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Effects of Ultrasound Pretreatment on the Enzymatic Hydrolysis of Soy Protein Isolates and on the Emulsifying Properties of Hydrolysates

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ABSTRACT: Soy protein isolate (SPI) was modified by ultrasound pretreatment (200 W, 400 W, 600 W) and controlled papain hydrolysis, and the emulsifying properties of SPIH (SPI hydrolysates) and USPIH (ultrasound pretreated SPIH) were investigated. Analysis of mean droplet sizes and creaming indices of emulsions formed by SPIH and USPIH showed that some USPIH had markedly improved emulsifying capability and emulsion stabilization against creaming during quiescent storage. Compared with control SPI and SPIH-0.58% degree of hydrolysis (DH), USPIH-400W-1.25% (USPIH pretreated under 400W sonication and hydrolyzed to 1.25% DH) was capable of forming a stable fine emulsion (d_{43} = 1.79 μm) at a lower concentration (3.0% w/v). A variety of physicochemical and interfacial properties of USPIH-400W products have been investigated in relation to DH and emulsifying properties. SDS−PAGE showed that ultrasound pretreatment could significantly improve the accessibility of some subunits (α-7S and A-11S) in soy proteins to papain hydrolysis, resulting in changes in DH, protein solubility (PS), surface hydrophobicity (H_0), and secondary structure for USPIH-400W. Compared with control SPI and SPIH-0.58%, USPIH-400W-1.25% had a higher protein adsorption fraction (F_{ads}) and a lower saturation surface load (Γ_{sat}), which is mainly due to its higher PS and random coil content, and may explain its markedly improved emulsifying capability. This study demonstrated that combined ultrasound pretreatment and controlled enzymatic hydrolysis could be an effective method for the functionality modification of globular proteins.

KEYWORDS: Soy protein isolates, enzymatic hydrolysis, ultrasound pretreatment, emulsifying properties, hydrolysates

INTRODUCTION

The current popularity of soy proteins, due to their low cost and high nutritional value, continues to drive soy research and commercial development of new food products. Soy protein isolate (SPI) is the most refined soy protein product, containing 90% protein on a moisture-free basis, and possesses some desirable functionalities. One of the most important functional uses of SPI is as an emulsifier or emulsion stabilizer, ranging from meat emulsions and baby foods to liquid diets. However, soy proteins as emulsifiers are generally recognized to be less efficient when compared with other food proteins, such as casein.[1] This may be mainly due to the complexity of the tertiary and quaternary structures of the two major components, the 7S and 11S globulins, which account for ca. 70% of the total soy proteins.[2] In addition, commercial SPI undergoes harsh processing conditions including spray drying and high temperature sterilization, which can cause total denaturation and aggregation of protein molecules, and may lose much of its inherent functionalities.[3,4] The improvement of the emulsifying properties of SPI will facilitate its use in the production of emulsion-based foods with desired functionalities and will therefore increase demand.

Two fundamental processes occur during emulsification: droplet breakup and droplet recoalescence (or flocculation). The final droplet size of a newly formed emulsion system therefore depends on (i) the initial generation of droplets of small size and (ii) the rapid stabilization of these droplets against flocculation and recoalescence once they are formed.[5] Surfactants affect both processes: they reduce the interfacial tension, thereby promoting droplet breakup, and they form a physical barrier at the oil−water interface, protecting the newly formed droplets against flocculation or recoalescence.[6] Emulsifying capability therefore refers to the ability of an emulsifier to form and stabilize small droplets during homogenization.[7] Proteins are commonly used as emulsifiers in most food emulsions,[8] and the emulsifying capability of proteins depends on their molecular and physicochemical characteristics. Solubility, surface hydrophobicity, and molecular flexibility are seen to be the major factors determining the emulsifying capability of proteins.[8,9] Once emulsions are formed, they are subjected to several forms of instability, including creaming, flocculation, coalescence, and Ostwald ripening. The creaming profiles often provide indirect information about the extent of droplet aggregation (flocculation and coalescence) in an emulsion: the faster the creaming, the larger the droplet (or droplet flocs).[7]

Modifications of soy proteins by enzymatic hydrolysis for improved functionalities are a well accepted and safe method.[10] Hydrolysis of proteins causes changes such as an increase in protein solubility, a decrease in molecular size, and exposure of hydrophobic groups, factors that influence emulsifying capability and emulsion-stabilizing ability of protein hydrolysates.[11] Many studies have shown that effects of enzymatic hydrolysis on protein functionalities are mainly dependent on the substrate

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characteristics, enzyme specificity, and DH.[12−14] DH is defined as the percentage of peptide bonds cleaved per gram of protein compared with the total number of peptide bonds per gram of protein.[15] We should note that DH is a key parameter that needs to be controlled to obtain reproducible results and to avoid excessive protein hydrolysis that can impair functionalities and/or cause bitter flavours.[15,16] Controlled enzymatic hydrolysis therefore means that the hydrolysis modification was regulated with a combination of enzyme/substrate (E/S) ratios, time, temperature, etc. for a desirable DH value. Papain is a vegetable-derived enzyme and has maximum activity around neutral pH.[15] Because of the broad specificity to peptide bond cleavage, papain is commonly used for protein hydrolysis to improve their functionalities.

However, previous studies have found that soy proteins are generally resistant to enzymatic hydrolysis due to their compact quaternary and tertiary structures that protect many of the peptide bonds.[17,18] Therefore, pretreatment is a necessary step to alter the structural characteristics of soy proteins, increasing their accessibility to enzymatic hydrolysis and obtaining enhanced functionalities.[19,20]

High intensity ultrasound (10−100 W/cm^2, with the frequency ranging from 20 to 100 kHz) is a cost-effective and quick technology that has been used to modify the structural and functional properties of globular proteins.[21−23] The beneficial use of ultrasound is achieved through the chemical, mechanical, and physical effects of acoustic cavitation. This involves the formation, growth, and violent collapse of small bubbles in liquids as a result of acoustic pressure fluctuations.[24] Recently, several papers have reported on the use of ultrasound pretreatment to increase the enzymatic hydrolysis of proteins.[25,26] It has been suggested that high intensity ultrasound treatment could modify protein conformation by affecting hydrogen bonds and hydrophobic interactions, disrupting the quaternary and/or tertiary structure of globular proteins, and more hydrolysis sites were then exposed and could be accessible by protease.[23,25,26] Therefore, a combination of ultrasound pretreatment and controlled enzymatic hydrolysis might be a promising way for the functionality modification of globular proteins. However, limited information is available concerning the effects of ultrasound pretreatment on the enzymatic hydrolysis pattern of SPI or on the emulsifying properties of its hydrolysates.

Hence, the objective of this work was to study the effects of combined ultrasound pretreatment and controlled enzymatic hydrolysis using papain on the emulsifying properties of SPI. It was also hoped that by measuring some physicochemical properties and interfacial properties, the underpinning mechanisms of improved emulsifying properties for SPIH and USPIH can be better understood.

MATERIALS AND METHODS

Materials and Chemicals. Spray-dried SPI (93.2% ± 0.7 (w/w) dry protein, 5.13% ± 0.18 (w/w) moisture, 1.06% ± 0.07 (w/w) fat, and ash by difference) was obtained from Wonderful Industrial Group (Yantai, China). Papain (EC 3.4.22.2), specified as 2000 gelatin digestion units (GDU)/g protein, was purchased from Baiao Biochemistry Co. (Jiangmen, China). Sunflower oil was purchased from Quest International (Swindon, UK). 1-Anilino-8-naphthalenesulfonate (ANS), sodium azide, and Nile Red were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals used in the present study were of analytical grade. Water was purified using a Milli-Q apparatus (Millipore, Bedford, UK) and was used to prepare all the aqueous solutions.

Ultrasound Pretreatment of SPI. SPI dispersions (5.0%, w/v) were prepared by adding SPI powder into Millipore water and then gently stirred overnight at ambient temperature. An ultrasound processor (XingDongLi Ultrasonic Electron Equipment Co. Ltd., Guangzhou, China) with a 1.5 cm diameter titanium probe was used to sonicate 100 mL of SPI dispersions in 150 mL flat bottom conical flasks that were immersed in a temperature-controlled (2 °C) water bath (Lauda RM6, Berlin, Germany). Samples were treated at 25 kHz at different levels of power output (0 W, 200 W, 400 W, and 600 W) for 15 min (pulse durations of on-time 9 s and off-time 1 s). The ultrasonic intensity of the generated ultrasonic wave was determined calorimetrically by measuring the temperature rise of the sonicated dispersion under adiabatic conditions at room temperature. The ultrasonic intensity measured in this experiment was 19 ± 2 W/cm^2, 31 ± 4 W/cm^2, and 42 ± 3 W/cm^2 for 200 W, 400 W, and 600 W, respectively. After ultrasound treatment, the protein dispersions were adjusted to pH 7.0 with 1 M NaOH. The subsequent enzymatic hydrolysis was carried out immediately after ultrasound pretreatment.

Preparations of SPIH and USPIH with Various DH Values. DH were controlled and determined using the pH-stat method, which determines the percent DH on the basis of the number of free titratable amino groups produced by the hydrolysis of peptide bonds.[15] DH was calculated as eq 1:

$$DH(\%) = \frac{B \times N_b}{\alpha \times M_p \times h_{tot}} \times 100\% \quad (1)$$

where B is the base consumption in mL, N_b is the normality of the base, α is the degree of dissociation of the α-amino groups, M_p is the mass of protein being hydrolyzed (g), and h_{tot} is the total number of peptide bonds in the protein substrate (meqv/g protein). For SPI, h_{tot} is 7.75 (meqv/g protein), and α from the literature is taken as 0.44 for SPI at pH 7.0.[15]

Hydrolysates with various DH values were prepared from 5.0% (w/v) SPI and USPI dispersions (200 W, 400 W, and 600 W) by hydrolysis at pH 7.0 and 55 °C using papain. The hydrolysis pH was controlled using a TIM840 autotitrator (Radiometer Analytical co., Villeurbanne, France). The E/S ratios varied from 0.05−0.5% (w/w), and the molarity of the NaOH solution used to maintain the pH varied from 0.1 to 2.0 M to minimize dilution effects. In preliminary experiments, DH as a function of the E/S ratio and hydrolysis time was investigated for both SPI and USPI-200−600W, and on the basis of the preliminary experiments, the hydrolysis time was set at 180 min so that the hydrolysis for each enzyme/substrate combination can reach a DH plateau. After hydrolysis, the papain was inactivated by placing in boiling water for 15 min, which results in a slight decrease in pH. NaOH (0.1 M) was used to readjust the hydrolysate dispersions to pH 7.0. The control SPI and USPI dispersions were prepared using the same incubation conditions and heat inactivation treatment, but without papain added and also required minimal NaOH addition. The hydrolysates were lyophilized and then preserved at −18 °C before use. Sample codes are subsequently composed of the name of the protein source, the power output of the ultrasound pretreatment, and the DH reached. For example, USPIH-400W-1.25% means the soy protein isolate hydrolysate was pretreated under 400 W sonication and hydrolyzed to a DH value of 1.25%.

Preparations of Emulsions. Emulsifier solutions were prepared by dispersing the desired amount (0.2−10.0%, w/v) of protein powder into Millipore water and then gently stirring overnight at ambient temperature. The pH of the resulting emulsifier solutions was adjusted to pH 7.0 with 1 M NaOH or 1 M HCl. Subsequently quoted pH values and emulsifier concentrations referred to those of the aqueous phase

before emulsification. Sodium azide (0.02%, w/v) was added to the dispersions as an antimicrobiological agent. Oil-in-water emulsions containing 20% (v/v) sunflower seed oil and 80% (v/v) emulsifier solutions were prepared at ambient temperature using a custom built single-pass laboratory-scale jet homogenizer operating at the starting pressure of approximately 300 bar.[27]

Determination of Droplet Size Distribution and Mean Droplet Size. Droplet size distributions of emulsion samples were determined by static multiangle light scattering using Mastersizer 2000 (Malvern Instruments Ltd., Worcestershire, UK). Refractive indices of sunflower seed oil and water were taken as 1.462 and 1.330, respectively; absorption index: 0.001. The mean droplet size was characterized in terms of the surface area mean diameter d_{32} and volume mean diameter d_{43}. The d_{32} value was used to estimate the specific surface area of freshly made emulsions, and the d_{43} value was used to monitor changes in droplet size.

Visual Assessment of Creaming. Creaming stability of emulsions was determined according to the method of Moschakis et al.,[28] with minor modifications. Emulsion samples were poured into 5 mL flat bottomed glass tubes (height 75 mm, diameter 9 mm) immediately after preparation. Subsequently, the tubes were capped with a plastic top to prevent evaporation. The tubes were then inverted and left inverted. The emulsion samples were stored quiescently at laboratory temperature in a cabinet, and the movement of any creaming boundaries was followed with time. The extent of creaming was characterized by the creaming index (CI, %): $CI = (H_S/H_E) \times 100\%$, where H_S is the height of serum layer, and H_E is the total height of the emulsion.

Confocal Laser Scanning Microscopy (CLSM) Observations. A Leica TCS SP2 confocal laser scanning microscope (Leica, Heidelberg, Germany) was used to visualize the microstructure of emulsion samples. The CLSM was operated in fluorescence mode with a 63× water-immersion objective of numerical aperture 1.20. Emulsion oil phase was stained with Nile Red dye (20 μL of 0.01% w/v dye in polyethylene glycol added to 5 mL emulsion). The sample was excited with the 488 nm Argon laser line. The stained emulsions were immediately placed into a laboratory-made welled slide, filling it completely. The well was 8 mm in diameter and 1.6 mm in depth (volume ~80 μL). Microimages were recorded at 24 °C at a resolution of 1024 × 1024 pixels and processed to determine the scale bars (Leica Qwin software). As Nile Red stains the oil phase, individual large oil droplets and regions rich in emulsion droplets appear as bright patches, whereas the aqueous (water/protein) phase appears dark in the microimages. The position of the confocal plane was held constant throughout the whole sequence of image recording. Eight scans were averaged to create each image.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was done to determine the effects of combined ultrasound pretreatment and enzymatic hydrolysis using papain on the protein polypeptide profiles. SDS−PAGE was done using a discontinuous SDS-Tris-glycine buffer system with 4% stacking gels and 15% resolving gels according to the method of Laemmli.[29] The protein samples were dissolved in sample buffer (0.0625 M Tris-HCl buffer (pH 6.8), containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol, and 0.01% (w/v) bromophenol blue) to a concentration of 10 mg/mL. These samples were heated in boiling water for 5 min and then were centrifuged at 10,000*g* for 10 min at 20 °C in a CR22G centrifuge (Hitachi Co., Tokyo, Japan). A wide-range molecular weight marker (M8445, Sigma Chemical Co.) ranging from 6.5 to 200 KDa was used as the standard. Then 10 μL of supernatant and 5 μL of marker (10 mg/mL) were applied to the lanes. All gels were run in a Mini-protean III system (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the gels were dyed in 0.25% Coomassie blue in 50% trichloroacetic acid and then destained in methanol/acetic acid/water (1:1:8, v/v/v) overnight.

Protein Solubility (PS) Measurements. PS was determined according to the method of Petruccelli et al.,[30] with minor modifications. Sample dispersions (1.0%, w/v) were gently stirred overnight at ambient temperature and then were centrifuged at 12,000*g* for 20 min at 20 °C to obtain the supernatants. Protein content of the supernatants was measured by the micro-Kjeldahl method (N × 6.25). Protein solubility was calculated as nitrogen solubility index (NSI, %) = (protein content of supernatant/amount of protein added) × 100%.

Surface Hydrophobicity (H_0) Measurements. H_0 was determined using 1-anilino-8-naphthalenesulfonate (ANS) as a fluorescence probe according to the method of Haskard et al.,[31] in the absence of SDS. Samples were dispersed into buffer solution (0.01 M sodium phosphate buffer, pH 7.0) to reach different concentrations (0.004−0.02%, w/v), followed by stirring overnight at ambient temperature. After centrifugation (12,000*g*, 20 min, 20 °C), the supernatants were collected. Then aliquots of ANS solution (20 mL, 8 mM in the same phosphate buffer) were added to 4 mL of sample. Fluorescence intensity was measured using a RF-5301 PC spectro-fluorometer (Shimadzu Corp., Kyoto, Japan) at an excitation wavelength of 390 nm and an emission wavelength of 470 nm. The initial slope of fluorescence intensity vs protein concentration (mg/mL) was calculated by linear regression analysis and used as the index of H_0.

Analysis of Circular Dichroism (CD) Spectrum. A MOS-450 circular dichroism spectrometer equipped with a Peltier element (Bio-Logic Science Instruments, Grenoble, France) was used for CD analysis. The far-UV CD spectroscopic measurements were done in a 2 mm path length quartz cuvette with a sample concentration of 0.1 mg/mL. All of the protein samples were centrifuged at 12,000*g* for 20 min at 20 °C, prior to the analysis of the supernatant. The samples were scanned from 190 to 250 nm, with a scan rate of 50 nm/min. A mean value of 112 for amino acid residue was assumed in all calculations, and CD measurements were expressed as mean residue ellipticity (θ, deg·cm^2/dmol). The secondary structure compositions of the samples were calculated from the mean residue ellipticity using CDPro software (Bio-Logic Science Instruments) with the CONTIN/LL program assuming small peptide structure parameters are the same as those for proteins.

Interfacial Tension Measurements. An OCA20 optical contact angle meter (DataPhysics, GmbH, Berlin, Germany) was used for interfacial tension measurement on the basis of the axisymmetric pendant drop shape analysis. Samples were dispersed into buffer solution (0.01 M sodium phosphate buffer, pH 7.0) to reach different concentrations (0.001−5.0%, w/v), followed by stirring overnight at ambient temperature. After centrifugation (12,000*g*, 20 min, 20 °C), the supernatants were filtered using a 0.2 μm membrane (PVDF) to eliminate the insoluble material. The densities of supernatants and sunflower seed oil were determined using a DMA 35N density meter (AntonPaar Instruments, Graz, Austria). An aqueous drop of 10 μL using the supernatants was added to a cuvette containing sunflower seed oil. The instrument recording started immediately after drop formation. The interfacial tension was studied at ambient temperature for up to 180 min, and the data was calculated using the SCA 20 software (DataPhysics, GmbH). The interfacial pressure (π) was calculated from $\pi = \gamma_0 - \gamma$, where γ_0 is the interfacial tension at the sunflower seed oil−pure buffer solution interface ($\gamma_0 = 30.3 \pm 0.5$ mN/m), and γ is the interfacial tension in the presence of the emulsifier. The saturation interfacial pressure (π_{sat}) was determined by averaging the measured interfacial pressure values over the range of emulsifier concentrations once π remained relatively constant as the emulsifier concentration increased indicating saturation.

Determination of Protein Adsorption Fraction (F_{ads}) and Surface Load (Γ_{sat}). The amount of nonadsorbed protein in the aqueous phase of the emulsions was determined according to the method described by Paton et al.,[32] with some modifications. Emulsions were centrifuged at 12,000*g* for 2 h at 20 °C to separate the oil droplets

from the serum phase (including precipitates). The oil phase was carefully removed using a syringe. The amount of nonadsorbed protein remaining in the serum phase and precipitates was determined by the micro-Kjeldahl method (N × 6.25). The surface load (Γ_{sat}, mg/m^2) was calculated using eq 2:[33]

$$\Gamma_{sat} = \frac{(C_i - C_{eq}) \times d_{32}}{6\emptyset} \quad (2)$$

where C_i is the initial protein concentration per unit volume of emulsion (kg/m^3), and C_{eq} is the nonadsorbed protein concentration per unit volume of emulsion (kg/m^3). In the current study, d_{32} of the emulsion was determined by static light scattering (Mastersizer); Ø is the oil volume fraction (= 0.2).

The protein adsorption fraction (F_{ads}, %) referring to the fraction of protein adsorbed onto the droplets during homogenization was calculated using eq 3:

$$F_{ads} = \frac{C_i - C_{eq}}{C_i} \times 100\% \quad (3)$$

where C_i and C_{eq} are the same as those in eq 2.

Statistical Analysis. Unless otherwise stated, all the tests were conducted in triplicate, and the results are given as the means ± standard deviations. The results obtained were subjected to one-way analysis of variance (ANOVA). Duncan's new multiple range test was performed to determine the significant difference between samples within the 95% confidence interval using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Effects of Ultrasound Power and E/S Ratio on DH and Emulsifying Properties of SPIH and USPIH-200−600W. The effects of ultrasound power and E/S ratio on DH and emulsifying properties of SPIH and USPIH-200−600W are shown in Table 1. DH values of both SPIH and USPIH increased with increasing E/S ratios. However, it should be noted that an enhancement of hydrolysis was observed for USPI-200−600W when compared with that of SPI. Because when the same E/S ratios were introduced, USPIH-200−600W had higher DH values than SPIH. In addition, the power level of the ultrasound pretreatment significantly influenced the DH of USPIH. Significantly higher ($P < 0.05$) DH values were found for USPIH-400W and USPIH-600W samples compared to that for USPIH-200W. In general, these results show that ultrasound pretreatment enhanced the hydrolysis of SPI.

The emulsifying properties of SPIH and USPIH were investigated using 20% (v/v) sunflower seed oil-in-water emulsions at pH 7.0 with an emulsifier concentration of 1.6% (w/v) based on preliminary experiments. Because the control SPI had a relatively large droplet size ($d_{43} \approx 30.95$ μm), there was scope for substantial improvement of emulsifying capability. As can be seen in Table 1, USPI-200−600W emulsions showed large d_{43}, ranging from 19.61 to 24.69 μm, although significantly smaller than that of the control SPI emulsion, indicating that the ultrasound pretreatment used gave limited improvement to the emulsifying capability of SPI. With the increase of DH value, the changes in d_{43} for emulsions formed by SPIH and USPIH-200−600W were similar, decreasing initially and then increasing at high DH values. Hydrolysed SPI showed limited improvement, with a minimum d_{43} of 17.21 μm (SPIH-0.58%). In contrast, some USPIH samples showed much greater improvements. It should be noted that the power level of the ultrasound pretreatment significantly influenced the emulsifying capability of USPIH-200−600W. USPIH-400W-1.25% showed the best emulsifying capability, in terms of its significantly smallest d_{43} (7.16 μm). These results suggest that ultrasound pretreatment and papain hydrolysis had a marked effect on improving the emulsifying capability of SPI.

Table 1. Effects of E/S Ratio and Ultrasound Power on the DH, Emulsifying Properties (d_{43}), and Emulsion Creaming Stability (Ci) of SPIH and USPIH-200−600W[a]

papain, E/S (%)	ultrasound power (W)	DH (%)	d_{43} (μm)	CI (%)
0	0		30.95 (±0.76) f	33.92 (±0.95) f
	200		24.69 (±1.27) g	25.35 (±1.16) h
	400		19.61 (±0.28) i	21.24 (±0.45) ijk
	600		21.83 (±0.55) h	21.73 (±0.66) ij
0.05	0	0.42 (±0.02) l	17.86 (±0.4) ij	23.34 (±1.04) hi
	200	0.54 (±0.03) k	15.94 (±0.63) jkl	18.26 (±2.17) klm
	400	0.81 (±0.01) i	13.40 (±1.03) mn	15.21 (±0.68) m
	600	0.87 (±0.02) h	14.75 (±0.41) lm	15.48 (±1.36) lm
0.1	0	0.58 (±0.02) k	17.21 (±0.32) jk	18.76 (±1.74) jkl
	200	0.71 (±0.04) j	16.27 (±0.29) jkl	15.23 (±0.76) m
	400	0.99 (±0.02) g	9.62 (±0.11) p	0
	600	1.05 (±0.03) f	11.14 (±0.09) op	8.72 (±0.52) n
0.2	0	0.73 (±0.01) j	33.64 (±1.78) e	39.61 (±3.56) de
	200	0.82 (±0.02) i	25.67 (±1.23) g	37.5 (±2.13) e
	400	1.25 (±0.02) d	7.16 (±0.06) q	0
	600	1.31 (±0.03) c	13.28 (±0.30) mn	15.46 (±0.35) lm
0.3	0	0.87 (±0.03) h	43.85 (±2.65) c	45.83 (±2.61) b
	200	0.96 (±0.02) g	40.87 (±1.86) d	44.17 (±3.15) bc
	400	1.43 (±0.02) b	12.54 (±0.1) no	10.07 (±0.49) n
	600	1.45 (±0.03) b	15.63 (±0.22) kl	20.85 (±1.63) ijk
0.5	0	0.98 (±0.02) g	66.02 (±1.91) a	54.17 (±1.86) a
	200	1.19 (±0.05) e	52.67 (±2.74) b	53.28 (±3.42) a
	400	1.82 (±0.03) a	26.11 (±0.35) g	29.83 (±2.74) g
	600	1.86 (±0.04) a	31.44 (±0.91) f	41.27 (±1.34) cd

[a] Results within a column with the same letters are not significantly different ($p < 0.05$).

In terms of creaming stability, Table 1 shows that creaming was not observed in the emulsions formed by USPIH-400W-0.99% and USPIH-400W-1.25% after 5 days of quiescent storage, whereas it was significant for the other emulsions. We can see that there was a positive correlation between d_{43} values and CI for SPIH and USPIH emulsions: the bigger the d_{43}, the larger the CI.

Since some USPIH-400W exhibited better emulsifying capability and emulsion stabilization against creaming, this system was chosen for further investigations on the changes in physicochemical properties and interfacial properties in relation to its emulsifying properties.

Microstructure Observation of Fresh SPIH and USPIH-400W Emulsions. Microstructures of some SPIH and USPIH-400W emulsions were obtained using CLSM. Droplet size distributions determined by light scattering (Mastersizer) were superimposed on the microimages to better characterize the emulsion microstructure. Figure 1A−C show the CLSM images and droplet size distributions of emulsions formed by SPIH with different DH values. As shown in Figure 1A, the emulsion formed by control SPI appeared to be highly flocculated. The reason for this instability might be that there was insufficient emulsifier present at the newly created oil−water interface for full coverage. Therefore, bridging flocculation of droplets, which meant that

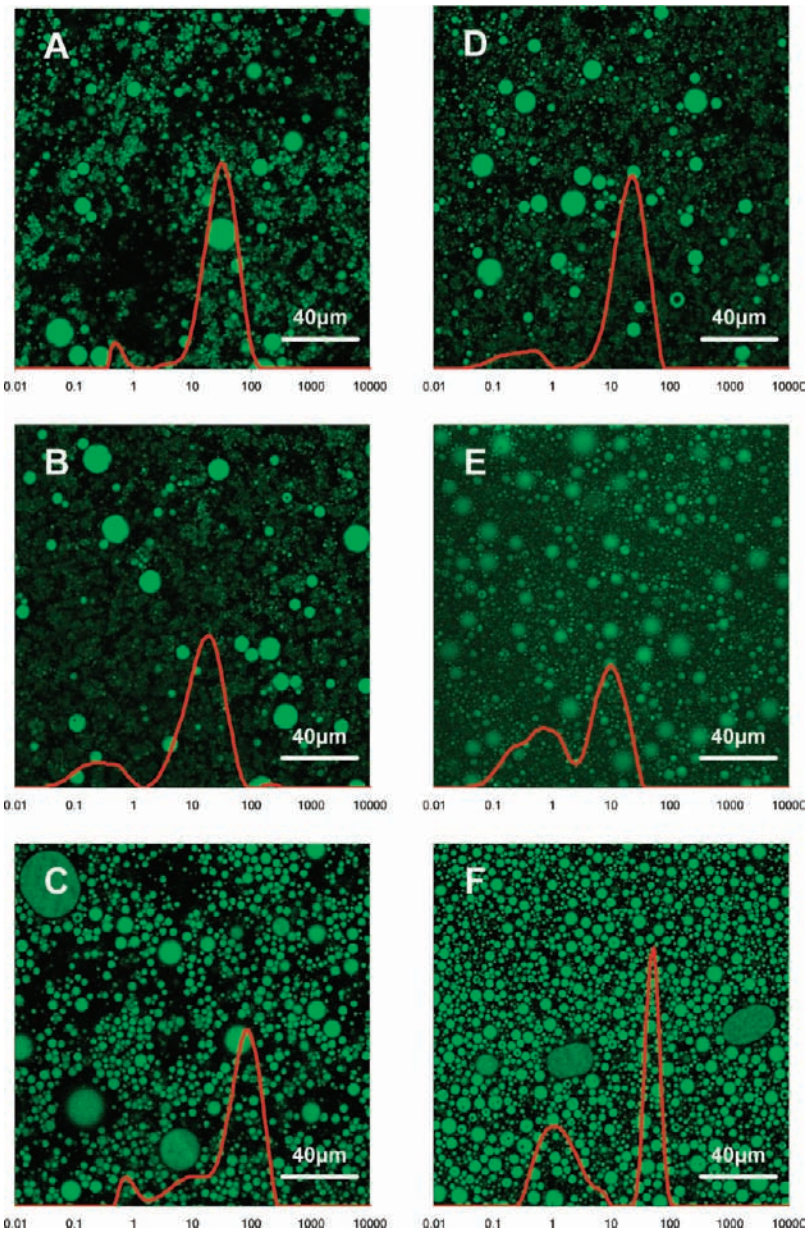

Figure 1. Microstructure of fresh emulsions (20% v/v oil, 1.6% w/v emulsifier, and pH = 7.0) formed by SPIH and USPIH-400W with selected DH values: (A), control SPI; (B), SPIH-0.58%; (C), SPIH-0.98%; (D), control USPI-400W; (E), USPIH-400W-1.25%; (F), USPIH-400W-1.82%. Droplet size distributions of diluted emulsions determined by light scattering (Mastersizer) are superimposed on the micrographs, with horizontal scale indicating particle size in micrometers.

protein on one droplet became shared with the initially uncoated surface of another, may occur. The droplet size distribution of the control SPI emulsion was mainly in the range of 10−100 μm. For the emulsion formed by SPIH-0.58% (Figure 1B), although less strongly flocculated than in the control SPI emulsion, flocculation was still obvious. However, the appearance of some small droplets (ranging from 0.1 to 1 μm) was clearly evident. This may suggest that SPIH-0.58% contained some peptides that were very surface active, enabling the generation and stabilization of some small droplets, but still the content of the emulsifier may not have been sufficient for full coverage of the droplet surface. For emulsion formed by SPIH-0.98% (Figure 1C), flocculation became stronger, and droplet size distribution showed a shift to larger sizes in contrast with that of SPIH-0.58% emulsion.

Figure 1D−F shows the CLSM images and droplet size distributions for emulsions formed by USPIH-400W with different DH values. For the emulsion formed by USPI-400W (Figure 1D), flocculation was less stronger than that in the control SPI emulsion, and some small droplets (ranging from 0.1 to 1 μm) were stabilized. For the emulsion formed by USPIH-400W-1.25% (Figure 1E), the most interesting observation was the disappearance of emulsion flocculation. Moreover, in contrast with emulsions formed by SPIH-0.58% and control USPI-400W, many more small droplets were stabilized, which may suggest that USPIH-400W-1.25% contained more surface active peptides. For emulsion formed by USPIH-400W-1.82% (Figure 1F), droplets are large, coarse, and flocculated; droplet size distributions showed a shift to larger sizes in contrast with that of

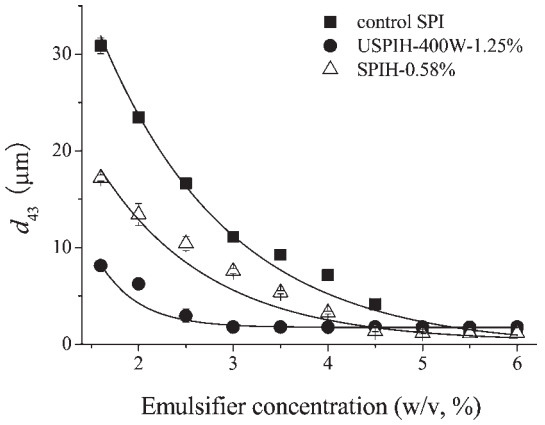

Figure 2. Influences of emulsifier concentration on the mean droplet sizes of fresh emulsions (20% v/v oil, pH = 7.0) formed by control SPI, SPIH-0.58%, and USPIH-400W-1.25%, respectively.

USPIH-400W-1.25% emulsion. This suggests that excessive enzymatic hydrolysis caused a decrease in the emulsifying capability for USPIH-400W, as it did for SPIH. In addition, some elliptically shaped droplets, i.e., deformed, can be observed in the USPIH-400W-1.82% emulsion. It has been reported that emulsion droplets formed by highly hydrolyzed proteins have a weak, loose and mobile interfacial membranes, and are easy to deform and coalescence.[34]

Influences of Emulsifier Concentration on the Mean Droplet Size of Fresh Emulsions Formed by SPIH and USPIH-400W. The d_{43} values of fresh emulsions formed by control SPI, SPIH-0.58%, and USPIH-400W-1.25% as a function of emulsifier concentration are shown in Figure 2. As expected, for all the protein emulsifiers used, when the emulsifier is limiting, d_{43} decreased with an increase in emulsifier concentration. Once there was sufficient emulsifier present to fully cover the surfaces of the oil droplets formed in the homogenizer, the d_{43} values will remain relatively constant. The minimum amount required to form stable emulsions for control SPI, SPIH-0.58%, and USPIH-400W-1.25% was 5.0% (w/v), 4.5% (w/v), and 3.0% (w/v), respectively. The measured d_{43} values of these stable emulsions were 1.48, 1.15, and 1.79 μm, respectively. These results indicated that USPIH-400W-1.25% required less protein to form stable emulsions with small droplet sizes under similar conditions.

SDS−PAGE Profiles of SPIH and USPIH-400W. The effect of papain hydrolysis on protein breakdown in SPIH and USPIH-400W was analyzed by SDS−PAGE. Figure 3A and B shows the progressive changes in the peptide profiles of SPIH and USPIH-400W prepared with papain at different E/S ratios (0−0.5%, w/w). The control SPI and USPI-400W displayed similar peptide profiles, with α and β subunits present representing the 7S, and acidic (A) and basic (B) subunits present representing the 11S. This observation agreed with previous studies that observed no effect of ultrasound on the peptide profile of soy proteins.[29] After papain hydrolysis, for both SPIH and USPIH-400W, all of the subunits degraded gradually with the increase of E/S ratio. For SPIH, α-7S disappeared at E/S = 0.5% (w/w); β-7S disappeared at E/S = 0.05% (w/w); A-11S and B-11S both disappeared at E/S = 0.2% (w/w). In contrast, α-7S and A-11S in USPI-400W appeared to be more readily hydrolyzed because they both disappeared at E/S = 0.1% (w/w). However, the progressive degradation pattern of β-7S and B-11S with the increase of E/S

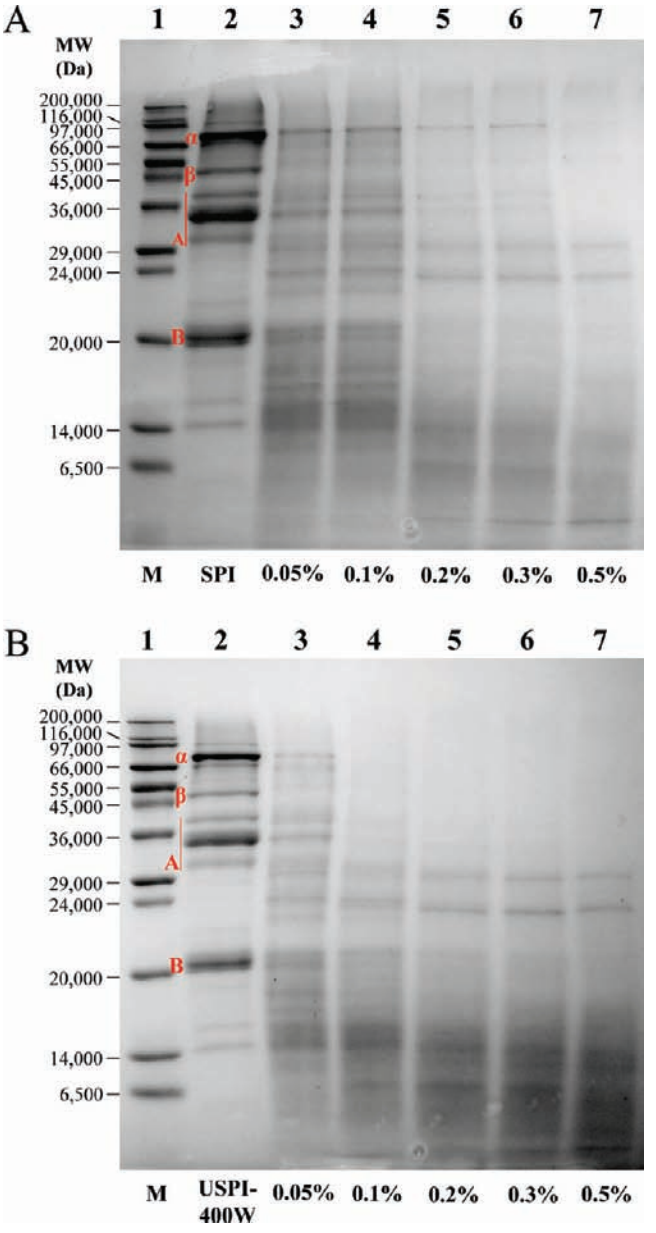

Figure 3. SDS−PAGE electrophoretic profiles of SPIH (A) and USPIH-400W (B) prepared with papain at various E/S ratios shown on the horizontal scale (0.05−0.5%); molecular weight markers (M) are also shown in the far left column for reference.

ratios in USPIH-400W was similar to those in SPIH. These results suggest that, in USPI-400W, the accessibility of α-7S and A-11S to papain hydrolysis was improved. This finding may explain the enhanced hydrolysis of USPI-400W as compared with control SPI in terms of DH values. According to Jambrak et al.,[23] appropriate ultrasound treatment can dissociate the globular proteins; therefore, more hydrolysis sites are exposed and could be accessible by proteases.

However, some small peptides (MW < 20.0 kDa) were formed in both SPIH and USPIH-400W. As the E/S ratio increased, these peptide bands showed a tendency to shift toward smaller molecular weights. This finding suggests that papain hydrolysis will cause a decrease in molecular weight for soy proteins, as reported previously.[35]

Table 2. PS and H_0 of SPIH and USPIH-400W Prepared with Papain at Different E/S Ratios[a]

	SPIH			USPIH-400W		
E/S ratio (%, w/w)	DH (%)	PS (%)	H_0	DH (%)	PS (%)	H_0
0	0	15.8 (±0.3)	1109.8 (±21.3)	0	26.3 (±0.8)	1326.2 (±17.6)
0.05	0.42	24.5 (±0.4)	1458.6 (±36.5)	0.81	45.8 (±1.1) a	1554.6 (±22.4)
0.1	0.58	31.9 (±0.8)	1834.8 (±12.9)	0.99	56.4 (±1.4)	1014.2 (±32.7)
0.2	0.73	36.7 (±1.4)	947.2 (±18.2)	1.25	68.5 (±0.7)	854.7 (±19.3)
0.3	0.87	40.2 (±0.7)	525.3 (±18.7)	1.43	73.6 (±0.9)	642.5 (±15.6)
0.5	0.98	45.9 (±1.6) a	206.4 (±9.6)	1.82	81.2 (±0.5)	253.8 (±21.5)

[a] Results having the same letter are not significantly different ($p < 0.05$).

Protein Solubility of SPIH and USPIH-400W. Solubility in the aqueous phase is a prerequisite for a protein to be a good emulsifier. Because emulsions were prepared at pH 7.0 in this work, PS of SPIH and USPIH-400W with different DH values were investigated at this same pH. Table 2 shows the PS of SPIH and USPIH-400W prepared with papain at different E/S ratios (0−0.5%, w/w). Control SPI showed a poor PS of 15.8%, which might be due to the protein aggregation induced by the harsh processing conditions.[4] After ultrasound pretreatment, the PS of USPI-400W increased to 26.3%. This result suggests that the ultrasound pretreatment used here showed a favorable effect on improving the PS of SPI. Upon hydrolysis, the PS of both SPIH and USPIH-400W increased markedly. It is well known that enzymatic hydrolysis can improve the PS of protein by decreasing molecular weight and increasing charged groups.[11] However, it should be noted that when the same E/S ratios were used, USPIH-400W had a significantly higher PS than SPIH. This finding could be explained by the fact that ultrasound pretreatment improved the enzymatic accessibility of SPI; therefore, more soy proteins in USPI-400W can be readily hydrolyzed and become soluble.

Surface Hydrophobicity of SPIH and USPIH-400W. Protein surface hydrophobicity is an index of the number of hydrophobic groups on the surface of a protein in contact with the polar aqueous environment and is closely related to its emulsifying properties. H_0 of SPIH and USPIH-400W prepared with different E/S ratios (0−0.5%, w/w) is shown in Table 1 and is plotted as a function of their DH in Figure 4. USPI-400W showed a higher H_0 (1326.2) than control SPI (1109.8). This finding was consistent with previous studies,[20] which showed that ultrasound treatment can cause the increase of H_0 for globular proteins. Upon hydrolysis, as shown in Figure 4, H_0 of SPIH and USPIH-400W both increased initially and then decreased at high DH values. SPIH showed a maximum H_0 of 1834.8 at DH 0.58%, and USPIH-400W showed a smaller maximum H_0 of 1554.6 at DH 0.81%. It is well known that controlled enzymatic hydrolysis can expose hydrophobic groups hitherto concealed in globular proteins.[11,35] However, further hydrolysis caused a drastic decrease in H_0 for SPIH, which may be due to two factors: (i) enzymatic cleavage to free hydrophobic amino acids; and (ii) protein aggregation caused by the increased hydrophobic interaction.[35] As a result, at high DH values, SPIH had high PS, but very low H_0, and exhibited an even worse emulsifying capability. In contrast, further hydrolysis caused a relatively moderate decrease in H_0 for USPIH-400W. The reason for this is not clear, but it may be related to the increased enzymatic accessibility of USPI-400W, which made the exposure and breakdown of hydrophobic groups more evenly during hydrolysis. We should note that some USPIH-400W (DH = 1.0−1.5%) had much increased PS and slightly decreased H_0, and exhibited much improved emulsifying capability.

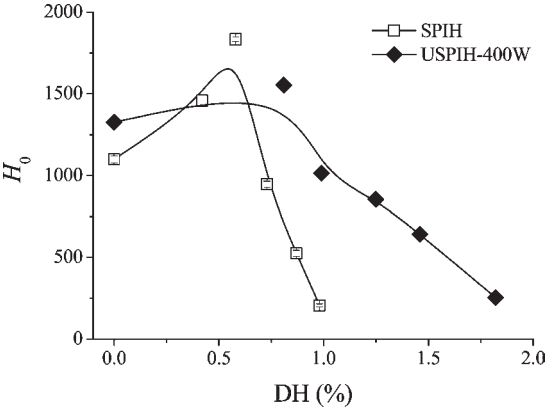

Figure 4. Influence of DH on the surface hydrophobicity (H_0) of SPIH and USPIH-400W at pH 7.0.

Secondary Structure Composition of SPIH and USPIH-400W. The secondary structure composition of dispersions of control SPI, SPIH-0.58%, and USPIH-400W-1.25% are shown in Table 3. Control SPI calculations showed the presence of the 8.8% α-helix, 36.2% β-sheet, 20.6% β-turn, and 34.4% random coils. SPIH-0.58% showed a calculated decrease in α-helix (to 7.8%) and β-turn (to 16.8%), and an increase in β-sheet (to 38.1%) and random coils (to 37.3%). In contrast, USPIH-400W-1.25% showed a greater change in secondary structure. The content of α-helix (4.6%), β-sheet (25.2%), and β-turn (10.5%) all decreased, as the random coil (59.7%) increased markedly. The implication of the increase in random coil content is that the flexibility of USPIH-400W-1.25% was increased. Structure and functionality were highly correlated. Previous research has shown that a more flexible structure benefited the adsorption and unfolding of protein molecule at the oil−water interface.[36] The increase in flexibility for USPIH-400W-1.25% evidently contributes to the improvement of its emulsifying properties.

Interfacial Pressure of SPIH and USPIH-400W at an Oil−Water Interface. As shown before, combined ultrasound pretreatment and controlled papain hydrolysis caused significant changes in both physicochemical and emulsifying properties for SPI. To better understand the relationship between them, some interfacial properties, including saturation interfacial pressure (π_{sat}) at sunflower seed oil−water interface, protein adsorption fraction during homogenization (F_{ads}), and saturation surface load (Γ_{sat}), were investigated for some SPIH and USPIH-400W.

Table 3. Secondary Structure Composition of Control SPI, SPIH-0.58%, and USPIH-400W-1.25%[a]

sample	α-helix (%)	β-sheet (%)	β-turn (%)	random coil (%)
control SPI	8.8 (±0.3)	36.2 (±0.2)	20.6 (±0.4)	34.4 (±0.3)
SPIH-0.58%	7.8 (±0.1)	38.1 (±0.5) a	16.8 (±0.4)	37.3 (±0.4) a
USPIH-400W- 1.25%	4.6 (±0.2)	25.2 (±0.2)	10.5 (±0.4)	59.7 (±0.3)

[a] Results having the same letter are not significantly different ($p < 0.05$).

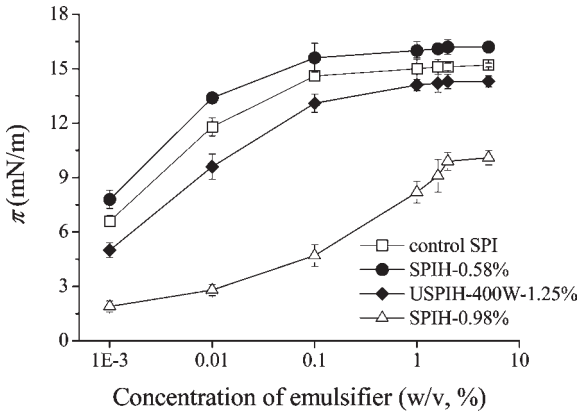

Figure 5. Interfacial pressure at the sunflower oil−water interface as a function of emulsifier concentration for SPIH and USPIH-400W with selected DH values.

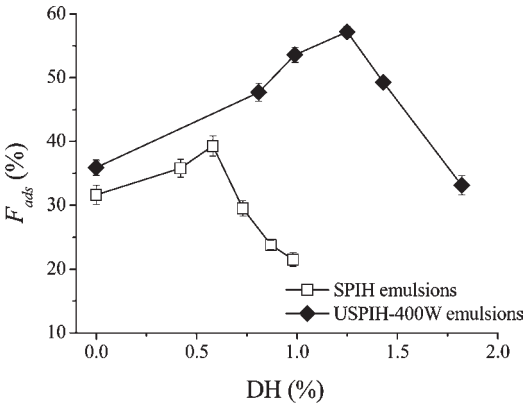

Figure 6. Influences of DH on the protein adsorption fraction (F_{ads}) of SPIH and USPIH-400W during homogenization (20% v/v oil, 1.6% w/v emulsifier, and pH = 7.0).

The interfacial pressures at the sunflower seed oil−water interface as a function of emulsifier concentration for control SPI, SPIH-0.58%, SPIH-0.98%, and USPIH-400W-1.25% are shown in Figure 5. As the emulsifier concentration increased, the interfacial pressure increased to a constant value (π_{sat}) when the interface became saturated with emulsifier. The value of π_{sat} is a measure of how effectively the adsorbed solute molecules are able to minimize the thermodynamically unfavorable interactions between oil and water phases at saturation, and has important consequences for the formation and stability of food emulsions.[7] The measured values of π_{sat} were 16.9 mN/m, 15.2 mN/m, 14.6 mN/m, and 10.5 mN/m for SPIH-0.58%, control SPI, USPIH-400W-1.25%, and SPIH-0.98%, respectively. The higher the value of π_{sat} for a particular interface, the better the solute is at minimizing thermodynamically unfavorable interactions at that interface, and therefore, the smaller the droplet could be generated during homogenization.[7] The π_{sat} of an emulsifier depends on the packing of the solute molecules at the interface, as well as their interactions with the other molecules present there, i.e., oil and water. Contrasting the π_{sat} with H_0 for SPIH and USPIH-400W-1.25%, a positive correlation can be found between the two parameters.

Fraction of Protein Adsorption and Surface Load of SPIH and USPIH-400W. F_{ads} of SPIH and USPIH-400W plotted as a function of the DH value is shown in Figure 6. Control SPI and USPI-400W had F_{ads} of 31.6% and 35.9%, respectively. With the increase of DH, F_{ads} of both SPIH and USPIH-400W increased initially and decreased at high DH values. SPIH showed a maximum F_{ads} of 39.3% at DH 0.58%. In contrast, USPIH-400W showed a larger maximum F_{ads} of 57.2% at DH 1.25%. A comparison of Figure 6 with Table 1 shows that a close relationship existed between mean droplet sizes and F_{ads} for emulsions formed by both SPIH and USPIH-400W. In most cases, the higher the F_{ads} is, the smaller the d_{43} will be.

The effectiveness of a protein as an emulsifier is also closely related to its saturation surface load (Γ_{sat}). The smaller the value of Γ_{sat}, the greater the area of oil−water interface that can be covered per gram of emulsifier, and therefore the smaller the size of droplets that can be effectively stabilized by the same amount of emulsifier.[33] Emulsions formed by 5.0% (w/v) control SPI, 4.5% (w/v) SPIH-0.58%, and 3.0% (w/v) USPIH-400W-1.25% were chosen as representative systems for saturation surface load studies. The measured values of Γ_{sat} were 4.6 mg/m^2, 4.0 mg/m^2, and 3.1 mg/m^2, respectively. These results indicated that, as compared with the control SPI and SPIH-0.58%, less amounts of USPIH-400W-1.25% are required to cover a unit area of droplet surface. It has been showed that USPIH-400W-1.25% contained a more flexible structure and therefore can rapidly alter its conformation. However, globular soy protein molecules have a slow conformation change due to kinetic constraints. Biopolymers capable of undergoing fast conformational changes at the interface tended to have a lower surface load because of significant spreading of the biopolymer molecules.[37]

In summary, this study found that combined ultrasound pretreatment and controlled papain hydrolysis caused significant changes in physicochemical properties and interfacial properties of SPI. It was encouraging to find that some USPIH exhibited markedly improved emulsifying capability and emulsion stabilization against creaming during quiescent storage. As has been demonstrated, SPI and SPIH-0.58% can substantially decrease the interfacial tension between oil and water and therefore generate small oil−water droplets during homogenization. Nevertheless, probably because of their low F_{ads}, the newly generated droplets tended to be bridging flocculated. In contrast, USPIH-400W-1.25% had a slightly smaller π_{sat}, mainly because of its lower H_0, but had a much higher F_{ads} (57.2%) and a significantly lower Γ_{sat} (3.1 mg/m^2). These may explain the effective use of USPIH-400W-1.25% in protecting emulsions

against bridging flocculation during homogenization. The current study found that papain hydrolysis of USPI-400W was more intensive and extensive than that of SPI because ultrasound pretreatment could significantly improve the accessibility of some subunits in soy proteins (α-7S and A-11S) to papain. As a result, compared with SPIH, USPIH-400W showed a stronger increase in PS and a more moderate change in H_0. Therefore, some USPIH-400W (e.g., USPIH-400W-1.25%) had much increased PS and slightly decreased H_0, which may be the main causes for the increase in their F_{ads} and emulsifying capability. Moreover, the increase in random coil content for USPIH-400W-1.25% probably caused an increase in its molecular flexibility, which benefited the adsorption and unfolding of peptide molecules at the droplet interface. However, it should be noted that excessive enzymatic hydrolysis caused strong decreases in π_{sat} and F_{ads} for both SPIH and USPIH-400W, eventually resulting in a drastic decrease in their emulsifying capability. The reason for this might be attributed to the strong decrease in H_0 at high DH.

In conclusion, modified soy protein could be an excellent emulsifying agent for food and other applications with combined ultrasound pretreatment and enzymatic hydrolysis leading to desirable functional modifications of globular proteins.

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ABBREVIATIONS USED

SPI, soy protein isolates; SPIH, SPI hydrolysates; USPIH, ultrasound pretreated SPIH; E/S, enzyme/substrate ratio; DH, degree of hydrolysis; CI, creaming index; CLSM, confocal laser scanning microscopy; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PS, protein solubility; ANS, 1-anilino-8-naphthalenesulfonate; CD, circular dichroism.

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