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Stable Oil-in-Water Emulsions Prepared from Soy Protein−Dextran Conjugates

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The solubility of acid soluble soy protein (ASSP) is poor in the pH range of 4−7. ASSP−dextran conjugates were prepared through the Maillard reaction to increase the solubility and/or dispersibility of ASSP. By using the conjugates and ultrasonication emulsification, stable oil-in-water emulsions with submicrometer-sized droplets were obtained. The factors, such as ASSP conjugation degree, that influence the emulsion stability were investigated. The emulsions were characterized using dynamic light scattering, ζ-potential, and atomic force microscopy. In the ultrasonication process, the increases of surface activity and aggregation of protein result in the formation of stable oil−water interface films. The dextran molecules conjugated to the protein effectively enhance the hydrophilicity and steric repulsion of the oil droplets, and therefore the emulsions are stable against heat treatment, long-term storage, and changes of pH and ionic strength. It was further verified that using protein−polysaccharide conjugates and ultrasonication emulsification is a versatile method for preparing stable emulsions.

Introduction

Proteins have been used as emulsifiers in food products for many years.[1] The stability of protein-containing oil-in-water emulsions depends strongly on the density and structure of the protein adsorption layers on the drop surface.[2,3] The protein adsorption layers prevent drop−drop coalescence by stabilizing the emulsion films. However, protein-stabilized emulsions are highly sensitive to environmental stresses such as pH, ionic strength, and temperature.[4] When the pH approaches the isoelectric point of the protein and/or the salt concentration is higher in the emulsion, the electrostatic repulsion of the protein adsorption layers decreases, and therefore coalescence and creaming happen.[5] When emulsion is subjected to heat treatment, for pasteurization or sterilization purposes, aggregation happens because of the denaturation of the protein that holds the droplets together.[6]

The emulsion stability can be improved by protein−polysaccharide complexes produced through electrostatic attraction or covalent binding.[5] McClements and co-workers found that the emulsion stability can be improved by adding polysaccharide forming an interfacial complex with the adsorbed protein layer after homogenization.[4,7] Maillard-type protein−polysaccharide conjugates have excellent emulsifying and steric stabilizing properties, especially under the conditions where the protein alone is poorly soluble.[5] Akhtar and Dickinson reported the emulsifying properties of covalent complexes of maltodextrin with whey protein isolate.[8] The covalent complexes lead to a very substantial enhancement in the protein emulsifying behavior under both acidic and neutral conditions. However, analogous covalent complexes of maltodextrin with soy protein do not have this positive effect.

Proteins of plant origin do not function effectively at acidic pH environments since they precipitate at pH values close to their isoelectric point.[9] Most foods and beverages are acidic. Therefore, the poor emulsion stability at the isoelectric point limits protein application in food and beverage industries. Among proteins of vegetable origin, soy protein is an abundant byproduct of the soybean-oil industry and has good functional properties for food processing because of the high nutritional value and the contribution to food texture and emulsifying properties.[10,11] Kobayashi et al. first reported soy protein−polysaccharide conjugates prepared through the Maillard reaction in 1990.[12] The studies of Kiosseoglou and co-workers demonstrated that soy protein−polysaccharide conjugates can improve emulsifying properties, especially in the reduction of oil droplet size and emulsion stabilization against creaming.[6,9,13,14] The conjugates adsorb at the interface together with unreacted protein constituents, enhancing steric stabilization forces of oil droplets. However, to the best of our knowledge, long-term stable emulsions stabilized by soy protein at acidic conditions have not been reported. The reason may be that, as pointed by Diftis et al., the protein aggregates adsorb at the interface during homogenization, and they probably act as "bridging" material in the dispersed system to cause droplet interactions.[9] Velev et al. reported oil-in-water emulsions stabilized by vegetable proteins, and they also suggested the presence of protein aggregates inside the film.[15]

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Ultrasound emulsification is one of high-energy emulsification techniques.[16] The power ultrasound can improve the solubility of soy protein.[17] In sonication, as pointed out in the literature, mechanical vibration causes the formation and collapse of bubbles. As a result of this cavitation, the media may experience extreme local effects: heating (10 000 K), high pressure (200 bar), and high strain rates (10^7 s^{-1}).[18] These extreme local effects can increase surface hydrophobicity, surface activity, and aggregation of protein.[19] Ultrasound emulsification can produce stable interface protein films. Suslick et al. reported microspheres with an oil core and a bovine serum albumin (BSA) shell formed by applying ultrasound to a BSA aqueous solution and vegetable oil.[20,21] The BSA molecules in the shell are not significantly denatured and are held together by the sonochemical formation of disulfide bonds via interprotein cysteine oxidation. Suslick et al. also proved that the stability of polyglutamate microspheres formed by ultrasound emulsification is due to hydrogen bonding networks.[22] Ultrasound emulsification can produce submicrometer emulsions.[23] Emulsion droplet size plays a key role in many emulsion properties such as stability, color, appearance, texture, and rheology. The aim of emulsification is usually to produce emulsion droplets as small as possible.[2,16]

In this study, stable emulsions were obtained by use of soy protein−dextran conjugates and ultrasonication emulsification. The effects of conjugation degree on the stability of the emulsions against long-term storage, heat treatment, and pH and ionic strength changes were investigated. The emulsions were characterized by dynamic light scattering (DLS), ζ-potential, and atomic force microscopy (AFM).

Materials and Methods

Materials. Acid soluble soy protein (ASSP, Soyasour 4000K, protein content ≥88%, moisture ≤7.5%, fat ≤1.5%, pH 3.6−4.4) was from Jilin Fuji Protein Co., Ltd. Dextran (62 kDa) was from Amersham Pharmacia Biotech. Corn oil was from a local market. All solutions were prepared using deionized water.

Preparation of ASSP−Dextran Conjugates. ASSP was dissolved in water. After 3 h of stirring, the pH of the solution was adjusted to 8.5. NaN_3 with a final concentration of 0.02% was added to inhibit microbial growth. The solution was stirred overnight, and undissolved protein was removed by centrifugation at 10 000 rpm for 15 min. Dextran was added into the supernatant in a desired weight ratio, and the mixture was lyophilized. The lyophilized powder was reacted at 60 °C under 79% relative humidity in a desiccator containing saturated KBr solution for a desired time. The conjugation degree of the resultant Maillard reaction product was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The gel was stained with Coomassie brilliant blue, and the protein content in each track was analyzed using a standard gray scale analysis method by Bandscan software (Glyko, Inc.) The Maillard reaction products were denoted as ASSP−dextran conjugates and were used without separation.

Preparation of Emulsions. The conjugate was dissolved in water. After pH adjustment, corn oil was added into the conjugate solution with a desired volume ratio. The mixture was pre-emulsified at room temperature with a homogenizer (FJ200-S, Shanghai Specimen Model Co.) at 10000 rpm for 1 min, then was immediately emulsified in a ice−water bath using ultrasonication (Scientz-IID, Scientz Biotechnology Co., Ltd.) at a power of 450 W for 6 min (on 2.5 s/off 2 s).

Extractable Free Oil Analysis. Uncapsulated oil in 13.33 mL emulsion, which was prepared from 3.33 mL oil and 10 mL conjugate solution, was extracted by 15 mL of petroleum ether twice.[24] The extraction was evaporated at 70 °C under vacuum to remove the solvent, was dried in an oven at 105 °C for 1 h to remove moisture, and was weighed in succession. As a control, 3.33 mL of oil was added into 30 mL of petroleum ether, and 99.95% of the oil remained after the same process. Parallel samples were analyzed, and average data were reported.

DLS Measurements. DLS measurements were carried out on a Malvern Autosizer 4700 (Malvern Instruments) equipped with a multi-τ digital time correlator (Malvern PCS7132) and a solid-state laser (Compass 315M-100, Coherent, Inc.; output power 100 mW at λ 532 nm). In DLS measurements, the line width distribution $G(\Gamma)$ was calculated from the Laplace inversion of the measured intensity−intensity time correlation function. $G(\Gamma)$ can be converted to the translational diffusion coefficient distribution $G(D)$ and to the hydrodynamic diameter distribution $f(D_h)$ by the Stokes−Einstein equation.[25] The measurements were performed at 25 °C and a fixed scattering angle of 90°. The refractive index is 1.333 and 1.472 for water and corn oil, respectively. The measured time correlation functions were analyzed by Automatic Program provided by Malvern. Apparent z-average hydrodynamic diameter (D_h) and polydispersity index (PDI, $\langle \mu_2/\Gamma^2 \rangle$) were obtained by CONTIN-mode analysis.[25] Emulsions diluted freshly with the same pH aqueous solution were used for DLS measurement, in which the ASSP concentration was 0.0245 mg/mL. If there is no specific indication in this report, the emulsions were diluted without SDS. Two batches of samples were measured, and average data were reported.

ζ-Potential Measurements. ζ-Potentials were measured at 25 °C on a ZetaSizer Nano ZS90 (Malvern Instruments) equipped with a 4 mW He−Ne Laser (λ 633 nm) using the technique of laser Doppler electrophoresis. Emulsions were changed to different pH values, and then were diluted to an ASSP concentration of 0.03 mg/mL with the same pH solution containing 5 mmol/L NaCl. ζ-Potentials were calculated by Dispersion Technology software provided by Malvern according to the Smoluchowski approximation in an automatic mode.[26] Each sample was analyzed three times, and average data were reported.

AFM Measurements. AFM images were acquired in tapping mode on a Digital Instruments Nanoscope IV (Veeco Instruments) equipped with a silicon cantilever of 125 μm and an E-type vertical engaged piezoelectric scanner. AFM samples were prepared by drying the solution on a freshly cleaved mica surface in a desiccator containing dried silica gel at room temperature for at least 2 days.

Results and Discussion

Solubility of ASSP. The solubility of ASSP in the pH range of 1−10 was investigated by centrifugating 22 mg/mL of ASSP solution at 10 000 rpm for 15 min. ASSP in the supernatants was lyophilized and weighed. The result shows that the soluble portion of ASSP is around 20 mg/mL in the pH ranges of 1−3 and 8−10, and around 5 mg/mL in the pH range of 5−7. The ASSP portion in the supernatant of pH 4 solution is 17.3 mg/mL, but the

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Table 1. Absorbance at 500 nm of ASSP−Dextran Conjugate Solutions[a]

WR 1:3		WR 1:6		WR 1:9	
reaction time (h)	absorbance	reaction time (h)	absorbance	reaction time (h)	absorbance
0	0.15 ± 0.01	0	0.12 ± 0.01	0	0.11 ± 0.01
9	0.34 ± 0.01	30	0.23 ± 0.01	72	0.53 ± 0.01
12	0.38 ± 0.04	40	0.31 ± 0.01	96	0.58 ± 0.03
15	0.47 ± 0.08	50	0.35 ± 0.03	120	0.66 ± 0.03

[a] The conjugates were prepared from WR 1:3, 1:6, and 1:9 mixtures through different times of the Maillard reaction. The concentration of ASSP in the conjugate solutions was 2.5 mg/mL. The absorbance of individual ASSP is 0.16.

supernatant is heavily turbid. These data indicate a poor solubility of ASSP in the pH range of 4−7. The behaviors of ASSP and dextran physical mixtures are the same as the individual ASSP, i.e., the precipitates or turbidity largely increase in the pH range of 4−7. In this paper, ASSP−dextran conjugates were prepared through the Maillard reaction in order to increase ASSP solubility in the pH range of 4−7 and therefore to enhance the emulsifying and stabilizing properties.

Maillard Reaction and the Solubility of the Conjugates. Maillard reaction is a natural and nontoxic process, which conjugates polysaccharide and protein by linking the reducing end carbonyl group in the former to the amino group in the latter in the early stage of the reaction.[27] Mixtures with weight ratios (WRs) of ASSP to dextran of 1:0.5, 1:1, 1:2, 1:3, 1:6, 1:9, and 1:12 were used to prepare the conjugates with different conjugation degrees. The resultant conjugates were dissolved in water with ASSP concentration of 2.5 mg/mL, and then the absorbance at 500 nm was measured. We found that the turbidity of the solutions increases, i.e., the solubility decreases for all of the mixtures after the reaction, which is consistent with the report of Akhtar and Dickinson.[8] For WR 1:0.5, 1:1, 1:2, 1:3, 1:6, and 1:9 mixtures, the products cannot completely disperse in water, i.e., precipitates exist after 3, 6, 20, 25, 50, and 144 h of reaction, respectively. After the same time of the reaction, a higher dextran ratio in the mixture leads to a higher solubility of the product. For each WR mixture, a longer reaction results in a poorer solubility and darker color of the product. These results imply that ASSP molecules cross-link even in the early stage of the reaction, and the cross-linking decreases with the increase of dextran ratio in the mixture.

In this study, we did not further study the conjugates prepared from WR 1:0.5, 1:1, and 1:2 mixtures because of the low conjugation degree and poor solubility after the reaction. We also did not further study the WR 1:12 conjugate. The reasons are that the solution is viscous, and too many dextran molecules in the solution can reduce the emulsification ability of ASSP, which will be demonstrated below. For WR 1:3, 1:6, and 1:9 mixtures, Table 1 shows the absorbance at 500 nm of the resultant solutions. The absorbance increases with the increase of reaction time, indicating an increase of dispersible particles and a decrease of soluble molecules in the conjugate solutions. In this study, we chose 12, 48, and 72 h of Maillard reaction for WR 1:3, 1:6, and 1:9 mixtures, respectively, to prepare the conjugates. The resultant products were separately denoted as WR 1:3, 1:6, and 1:9 conjugates.

Figure 1 shows SDS−PAGE analysis of ASSP and WR 1:3, 1:6, and 1:9 conjugates. Before the SDS−PAGE analysis, the conjugate samples were reacted with the loading buffer containing SDS and β-mercaptoethanol to destroy the disulfide-bond cross-links and hydrophobic aggregation in the conjugates and ASSP. Compared with individual ASSP, the new smear band with much higher molecular weight indicates the formation of ASSP−dextran conjugate.[14] Figure 1 also exhibits that the conjugates contain free proteins. The conjugation degree was analyzed through the standard gray scale analysis method. Compared with the gray in each whole lane, the gray of the smear band is 9.1%, 32.0% and 34.4% for WR 1:3, 1:6, and 1:9 conjugates, respectively. This result means that about 9.1%, 32.0% and 34.4% of ASSP were conjugated with dextran, i.e., there are about 90.9%, 68.0%, and 65.6% of free proteins after the Maillard reaction in WR 1:3, 1:6, and 1:9 conjugates, respectively.

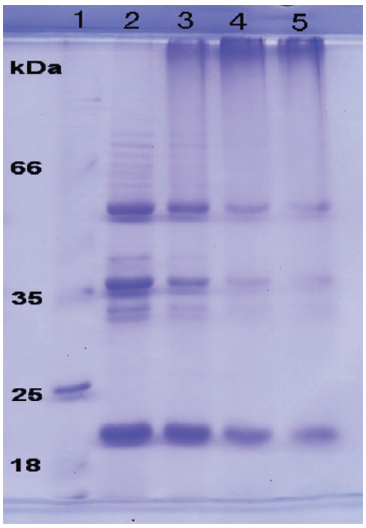

Figure 1. SDS−PAGE analysis of protein marker (lane 1), ASSP (lane 2), and ASSP−dextran conjugates of WR 1:3 (lane 3), 1:6 (lane 4), and 1:9 (lane 5) prepared by 12, 48, and 72 h of the Maillard reaction, respectively. In each of lanes 2−5, 20 μL of sample with an ASSP concentration of 2.5 mg/mL was loaded.

WR 1:9 conjugate was dissolved in water, the solution was adjusted to different pH values, and the final ASSP concentration was 15 mg/mL in each sample. No precipitation occurred after overnight storage for all the samples of pH 1−10. The solutions were centrifuged at 10000 rpm for 15 min, and then the supernatants were lyophilized and weighted. The dry weights of the supernatants are almost the same for all the samples of pH 1−10, but the turbidities of pH 4, 5, and 6 solutions are much higher than those of the solutions of pH 1−3 and 7−10. These phenomena indicate that conjugation with dextran can effectively increase the solubility and dispersibility of ASSP in the pH range of 4−7. The free proteins in the solution can bind with the conjugates, forming dispersible particles in the pH range of 4−7 that suppresses the precipitation.

Emulsion Preparation. A 3 mL portion of WR 1:3 conjugate solution having an ASSP concentration of 20 mg/mL and pH 8.0 was mixed with 1 mL of corn oil, i.e., oil volume fraction $\phi = 0.25$. The mixture was emulsified at room temperature by means of homogenizing at 20 000 rpm for 1.5 min. A cream layer occurred in the emulsion almost immediately after the emulsification,

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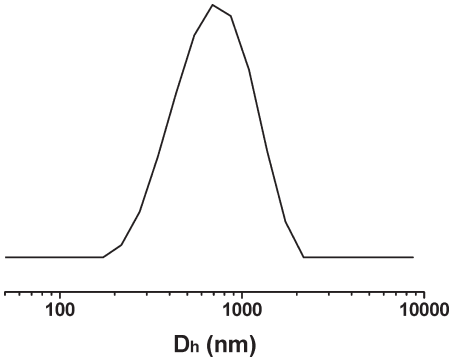

Figure 2. Size distribution of the emulsion prepared by ultrasonication and WR 1:3 conjugate with an ASSP concentration of 20 mg/mL and $\phi = 0.25$.

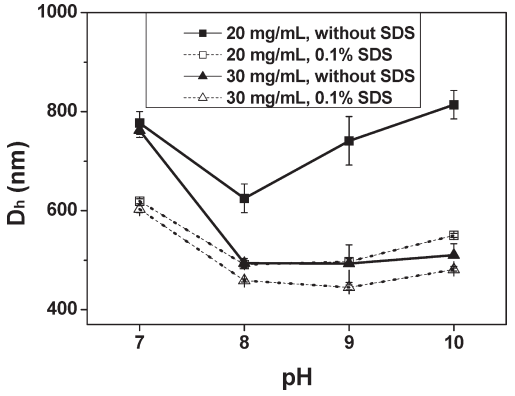

Figure 3. Droplet sizes of the emulsions prepared from WR 1:3 conjugate at different pH values with ASSP concentrations of 20 and 30 mg/mL as well as $\phi = 0.25$. The emulsions were diluted by the same pH aqueous solutions with and without 0.1% SDS for DLS measurement.

indicating that the oil droplets are not small enough to have long-term stability. Oil layer occurred in the emulsion after a month of storage at 4 °C, implying that the protein adsorption layers on the oil−water interface are not perfect. DLS was used to measure the size distribution of the oil droplets freshly prepared. The DLS result shows multiple peaks with a z-average D_h of about 2 μm (data not shown) for the emulsion diluted with pH 8.0 aqueous solution. We also used pH 8.0 solution containing 0.1% SDS to dilute the emulsion and obtained a similar DLS result.

In order to obtain smaller droplets with stable interface protein films, ultrasound emulsification was applied after pre-emulsification with a homogenizer. For the emulsion prepared by ultrasound from the WR 1:3 conjugate with an ASSP concentration of 20 mg/mL and $\phi = 0.25$, the size distribution of the droplets (Figure 2) exhibits a single peak with a z-average D_h of 613 nm. The DLS result demonstrates that ultrasound emulsification can effectively decrease the size of the oil droplets. The effect of ultrasonication time on the emulsification was investigated. The size distributions of the droplets are similar for the emulsions prepared in the ultrasonication time range of 1−10 min (data not shown). In this study, we used 6 min of ultrasonication to prepare the emulsions.

Effects of pH and Concentration of Conjugate Solution. Figure 3 shows the D_h values of the emulsions prepared from WR 1:3 conjugate at pH 7, 8, 9, and 10. In order to investigate the aggregation of the droplets, the emulsions were diluted by the same pH aqueous solutions with and without 0.1% SDS to reach an ASSP concentration of 0.0245 mg/mL before DLS measurement. As we know, excess SDS can destroy the aggregation of the droplets. The D_h values of the droplets in the presence of 0.1% SDS are significantly smaller than those without SDS, suggesting that the aggregates of the droplets exist in the emulsions produced by ultrasonication. ASSP carries about zero net charge at pH ~4.8, which will be proven by ζ-potential below. ASSP carries more negative charges when the pH of the solution changes from 7 to 10. The increase of the electrostatic repulsion between ASSP molecules can decrease the self-aggregation of the conjugate that enhances the emulsification ability of the conjugate. On the other hand, the increase of the electrostatic repulsion can depress the formation of protein layers on oil−water interfaces that reduces the emulsification ability of the conjugate. These two opposite factors lead the droplet sizes to decrease first and then increase when the pH was changed from 7 to 10. In this paper, we chose to prepare the emulsions at pH 8 because the viscosities of the emulsions prepared at pH 8 are much smaller than those prepared at pH 7.

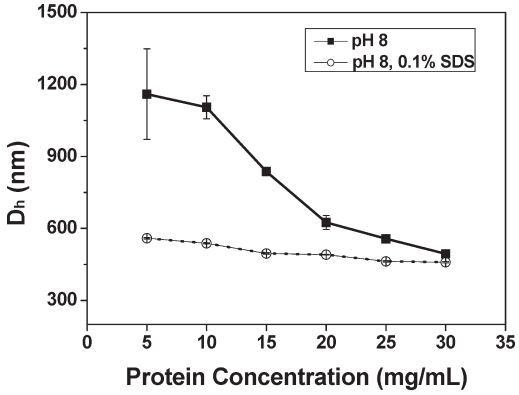

Figure 4. Droplet sizes of the emulsions prepared from WR 1:3 conjugate at pH 8.0 with $\phi = 0.25$ and different ASSP concentrations. The emulsions were diluted by pH 8 aqueous solutions with and without 0.1% SDS for DLS measurement.

Figures 3 and 4 show the influence of the conjugate concentration on the emulsification. At $\phi = 0.25$, decreasing conjugate concentration results in larger droplets. Further decreasing conjugate concentration to 1 mg/mL, oil and emulsion phases coexist in the solution. Figure 4 also shows the D_h values decrease in the presence of SDS, indicating that droplet aggregates exist in the emulsions. The individual droplet size decreases from 559 to 459 nm when ASSP concentration increases from 5 to 30 mg/mL. Although the droplet aggregates exist in the emulsions produced by ultrasonication, in the following study of this work, we only present the droplet sizes in aqueous solutions without SDS, the real media of the oil droplets.

Effect of Oil Volume Fraction ϕ and Heating. Table 2 shows that, at the conjugate concentration of 20 mg/mL, increasing the volume ratio of the conjugate solution to oil from 2:1 to 3:1, 5:1, and 10:1, i.e., changing ϕ from 0.333 to 0.091, results in a decrease of D_h, which is similar to the effect of increasing conjugate concentration. These results can be explained by the fact that more conjugate molecules can stabilize larger oil−water interface area and therefore can produce smaller droplets.

We also investigated the emulsions after a heat treatment at 90 °C for 30 min. As mentioned above, when protein stabilized emulsion is subjected to a heat treatment, for pasteurization or sterilization purposes, aggregation happens because of the denaturation of the protein that holds the droplets together.[6] Table 2 shows that the droplet sizes of the emulsions do not change much after the heat treatment compared with the emulsions without the treatment. Furthermore, the emulsions are homogeneous, and the droplet sizes do not change much after the heat treatment and

Table 2. Droplet Size and Extractable Free Oil Results of Heated and Unheated Emulsions[a]

	before heating		after heating	
ϕ	D_h (nm)	extractable free oil (%)	D_h (nm)	extractable free oil (%)
0.333	916 ± 4	0.3 ± 0.1	1060 ± 29	0.3 ± 0.1
0.250	611 ± 74	0.3 ± 0.1	712 ± 110	0.3 ± 0.1
0.167	552 ± 12	0.4 ± 0.1	504 ± 10	0.3 ± 0.1
0.091	395 ± 2	1.0 ± 0.3	362 ± 7	0.8 ± 0.2

[a] The emulsions were prepared from WR 1:3 conjugate at pH 8 with an ASSP concentration of 20 mg/mL and different ϕ values.

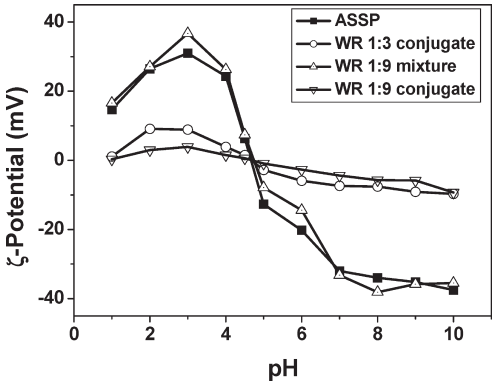

Figure 5. ζ-Potentials of the emulsions as a function of pH. ASSP concentration in all the samples was 0.03 mg/mL.

then 2 months of storage at room temperature (data not shown). This result indicates that droplet aggregation upon heating is effectively prevented. It is obvious that the adsorption of the conjugate on the droplet surface leads to an increase of surface hydrophilicity and an enhancement of steric repulsion, as reported by Difits and Kiosseoglou.[6] One of the important parameters for emulsification is the efficiency of oil encapsulated in the droplets. The data in Table 2 show that about 99% of the oil was encapsulated in the droplets before and after the heat treatment. The high encapsulation efficiency and the high stability of the emulsions indicate that ultrasonic emulsification produces stable oil−water interface films by forming intermolecular disulfide bonds and hydrogen bonding networks.[19−22]

If there is no specific indication, in the following study, for WR 1:3 and 1:6 conjugates, the emulsions were prepared in the condition of ASSP concentration of 20 mg/mL and $\phi = 0.25$; for WR 1:9 conjugate, the emulsions were prepared with an ASSP concentration of 15 mg/mL and $\phi = 0.20$ to reduce the viscosity of the emulsions.

ζ-Potentials of the Emulsions. ζ-Potential directly relates to the net charges on the surface of the macromolecules and particles. Figure 5 shows the ζ-potentials of the emulsions as a function of pH. All emulsions exhibit positive and negative ζ-potentials when the pH of the emulsions is lower and higher than 4.8, respectively, which indicates that the isoelectric point of ASSP is around pH 4.8. Figure 5 shows that the ζ-potential curve of the WR 1:9 mixture emulsion is different from the curve of the WR 1:9 conjugate emulsion, but is similar to the curve of the ASSP emulsion. This result reveals that the dextran in the mixture does not adsorb on the droplet surface. The absolute ζ-potential values of the conjugate emulsions are close to zero in the pH range of 1−10. This result demonstrates that the bulky hydrophilic chains of dextran on the droplet surface greatly decrease the movement of the droplets as they were subjected to the electric field. Compared with the emulsions prepared from WR 1:3 conjugate, WR 1:9 conjugate results in more dextran molecules on the droplet surface and therefore results in smaller ζ-potentials.

Table 3. Droplet Sizes (D_h, nm) of the Emulsions After Long-Term Storage

WR		0 month	2 months	4 months
1:3	mixture	816 ± 14	842 ± 14	880 ± 9
	conjugate	613 ± 2	628 ± 20	666 ± 7
1:6	mixture	803 ± 51	819 ± 10	947 ± 12
	conjugate	522 ± 18	546 ± 27	610 ± 52
1:9	mixture	647 ± 2	636 ± 10	680 ± 22
	conjugate	419 ± 20	445 ± 5	500 ± 4

Stability of the Emulsions against Long-Term Storage and pH and Ionic Strength Changes. The emulsions prepared from WR 1:3, 1:6, and 1:9 mixtures and conjugates were left at 4 °C for 4 months. Then, the droplet sizes were measured, and they are shown in Table 3. The D_h values of the droplets do not change much after the storage. This result further demonstrates that the irreversible adsorption of ASSP on the droplet surface effectively prevents drop−drop coalescence by stabilizing the interface films. However, creaming occurred for all the emulsions prepared from the mixtures. The increase of dextran WR in the mixture, and therefore the increase of the viscosity of the solution, can increase the creaming stability. For the emulsions prepared from 1:3, 1:6, and 1:9 conjugates, the emulsions were homogeneous after 4 months of storage, i.e., no creaming and flocculation layers occurred. This result can be explained by the fact that the dextran conjugated to ASSP effectively increases the steric repulsion and hydrophilicity of the droplets, and thus the emulsions are stable against long-term storage.

The size changes of the emulsions at different pH values were investigated. The emulsions were prepared at pH 8.0, then were changed to pH 1−7. Except the WR 1:9 conjugate emulsion, all emulsions exhibited pH-dependent behaviors: the emulsions were homogeneous after pH change and storage, but creaming and flocculation appeared when the emulsions of pH 4−6 were diluted to 0.0245 mg/mL of ASSP concentration with the same pH solution for DLS measurement. The creaming and flocculation of the conjugate emulsions are much less compared with the mixture emulsions. The data in Table 4 show that the D_h values increase when the pH of the emulsions was changed from 8 to 5. Then, the D_h values decrease with the change of pH from 5 to 1. The pH-dependent changes of D_h can be ascribed to the aggregation of the droplets when the pH of the emulsions is close to 4.8 where the ζ-potential of ASSP is about zero and the solubility of ASSP is minimal. ASSP carries more positive charges when further decreasing pH from 4.8, which causes the aggregates of the droplets to redisperse, so the droplet sizes decrease. For the WR 1:9 conjugate, the interactions of ASSP between different droplets are greatly suppressed by highly hydrated dextran on the droplet surface, and therefore the creaming and flocculation at pH around 4.8 is prevented. The stability of WR 1:9 conjugate emulsions against long-term storage was investigated. The droplet sizes after 2 months of storage are very similar to the sizes of the emulsions prepared freshly. It is obvious that the steric repulsion and hydrophilicity of WR 1:9 conjugate layers on the droplet surface lead to a higher emulsion stability.

WR 1:12 conjugate emulsions also exhibit pH-dependent behavior (Table 4): creaming and flocculation appeared after diluting the emulsions of pH 1−6. Elizalde et al. reported the effect of pH on the relationship between hydrophilic/lipophilic characteristics and emulsification properties of soy protein.[28]

(28) Elizalde, B. E.; Bartholomai, G. B.; Pilosof, A. M. R. *Lebensm. -Wiss. - Technol.* **1996**, *29*, 334–339.

Table 4. Droplet Sizes (D_h, nm) of the Emulsions After Changing Emulsion pH[a] to Different Values

				1:9			
pH	ASSP	1:3 conjugate	1:6 conjugate	mixture	conjugate	conjugate (after 2 months)	1:12 conjugate
8	846	613 ± 2	522 ± 18	647 ± 2	419 ± 20	445 ± 5	627 ± 8
7	1204	599	536 ± 6	706 ± 9	549 ± 15	532 ± 34	649 ± 2
6	2555[b]	1069[b]	715 ± 21[b]	2606 ± 101[b]	547 ± 35	540 ± 12	1150 ± 63[b]
5	2865[b]	1473[b]	1069 ± 34[b]	4281 ± 424[b]	584 ± 26	551 ± 21	1912 ± 142[b]
4	2519[b]	1082[b]	792 ± 22[b]	1818 ± 309[b]	585 ± 14	514 ± 14	1248 ± 10[b]
3	1797	986	720 ± 31	1017 ± 86	575 ± 24	510 ± 11	1201 ± 5[b]
2				859 ± 84	469 ± 61	513 ± 6	1158 ± 6[b]
1				877 ± 91	470 ± 50	495 ± 37	1166 ± 9[b]

[a] The emulsions were prepared at pH 8.0. [b] Creaming and flocculation existed after dilution; data for rough reference only.

Table 5. Droplet Size (D_h, nm) Changes of WR 1:9 Conjugate Emulsions in the Presence of Different Concentrations of NaCl After 4 Months of Storage

	adding NaCl before emulsification			adding NaCl after emulsification[a]		
NaCl concentration (mol/L)	0 month	2 months	4 months	0 month	2 months	4 months
0	419 ± 20	445 ± 5	500 ± 4	419 ± 20	445 ± 5	500 ± 4
0.05	515 ± 11	528 ± 4	587 ± 5	638 ± 18	532 ± 31	633 ± 24
0.10	562 ± 22	632 ± 5	712 ± 26	657 ± 10	576 ± 33	694 ± 22
0.20	606 ± 22	698 ± 18	796 ± 4	688 ± 7	598 ± 16	737 ± 18

[a] Droplet sizes were measured after adding NaCl into the emulsions and then storing for different months.

They proved that relatively high hydrophobicity is needed to enhance emulsion capacity, and high hydration of the interfacial film is necessary for increasing emulsion stability. Compared with the WR 1:9 conjugate, in this study, the increase of dextran molecules in the WR 1:12 conjugate reduces the hydrophobicity of ASSP and therefore reduces the emulsification ability of ASSP.

Salt can screen the electrostatic repulsion of ASSP layers on the droplet surface and therefore can decrease the stability of the emulsions.[5] For the aqueous solutions of WR 1:3, 1:6, and 1:9 mixtures, precipitation appeared when increasing NaCl concentration to 0.1 mol/L. In the aqueous solutions of WR 1:3, 1:6, and 1:9 conjugates, no precipitation appeared when NaCl concentration reached 0.2 mol/L. For the emulsions, however, only WR 1:9 conjugate emulsions are stable in the presence of NaCl. Regardless of whether NaCl was added before or after the emulsification, WR 1:9 conjugate emulsions were homogeneous; neither creaming nor flocculation was observed when the emulsions were diluted by the solutions with the same pH and NaCl concentration. Table 5 shows that the D_h values of WR 1:9 conjugate emulsions do not change much in the presence of 0.05, 0.10, and 0.20 mol/L of NaCl after 4 months of storage. This result indicates that WR 1:9 conjugate on the droplet surface can effectively prevent the aggregation of the emulsions induced by NaCl.

Morphologies of the Conjugate Aggregates and Emulsions. Figure 6A shows the morphology of WR 1:9 conjugate aggregates. The conjugate aqueous solution is turbid (Table 1). The AFM image of Figure 6A verifies that some of ASSP aggregated after the Maillard reaction. Aggregates still exist in the aqueous solution without oil after the ultrasonication that is demonstrated in Figure 6B. The morphologies of the conjugate aggregates before and after the ultrasonication are different. The particles are spherical with smooth surface after the ultrasonication (Figure 6B). The heights of the particles in Figure 6A are in the range of 2−6 nm, while in Figure 6B they are 4−9 nm. Liu et al. reported that ultrasound can improve the solubility of soy protein.[17] On the other hand, Gulseren et al. reported that the increased particle size and decreased number of free sylfhydryl groups in BSA solution after ultrasonication may be attributed to the formation of protein aggregates.[19] The study of silk fibroin by Wang et al. found that ultrasonication initiates the formation of β-sheets by alteration in hydrophobic hydration, thus accelerating the formation of physical cross-links responsible for gel stabilization.[18] Suslick group also proved that ultrasound can promote the formation of disulfide bonds and hydrogen bonding networks.[20−22] Recently, Li et al. reported that ultrasound can destroy the aggregates of glutamic dendron and induce gel formation.[29] In our study, the difference shown in Figure 6A,B demonstrates that the ultrasonication destroys the original aggregates in the conjugate solution and induces the formation of new aggregates.

The morphology of the emulsion droplets prepared from WR 1:9 conjugate was observed by AFM. Figure 6C,D exhibits two kinds of droplets: larger and smaller ones. The largest droplet in Figure 6C is elliptoid, having two axes of 2.3 and 3.0 μm and a height of 30 nm. The smaller droplets in Figure 6C have an average diameter about 80 nm and an average height about 21 nm, which are larger than the conjugate aggregates shown in Figure 6A,B. Figure 6C,D shows that a few smaller droplets are on the larger ones. This result verifies that the droplet aggregates exist in the emulsion, which is consistent with the results shown in Figures 3 and 4. The droplet surface contains a few very small particles that may be the conjugate aggregates adsorbed in the ultrasonication emulsification process.

We also used egg ovalbumin to prepare ovalbumin−dextran conjugates and emulsions (Supporting Information). The results demonstrate that the emulsions having D_h values of about 360 nm are very stable in the pH range of 1−7 investigated, further verifying that ultrasonication is an effective method to prepare stable emulsions from protein−polysaccharide conjugates. During the ultrasonication emulsification process, the increase of surface activity and aggregation of protein can increase the adsorption and irreversible attachment of the protein molecules on the droplet surface that effectively improve emulsification capability and stability. The conjugates with highly hydrated dextran adsorbed at the interface together with unreacted protein

(29) Li, Y. G.; Wang, T. Y.; Liu, M. H. *Tetrahedron* **2007**, *63*, 7468–7473.

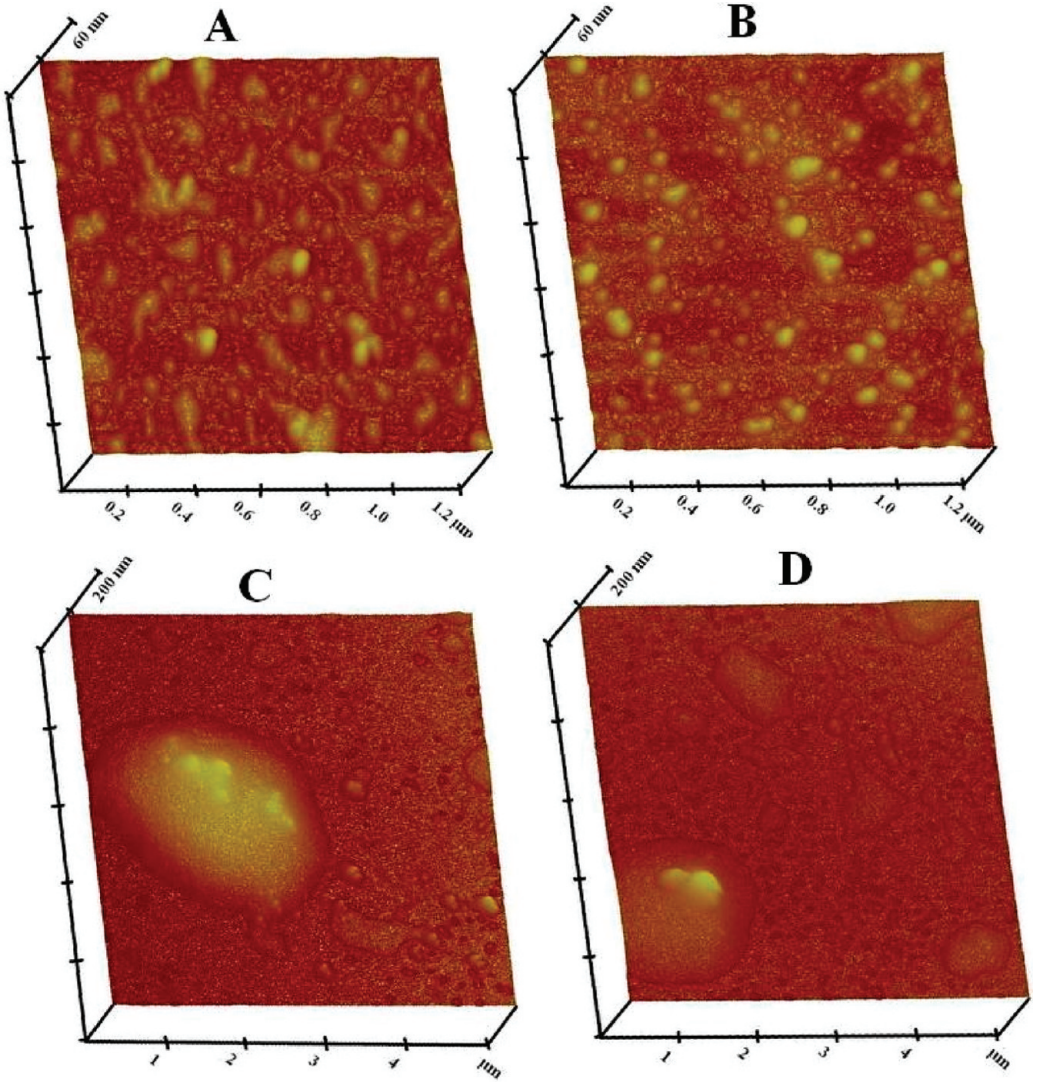

Figure 6. AFM images of WR 1:9 conjugate aggregates formed before (A) and after (B) ultrasonication. AFM images of WR 1:9 conjugate stabilized oil droplets formed after ultrasonication (C and D).

further enhance the stabilization of the oil droplets. Once the stable interface films formed at neutral pH, the dextran molecules on the surface make the oil droplets stable against heat treatment, long-term storage and changes of pH and ionic strength.

Conclusion

Dextran was conjugated to ASSP in this report to improve the solubility and/or dispersibility of ASSP in the pH range of 4−7 through the Maillard reaction. Ultrasonication was applied to prepare stable emulsions with submicrometer droplets. Ultrasonication can increase the surface activity and protein aggregation that result in the formation of stable oil−water interface films. The dextran molecules conjugated to the protein enhance the hydrophilicity and steric repulsion of the emulsions. The emulsions prepared from WR 1:9 conjugate at neutral pH are very stable against heat treatment, long-term storage, and changes of pH and ionic strength. The knowledge obtained in this study can be effectively applied to prepare other stable protein−polysaccharide conjugate emulsions through ultrasonication emulsification.

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Supporting Information Available: The preparation and characterization of the emulsions stabilized by ovalbumin−dextran conjugate. This material is available free of charge via the Internet at http://pubs.acs.org.