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Adsorption and Dilatational Rheology of Heat-Treated Soy Protein at the Oil–Water Interface: Relationship to Structural Properties

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ABSTRACT: We evaluated the influence of heat treatment on interfacial properties (adsorption at the oil–water interface and dilatational rheology of interfacial layers) of soy protein isolate. The related structural properties of protein affecting these interfacial behaviors, including protein unfolding and aggregation, surface hydrophobicity, and the state of sulfhydryl group, were also investigated. The structural and interfacial properties of soy protein depended strongly on heating temperature (90 and 120 °C). Heat treatment at 90 °C induced an increase in surface hydrophobicity due to partial unfolding of protein, accompanied by the formation of aggregates linked by disulfide bond, and lower surface pressure at long-term adsorption and similar dynamic interfacial rheology were observed as compared to native protein. Contrastingly, heat treatment at 120 °C led to a higher surface activity of the protein and rapid development of intermolecular interactions in the adsorbed layer, as evidenced by a faster increase of surface pressure and dilatational modulus. The interfacial behaviors of this heated protein may be mainly associated with more flexible conformation and high free sulfhydryl group, even if some exposed hydrophobic groups are involved in the formation of aggregates. These results would be useful to better understand the structure dependence of protein interfacial behaviors and to expand utilization of heat-treated protein in the formulation and production of emulsions.

KEYWORDS: *soy protein, heat treatment, structural properties, oil–water interface, adsorption, surface dilatational rheology*

INTRODUCTION

Proteins are usually used as emulsifiers and foam stabilizers in the food industry because of their amphiphilic structure. During emulsification, proteins undergo diffusion from the bulk to the interface, actual molecular adsorption, change and reorganization of protein conformation, and formation of viscoelastic films around oil droplets to stabilize the droplets against flocculation and coalescence.[1,2] The interfacial properties of protein, such as the ability to lower interfacial tension and the establishment of interfacial architecture, have attracted considerable attention based on a possibility of providing useful insight into the formation and stabilization of foam and emulsion.[3] In fact, it is widely believed that these interfacial properties depend strongly on structural properties of protein (e.g., molecular flexibility, surface hydrophobicity, and state of sulfhydryl groups) and bulk conditions (e.g., ionic strength, pH, and protein concentrations).[4−6] Freer et al.[4] reported that, at the hexadecane–water interface, globular lysozyme exhibited less surface activity and high dilatational storage modulus than a disordered protein with a flexible structure such as β-casein.

The structural modification caused by additional treatments also affects interfacial properties of protein, especially rigid globular proteins. Heat treatment, a widespread unit operation in the food industry, has been extensively attempted to modify structural and functional properties of globular proteins, such as whey protein and β-lactoglobulin.[7] Moreover, heat-denatured protein or formed aggregates have been successively used for the stabilization of foams and emulsion.[8,9] To further understand the relationship between protein structure and some functional properties (e.g., foaming and emulsifying properties), the adsorption behaviors of heat-treated protein and interfacial rheology of adsorbed layers have received increasing attention recently.[10−15] These properties were strongly influenced by some factors such as the shape and size of aggregates, the charge screening, and the ratio of denatured native-like monomers.[14−16] For some proteins, for example, whey protein isolate,[5] α-lactalbumin,[11] and β-lactoglobulin,[12] heat treatment causes the enhancement of both surface activity and rheological viscoelasticity of absorbed layers at the air–water interface. However, ovalbumin heated at 80 °C exhibits lower shear elastic constant on aging than native protein even if protein molecules can establish rapid adsorption in the interfacial layer.[13] At the oil–water interface, long fibers of β-lactoglobulin induced by heat treatment at acidic condition show the highest interfacial shear modulus.[15] The increases in surface hydrophobicity and exposed sulfhydryl groups due to protein unfolding are considered as key parameters for heat-induced changes in protein adsorption and rheological viscoelasticity of absorbed layers.[11−14]

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Being surface active, soy proteins, including soy protein isolate (SPI), glycinin, and β-conglycinin, have functioned as foaming and emulsifying agents. In the presence of salt, heat-induced denaturation of SPI increases the emulsion stability against creaming due to the formation of rigid flocs.[10] More recently, heat treatment of SPI improves the freeze−thawing stability of oil-in-water emulsions.[17] Interfacial properties of soy protein with native structure at the air−water interface have been widely tested at varying protein concentrations, ionic strengths, and pH values.[6,18] Contrastingly, this topic at the oil−water interface has attracted much less interest, especially for heat-treated soy protein. Palazolo et al.[10] reported that heat treatment at 95 °C leads to an increase in interfacial pressure due to high dissociation degree of SPI and high surface hydrophobicity.

To date, more attention on the heat-induced changes in structural and functional properties of protein has been directed to heating temperature in the range below 100 °C. Recently, a higher temperature (above 100 °C) in the hydrothermal cooking process has been used to efficiently improve the solubility and emulsifying properties of heat- and alcohol-denatured soy proteins.[19,20] However, structural changes upon high temperature (>100 °C) and interfacial properties of heat-treated soy protein have not been characterized. The main objective of the present study was to evaluate the influence of heat treatment (90 and 120 °C) on structural and interfacial properties of SPI. The degree of protein unfolding and aggregation, surface hydrophobicity, and sulfhydryl group were investigated, and dynamic surface pressure and dilatational rheology of adsorbed layers at the oil−water interface were monitored using an automatic pendant drop tensiometer. Results were discussed to better understand the relationship between structural characteristics of protein and their interfacial properties.

MATERIALS AND METHODS

Materials. Defatted soy flour was provided by Shandong Yuwang Industrial and Commercial Co., Ltd. (Shandong, China). SPI was prepared from flour by alkaline extraction (pH 8.0) followed by precipitation at pH 4.5. The precipitate was redissolved in distilled water and then neutralized to pH 7.5 with 2 M NaOH. Subsequently, a protein solution was dialyzed against distilled water at 4 °C for 48 h and lyophilized. The protein content of SPI was 85.79 ± 0.70%, determined by Dumas method ($N \times 5.71$, wet basis) in a Rapid N Cube (Elementar France, Villeurbanne, France).

Bovine thyroid (669 kDa), rabbit muscle (158 kDa), and chicken egg white (75 kDa) were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). 1-Anilino-naphthalene-8-sulfonic acid (ANS) and 5,50-dithio-bis 2-nitrobenzoic acid (DTNB) reagents were purchased from Sigma-Aldrich (St. Louis, MO). Corn oil was obtained from a local supermarket and purified with Florisil (60−100 mesh, Sigma Aldrich) to remove surface-active impurities as described elsewhere,[21] since the surface tension of commercial corn oil with water decreased with time. All other reagents were of analytical grade.

Heat Treatment. The SPI dispersions (1%, w/v) were prepared by dissolving lyophilized SPI in 5 mM phosphate buffer (pH 7.0) and hydration for 12 h at 4 °C. Insoluble proteins were removed by centrifugation at 10000*g* for 20 min and filtration through 0.22 μm filters (Millipore, Fisher Scientific). Native SPI exhibited high protein solubility (97%). Protein solutions (2 mL) were placed in hermetic bottles (volume 4 mL) and then heated at different temperatures (90 and 120 °C). Heat treatments at 90 and 120 °C were performed in a water bath (TW12; Julabo, Seelbach, Germany) and in an autoclave (YX280D, Huatai Co. Ltd., Hefei, China), respectively. The sample temperature reached required values within 3 min. After they were held for 20 min at the required temperature, protein samples were immediately cooled in an ice bath for further analysis. To explore if heat treatment induces the formation of insoluble aggregates, heat-treated samples were centrifuged at 10000*g* for 20 min, and the protein concentration of supernatant was determined according to Lowry's method[22] using bovine serum albumin as the standard.

Small-Angle X-ray Scattering (SAXS). SAXS experiments were performed using a SAXSess camera (Anton-Paar, Graz, Austria), equipped with a PW3830 X-ray generator with a long fine focus sealed glass X-ray tube (PANalytical, Almelo, NL). The X-ray tube generator was operated at 40 kV and 50 mA. A focusing multilayer optics and a block collimator provide an intense monochromatic primary beam (Cu Kα, λ = 0.1542 nm), and a semitransparent beam stop enables measurement of attenuated primary beam at zero scattering vector (q = 0).

The samples (10 mg/mL) were filled into a vacuum-tight thin quartz capillary and placed in a TCS 120 temperature-controlled sample holder unit. The sample-to-detector distance was 261.2 mm, and the temperature was kept at 26.0 °C. The 2D scattered intensity distribution recorded by an imaging-plate detector was read out by a Cyclone storage phosphor system (Perkin-Elmer, Boston, MA). The 2D data were integrated into 1D scattering function $I(q)$ as a function of the magnitude of scattering vector q ($q = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle). Each measurement was collected for 30 min. All $I(q)$ data were normalized to have the uniform primary intensity at q = 0 for transmission calibration. The background scattering contributions from capillary and solvent were corrected, and desmearing was necessary because of the line collimation.

Dynamic Light Scattering. Protein samples were diluted to 1 mg/mL with 5 mM phosphate buffer (pH 7.0) filtered through a 0.22 μm filter. DLS analysis was carried out at a fixed angle of 173° using a Zetasizer Nano-ZS instrument (Malvern Instruments, Worcestershire, United Kingdom) at 25 °C. The appropriate viscosity and refractive index parameters for each solution were set. The apparent hydrodynamic radius (R_h) of protein samples was analyzed by means of "cumulants" method and Stokes−Einstein equation using Dispersion Technology Software (DTS) (V4.20).[23]

Size Exclusion Chromatography (SEC). SEC analysis was performed on a Waters Breeze system equipped with a 1525 pump and 2487 UV detector (Waters Corp., Milford, MA). Aliquots (10 μL) of native and heat-treated protein solution were filtered through 0.22 μm filters and injected into a prepacked TSK G4000SWxl column (TOSOH, Japan). The elution was conducted with 50 mM sodium phosphate containing 50 mM NaCl (pH 7.2) as the mobile phase. The flow rate was 0.7 mL/min, and the absorbance was monitored at 280 nm. Bovine thyroid (669 kDa), rabbit muscle (158 kDa), and chicken egg white (75 kDa) were used as standard proteins for calibration.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Aliquots (40 μL) protein solution were mixed with SDS sample buffer [40 μL, 150 mM Tris-HCl, 20% glycerol (v/v), 1% SDS (w/v), 8 M urea, and 2% 2-mercaptoethanol (2-ME, v/v), pH 6.8] and heated at 95 °C for 5 min. The protein samples for nonreducing SDS-PAGE were prepared by same process, just without the presence of 2-ME. SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli[24] using 12.5% separating gel and 5% stacking gel. The gel was stained using Coomassie brilliant blue (R-250) stain solution (45% methanol, 10% acetic acid, and 0.25% R-250) and destained in methanol−water solution containing 10% acetic acid (methanol/acetic acid/water = 1:1:8, v/v/v).

Free Sulfhydryl Group. The free sulfhydryl group (SH) contents of protein samples were determined by the method of Beveridge et al.[25] Protein samples were diluted to 2 mg/mL with Tris-Gly buffer (86 mM Tris, 90 mM glycine, and 40 mM EDTA, pH 8.0) containing 8 M urea. Aliquots (80 μL) of Ellman's reagent (DTNB in Tris-Gly buffer, 4 mg/mL) were added to 2.5 mL sample solution, and the absorbance was measured at 412 nm after 15 min. The calculation was as follows:

$$\mu\text{M SH/g} = 73.53 \times A_{412}/C \quad (1)$$

where A_{412} is the absorbance at 412 nm, C is the sample concentration (mg/mL), and 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 of solids to g of solids).

Surface Hydrophobicity. The protein surface hydrophobicity was determined by titration with ANS according to the method of Liu et al.,[26] with modifications as described below. The aliquots (1 mL) of protein solutions (0.2 mg/mL) were placed in the cell of an F7000 fluorescence spectrophotometer (Hitachi Co., Japan), and then, aliquots (10 μL) of ANS (5 mM in 5 mM phosphate buffer, pH 7.0) were titrated to reach a final concentration of 50 μM. The molar coefficient (5000 M^{-1} cm^{-1} at 350 nm) was used to calculate ANS concentration. The relative fluorescence intensity (F) was measured at 390 (excitation; slit width, 5 nm) and 470 nm (emission; slit width, 5 nm). Data were elaborated using the Lineweaver−Burk equation (eq 2):

$$1/F = 1/F_{max} + (K_d/L_0)(1/F_{max}) \quad (2)$$

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

Dynamic Surface Properties. The dynamic surface properties of protein at the corn oil−water interface were monitored by recording temporal evolution of surface pressure and surface dilatational parameters using an optical contact angle meter (OCA-20, Data-physics Instruments GmbH, Germany) equipped with oscillating drop accessory (ODG-20). The experiments were carried out at 25 °C. In view of protein concentration dependence of interfacial properties, protein solutions (0.01−1%, w/v) were placed in the syringe, and then, a drop of protein solution was delivered and allowed to stand for 3 h to achieve protein adsorption at the oil−water interface. Surface tension measurements were performed to check the absence of surface-active contaminants in the buffer solutions.

The surface tension (σ) was calculated according to fundamental Laplace equation. The surface pressure is $\pi = \sigma^0 - \sigma$, where σ^0 is the surface tension of distilled water. During the first step, at relatively low pressure when diffusion is the rate determining step, a modified form of the Ward and Tordai equation can be used to correlate the change in interfacial pressure with time defined by eq 3.[27]

$$\pi = 2C_0KT(D\theta/3.14)^{1/2} \quad (3)$$

where C_0 is the concentration in the bulk phase, K is the Boltzmann constant, T is the absolute temperature, D is the diffusion coefficient, and θ is adsorption time (s). If the diffusion of proteins at the interface controls the adsorption process, a plot of π against $\theta^{1/2}$ will then be linear, and the slope of this plot will be the diffusion rate (k_{diff}).

To obtain surface dilatational parameters, sinusoidal interfacial compression and expansion were performed by decreasing and increasing the drop volume at 10% of deformation amplitude ($\Delta A/A$) and 0.1 Hz of frequency. Details of this experiment are given elsewhere.[28] The surface dilatational modulus (E) derived from the change in interfacial tension (σ), resulting from a small change in surface area (A), can be described by eq 4.[29]

$$E = d\sigma/(dA/A) = -d\pi/d \ln A = E_d + iE_v \quad (4)$$

The dilatational modulus (E) is a complex quantity and is composed of real and imaginary parts ($E = E_d + iE_v$). The real part of dilatational modulus or storage component is dilatational elasticity (E_d). The imaginary part of dilatational modulus or loss component is surface dilatational viscosity (E_v).

Statistics. Unless specified otherwise, all tests were carried out in duplicate or triplicate. An analysis of variance (ANOVA) of the data was performed using the SPSS 13.0 statistical analysis system. A least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Structural Properties. Glycinin and β-conglycinin are the two main protein fractions of soy protein. Preliminary studies confirmed that glycinin subjected to heat treatment at 120 °C changed to insoluble precipitate. However, heat treatments at 90 and 120 °C did not significantly decrease the solubility of SPI. In previous studies, β-conglycinin in SPI has been confirmed to inhibit the formation of insoluble glycinin aggregates.[30] In view of the theme of this study, SPI were integrally considered to evaluate the effects of heat treatment on interfacial properties of soy protein. Understanding structural properties of protein continues to be an important focus to elucidate its interfacial behaviors at the oil−water interface. Therefore, we characterized the structural properties of native and heat-treated SPI, including protein unfolding, formation of aggregates, surface hydrophobicity, and sulfhydryl groups.

Protein Unfolding and Aggregation. Heat treatment above the denaturation temperature usually causes partial unfolding and subsequent aggregation of protein. The SAXS approach was performed to give information about shape, compactness, and size of native and heat-treated proteins. Figure 1 shows the

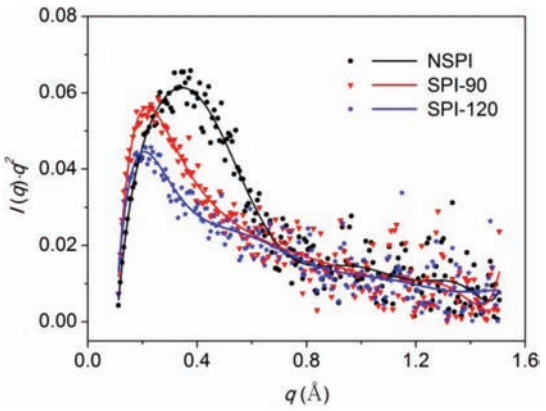

Figure 1. SAXS Kratky profiles of native and heat-treated SPI. NSPI, native SPI; SPI-90, SPI heated at 90 °C; and SPI-120, SPI heated at 120 °C.

Kratky plot derived from SAXS data, namely, $q^2 \cdot I(q)$ versus q, a qualitative dependence on molecular globularity and conformation.[31] It is well-known that the Kratky plot for native conformation has a maximum peak, whose magnitude depends on the compactness degree of protein, while unfolded polypeptide exhibits a plateau and then rises monotonically.[32] The Kratky plot of native SPI displayed the typical bell shape, with the presence of a characteristic maximum, confirming its spherical morphology (Figure 1). In the case of heat-treated SPI, the presence of maximum peaks with decreased magnitude not only suggests the maintenance of globular shapes but also confirms partially unfolded structures of rigid glycinin and β-conglycinin. The spherical-like shape of SPI heated at 90 and 120 °C was also directly observed by atomic force microscopy (AFM, data not shown). Moreover, SPI treated at 120 °C exhibited a lower magnitude of maximum peak than protein heated at 90 °C, indicating the formation of a more flexible conformation at a higher temperature (Figure 1).

The extrapolation of intensity to an angle of zero $I(0)$ and the gyration radius of protein R_g, the two most fundamental structural parameters, were calculated by Guinier approxima-

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tion derived from SAXS data, ln $I(q)$ versus q^2.[33] These structural parameters of native and heat-treated SPI are summarized in Table 1. $I(0)$ is proportional to the molecular weight of scattering species, while R_g represents the size or molecular compactness. As expected, $I(0)$ and R_g of heat-treated SPI were higher than those of native protein, further suggesting that protein unfolding and subsequent aggregation occurred between subunits of glycinin and β-conglycinin. It is noteworthy that a decrease in $I(0)$ and an increase in R_g were observed with increasing heating temperature from 90 to 120 °C. These results further reveal a more flexible conformation of protein upon heat treatment at a higher temperature (120 °C), consistent with the Kratky plot (Figure 1). To aid in clarifying these points, a DSL experiment was also carried out. Similar to the changes in R_g, heat treatment, especially at 120 °C, led to an increase of hydrodynamic radius R_h (Table 1). It should be pointed out that, in all cases, R_h was obviously larger than R_g, especially for native SPI. Generally, R_g/R_h = 0.775 represents uniform hard spheres. The different q range used in DLS and SAXS experiments could largely account for this discrepancy. The large aggregates mainly contribute to determine R_h at low q values during DLS experiment. At the q values seen by SAXS, this contribution was strongly reduced, and thus, R_g mostly came from the contributions of monomers. Similar observations were reported for β-lactoglobulin microgels using SAXS and DSL as testing methods.[34]

Table 1. Structural Parameters of Native and Heat-Treated SPI Derived from SAXS and DLS Data

samples	$I(0)^a$	R_g (nm)^a	R_h (nm)^b
NSPI	1.75 ± 0.02	5.70 ± 0.05	16.95 ± 0.16
SPI-90	2.89 ± 0.04	7.49 ± 0.04	17.60 ± 0.13
SPI-120	2.58 ± 0.04	7.91 ± 0.05	20.37 ± 0.06

[a]Guinier approximation of SAXS data; $I(0)$, the extrapolation of intensity to an angle of zero; R_g, the gyration radius of protein. [b]From DLS experiments; R_h, the hydrodynamic radius of protein.

SEC was conducted to better understand the aggregation state of proteins subjected to heat treatment at 90 and 120 °C, as shown in Figure 2. The elution profile of native SPI showed major eluting peaks appeared at retention times of 13.5 and 17.5 min, corresponding to glycinin and β-conglycinin, respectively. A low fraction of aggregates (8 min) also appeared in native SPI. In contrast, heat treatment at 90 °C caused the increases in eluting peaks at 8 and 10 min, with the decreases in integrated areas of typical peaks, especially at 13.5 min. These results indicate the partial transformation of native glycinin and β-conglycinin to soluble aggregates with high molecular weight (Figure 2). Seemly, heat-treated proteins were constituted of soluble aggregates and residual nonaggregated proteins. Heat treatment at 120 °C led to an increase in relative integrated areas of peak at 8 min, revealing an increase in the size of aggregates, consistent with SAXS and DLS data (Figure 1 and Table 1).

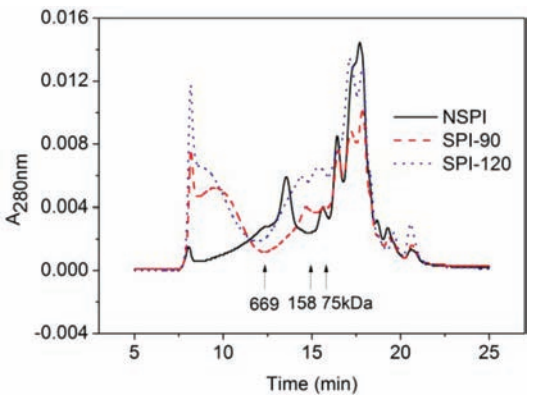

Figure 2. SEC profiles of native and heat-treated SPI (SPI-90 and SPI-120).

Surface Hydrophobicity. The surface hydrophobicity of protein has long been used to identify the structural changes and recognized as a related factor controlling surface activity of protein. Table 2 summarizes surface hydrophobicity parameters of native and heated SPI. The overall surface hydrophobicity index (PSH) of all heated SPI, defined as the number and affinity of hydrophobic sites, were significantly ($p < 0.05$) higher than that of native protein. These results are related to the transformation of more hydrophobic clusters in the interior of molecule to protein's surface due to partial unfolding, as demonstrated by SAXS and DSL data (Figure 1 and Table 1). Moreover, an increase in fluorescence intensity at saturating concentrations of ANS probe (F_{max}), with an increase of apparent dissociation constant (K_d), were observed for heat-treated SPI (Table 2). These phenomena suggest the generation of new hydrophobic binding sites on the molecular surface for ANS and the decrease of the binding affinity of ANS to protein, consistent with previous papers.[35] Interestingly, as compared with SPI treated at 90 °C, PSH, F_{max}, and K_d values significantly ($p < 0.05$) decreased with increasing temperature to 120 °C (Table 2), suggesting a decrease in surface hydrophobicity and an increase in accessibility of the binding sites for ANS. Hence, we could speculate that these hydrophobic sites in unfolded SPI upon heating at 120 °C could easily be involved in protein−protein interactions.

Sulfhydryl Group. As one of the factors affecting interfacial behaviors of protein, free SH contents of various samples were measured to gain insight into the effects of heat treatment on covalent interactions of protein, as presented in Table 2. Heat treatment at 90 °C resulted in a significant ($p < 0.05$) reduction in the free SH, indicative of the inaccessibility to DTNB, which may be attributed to the oxidation of SH groups into disulfide bond (SS) during heating and/or SH−SS−exchange reactions.[30] However, a significant ($p < 0.05$) increase in free SH contents was found when the heating temperature was increased to 120 °C, suggesting the cleavage of a disulfide bond and/or the inhibition of disulfide bond formation. It has been recognized that the SS linking acidic and basic polypeptides of glycinin are broken during heat denaturation.[36] Upon cooling from 120 °C, heat-shocked proteins may participate in severe aggregation caused by strong hydrophobic interactions, as demonstrated by the decrease of surface hydrophobicity (Table 2). Some free SH may be buried in the interior of these aggregates, leading to the reduction in the accessibility to oxygen and other SH groups.

SDS-PAGE profiles under nonreducing and reducing conditions are shown in Figure 3. Glycinin consists of an acidic subunit and a basic one linked by a single disulfide bridge. β-Conglycinin is composed of three subunits: α, α', and β subunits. Native SPI showed typical bands containing β-conglycinin and glycinin. Under nonreducing, heat-treated SPI exhibited the bands with large molecular weight at the top of stacking and separating gel, and concomitantly, the intensity of

Table 2. Protein Surface Hydrophobicity and SH Accessibility of Native and Heat-Treated SPI[a]

	surface hydrophobicity			
samples	F_{max}	K_d (μM)	PSH (F/mg μM)	free SH
NSPI	269.39 ± 2.25 c	21.46 ± 0.67 c	62.79 ± 1.45 c	4.38 ± 0.07 a
SPI-90	682.91 ± 3.03 a	30.98 ± 0.65 a	110.25 ± 2.51 a	2.18 ± 0.02 c
SPI-120	501.98 ± 6.68 b	28.38 ± 0.93 b	88.49 ± 2.10 b	3.56 ± 0.01 b

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

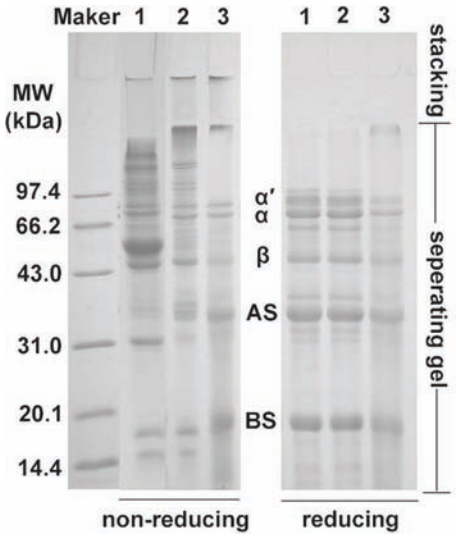

Figure 3. SDS-PAGE profiles of native and heat-treated SPI under nonreducing and reducing conditions. Lane 1, NSPI; lane 2, SPI-90; lane 3, SPI-120; α, α', and β, three subunits of β-conglycinin; AS, acidic subunit of glycinin; and BS, basic subunit of glycinin.

β-conglycinin and glycinin bands decreased (Figure 3, lanes 2 and 3). Moreover, the acidic and basic polypeptides of glycinin were released by thermal dissociation of the disulfide bridge.[36] As compared with SPI heat at 90 °C (lane 2), heat treatment at 120 °C caused an increase in the intensity of bands at the top of stacking gel (lane 3), accompanied by an increase in the intensity of acidic and basic subunits. These results support the viewpoint that a higher temperature (120 °C) inhibits the formation of aggregates linked by a disulfide bond, as demonstrated by increased free SH (Figure 1 and Table 2). In the presence of 2-ME, SPI aggregates formed at 90 °C were dissociated into subunits, suggesting the presence of intermolecular disulfide bonds due to the oxidation and/or SH–SS interchange reactions.[30] By contrast, upon heat treatment at 120 °C, a part of aggregates at the top of separating gel were still observed although 8 M urea was added to sample buffer. Different mechanisms of covalent cross-links in protein during heat treatment, such as cross-links caused by β-elimination,[37] may be responsible for this result, which are investigated elsewhere based on the focus in this study.

In summary, SPI was involved in different pathways of structural changes upon heating at 90 and 120 °C. Heat treatment at 90 °C led to partial unfolding of protein and an increase in surface hydrophobicity, accompanied by the involvement of disulfide bond in the formation of soluble aggregates. Comparatively, more flexible protein conformation and higher free SH were found with increasing the heating temperature to 120 °C. The soft flexibility structure of β-casein was believed to be related to rapid kinetics of adsorption at the hexadecane/water interface, and the developing interactions with other protein molecules in the adsorbed layers were significantly different.[4] On the basis of structural characters of heat-treated protein, protein unfolding may be helpful to improve adsorption and rearrangement of protein at the oil–water interface. More details in adsorption kinetic of protein at the interface and interfacial rheology of adsorbed layers are desired to better understand the structure dependence of protein interfacial behaviors.

Adsorption Rheokinetics. Generally, the adsorption kinetics of protein at the oil–water interface is described by different stages:[1] (i) Diffusion from the bulk to the proximity of the interface, (ii) actual molecular adsorption to the interface, and (iii) reorganization of protein at the interface. The adsorption process may involve an energy barrier generally related to several factors, such as protein conformational changes with more accessibility of hydrophobic fragments, protein reorientation to locate binding sites closer to the interface, and exploration of available adsorption sites at the interface.[3] The evolution of surface pressure (π) for native and heated SPI is shown in Figure 4. The π values gradually increased with adsorption time (θ), which can be associated with protein adsorption at the interface.

It appeared that strongly depending on protein concentration in the bulk phase, adsorption kinetics considerably varied among various samples. At low protein concentration (0.001 and 0.01%), the diffusion of protein controls the adsorption process, and the diffusion rate (k_{diff}) can be calculated from a linear plot of π against $\theta^{1/2}$ in the initial of π increase (Figure 4A,B).[27] The k_{diff} values increased with the elevation of protein concentration, revealing that protein diffusion is driven by the concentration gradient. SPI heated at 90 °C showed similar adsorption kinetics with slightly increased k_{diff} as compared to native protein, whereas heat treatment at 120 °C distinctly led to higher k_{diff} and a rapid increase of π value, especially at a protein concentration of 0.001% (Figure 4A), indicating a higher surface activity of protein. These observations may be associated with flexible conformation of heat-treated protein. It is noteworthy that heat-treated proteins consist of aggregates and residual nonaggregated proteins, as confirmed by SEC data (Figure 2). The residual proteins are generally assumed to rapidly reach the oil–water interface because they exhibit higher diffusion coefficients due to smaller size as compared to aggregates.[13,16] The adsorption of residual proteins could be considered to cause the increase of π value in the initial of adsorption. Upon heat treatment at 120 °C, a more flexible conformation of protein evidenced by SAXS experiment (Figure 1) may be speculated to be mainly responsible for high initial k_{diff} and π value at long-term adsorption (Figure 4A). The exposure of hydrophobic patches on the protein's surface, a result of protein unfolding, could reduce the energy barrier for adsorption (Table 2), leading to the improvement of adsorption efficiency.[13] Depending on the heating temperature

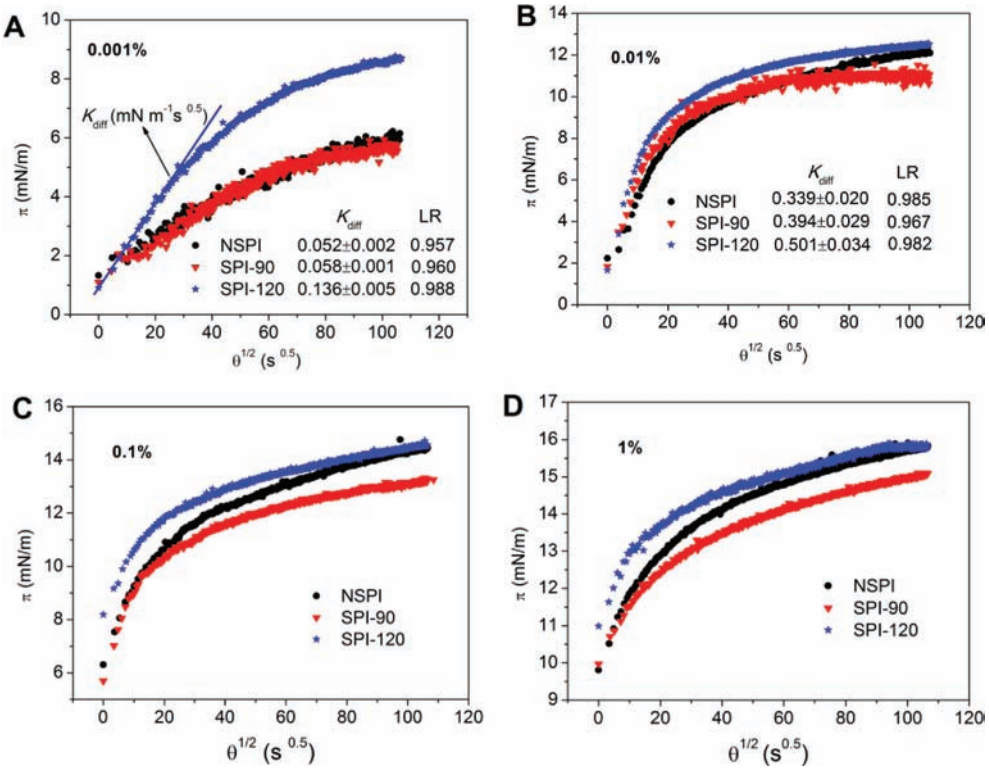

Figure 4. Square root of time ($\theta^{1/2}$) dependence of surface pressure (π) for native and heat-treated protein (SPI-90 and SPI-120) adsorbed layers at the oil–water interface. Protein concentrations in the bulk phase: (A) 0.001, (B) 0.01, (C) 0.1, and (D) 1%. The insets show the apparent rate of diffusion to the interface (k_{diff}) and linear regression coefficient (LR). The k_{diff} under high protein concentrations (0.1 and 1%) cannot be fit by the Ward and Tordai equation (eq 3) because of high π values as soon as data collection.

(90 and 120 °C), this fact may compensate the decreased diffusion coefficient of aggregates. Previous studies support these hypotheses by the observations of a faster increase in the surface pressure of heated proteins at the interface, as compared to native proteins.[5,13]

With increasing protein concentrations (0.1 and 1%) (Figure 4C,D), protein adsorption cannot be commonly diffusion-controlled process because of high π value as soon as data collection.[27] It is noteworthy that a higher π value at the beginning of adsorption was observed for protein heated at 120 °C (Figure 4C,D), further supporting the viewpoint of higher surface activity and rapid adsorption at the interface, as demonstrated by k_{diff} data (Figure 4A,B). Overall, with increasing adsorption time, protein heated at different temperatures displayed similar profiles of π increase except that higher π values were found for protein heated at 120 °C. As compared to native protein, a shorter time was needed to reach relative equilibrium adsorption for heat-treated proteins. These observations may be related to the adsorption behaviors of heat-induced aggregates at long adsorption times. In fact, the presence of steric hindrance derived from residual protein molecules could prevent some of the aggregated molecules from reaching the oil–water interface. Moreover, first adsorbed proteins at the oil–water interface produce a barrier to reduce the adsorption of large molecules, which are anchored into the interface only by a small part of their surface.[13] In brief, the differences in adsorption kinetics at the oil–water interface appeared to be attributed to different structural properties. A more flexible conformation of SPI heated at 120 °C may account for the enhancement of surface activity upon heat treatment.

Interfacial Dilatational Rheology. Interfacial rheology of adsorbed layers is considered as indicators of structural state of proteins adsorbed at the oil–water interface and macromolecule interactions.[38] The evolution of surface dilatational modulus (E) with a π value in the surface layer for the adsorption of native and heat-treated SPI is shown in Figure 5.

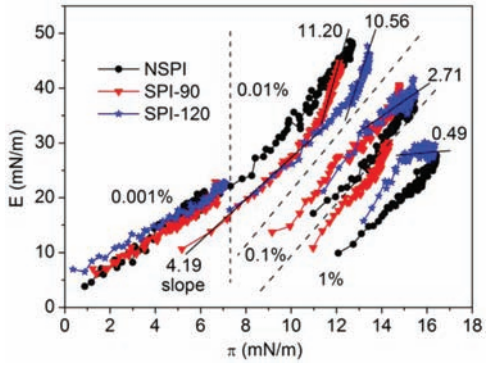

Figure 5. Surface dilatational modulus (E) as a function of surface pressure (π) for native and heat-treated SPI (SPI-90 and SPI-120) at the oil–water interface.

The curve of E versus π can give the information on the surface load of the protein and/or the degree of macromolecule interactions.

In all cases, the E increased almost immediately following protein adsorption at the interface (increase in π value), revealing the existence and development of interactions between adsorbed molecules, consistent with other globular

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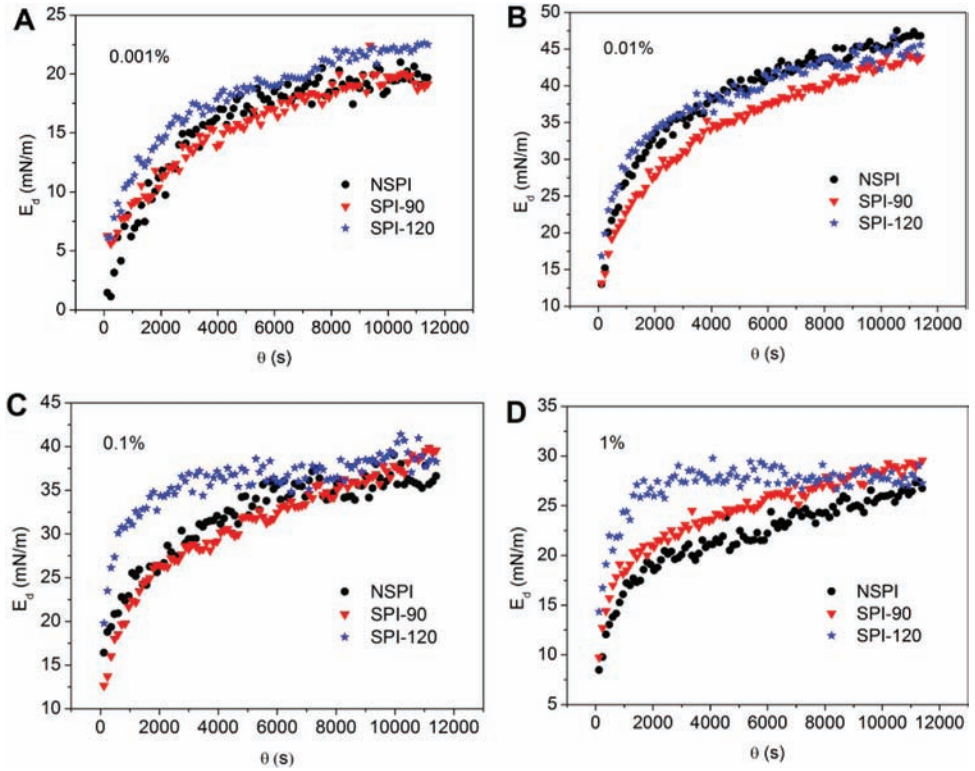

Figure 6. Time-dependent dilatational elasticity (E_d) for native and heat-treated SPI (SPI-90 and SPI-120) adsorbed layers at the oil−water interface. Protein concentrations in the bulk phase: (A) 0.001, (B) 0.01, (C) 0.1, and (D) 1%. Frequency, 0.1 Hz. Amplitude of compression/expansion cycle, 10%.

proteins.[5] Moreover, dilatational storage modulus (E_d) of all samples was evidently larger than loss modulus (E_v), suggesting a weakly dissipative viscoelastic system at the interface and primarily elastic in nature (data not shown).[16] Overall, native and heated SPI show a maximum in E value at the protein concentration of 0.01% and gradually decreased with increasing concentration to 1% (Figure 5). Similar observations have been reported previously for many other proteins.[28] This phenomenon may be explained by the presence of a compact packed layer after protein adsorption to the interface. The inaccessibility of more protein molecules to the interface prevents the actual adsorption of protein and the occurrence of intermolecular interactions.

Similar slopes derived from E versus π line were found for native and heat-treated protein at the protein concentration of 0.001% in the bulk phase (Figure 5). In all cases, the slopes significantly increased when the π value reached 9 mN/m (at protein concentrations of 0.01−1%), implying that higher protein amounts at the interface were required to establish intermolecular interactions. The curve of E versus π seemed to considerably vary among various samples at the protein concentrations of 0.01−1%, especially at high π value. At 0.01%, as compared to native protein, SPI heated at 90 °C showed an increase of slope from 4.19 to 11.20 with increasing π value as a great quantity of protein molecules had been adsorbed at the interface (above 11 mN/m). A similar change was observed for SPI heated at 120 °C when the π value reached 12 mN/m. These results indicate the enhancement of macromolecule interactions. Interestingly, at high protein concentration (0.1−1%), the slope of protein heated at 120 °C decreased at the later stage of adsorption (2.71 and 0.49 at protein concentrations of 0.1 and 1%, respectively). These observations may be mainly attributed to the rearrangement of adsorbed primary layer and/or multilayer formation at a surface pressure higher than the equilibrium surface pressure.[13] The increase of the protein amount adsorbed on emulsion droplets and the formation of multilayers have been reported for denatured β-lactoglobulin caused by heat treatment at 80 °C.[39]

The dynamic elastic modulus of interfacial layers during protein adsorption is presented in Figure 6. In all cases, the gradual increase in E_d with adsorption time should be attributed to protein adsorption and developing intermolecular contacts at the interface. Native protein and SPI heated at 90 °C showed similar E_d versus θ plot regardless of protein concentrations. In contrast, heat treatment at 120 °C led to a distinct curve of E_d versus θ, especially at high protein concentrations (0.1 and 1%). As compared with native sample, protein heated at 120 °C showed really higher E_d values as soon as proteins started to adsorb (few minutes), which could be associated with the fast diffusion of SPI heated at 120 °C to the interface (Figure 4). Moreover, a faster increase in E_d values was found for this heated protein at high protein concentrations (0.1−1%), and a plateau value was reached very rapidly (Figure 6C,D), revealing the rapid establishment of intermolecular contacts in the adsorbed layer. Attractive interactions (hydrophobic interactions and to a lesser extent disulfide bonds) may contribute to the development of interfacial rheology following the formation of an interfacial layer, which is one largely accepted mechanism.[40] A more flexible structure of protein caused by heating at 120 °C increased the accessibility of active sites of protein molecules upon adsorption, accounting for a faster increase in dilatational modulus (Figure 1). The actual molecular adsorption to the oil−water interface is believed to be accompanied by protein unfolding, and the consequent

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formation of intermolecular disulfide cross-links may be a possible reason for this because of the evidence for increased free SH of protein (Table 2).

In conclusion, depending on heat temperature, heat treatment significantly influenced interfacial behaviors of soy protein at the oil−water interface. As compared to native protein, heat-treated protein exhibited a higher surface activity due to protein unfolding and the increase in surface hydrophobicity, especially SPI heated at 120 °C. Moreover, heat treatment at 120 °C led to rapid formation of viscoelastic protein films at the oil−water interface. Taking the results of structural properties together, the flexibility and surface properties of heated SPI are probably two important parameters to explain the difference in protein adsorption and interfacial modulus. The interfacial properties of heat-treated protein could contribute to a better understanding of complex mechanisms involved during emulsion formation and stabilization.

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Notes

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