JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Role of Conformational Flexibility in the Emulsifying Properties of Bovine Serum Albumin

Chuan-He Tang*[,†,‡] and Lan Shen[†]

[†]Department of Food Science and Technology and [‡]State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, People's Republic of China

ABSTRACT: Although it is well recognized that the conformation state of a protein affects its surface properties, the importance of conformation flexibility to its functionality is still not well understood. This study systemically investigated the influence of protein concentration (c) and disulfide bond (S−S) cleavage with a reducing agent, β-mercaptoethanol (2-ME), on the conformation and emulsifying properties of an ideal globular protein, bovine serum albumin (BSA), with the aim to unravel the role of conformational flexibility in the functionality. The conformations were evaluated using size exclusion chromatography, dynamic light scattering (DLS), extrinsic fluorescence, and derivative UV spectroscopy. The emulsifying properties, including emulsifying ability, extent of droplet flocculation at a specific period of storage, and stability against flocculation and/or coalescence as well as creaming, were characterized using droplet size and creaming index analyses. The results indicated that the tertiary conformation of native BSA was closely dependent on its c (in the range of 0.05−1.0%), and increasing c resulted in a more compacted and rigid conformation. The c dependence largely determined the susceptibility of S−S bridges to reduction and even refolding of reduced BSA molecules. Interestingly, there was approximately a critical c (e.g., 0.25−0.5%) below which the S−S cleavage resulted in a gradual structural unfolding of the molecules and above which the situation was the reverse. On the other hand, the alteration with protein and 2-ME concentrations led to a variety of changes in emulsion size ($d_{4,3}$; in water or 1% SDS) at 4 and 24 h and creaming index (up to 2 weeks). In general, at a low c value (e.g., 0.25%) increasing the S−S cleavage progressively improved the emulsifying ability and emulsion stability (especially against coalescence and creaming), whereas at c = 0.5 or 0.75%, the S−S cleavage, on the contrary, impaired the emulsifying properties, especially emulsion stability against flocculation and/or coalescence. These results suggest that the conformational flexibility of the protein (in solution) plays a vital role in different aspects of its emulsifying properties, for example, ease of structural unfolding at the interface, lateral interactions between adsorbed proteins, and formation of a viscoelastic interfacial layer (or multilayers). This knowledge could provide an in-depth understanding of the relationships between tertiary conformational flexibility and emulsifying properties of globular proteins.

KEYWORDS: *structure−function relationship, emulsifying ability, emulsion stability, bovine serum albumin (BSA), disulfide bond cleavage, globular proteins*

INTRODUCTION

Proteins play a crucial role in the qualities of a wide variety of foods, in terms of gels, emulsions, or foams. In the past decades, knowledge about the functionality of food proteins, especially from milk sources, had progressively increased; however, understanding of their structure−function relationships, as well as the molecular bases of the functionalities in foods, is still very limited.[1] The lack of this understanding greatly limits the direct use of novel proteins (e.g., vegetable proteins) in foods. This is remarkably contrasting from the critical demand from the food industry for proteins from cheaper sources and with desirable targeted functionalities. To overcome this limitation, the importance of structural characteristics (e.g., conformational flexibility or rigidity) to the functionalities of proteins needs to be revisited and better understood.

Food proteins have been widely used as emulsifiers to form and stabilize emulsions in the food industry. During emulsification, proteins can facilitate breakage of oil drops by lowering oil−water interfacial tension and stabilize the droplets against coalescence during emulsification as well as upon subsequent storage of the formed emulsions.[2] The potential of a protein to be an effective emulsifier is closely related to its intrinsic physical, chemical, and structural properties. Surface hydrophobicity and the hydrophobicity/hydrophilicity balance of proteins are important parameters affecting their emulsifying properties. For example, many previous works observed positive correlations between surface hydrophobicity and emulsifying properties (e.g., interfacial tension and emulsifying activity index) of proteins.[1] Although solubility is often considered to be a prerequisite for good emulsification performance of proteins, there is no direct relationship between their solubility and emulsifying activities (e.g., emulsifying ability). For globular proteins, the conformational flexibility sometimes plays a more important role in the interfacial and emulsifying properties. There is accumulating evidence showing close relationships between the interfacial behaviors and emulsifying properties of proteins.[3−8] The interfacial behaviors of proteins involve many aspects, including rate of adsorption at the interface, amount of protein adsorbed, extent of conforma-

Received: August 6, 2012
Revised: March 4, 2013
Accepted: March 6, 2013
Published: March 6, 2013

tional rearrangement at the interface, extent of reduction in interfacial tension, and ability to form a cohesive and viscous film.[1] The adsorption of a protein onto an interface induces conformational changes of the protein.[9] To better understand the structure and dynamics of proteins at the interface, Dickinson and Matusumura[10] introduced the concept of "molten globule state" to describe the state of an adsorbed globular protein at a liquid interface, thus providing an insight into the relationship between protein structure and the properties of adsorbed layers. It has been indicated that α-lactalbumin in the molten state is preferentially adsorbed at the oil−water interface, and the adsorbed layer structure produced by adsorption from the molten globule state seems to be rather different from that from the native state.[10] Thus, it can be reasonably expected that a globular protein with higher conformational flexibility or greater ease of conformational change exhibits better interfacial activities and emulsifying properties.

On the other hand, conformations of proteins are dynamically stable, which vary with the history of their preparations and/or treatments, environment conditions (e.g., temperature, pH, ionic strength, and presence of other components), and even protein concentration (c). Some previous work had pointed out that c could affect the interfacial properties of globular protein-stabilized emulsions.[11] However, surprisingly, few works have addressed the influence of c on the emulsifying properties of globular proteins from the view of conformation state in relation to c.

Bovine serum albumin (BSA) is a highly soluble multidomain protein without bulky appending carbohydrates, which is stable and available at high purity and low cost. It is constituted by 585 amino acid residues, including 35 cysteines (17 disulfide bond bridges), which confer a relatively strong stability to the protein.[12] Although the crystallographic structure of BSA has not yet been resolved, more scientists tend to agree that the tertiary conformation of BSA is similar to the heart-shaped structure observed in human serum albumin.[13] More recently, Barbosa et al.[14] investigated the effects of c (1.0, 2.0, and 5.0%) and pH on the conformational states of BSA using small-angle X-ray scattering and confirmed that BSA overall shape (or conformation) changes as a function of c. Furthermore, reduction of disulfide bond (S−S) bridges might result in changes in the conformation state of BSA.[15,16] On the other hand, BSA has been well confirmed to exhibit good emulsifying properties, with emulsifying ability (EA) superior to that of soy proteins, β- or κ-casein, ovalbumin, and β-lactoglobulin.[17,18] This superior EA is probably due to the unique balance of its chemical and structural properties. Thus, BSA seems to be an ideal model globular protein to investigate the structure−function (emulsifying properties) relationships of globular proteins.

Conformational flexibility or ease of conformational changes for a globular protein is difficult and even impossible to directly characterize. In most cases, only relative changes in conformational flexibility of a protein under different conditions or of different proteins can be characterized. Thus, there are two strategies to study the importance of conformational flexibility for the emulsifying properties of a globular protein. One is to characterize the changes in emulsifying and/or structural properties of a specific protein by chemical modification. For example, Waniska et al.[17] investigated the influence of different modifications (including S−S reduction) on the EA (reflecting its ability to aid emulsion formation, as well as stabilization of the newly created emulsion) of BSA over the pH range of 2−10 and preliminarily indicated the importance of protein structure and charge on the emulsifying properties. Klemaszewski et al.[19] investigated the effects of controlled sulfitolysis on the emulsion characteristics of BSA at three oil volume fractions (0.22, 0.42, and 0.62) and found that at higher oil fractions, the modification resulted in smaller emulsion size and greater surface areas, indicative of enhanced emulsifying activity possibly by increased flexibility. In a previous work about the influence of S−S cleavage on interfacial (foaming) properties of whey proteins, a good correlation between foaming properties and unfolding ability (or conformational flexibility) of proteins was observed.[20]

Another strategy is to characterize the differences in emulsifying properties between different proteins, with different conformational characteristics. For example, Kato et al.[18] compared the interfacial properties of proteins with different susceptibilities to protease digestion and observed good relationships between the foaming power and emulsifying activity and the digestion velocity of proteins. Because the susceptibility to protease digestion is an indication of the conformational flexibility of proteins, they thus suggested that the flexibility of protein structure might be an important structural factor governing foam formation and emulsification. Poon et al.[21] similarly investigated the relationships between protein flexibility and emulsifying activity of proteins using different model proteins by means of selected disruption of S−S bridges and/or noncovalent interactions of the proteins. They generally found that the enhanced protein flexibility (induced by denaturants) led to an improvement of emulsifying activity.

The major shortfall of the above-mentioned works is a lack of knowledge about the conformational characteristics of BSA, for example, the c dependence of its tertiary conformation, even under reducing conditions. Furthermore, a challenge still exists to relate the conformational flexibility of protein in solution to that in the adsorbed layers at the interface. The conformational state of proteins in solution remarkably changes with variation of many environmental conditions and, as a consequence, intermolecular interaction balance between proteins in solution may change. Lee and Hirose[22] tried to characterize the influence of S−S cleavage with β-mercaptoethanol (2-ME; a reducing agent) on the emulsifying properties of BSA. As expected, they observed a progressively increased extent of S−S cleavage upon increasing 2-ME concentration (from 0 to 200 mM), but the S−S cleavage at 2-ME concentrations above 30 mM, on the contrary, greatly impaired the emulsifying properties. Although they observed the formation of linear aggregates (several hundreds of nanometers in length) in the BSA samples treated by 2-ME at high concentrations, they did not realize that the removal of 2-ME using gel filtration had dramatically changed the environment that kept the "S−S cleaved or reduced" BSA molecules dynamically stable in solution. In fact, the impairment of the emulsifying properties of BSA observed in this previous work indirectly supports the importance of conformational flexibility to the emulsifying properties of globular proteins.

Thus, the present work was to systematically characterize the influence of conformational flexibility (of protein in solution) on the emulsifying properties of BSA, with the aim of an in-depth understanding of the importance of conformational flexibility to emulsifying functionality of globular proteins. To achieve this objective, we applied the aforementioned first strategy to study the relationships between the conformational

characteristics and emulsifying properties of BSA. In the first part, we investigated the conformational characteristics of BSA at different c values as affected by the S−S cleavage with 2-ME and luckily found that the tertiary conformation of this protein in solution could be delicately modulated by variation in c and 2-ME concentrations. This provided the possibility to establish the relationships between the conformation flexibility and emulsifying properties (e.g., emulsifying ability, emulsion stability against flocculation, coalescence, and even creaming). The conformational characteristics were characterized using size exclusion chromatography, dynamic light scattering (DLS), extrinsic fluorescence, and second-derivative ultraviolet (UV) spectroscopy, whereas the emulsifying properties were characterized in terms of droplet size analysis and creaming experiments. According to the authors' knowledge, this is the first paper to indicate that modulation of conformational flexibility and emulsifying properties of globular proteins by S−S cleavage is dependent on c.

MATERIALS AND METHODS

Materials. BSA (~98% purity with essentially no fatty acid) was purchased from Capital Bio Co. (Beijing, China) and used without further purification. Soy oil was purchased from a local supermarket in Guangzhou (China). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and 1,8-anilinonaphthalenesulfonate (ANS), as well as Nile Blue A, were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-ME was purchased from Shanghai DIN GUO Biotech. Co. Ltd. (China). All other chemicals used were of analytical or better grade.

Reduction Reaction of BSA. All of the BSA solutions at various c values (0.05−1.0%, w/v) were prepared in 10 mM phosphate buffer (pH 7.0). 2-ME was added to reach a specific concentration (0−50 mM), and then the mixtures were incubated at room temperature for 2 h under a mild magnetically stirring condition. After the incubation, the mixtures were subject to the following experiments.

Characterization of BSA Solutions. *Exposed Free Sulfhydryl Group (SH) Contents.* Determination of exposed free SH contents was according to the method of Beveridge et al.,[23] with a few modifications. BSA solutions containing different protein and 2-ME concentrations (5 mL) were mixed with 5 mL of Tris−Gly buffer (0.086 M Tris, 0.09 M glycine, 0.04 M EDTA, pH 8.0) containing 8 M urea to produce the test protein solutions. Each test protein solution (50 μL) was further mixed with 4.95 mL of the Tris−Gly buffer. Then 50 μL of Ellman's reagent (DTNB in the Tris−Gly buffer, 4 mg/mL) was added, and absorbance was measured at 412 nm after 5 min. For each determination, the same volume (50 μL) of deionized water with the same 2-ME concentration (as in the test protein solution) and 50 μL of Ellman's reagent were added to 4.95 mL of the Tris−Gly buffer, and the mixture after 5 min was used as the blank. Free SH content (mmol/mmol of BSA) was calculated with the equation

$$\text{free SH content (mmol/mmol)} = 73.53 \times 66.43 \times A_{412} \times D/c \quad (1)$$

where c is the protein sample concentration (mg/mL), D is the dilution factor, 73.53 is derived from $10^6/(1.36 \times 10^4)$, 1.36×10^4 is the molar absorptivity, and 10^6 is for the conversion from molar basis to μM/mL basis and from mg solids to g solids; 66.43 is the molar mass of BSA (66.43 g/mmol).

High-Performance Size Exclusion Chromatography (HPSEC). The protein−protein associations in BSA solutions were evaluated using a HPSEC technique. The HPSEC experiment was performed using a Waters HPLC 1525 system (Waters, Division of Millipore, Milford, MA, USA) fitted with a TSK-GEL G3000SWXL column (0.78 × 30 cm, Tokyo, Japan) preceded by a guard column TSK-GEL Guard Column SWXLne (Tokyo, Japan). Each sample containing about 0.1−0.75% (w/v) BSA with various concentrations of 2-ME (0, 10, 30, and 50 mM) in 50 mM phosphate buffer at pH 7.0 was centrifuged, and then the supernatants were filtered with PVDF Millipore membrane filters (0.22 μm). The following chromatographic conditions were applied: (1) injection volume, 15 μL; (2) eluting rate, 0.7 mL/min; (3) elution solvent, 50 mM phosphate buffer (pH 7.0) containing 0, 10, 30, and 50 mM 2-ME, for the corresponding samples with 0, 10, 30, and 50 mM 2-ME, respectively. The absorbance was recorded at 280 nm. All data were collected and analyzed by Breeze software (Waters).

Dynamic Light Scattering. The hydrodynamic radius (R_h) of BSA molecules in solution was evaluated using the DLS technique. DLS analysis was performed at a fixed angle of 173° using a Zetasizer Nano-ZS instrument (Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He−Ne laser (633 nm wavelength) at 25 °C. The BSA solutions with various concentrations of 0.1−0.75% (w/v), in the presence of various concentrations (0−50 mM) of 2-ME, in 50 mM phosphate buffer at pH 7.0 were used for the determination. All of the samples were centrifuged at 10000g for 20 min to remove any insoluble in the solutions and further filtered with PVDF Millipore membrane filters (0.22 μm). The filtered samples were placed in a 1 × 1 cm cuvette and in a sample compartment self-enclosed. The information on the proteins obtained with DLS is given by the scattered light intensity, the z-average diameter, and the diameter size distribution of the particles in solution. The mean size (z-average diameter; 2 times the R_h) was obtained with a single exponential fitting, whereas the size distribution obtained is a plot of the relative volume ratio of particles with their mean size.

Extrinsic Fluorescence Spectroscopy. The extrinsic fluorescence spectroscopy of BSA solutions at c values of 0.05 and 0.1% (w/v), in the presence of 0−50 mM 2-ME, was determined using the fluorescence probe ANS^-, on an F4500 fluorescence spectrophotometer (Hitachi Co., Japan). ANS^- solution (8 mM; 20 μL) was added to 4 mL of each solution, and extrinsic fluorescence intensity of the mixture was measured at 370 nm (excitation) and 470 nm (emission) at a constant slit of 5 nm for both excitation and emission, respectively.

Second-Derivative Ultraviolet (UV) Spectroscopy. The BSA solutions of various concentrations (0.05, 0.1, and 0.25%, w/v), in the presence of various concentrations (0−50 mM) of 2-ME in 10 mM phosphate buffer (pH 7) were used for the determination of UV spectroscopy. Baseline-corrected UV spectra were recorded between 250 and 300 nm at medium speed in double-beam mode using a UV2300 spectrophotometer (Techcomp Co., China), with the phosphate buffer with comparable concentrations of 2-ME as the blank. The data interval was 0.1 nm. The second-derivative treatment of UV spectra was performed using Origin 8.0 software (Origin-Lab Corp., Northampton, MA, USA).

Preparation of BSA-Stabilized Emulsions. The emulsions stabilized by BSA at various concentrations of 0.1−1.0% (w/v), in the presence of various concentrations (0−50 mM) of 2-ME, in 50 mM phosphate buffer at pH 7.0 (containing 0.02% sodium azide) were prepared as follows. In brief, 10 mL of soy oil and 40 mL of each BSA solution were first prehomogenized using a high-speed dispersing unit (model IKA-ULTRATURRAX T25 basic, IKA 190 Works, Inc., Wilmington, NC, USA) at 10000 rpm for 2 min. Then, the resultant dispersions were further homogenized though a Microfluidizer (M110EH model, Microfluidics International Corp., Newton, MA, USA), one pass with an overall pressure at 40 MPa at room temperature. Unless stated otherwise, all of the preparations were performed at room temperature.

Characterization of BSA-Stabilized Emulsions. *Droplet Size Distribution and Volume Average Size (d_{43}).* The size distribution and volume average size (d_{43}) of droplets in the emulsions after storage periods of 4 and 24 h, dispersed in deionized water or 1% SDS, were determined using a Malvern Mastersizer 2000 instrument (Malvern Instrument Ltd.). The refractive index of the soy oil was 1.456, and the imaginary part of the refractive index (due to absorption) was fixed at 0.001. The d_{43} values obtained with deionized water and with 1% SDS as the dispersants were denoted d_{43-SDS} and d_{43+SDS}, respectively. During measurement, all of the emulsion samples (1−2 mL) were diluted with about 800 mL of recirculating deionized water or 1% SDS solution to avoid the impact of multiple scattering.

Flocculation index (FI) of the emulsions after quiescent storage of 4 or 24 h was evaluated according to the method described by Palazolo et al.[24] Percentage of FI was calculated with the following equation:

$$\mathrm{FI}\ (\%) = [(d_{43-\mathrm{SDS}})/(d_{43+\mathrm{SDS}}) - 1.0] \times 100 \quad (2)$$

Stabilities of coalescence plus flocculation ((C+F)%), coalescence (C%), and flocculation (F%) of the emulsions during the storage period of 4−24 h were also calculated according to the method by Palazolo et al.,[24] using the following equations:

$$(\mathrm{C+F})\% = [(d_{43-\mathrm{SDS},24\mathrm{h}} - d_{43+\mathrm{SDS},4\mathrm{h}})/d_{43+\mathrm{SDS},4\mathrm{h}}] \times 100 \quad (3)$$

$$\mathrm{C}\% = [(d_{43+\mathrm{SDS},24\mathrm{h}} - d_{43+\mathrm{SDS},4\mathrm{h}})/d_{43+\mathrm{SDS},4\mathrm{h}}] \times 100 \quad (4)$$

$$\mathrm{F}\% = (\mathrm{C+F}\%) - \mathrm{C}\% \quad (5)$$

Creaming Index. The creaming index was determined by macroscopic observations of emulsions, under a quiescent condition at room temperature. Each emulsion (10 mL) was filled into a glass test tube (1.5 cm internal diameter × 12 cm height), and the height of the bottom serum layer (H_s) as well as that of the total emulsion (H_t) were recorded after storage at ambient temperature for up to 2 weeks. The mean and standard deviation (SD) of three replicates is reported. The creaming index (%) was reported as the following equation:

$$\text{creaming index}\ (\%) = (H_s/H_t) \times 100 \quad (6)$$

Microstructure. The microstructures of the emulsions formed at c = 0.5% were evaluated by confocal laser scanning microscopy (CLSM) using a Leica TCS SP5 confocal laser scanning head mounted on a Leica DMRE-7 (SDK) upright microscope (Leica Microsystems Inc., Heidelberg, Germany) equipped with a 20-HC PL APO/0.70NA oil immersion objective lens. The various emulsion samples were mixed with an appropriate amount of 1.0% (w/v) Nile Blue A (fluorescent dye) in deionized water for at least 2 min under mild shaking conditions and left to allow the staining to reach completion for at least 1 h, before the observation. The stained emulsions were placed on concave confocal microscope slides (Sail; Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), covered with glycerol-coated coverslips, and examined with a 100 magnification lens and an argon/krypton laser having an excitation line of 514 nm and a helium neon laser (He−Ne) with excitation at 633 nm. The CLSM images were obtained with green color for oil phase and red color for protein phase, respectively.

Statistics. An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) with a confidence interval of 95% was used to compare the means.

RESULTS

Conformational Characteristics of BSA in Solution. *Exposed Free SH Contents.* Figure 1 shows the influence of 2-ME concentration (0−50 mM) on exposed free SH contents of BSA at different concentrations (0.1−1.0%). We can observe that at a specific protein or 2-ME concentration, the free SH content progressively increased with increasing 2-ME or protein concentration. 2-ME is a reducing agent that can cleave disulfide bonds; the progressive increase in free SH contents upon increasing 2-ME concentration could thus reflect the increasing extent of S−S cleavage by reduction. Interestingly, the S−S cleavage was highly dependent on the applied c, with higher extent of S−S cleavage observed at lower c (at a given 2-ME concentration; Figure 1). For example, at a 2-ME concentration as high as 50 mM, the free SH contents were 26, 10, 5, 3, and 2.4 mmol/mmol of BSA for c values of 0.1, 0.25, 0.5, 0.75, and 1.0%, respectively. There are 17 intramolecular disulfide bonds and one cysteine sulfhydryl in a BSA molecule, which correspond to 35 half-cysteines. Thus, about 73.5, 29.4, 14.7, 8.8, and 7.0% of the disulfide bonds were reductively cleaved at 50 mM 2-ME for the tested c values in an increasing order. This seems to be the first observation that the S−S cleavage of proteins by reduction is closely associated with their c. One of the possible reasons for this phenomenon is that the tertiary conformation of BSA may exhibit a concentration-dependent behavior.

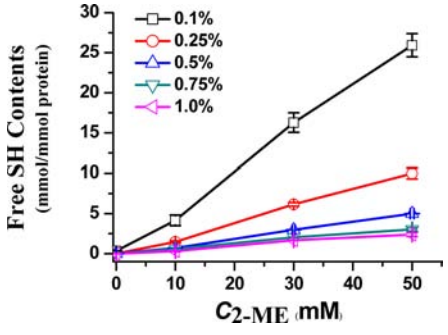

Figure 1. Exposed free SH contents of BSA as a function of protein (0.1−1.0%) and 2-ME (0−50 mM) concentrations. Each datum is the mean and standard deviation of triplicate measurements.

Protein−Protein Associations. When a BSA molecule is reduced, there may be an interchange reaction between the exposed free SH groups and the disulfide bonds. If new intermolecular S−S bridges are formed, some kinds of protein associations and even polymerization will occur. We analyzed the protein−protein interactions of BSA at various protein and 2-ME concentrations using HPSEC, and one set of typical HPSEC profiles at a given protein concentration of 0.1% is presented in Figure 2. In all of the cases, a prominent elution peak at 12.8 min and a minor peak at 11.3 min were observed, clearly attributed to monomeric and dimeric BSA, respectively.[15,25] In the presence of 2-ME, an aggregate peak at about 7.9 min was observed, but the magnitude of the peak was minor and unaffected by increasing 2-ME concentration (Figure 2). Similar results were observed at increasing c values (e.g., 0.25−0.75%; data not shown). These observations excluded the possibility that intermolecular S−S bridges might occur in 2-ME-reduced BSA. In other words, the observations indicated that at c values ranging from 0.1 to 0.75%, most of the BSA molecules were present in the monomeric form, even in the presence of 2-ME up to 50 mM. Actually, these observations are consistent with the previous findings that the overall conformation (especially tertiary conformation) of BSA at neutral pH is rigid; and even though S−S bridges cleaved, the secondary structure of BSA is still resistant to heat treatment up to 65 °C.[13,26]

However, Lee and Hirose[22] reported a contrasting observation about the influence of reduction on the size exclusion chromatographs of reduced BSA. They observed that with increasing 2-ME concentration, the monomer peak progressively decreased, whereas a new peak corresponding to aggregates that were eluted at the void volume of the column increased. Using electron microscopy, they confirmed that these aggregates were linear polymers with a length of about 50−160 nm. The formation of these linear aggregates was clearly associated with the changes of balance between intermolecular and intramolecular interactions by the 2-ME removal using gel filtration (used in the previous work before the emulsification). In contrast, in the present work, the 2-ME concentration did not change throughout the experiment, and

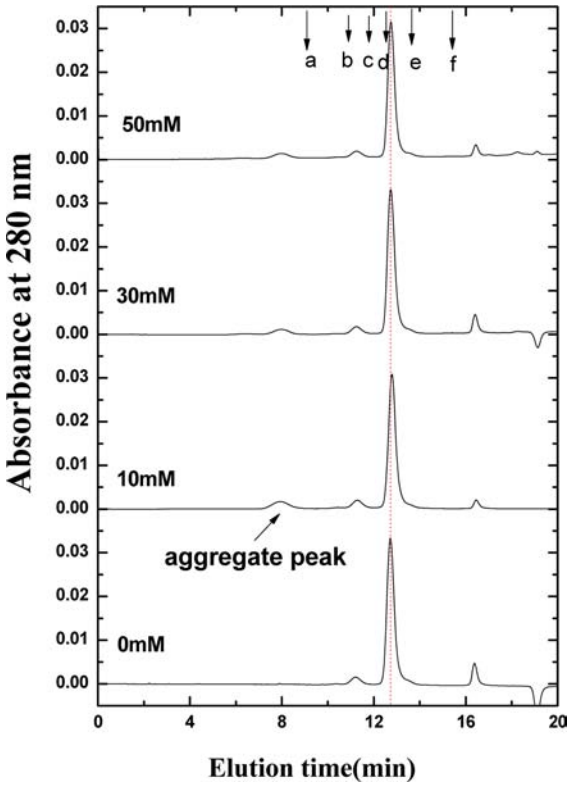

Figure 2. Typical SEC UV elution profiles of native (control) and reduced BSA. Only profiles of BSA at a concentration of 0.1% (w/v) and various 2-ME concentrations (0−50 mM) are presented. Standard protein molecular weights: a, 669 kDa; b, 440 kDa; c, 158 kDa; d, 75 kDa; e, 43 kDa; f, 13 kDa.

as a consequence, the reduced BSA molecules might be kinetically stable in the system.

Hydrodynamic Radius (R_h). The changes in conformation and diffusion coefficient of BSA have been well characterized using DLS.[27,28] Light-scattered intensity is usually proportional to mass, shape, and number of particles in the system.[29] Because there was basically no protein−protein association of BSA occurring at any tested c value in the presence of 2-ME (0−50 mM), as evidenced by unchanged SEC elution profiles (Figure 2), the changes in scattered intensity are thus indicative of changes in the shape of the molecules or their tertiary conformation. We determined the size distribution of BSA particles at various c values of 0.1−0.75%, in the presence of 0−50 mM 2-ME, and the results are presented in Figure 3. As expected, all of the BSA particles exhibited a similarly homogeneous Gaussian profile of size distribution, which is well consistent with the HPSEC observations (Figure 2), confirming that the S−S cleavage did not result in any kind of protein association.

However, it can be still observed that the S−S cleavage with 2-ME led to a slight shift of the distribution peak toward a lower or higher size, depending on the applied c (Figure 3). The influence of the S−S cleavage on the R_h of BSA molecules at various c values (0.1−0.75%) is summarized in Figure 4. In the absence of 2-ME, the R_h of native BSA molecules ranged from about 3.9 to 3.4 nm, as the c increased from 0.1 to 0.75%. The R_h of native BSA at c = 0.1% is well consistent with that (3.8 ± 0.2 nm) reported by Adel et al. at the same c using the same DLS technique.[28] The data are also in accordance with the theoretically calculated Stokes' radius (3.55 nm),[25] and the dimension size (8 nm) of the heart-shaped model[13] for monomeric BSA. The decreased R_h upon increasing c is similar to the DLS observation of Gaigalas et al.[27] about the c dependence of diffusion coefficient of BSA at pH 7.0, where the diffusion coefficient progressively increased with c increasing up to 4.0%. They attributed this phenomenon to Coulomb repulsion interaction of BSA (determined by surface charges),

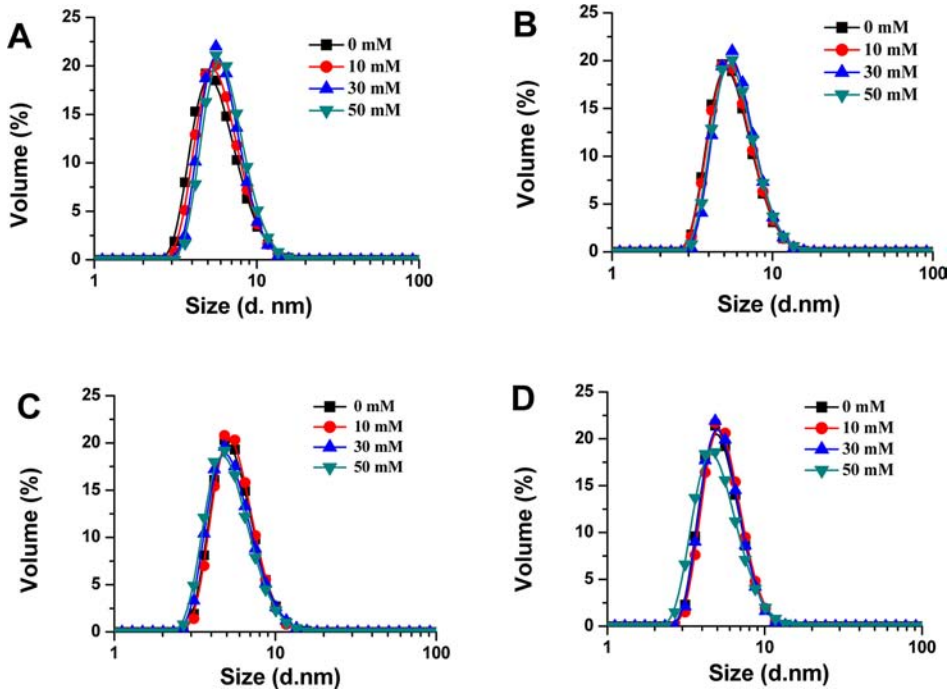

Figure 3. Typical size distribution profiles of native and reduced BSA at various c values of 0.1% (A), 0.25% (B), 0.5% (C), and 0.75% (D), respectively. BSA was reduced by 2-ME at 0 (native), 10, 30, and 50 mM, respectively.

dx.doi.org/10.1021/jf305471k | J. Agric. Food Chem. 2013, 61, 3097−3110

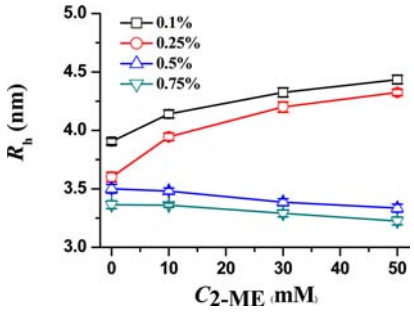

Figure 4. Changes in R_h values of BSA at various c values (0.1−0.75%) upon increasing 2-ME concentration in the range of 0−50 mM. Each datum is the mean and standard deviation of triplicate measurements.

because increasing c might greatly increase the probability of encounters during which the charged proteins come close enough to feel the Coulomb repulsion as well as other mutual interactions.[27] Using a small-angle X-ray scattering technique, recently Barbosa et al.[14] confirmed that c plays an important role in the pH-unfolded conformation of BSA, due to a compromise between repulsive protein−protein interaction and crowding effects. The importance of intermolecular repulsion forces has also been confirmed in the formation of BSA gel at pH 7.0 with a highly structured network.[30]

Upon increasing 2-ME concentration from 0 to 50 mM, we can interestingly observe that at c values of 0.1 and 0.25%, the R_h of BSA molecules progressively increased from 3.9 to 4.5 nm and from 3.6 to 4.3 nm, respectively, whereas at c values of 0.5 and 0.75%, the R_h on the contrary decreased (Figure 4). The observations clearly indicated that S−S cleavage-induced changes in R_h of molecules were closely dependent on the c. The increased R_h upon increasing 2-ME concentration observed at low c values (0.1−0.25%) may reflect that the S−S cleavage favored structural unfolding or formation of expanded and unfolded conformation of BSA. In contrast, S−S cleavage led to the formation of a more compact conformation, or structural refolding of BSA, at high c values (≥0.5%). This can be also explainable using the Coulomb repulsion,[27] because at high c values, once the S−S cleavage occurred, the intermolecular Coulomb repulsion would drive the partially unfolded BSA to undergo a conformational rearrangement to form a new, more compact, state.

Tertiary Conformation. (a) Extrinsic Fluorescence Spectroscopy. To further unravel the changes in tertiary conformation of BSA, as modulated by both c and S−S cleavage, we investigated the influence of 2-ME addition on the extrinsic fluorescence and UV spectra of BSA at various c values. One point should be to clarify that there is a limitation of c for the spectral determination, for example, a maximum of 0.1% for extrinsic fluorescence spectroscopy.

When the extrinsic fluorescent probes (e.g., ANS^-) are mixed with proteins, they will preferentially adsorb to the hydrophobic clusters on the proteins. As a consequence, their emission spectra suffer a large blue shift with a simultaneous increase in intensity.[31,32] Figure 5 shows the influence of increasing 2-ME concentration on typical emission fluorescence spectra of ANS^- in the presence of BSA at c = 0.05 and 0.1%. As the c increased from 0.05 to 0.1%, the maximal fluorescence intensity for native BSA increased by about 38%, indicating a nonlinear increase in the maximal intensity. This observation suggested that the number of hydrophobic cavities (or zone) in the protein decreased with increasing c. This is basically consistent with the R_h data (although in the latter case, the tested c values were much higher; Figure 4), confirming that the tertiary conformation of native BSA was more compact at higher c values.

Upon increasing 2-ME concentration, we can see that at the both c values the maximum fluorescence intensity of ANS^- gradually declined, with higher extent of reduction at c = 0.05% (Figure 5). The progressive reduction in maximum intensity indicated enhanced polarity of the microenvironment of the probe. This seems to be in contrast to the R_h data at c = 0.1% (Figure 4), because in the latter case, it was indicated that the conformation of BSA became more unfolded upon S−S cleavage. A similar contrasting phenomenon has also been observed for intrinsic fluorescence spectra of native and 2-ME-reduced BSA (at c = 0.01%), where the maximum fluorescence intensity progressively decreased with increasing 2-ME concentration from 0 to 50 mM.[33] The decreased intrinsic fluorescence intensity reflected enhanced polarity of the microenvironment of the Trp residues, indicating structural unfolding or loosening of tertiary conformation.[31] One of the possible reasons for this inconsistency may be that when the conformation of BSA (e.g., at c = 0.1%) became more unfolded, the effective amount of cationic groups (e.g., lysine, histidine, and arginine groups) available to bind ANS^- through ion pair formation[34] might decrease, due to increased electrostatic repulsion between the negatively charged groups on the surface of the protein molecules and the organic sulfonate group of

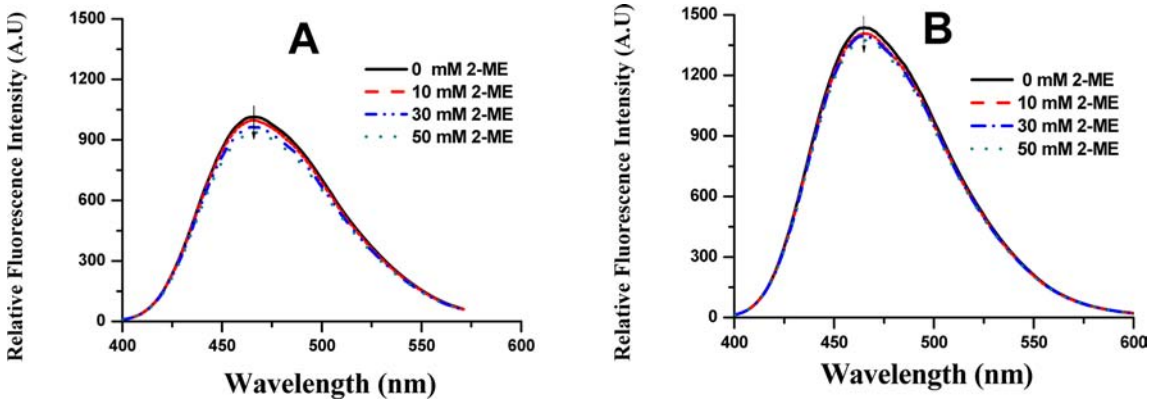

Figure 5. Typical emission fluorescence spectra of ANS^- in the presence of BSA at c = 0.05% (A) and 0.1% (B), with increasing concentrations (0−50 mM) of 2-ME.

ANS^-. As a consequence, the fluorescence intensity of ANS^- in the presence of 0.1% BSA decreased with increasing 2-ME concentration.

(b) Second-Derivative UV Spectroscopy. The S−S cleavage-induced changes in the conformation of BSA at three selected c values (0.05, 0.1, and 0.25%) were further characterized using second-derivative UV spectroscopy, as shown in Figure 6. This

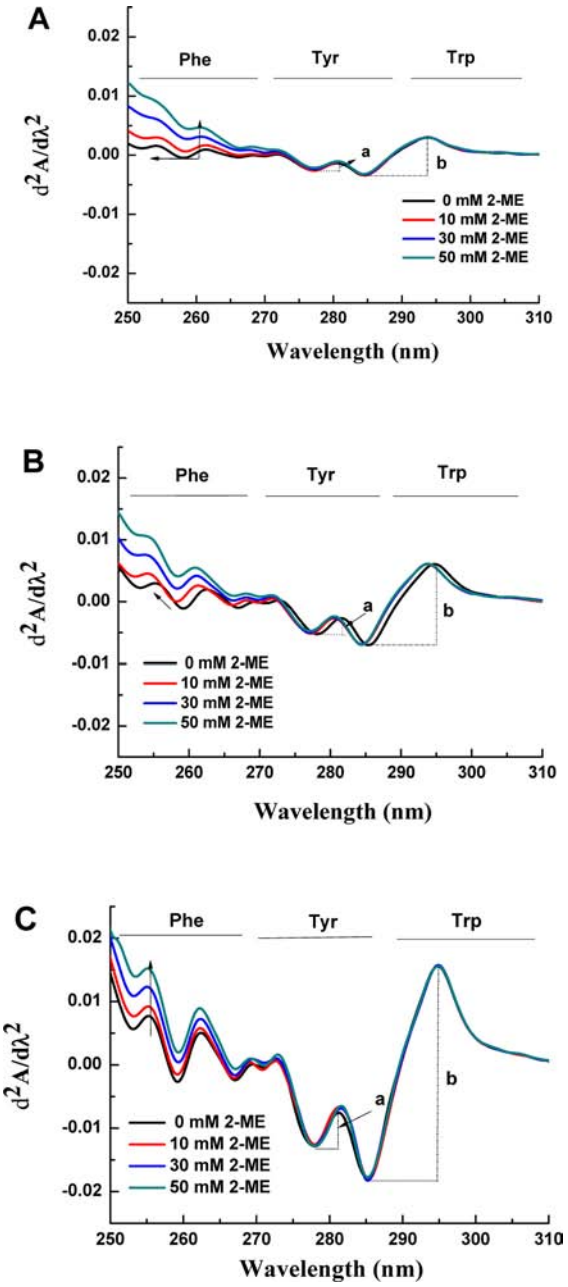

Figure 6. Typical second-derivation profiles of UV spectra of BSA at c values of 0.05% (A), 0.1% (B), and 0.25% (C) in the presence of 0−50 mM 2-ME.

spectroscopy, which can virtually eliminate interference from light scattering and from cystine, has been confirmed to well reflect the changes in polarity of the microenvironment of phenylalanine (250−270 nm), tyrosine (270−285 nm), and tryptophan (above 290 nm) in the proteins.[35] As usual, the derivative spectral bands suffer a blue shift with a concomitant increase in amplitude, when the microenvironment of aromatic acid residues becomes more exposed to solvent (relative to that when the residues are buried within the interior of a protein).[36] One point of interest is that at tested c values, only derivative spectral bands corresponding to Phe residues were considerably changed by 2-ME with increasing concentration (Figure 6). This observation suggested that the microenvironment of the Phe residues in BSA might be closely associated with the S−S bridges. In this respect, we can basically see that the S−S cleavage resulted in enhanced amplitude and, concomitantly, blue shifts of derivative bands with Phe residues, with higher extents of changes observed at higher 2-ME concentrations, indicating exposure of Phe hydrophobic clusters to the solvent or structural unfolding. Like the extrinsic fluorescence data (Figure 5), the changes of the Phe derivative bands were greater at low c values (Figure 6), further indicating the c dependence of tertiary conformation.

Although the polarity of the microenvironment of the Tyr and Trp residues at these tested c values was nearly unaffected by 2-ME with increasing concentrations up to 50 mM, as evidenced by slight changes in peak and trough positions and amplitudes of Tyr and Trp bands (Figure 6), it can be still seen that the second-derivative UV bands with Tyr and Trp residues varied with c. The change in amplitude, which is described by calculating the ratio ($r = a/b$) of the two peak-to-trough values marked in Figure 6, has been well applied to indicate the change in solvent polarity or in the microenvironment of the Tyr residues.[36] At c = 0.05%, the r was calculated to be about 0.232, much higher than that at c = 0.1 and 0.25% (0.185 and 0.201, respectively). In accordance with fluorescence spectroscopy, this suggests that for tyrosine the solvent polarity decreased as c increased; that is, the Tyr residues became more buried within the protein at higher c values. This indirectly confirmed the above argument that the tertiary conformation of BSA was more affected by c than by S−S cleavage.

Characteristics of BSA-Stabilized Emulsions. *Emulsifying Ability.* The droplet size distribution of BSA-stabilized emulsions formed at various protein (0.25, 0.5, and 0.75%) and 2-ME (0−50 mM) concentrations was evaluated, with deionized water or 1% SDS as the dispersant. Table 1 summarizes the mean droplet size (d_{43}; diluted in water or 1% SDS) of all the emulsions after storage periods of 4 and 24 h. As usual, the d_{43} of a freshly prepared emulsion in 1% SDS can reflect the ability of a protein to help dispersion of the oil phase into an aqueous medium, because the presence of 1% SDS may inhibit bridging flocculation of oil droplets, thus keeping individual droplets separate in the emulsions. In the present work, the d_{43} data of droplets (in 1% SDS) at 4 h were selected as an indication for the emulsifying ability of BSA, with lower d_{43} indicative of higher emulsifying ability.

In the absence of 2-ME, d_{43} (in 1% SDS) considerably decreased from 5.53 to 0.76 μm, as c increased from 0.25 to 0.5%; $d_{4,3}$ significantly but slightly decreased to 0.50 μm upon a further increase in c to 0.75% (Table 1). The data are basically consistent with the result for BSA in a model system reported by Waniska et al.,[17] reflecting that at c values of ≥0.5%, the total interfacial area nonlinearly increased with c, due to adequate quantity of protein available to aid formation and stabilization of the newly created emulsion. The d_{43} value at c = 0.75% was even distinctly lower than that at c = 1.78% (1.10 μm) for native BSA reported by Lee and Hirose.[22] The difference might be due to the difference in emulsification efficiency between two different homogenization techniques (ultrasonication vs microfluidization).

Table 1. Mean Droplet Size (d_{43}), Flocculation Index (FI), and Flocculation and/or Coalescence Stability of BSA Emulsions at Protein Concentrations of 0.25, 0.5, and 0.75% in the Presence of Various Concentrations of 2-ME ($C_{2\text{-ME}}$; 0−50 mM)[a]

		d_{43}[b] (μm)						emulsion stability during storage for 4−24 h[c]		
		4 h		24 h		FI%				
BSA concn (%)	$C_{2\text{-ME}}$ (mM)	water	1% SDS	water	1% SDS	4 h	24 h	(C+F)%	C%	F%
0.25	0	17.5 a	5.53 a	72.8 a	58.5 a	217.0 a	24.4	1216 b	958 b	258 b
	10	11.5 b	3.85 b	38.2 b	30.2 b	197.5 b	26.5	892 d	684 c	208 c
	30	10.8 c	3.70 c	17.5 c	16.4 c	192.6 c	6.7	373 g	343 e	30 f
	50	10.4 d	3.65 c	16.2 d	10.6 d	183.5 d	52.8	344 h	190 f	154 d
0.50	0	0.86 h	0.76 g	1.55 j	1.23 j	13.0 h	17.4	104 j	61.8 f	42 e
	10	3.71 f	3.20 e	4.32 h	3.30 i	15.6 g	30.9	35 k	3.1 j	32 f
	30	8.38 e	3.24 d	12.3 f	6.20 h	100.4 f	98.4 a	280 i	91.4 i	189 c
	50	10.6 c	3.78 b	15.2 e	7.61 g	179.9 e	99.7 a	402 f	101 h	301 a
0.75	0	0.55 j	0.50 i	1.41 j	1.09 j	10.4 k	29.4	282 i	118 g	164 d
	10	0.68 i	0.61 h	3.50 i	3.20 i	11.7 j	9.4 a	474 e	425 d	49 e
	30	0.68 i	0.60 h	9.64 g	7.86 f	12.9 i	22.6	1507 a	1210 a	297 a
	50	1.10 g	0.90 f	9.71 g	8.44 e	11.4 j	15.1	979 c	938 b	41

[a]Each datum is the mean of triplicate measurements. Different letters (a−k) represent significant difference at the $p < 0.05$ level withiin the same column. [b]The d_{43} was determined on the emulsions (after storage for 4 and 24 h), dispersed with deionized water and 1% SDS, respectively. [c](C+F)%, stability against coalescence plus flocculation; C%, stability against coalescence; F%, stability against flocculation.

d_{43} (in 1% SDS) was markedly affected by the presence of 2-ME, but to different extents depending on the applied protein and 2-ME concentrations (Table 1), indicating variation in emulsifying ability. Herein, we first see that the changing pattern of d_{43} upon increasing 2-ME concentration was closely dependent on c. Overall, d_{43} at c = 0.25% progressively decreased with 2-ME concentration increasing from 0 to 50 mM, although a considerable decrease was observed only at a 2-ME concentration of 10 mM, whereas at c = 0.5 or 0.75%, d_{43} changed in a contrary way (Table 1). Especially at c = 0.5%, d_{43} was considerably increased from 0.76 to 3.2−3.78 μm by the presence of 2-ME, even at a concentration as low as 10 mM; the d_{43} value at 50 mM 2-ME concentration (3.78 μm) was even significantly higher than that at c = 0.25% (3.65 μm). In contrast, the d_{43} (in 1% SDS) at c = 0.75% only slightly increased upon S−S cleavage compared with that at c = 0.5%. The observations clearly indicated that the emulsifying ability of BSA was improved by S−S cleavage at c = 0.25%, but impaired at $c \geq 0.5\%$.

Flocculated State of Oil Droplets in Emulsions. The flocculated state of oil droplets in emulsions after 4 h of storage was evaluated in terms of flocculation index percentage (FI%), as also included in Table 1. For native BSA emulsions, the d_{43} of droplets (17.5 μm; in deionized water) at c = 0.25% was remarkably higher than that (5.53 μm) with 1% SDS as the dispersant, indicating the high extent of bridging flocculation of oil droplets. In contrast, the FI% values at c = 0.5 and 0.75% (13.0 and 10.4, respectively) were considerably lower compared to that at c = 0.25% (217) (Table 1). This observation confirmed the above argument that for native BSA the protein amount at c = 0.5% was enough to form a homogeneous and fine emulsion.

As expected, the presence of 2-ME with increasing concentration led to changes in FI%, with c-dependent changing patterns similar to that for d_{43} (in 1% SDS). For example, the FI% at c = 0.25% gradually decreased with increasing 2-ME concentration, whereas in the case at c = 0.5 or 0.75%, a contrary changing pattern was observed (Table 1). By comparison, we can see that S−S cleavage induced changes in FI% were most dramatic at c = 0.5% among the three tested c values. In this case, d_{43} (in deionized water) progressively increased from 0.86 to 10.6 μm, as the 2-ME concentration increased from 0 to 50 mM; concomitantly, the FI% increased from 13 to 180, with a considerable increase observed between 2-ME concentrations of 10 and 30 mM (Table 1). This observation suggested that the flocculated state of oil droplets was to the highest extent affected by the S−S cleavage at c = 0.5% relative to the other two c values.

Flocculation and/or Coalescence Stability. The stability of various emulsions formed at various protein and 2-ME concentrations against flocculation and/or coalescence, during a storage period from 4 to 24 h, was evaluated in terms of (C+F)%, C%, and F%, as also included in Table 1. As usual, both flocculation and coalescence of oil droplets in emulsions simultaneously occurred during storage. As expected, the flocculation and/or coalescence stability of these emulsions varied with the applied protein and 2-ME concentrations. In the absence of 2-ME, highest (C+F)% was observed at c = 0.25% (1216), followed by 0.75% (282) and 0.5% (104) (Table 1). This observation is in accordance with the fact that at c = 0.25% the proteins were not enough to fully cover the interfacial area, so the interfacial area is weaker with regard to coalescence. The seemingly abnormal lower ability at c = 0.75% relative to c = 0.5% might reflect that once the amount of proteins was enough to fully cover the interfacial area, the conformational stability of the proteins would play a predominant role in the emulsion stability (because the BSA molecules were more compacted at c = 0.75% than at c = 0.5%). In the presence of 2-ME, we can generally observe that at c = 0.25% the (C+F)% progressively decreased with increasing 2-ME concentration, whereas in the case at c = 0.5 or 0.75%, the changing pattern was the reverse (with exceptions at c = 0.5% and 10 mM 2-ME or at c = 0.75% and 50 mM 2-ME; Table 1). The results indicated that at c = 0.25% increasing the S−S cleavage greatly improved the stability of the emulsions against flocculation plus coalescence, whereas at higher c values (0.5 and 0.75%), the

dx.doi.org/10.1021/jf305471k | J. Agric. Food Chem. 2013, 61, 3097−3110

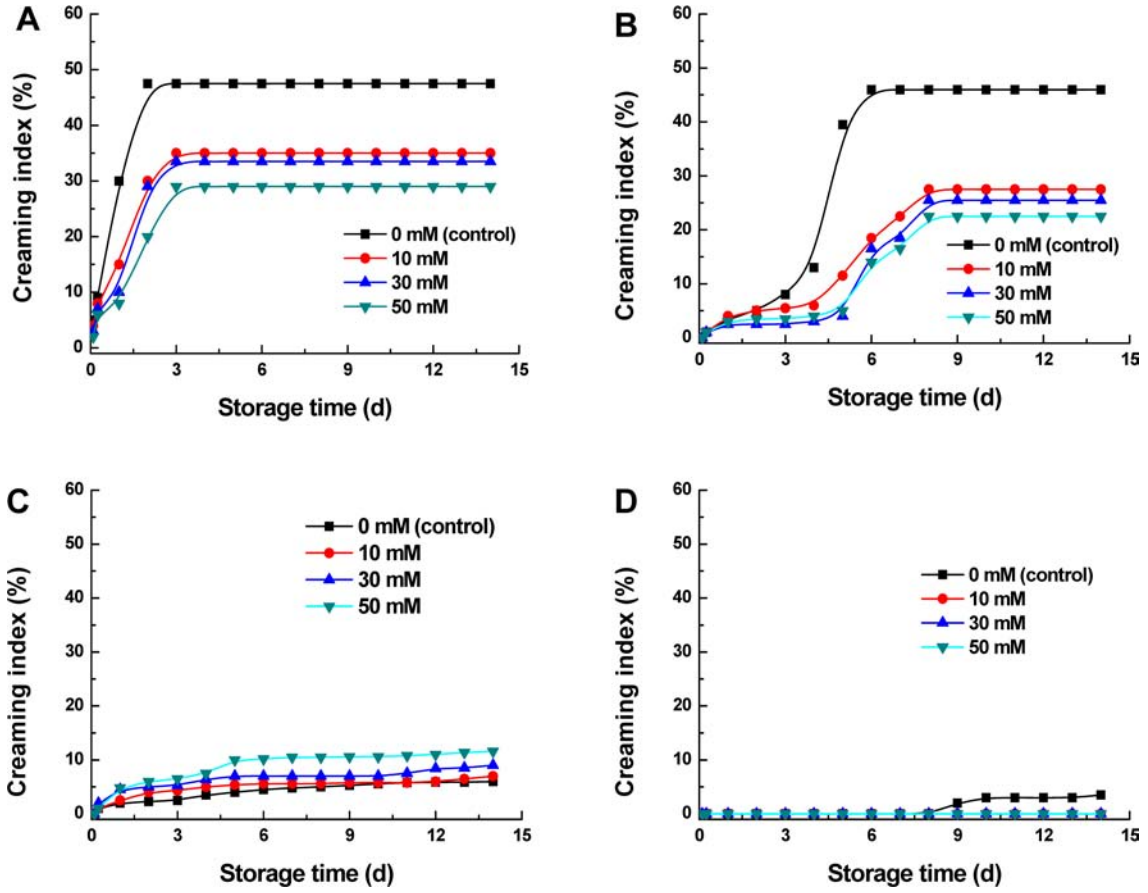

Figure 7. Time evolution profiles of creaming index of BSA-stabilized emulsions at c values of 0.10 (A), 0.25 (B), 0.50 (C), and 0.75% (D) in the presence of various concentrations (0−50 mM) of 2-ME. Each datum is the mean of duplicate measurements.

emulsion stability, on the contrary, decreased with the S−S cleavage. By comparison, the S−S cleavage induced changes in emulsion stability against flocculation plus coalescence were much more dramatic at c = 0.75% than at c = 0.5% (Table 1). This observation further supported the above argument that at high c values (e.g., ≥0.5%) the conformational flexibility of the proteins largely accounted for the emulsion stability.

At c = 0.25%, the oil droplets were much less flocculated at 24 h of storage than at 4 h, as evidenced by a significantly lower FI at 24 h relative to that at 4 h (Table 1). In this case, the C% at any 2-ME concentration was also be considerably higher than the F% counterpart. The observations reflected that a large portion of flocculated oil droplets was coalesced to form large size droplets, suggesting that the coalescence of oil droplets predominated the stability of BSA emulsions at c = 0.25%. Upon increasing 2-ME concentration from 0 to 50 mM, we can also see that the C% progressively decreased from 958 to 190, whereas the F% slightly changed (Table 1), indicating that at this low c the improvement of emulsion stability by S−S cleavage was mainly through enhanced stability against coalescence.

The situation at c = 0.75% seemed to be more complex. In the absence of 2-ME, the oil droplets became more flocculated when the storage was prolonged from 4 to 24 h (as evidenced by higher FI% at 24 h) and, concomitantly, the F% (164) was also distinctly higher than the C% (118) (Table 1), indicating that at this c both flocculation and coalescence contributed to the stability of native BSA emulsion, with flocculation as the major mechanism. However, the relative importance of flocculation and coalescence for the emulsion stability was completely changed by the S−S cleavage. In the presence of any concentration of 2-ME, the C% was remarkably higher than the F% counterpart (∼10−20 folds; Table 1), indicating the predominant role of coalescence in the emulsion stability. The changing pattern of C% upon increasing 2-ME concentration was basically the same as that for (C+F)%. Thus, the impairment of emulsion stability by the S−S cleavage was mainly by means of increased coalescence of oil droplets.

Contrasting from the situation at c = 0.75%, the decreased emulsion stability at c = 0.5% by the presence of 30 or 50 mM 2-ME was largely due to flocculation of oil droplets, as evidenced by much higher F% values than the C% counterparts (Table 1). This could be also applicable at a 2-ME concentration of 10 mM, although in this case, the emulsion stability was contrarily improved by the S−S cleavage relative to that in the absence of 2-ME.

The above observations are basically in accordance with the results about the oil fraction dependence (at a constant c in bulk solution) reported by Klemaszewski et al.,[19] who applied oxidative sulfitolysis to cleave disulfide bonds of BSA. In this previous work, they found that at a constant c (0.3%), S−S cleavage resulted in a gradual improvement of emulsifying properties (decreased droplet size and increased surface area) at high oil fractions (e.g., 0.42 or 0.62), whereas at a much lower oil fraction (0.22), the emulsifying properties were nearly unaffected, and even impaired, by S−S cleavage to an extent of 60%. They attributed the improvement of the EA of BSA to increased flexibility, as well as exposed hydrophobic segments

from the S−S cleavage. Waniska et al.[17] reported that the S−S reduction with another reducing agent (DL-dithiothreitol; DTT) reduced the EA of BSA in the pH range of 4−7; however, Poon et al.[21] contrarily pointed out that S−S cleavage with DTT slightly improved its EA. These contrasting observations about the emulsifying properties of reduced BSA can be well explained if the c dependence of the influence of S−S cleavage is taken into account.

Creaming Stability. The emulsion stability of BSA-stabilized emulsions, formed at various c values (0.10−0.75%) in the presence of 0−50 mM 2-ME, upon storage up to 2 weeks was further characterized using the creaming index as an indicator, as illustrated in Figure 7. As expected, the changing pattern of creaming index upon storage of the emulsions varied with the protein and 2-ME concentrations. In the absence of 2-ME, the creaming index at c = 0.1% increased quickly to about 48% upon increasing storage up to 3 days and then remained constant upon further storage (Figure 7 A). When the c was increased to 0.25%, the time needed to reach maximal creaming index was greatly enhanced (e.g., up to about 6 days), and the maximal creaming index was slightly reduced (Figure 7B), indicating partial inhibition of creaming. Further increasing c resulted in a further inhibition of creaming; especially at c = 0.75%, no creaming occurred up to 8 days (Figure 7C,D). These observations clearly indicated that increasing c (in the range of 0.10−0.75%) progressively improved the stability against creaming of native BSA emulsions.

The presence of increasing concentrations of 2-ME remarkably changed the creaming behavior of the emulsions, and the influence was closely dependent on the applied c (Figure 7). At low c values, for example, 0.10 or 0.25%, we can see that increasing 2-ME concentration resulted in a progressive inhibition of creaming. The improvement of the creaming stability by the S−S cleavage was better at c = 0.25% than at c = 0.10% (Figure 7A,B). In contrast, the situation at c = 0.50% seemed to be completely different. In this situation, increasing 2-ME concentration, on the contrary, progressively accelerated the instability against creaming, although to a limited extent (e.g., the creaming index after storage of 14 days increased from ∼5 to 10%, as the 2-ME concentration increased from 0 to 50 mM; Figure 7C). At c = 0.75%, the presence of any concentration of 2-ME completely inhibited the creaming (Figure 7D).

The creaming behavior at c = 0.25% is well consistent with the (C+F)% and/or C% data (Table 1), confirming that at c = 0.25% the coalescence of oil droplets during short periods (e.g., 24 h) facilitated the creaming of the emulsions, and decreasing coalescence by the S−S cleavage thus improved the creaming stability. The creaming behavior at c = 0.5% is much better related to the d_{43} data (in deionized water) at 4 h than the (C+F)%, C%, or F% data (Table 1), suggesting that at this c the progressive but slight decrease in creaming stability by the S−S cleavage was largely due to the increased size of oil droplets (and FI%) in freshly prepared emulsions. On the other hand, we can see that at high 2-ME concentrations, for example, 50 mM, the d_{43} data (in deionized water) at 4 or 24 h, as well as the (C+F)% for the emulsion at c = 0.5%, were similar to those at c = 0.25%, but the creaming index at prolonged storage periods (e.g., >8 days) in the former case was much less (Table 1). This observation suggested that besides the above-mentioned parameters, for example, size and flocculated state of droplets in fresh emulsions, and coalescence and flocculation stability, there are other parameters (e.g., nature and microstructure of droplets in the emulsions) contributing to the creaming stability. This can be to a more distinct extent reflected in the case at c = 0.75%, at which increasing 2-ME concentration remarkably decreased the stability against flocculation plus coalescence, or coalescence, but the creaming was completely inhibited by the presence of any concentration of 2-ME (Table 1).

To unravel the underlying mechanism for higher creaming stability at higher protein concentrations (e.g., 0.5−0.75%; relative to 0.1 or 0.25%), we evaluated the microstructure of the emulsions (4 h) at c = 0.5% and at various 2-ME concentrations (0−50 mM) using the CLSM technique (the fluorescence dye Nile Blue A was applied to indicate the protein (excitation at 633 nm; red color) and oil (excitation at 514 nm; green color) phases, respectively), as displayed in Figure 8. We can see that

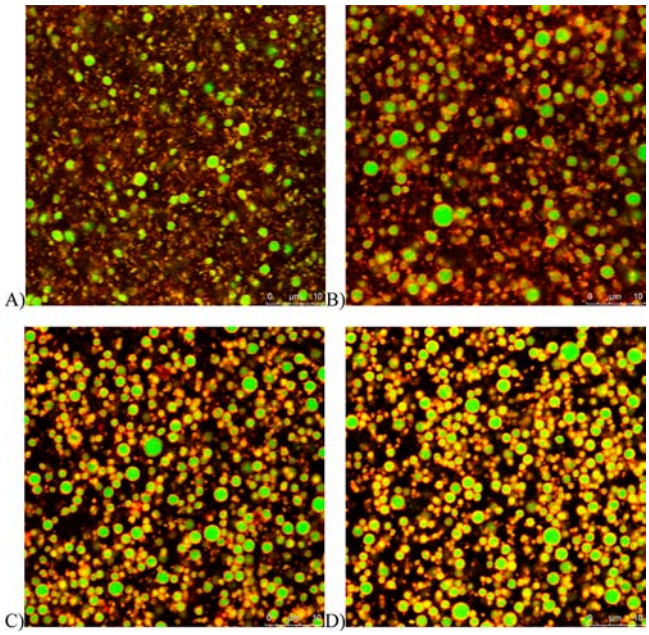

Figure 8. Typical CLSM images of the emulsions stabilized by BSA at c = 0.5% in the presence of 0 (A), 10 (B), 30 (C), and 50 (D) mM 2-ME, respectively.

the presence of 10−50 mM 2-ME resulted in an increase in mean droplet size, but there were no distinct changes in size distribution of oil droplets between different 2-ME concentrations. This observation is consistent with the d_{43} data (in 1% SDS; Table 1), confirming that the S−S cleavage impaired the emulsifying ability. On the other hand, we can see that upon increasing 2-ME concentration from 0 to 50 mM, the morphology of the droplets in the emulsions gradually became more vivid and visible (Figure 8), reflecting a gradual decrease in mobility of oil droplets or an increase in extent of bridging flocculation. At high 2-ME concentrations, for example, 30 or 50 mM, all of the oil droplets were associated with one another to form a weak gel-like network in the system, which seems to agree well with the description for a type of aggregated droplet emulsion gel.[37] Thus, the relatively higher creaming stability for the emulsions at c = 0.5% (relative to that at c = 0.25%) could be largely attributed to formation of a weak gel-like network.

DISCUSSION

Modulation of Conformational Flexibility of BSA by S−S Cleavage: Protein Concentration Dependence. In

general, the overall conformation of BSA in neutral solution is rigid and compact, very similar to the heart-shaped structure of human serum albumin observed in the crystal.[13] However, the conformation of native BSA is a dynamic structure, such that at any instant it may undergo a kind of structural unfolding/refolding (or expansion/compaction).[25] In the present work, it has been convincingly indicated that in the tested c range (0.1−0.75%), the tertiary conformation of native BSA is highly dependent on c, with conformation more compact and rigid at higher concentrations. This basically reconciles with the findings of Gaigalas et al.[27] and Barbosa et al.[14] that BSA overall shape changes as a function of c. The high c dependence of conformation of native BSA seems to be largely associated with its unique surface nature. The surface of this protein is highly charged, with a charge number per molecule of 13 and a density of surface charge of 4244 ($-$esu.cm^{-2}), respectively.[14,25] The high surface charge would cause a strong Coulomb repulsion interaction between proteins. Thus, the more compact conformation at higher c values was largely due to the higher effect of the Coulomb repulsion interaction, because the intermolecular repulsion interaction might change the balance of intramolecular repulsive and attractive interactions that maintain the conformation of native BSA. Interestingly, Barbosa et al.[14] indicated that in the c range of 1.0−5.0%, increasing the c leads to a reduction of attractive potential of BSA. This indirectly supported the above argument that the repulsion interaction strengthens as BSA concentration increases.

BSA monomer consists of three homologous domains (I, II, and III) encompassing the complete sequence (585 amino acid residues), with each domain consisting of two subdomains, and the 17 intrachain disulfide bonds contained in the whole molecule are almost equally allocated to the three domains.[15] Among the 17 S−S bridges of human serum albumin (HSA: 80% similarity to BSA), there are about 7 that are accessible to the solvent.[16] The accessibility of S−S bridges in BSA to the solvent is actually affected by its conformational state that highly depends on c. The high c dependence of S−S cleavage induced by the presence of 2-ME, with higher extent of cleavage at lower c values, has been confirmed in the present work (Figure 1).

The disulfide bridges between cysteine residues are a key structural element of many proteins and peptides. Their primary effect is to impose distance and angle constraints between joined cysteine residues, thus stabilizing the folded state with respect to the unfolded form. Partial S−S cleavage subsequently leads to the unfolding of the protein, accompanied by a reorganization of the protein structure.[16] For HSA, Lee and Hirose[15] pointed out that the conformation of reduced protein is highly variable, depending on pH and ionic strength; the disulfide-reduced state with partially folded variable conformation is involved in the reversible interconversion between urea-denatured reduced and native HSA forms. The refolding of urea-denatured BSA has been corroborated by a small-angle neutron scattering study at c = 1.0%, in which it was observed that the radius of gyration of the compact form of the protein in solution decreased as the urea concentration increased.[38] Thus, the conformational state of S−S-cleaved BSA at each equilibrium state in solution is determined by the reversible interconversion between structural unfolding/refolding, as affected by variation in the protein and 2-ME concentrations. This can be well applied to explain the observations for the c dependence of the influence of S−S cleavage on the conformation of the protein (Figures 4−6). For example, at low c values (e.g., 0.1 or 0.25%), increasing S−S cleavage favored the structural unfolding of the protein, whereas at high c values of ≥0.5%, the refolding of the reduced protein dominated the conformational state at equilibrium.

On the other hand, although the protein carries a net negative charge, there is a clearly defined patch (size = 2−3 nm) of positive charge on the BSA molecule surface at pH 7.0.[39] The changes in conformation of the protein clearly led to alteration of intramolecular interactions (especially electrostatic interactions between the domains and even between the subdomains). Thus, we tend to consider that in essence the conformational state of S−S-cleaved BSA at equilibrium results from a delicate compromise between intermolecular and intramolecular electrostatic interaction forces. At high c values (e.g., ≥0.5%), high intermolecular electrostatic repulsion interaction (Coulomb interaction effect) seemed to predominate the intramolecular interactions, thus favoring the refolding of S−S-cleaved BSA to form a more compact conformation, whereas at low c values, the Coulomb interaction effect was insignificant relative to the intramolecular interactions, and so the removal of the constraints between joined cysteine residues by the S−S cleavage would greatly favor the structural unfolding.

According to the present observations about the c dependence on the conformation of BSA, we reasonably hypothesize that there should be a critical c (between 0.25 and 0.5%) at which the conformation of the protein might be unaffected by increasing 2-ME concentration. It would be interesting to investigate the influence of S−S cleavage on the conformation flexibility of BSA at this critical c.

Understanding the Role of Conformational Flexibility in the Emulsifying Ability of BSA. All of the above-mentioned indicated that by means of varying the c and S−S cleavage, BSA is an ideal globular protein to be used for elucidating the importance of conformational characteristics to emulsifying properties of globular proteins. In the present work, the 2-ME concentration in each case was kept constant, which is very important to ensure that the conformational changes of the protein were predominately at the tertiary conformation level (as evidenced by the unchanged HPSEC profiles; Figure 2), or quaternary conformation may be involved.

The emulsification of a globular protein usually consists of at least three steps: (I) adsorption at the interface of oil droplets; (II) orientation at the interface and structural unfolding (to form a "molten globule state"); (III) structural rearrangement and formation of a viscoelastic interfacial layer or multilayers. The ability of a protein to form an emulsion with fine oil droplets is mainly associated with the first two steps (I and II). The rate of adsorption for a protein (with a net electrical charge) at the interface of oil droplets is related to the bulk concentration of the protein, the diffusion coefficient, and the electrical potential set up at the interface by the adsorbed protein.[20] The adsorption also depends on hydrophobic interactions between the surface and the amino acid side chains in the protein.[40] Following adsorption, orientation and conformation unfolding of adsorbed proteins occur, but the extent of globular protein unfolding upon adsorption is dependent on the protein structure and the degree of crowding in the adsorbed layer.[41] At high bulk concentrations of the protein (c), fast adsorption occurs, giving less time for protein molecules to orient and spread out at the surface. As a result, the area occupied per molecule is lower and the adsorbed

amount higher.[41] The protein molecules at low c values reach the interface at optimal conditions; they can fully adsorb at any locus and occupy a maximum interface area, because the interface is still free of molecules.[41] Thus, there is an optimal c of interfacial saturation for any protein, at which the interface is just fully covered by the protein. In the present work, we observed that the optimal c of native BSA was approximately 0.5%; this optimal c was slightly increased to about 0.75% by S−S cleavage with 10−50 mM 2-ME (Table 1). This is well consistent with the decreased conformational flexibility at c = 0.5 or 0.75% by S−S cleavage.

On the other hand, the importance of tertiary conformational flexibility to the emulsifying ability can be also well reflected in the influence of increasing 2-ME concentration at any tested c value in the range of 0.25−0.75%. However, the underlying mechanism at different c values seems to be different. At low c values (e.g., 0.25%), the improvement of the emulsifying ability of BSA by increasing the S−S cleavage might be largely due to increased ease of conformational changes at the interface. In this case, the protein with increased conformational flexibility in solution (as evidenced by increased R_h; Figure 4) might be more like the molten globule state upon adsorption at the interface[10] and subsequently lead to increased interfacial coverage per molecule. It has been confirmed that transformation from the native state to the molten globule state increases the relative affinity of α-lactalbumin for the interface during emulsification; the extent of adsorption of this protein at the more hydrophilic silica particle surface is much greater in the molten globule state.[10]

At c = 0.75% (above the optimal c), increasing S−S cleavage, on the contrary, led to a progressive impairment of the emulsifying ability (as evidenced by a progressive increase in d_{43} (in 1% SDS); Table 1); accordingly, the tertiary conformational flexibility of the protein in solution decreased. In this case, the impairment of the emulsifying ability might be largely due to decreased lateral protein−protein associations at the interface, because the decreased R_h of BSA by the S−S cleavage (Figure 4) would lead to increased surface charge density, thus favoring the intermolecular repulsion between the individual protein units within the layer. Increased electrostatic repulsion to a large extent inhibited interprotein conformational rearrangement at the interface.[43]

Interestingly, the importance of the conformational flexibility for the emulsifying ability is better reflected at c = 0.5% (close to the optimal c for native BSA). In this case, the emulsifying ability of BSA was seriously impaired by the S−S cleavage; the S−S cleavage also resulted in a similar decrease in conformational flexibility to that at c = 0.75% (Table 1 and Figure 4). The observations suggested that for the reduced BSA with more compact conformation the protein amount at c = 0.5% was not enough to fully cover the newly created interfacial area. Thus, the impairment of the emulsifying ability at this c mainly resulted from a lack in the amount of proteins to form an effective interface saturation coverage.

Understanding the Role of Conformational Flexibility in the Emulsion Stability of BSA. All emulsions are inherently unstable, despite the adsorption of proteins at the interface and subsequent formation of a protein interfacial layer or interfacial multilayers. Upon storage, the oil droplets in the emulsions may flocculate, coalescence, and even form a creaming layer. The coalescence stability of the emulsion is largely associated with the nature of interfacial protein film, for example, thickness and viscoelasticity. In this respect, the conformational stability of protein plays a vital role, because the protein with higher conformational flexibility will more easily form a viscoelastic protein film that can provide greater protection against coalescence.

Upon storage with a relatively short period (up to 24 h), in the present work, we can observe that the flocculation plus coalescence stability of the native BSA emulsion at c = 0.25% was much less than at c = 0.5 or 0.75%, and the decreased stability of the former emulsion was predominately contributed by the coalesence stability (Table 1). The much lower coalescence stability of the emulsion at low c values might be closely related to lower protein load at the interface. Britten and Giroux[11] observed that for whey protein emulsions the protein load increased with c, but leveled off at a concentration of 0.5%. However, we can interestingly observe that the flocculation and/or coalescence stability of the native BSA emulsion at c = 0.5% was, on the contrary, significantly higher than that at c = 0.75% (Table 1). The "abnormally" decreased emulsion stability at c = 0.75% compared with that at c = 0.5% might be due to formation of a more elastic and rigid interfacial layer, because the tertiary conformation of native BSA at c = 0.75% was more compacted that that at c = 0.5% (as evidenced by a lower R_h value; Figure 4). In another work,[44] Britten and Giroux indicated that the whey protein emulsions formed at a specific c were less stable to coalescence than casein emulsions. This previous observation indirectly confirmed the importance of conformational flexibility of the proteins to the emulsion stability, because it is well recognized that caseins are much more flexible in conformation (especially tertiary conformation) than whey proteins.

At low c values (e.g., 0.25%), increasing S−S cleavage resulted in a progressive increase in stability against flocculation plus coalescence (and coalescence alone) (Table 1). This is well in accordance with the above argument that increasing conformational flexibility greatly facilitated the structural unfolding and rearrangement of the protein at the interface and even formation of a viscoelastic interfacial layer. In contrast, at c = 0.75% the emulsions against flocculation plus coalescence progressively decreased with increasing 2-ME concentration up to 30 mM; unexpectedly, the coalescence also predominated the emulsion instability (Table 1). A similar decrease in stability against flocculation plus coalescence was observed at c = 0.5% as the 2-ME concentration increased above 10 mM, but in this case, the magnitude of the decrease was much less than at c = 0.75%, and the bridging flocculation played a major role in the storage instability (Table 1). These observations basically supported the role of conformational flexibility for the emulsion stability (especially against coalescence). The much higher susceptibility to destabilization by the S−S cleavage at c = 0.75% further suggested that the formed interfacial protein layer at this c was less viscoelastic than that at c = 0.5%. Wüstnek et al.[42] also observed that at c values exceeding the saturation of the adsorption layer, both dilational elasticity and viscosity of the interfacial film decrease, although the adsorption layer thickness increases. They attributed this to disturbance of optimal layer structure by a large amount of molecules that enter the interface at the same time and cannot be optimally arranged. However, this seems to be contrasting from the observation[8] for β-lactoglobulin emulsion that increasing c enhances the lateral interactions at the interface and/or the thickness of the interfacial multilayers, thus allowing improved emulsion stability. The difference might be associated with the difference between the surface net charges of these two

globular proteins, with BSA being much higher charged on the surface. From this point of view, the decreased emulsion stability at c = 0.75% relative to c = 0.5% could be partially attributed to the higher Coulomb repulsion interaction between lateral adsorbed proteins.

By comparison, the situation for the creaming stability upon prolonged storage (up to 2 weeks) is much more complex. In general, increasing the c progressively inhibited the creaming of BSA emulsions (Figure 7). Especially at c = 0.75%, no distinct creaming occurred for the emulsions even after storage of 2 weeks. This might reflect the fact that increasing c increases protein load at the interface and reduces water molecule orientation at the interface, thus possibly contributing to the increased emulsion stability.[11] On the other hand, we can still observe that at low c values, for example, ≤0.25%, increasing S−S cleavage progressively improved the creaming stability, whereas at c = 0.5%, the changing pattern was the reverse (Figure 7). This suggests that increasing/decreasing conformational flexibility of the protein in solution still produced a positive/negative influence on the creaming stability of the emulsions (upon prolonged storage). At c = 0.25%, the improvement of the creaming stability by S−S cleavage was largely attributed to the increased coalescence stability, whereas the impairment of the creaming stability at c = 0.5% was associated with both the size of the flocculated oil droplets in the fresh emulsions and the decreased stability against flocculation plus coalescence.

Another interesting point is noteworthy that at c = 0.75% the emulsions were stable against creaming, irrespective of the extent of S−S cleavage, although increasing S−S cleavage led to a progressive and significant destabilization against coalescence (Table 1). This seems to be in contrasting with the general viewpoint that the coalescence of oil droplets facilitates the creaming of the emulsions. Herein, we provided evidence (Figure 8) to show that the increased creaming stability at higher c values was related to the enhanced extent of interdroplet interactions in the emulsions. The increased interdroplet interactions (especially electrostatic repulsion interaction) greatly decreased the mobility of the droplets, thus slowing the rate of creaming.

Overall, the importance of conformation flexibility to the emulsifying properties of BSA, as related to the applied c, can be well illustrated in Figure 9. In the c range from 0.1 to 0.75%, R_h and conformational flexibility of native BSA progressively decreases with increasing the c. For native BSA, the solution with a concentration of 0.5% (w/v) probably corresponds to a system in the vicinity of the concentration of interfacial saturation. Below this interfacial saturation, for example, 0.25%, a portion of the interfacial area cannot be fully covered by the protein, and in this case, all of the molecules can spread out at the interface at maximal extent of interfacial coverage. Above the saturation, for example, 0.75%, a multilayered structure can be formed at the interface, but the orientation and protein−protein association of adsorbed proteins at the interface seem to be greatly limited. From this viewpoint, we can reasonably hypothesize that the interfacial protein layer at c = 0.25% is much thinner but more viscoelastic in nature, whereas at c = 0.75%, the layer is much thicker and predominately elastic and fragile in nature. Upon increasing S−S cleavage, the tertiary conformation of proteins at c = 0.25% becomes more expanded and flexible, whereas that at c = 0.5 or 0.75% becomes more compacted and rigid. When these reduced molecules are adsorbed at the interface during emulsification, the structural unfolding and rearrangement of the reduced BSA molecules at c = 0.25% become easier as compared with native BSA at a comparable c. As a result, the formed interfacial protein layer is more viscoelastic in nature (than that for native BSA), thus greatly favoring the emulsion stability against coalescence and/or creaming. In contrast, the situation at c = 0.75% is the reverse, where the formed interfacial layer becomes more fragile in nature and, accordingly, the coalescence stability is greatly impaired. The situation at c = 0.5% seems to be marginal, because this concentration is in the vicinity of interfacial saturation. In this case, the BSA molecules in solution also become more compacted and rigid upon S−S cleavage. The conformational changes may lead to the amount of proteins not being sufficient to fully cover the interfacial area; for example, some gaps at the interface might occur (Figure 9) and, as a consequence, the emulsion stability against coalescence and bridging flocculation, and even creaming, decreases.

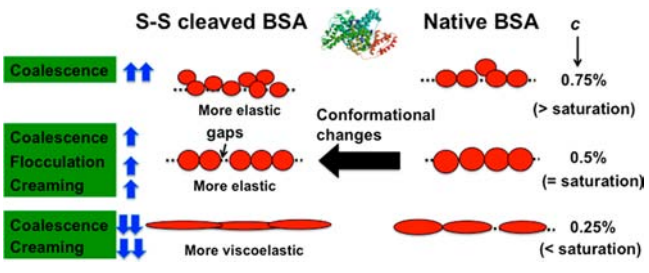

Figure 9. Schematic illustration for the c dependence of the influence of S−S cleavage on the conformational flexibility and emulsifying properties of BSA.

AUTHOR INFORMATION

Corresponding Author

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

Funding

This work is supported by the National Natural Science Foundation of China (Serial No. 30972049 and 31171632), the Fundamental Research Funds for the Central Universities (SCUT; Serial No. 2011ZG0019), and the Program for New Century Excellent Talents in University (NCET-10-0398).

Notes

The authors declare no competing financial interest.

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