits of the major storage protein of the bean *Phaseolus vulgaris* L. *Plant Physiol.* **1977**, *59*, 1122−1124.
(21) Castellani, O.; Belhomme, C.; David-Briand, E.; Guérin-Dubiard, C.; Anton, M. Oil-in-water emulsion properties and interfacial characteristics of hen egg yolk phosvitin. *Food Hydrocolloids* **2006**, *20*, 35−43.
(22) Puppo, M C.; Beaumal, V.; Speroni, F.; de Lamballerie, M.; Añón, M. C.; Anton, M. β-Conglycinin and glycinin soybean protein emulsions treated by combined temperature-high-pressure treatment. *Food Hydrocolloids* **2011**, *25*, 389−397.
(23) Liang, H. N.; Tang, C. H. pH-Dependent emulsifying properties of pea proteins. *Food Hydrocolloids* **2013**, *33*, 309−319.
(24) Güzey, D.; Gülseren, I.; Bruce, B.; Weiss, J. Interfacial properties and structural conformation of thermosonicated bovine serum albumin. *Food Hydrocolloids* **2006**, *20*, 669−677.
(25) Chu, B.; Zhou, Z. Laser light scattering of model casein solutions: Effects of high temperature. *J. Colloid Interface Sci.* **1995**, *170*, 102−112.
(26) Tulloch, P. A.; Blagrove, B. J. Electron microscopy of seed-storage globulins. *Arch. Biochem. Biophys.* **1985**, *241*, 521−532.
(27) Ruiz-Henestrosa, V. M.; Martinez, M. J.; Patino, J. M. R.; Pilosof, A. M. R. A dynamic light scattering study on the complex assembly of glycinin soy globulin in aqueous solutions. *JAOCS, J. Am. Oil Chem. Soc.* **2012**, *89*, 1183−1191.
(28) Dufour, E.; Hoa, G. H.; Haertlé, T. High-pressure effects of β-lactoglobulin interactions with ligands studied by fluorescence. *Biochim. Biophy. Acta* **1994**, *1206*, 166−172.
(29) Lange, R.; Balny, C. UV-visible derivative spectroscopy under high pressure. *Biochim. Biophys. Acta* **2002**, *1595*, 80−93.
(30) Deshpande, S. S.; Damodaran, S. Heat-induced conformational changes in phaseolin and its relation to proteolysis. *Biochim. Biophys. Acta* **1998**, *998*, 179−188.
(31) Arntfield, S. D.; Murray, E. D. The influence of processing parameters on food protein functionality. I. Differential scanning calorimetry as an indictor of protein denaturation. *Can. Inst. Food Sci. Technol. J.* **1981**, *14*, 289−294.
(32) Yin, S. W.; Tang, C. H.; Wen, Q. B.; Yang, X. Q. Conformational and Thermal properties of phaseolin, the major storage protein of red kidney bean (*Phaseolus vulgaris* L.). *J. Sci. Food Agric.* **2011**, *91*, 94−99.
(33) Meng, G. T.; Ma, C. Y. Thermal properties of *Phaseolus angularis* (red bean) globulin. *Food Chem.* **2002**, *73*, 453−460.
(34) Kinsella, J. E. Relationship between structure and functional properties of food proteins. In *Food Proteins*; Fox, P. F., Cowden, J. J., Eds.; Applied Science Publishers, London, UK, 1982; pp 51−103.
(35) Privalov, P. L.; Khechinashvili, N. N.; Atanssaov, B. P. Thermodynamic analysis of thermal transition in globular proteins. I. Calorimetric study of chymotrypsinogen, ribonuclease and myoglobin. *Biopolymer* **1971**, *10*, 1865−1890.
(36) Dickinson, E.; Matsumura, Y. Proteins at liquid interface: role of the molten globule state. *Colloids Surf., B* **1994**, *3*, 1−17.
(37) Chatterjee, A.; Mandal, D. K. Denaturant-induced equilibrium unfolding of concanavalin A is expressed by a three-state mechanism and provides an estimate of its protein stability. *Biochim. Biophys. Acta* **2003**, *1648*, 174−183.
(38) Castelain, C.; Genot, C. Conformational changes of bovine serum albumin upon its adsorption in dodecane-in-water emulsions as revealed by front-face steady-state fluorescence. *Biochim. Biophys. Acta* **1994**, *1199*, 59−64.
(39) Tang, C. H.; Liu, F. Cold, gel-like soy protein emulsions by microfluidization: Emulsion characteristics, rheological and micro-structural properties, and gelling mechanism. *Food Hydrocolloids* **2013**, *30*, 61−72.
(40) Patino, J. M. R.; Sánchez, C. C.; Ortiz, S. E. M.; Niño, M. R.; Añón, M. C. Adsorption of soy globulin films at the air-water interface. *Ind. Eng. Chem. Res.* **2004**, *43*, 1681−1689.
(41) Suttiprasit, F.; Krisdahasima, V.; McGuire, J. The surface activity of α-lactalbumin, β-lactoglobulin and BSA. *J. Colloid Interface Sci.* **1992**, *154*, 316−326.
(42) Perez, A. A.; Carrara, C. R.; Sánchez, C. C.; Santiago, L. G.; Patino, J. M. R. Interfacial dynamic properties of whey protein concentrate/polysaccharide mixtures at neutral pH. *Food Hydrocolloids* **2009**, *23*, 1253−1262.
(43) Mo, X.; Wang, D.; Sun, X. S. Physicochemical properties of β and α′α subunits isolated from soybean β-conglycinin. *J. Agric. Food Chem.* **2011**, *59*, 1217−1222.
(44) Britten, M.; Giroux, H. J. Interfacial properties of milk protein-stabilized emulsions as influenced by protein concentration. *J. Agric. Food Chem.* **1993**, *41*, 1187−1191.
(45) Dalgleish, D. G. Adsorption of protein and the stability of emulsions. *Trends Food Sci. Technol.* **1997**, *8*, 1−6.
(46) Kella, N. K. D.; Yang, S. T.; Kinsella, J. E. Effect of disulfide bond cleavage on structural and interfacial properties. *J. Agric. Food Chem.* **1989**, *37*, 1203−1210.
(47) Dickinson, E. Adsorbed protein layers at fluid interfaces: interactions, structure and surface rheology. *Colloids Surf., B* **1999**, *15*, 161−176.
(48) Pereira, L. G. C.; Théodoly, O.; Blanch, H. W.; Radke, C. Dilatational rheology of BSA conformers at the air/water interface. *Langmuir* **2003**, *19*, 2349−2356.