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ARTICLE

# Physicochemical and Emulsifying Properties of Whey Protein Isolate (WPI)−Dextran Conjugates Produced in Aqueous Solution

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The physicochemical and emulsifying properties of protein and polysaccharide conjugates prepared under mild conditions were investigated. The covalently linked conjugates of whey protein isolate (WPI) and dextran (DX, 440 kDa) were produced by incubating aqueous solutions containing 10% WPI and 30% DX at pH 6.5 and 60 °C for 48 h. After purification by anion-exchange chromatography and affinity chromatography, the conjugate had a weight-average molecular weight ($M_w$) of 531 kDa and a radius of gyration ($R_g$) of 30 nm as determined by size exclusion chromatography−multiangle laser light scattering (SEC-MALLS); the molar binding ratio of WPI to DX was calculated to be ∼1:1. The purified conjugate had significantly improved heat stability when subjected to 80 °C for 30 min and remained soluble over a range of pH from 3.2 to 7.5 and ionic strengths from 0.05 to 0.2 M in contrast to native WPI. The emulsifying ability and emulsion stability made with WPI−DX conjugate were also improved compared to WPI and gum arabic (an emulsifier containing naturally derived glycoproteins).

**KEYWORDS: Whey protein isolate; dextran; conjugate; functionality**

## INTRODUCTION

In the early 1980s, it was recognized that covalent coupling of polysaccharides to proteins via chemical cross-linking could greatly increase the solubility, heat stability, and emulsifying properties of proteins (*1*, *2*). However, this method is not appropriate for food applications because of the toxicity of these chemicals. Soon after that, the Maillard reaction, which involved dry heating of freeze-dried mixtures of protein and polysaccharides, was used to produce conjugates, and this attracted the attention of many researchers. It has been reported that the conjugates made by the dry-heating process and using various polysaccharides and proteins from whey (*3*−*6*), egg (*7*), soybean (*8*), and fish (*9*) have excellent emulsion stability with respect to creaming, flocculation, and coalescence, as compared to their respective native proteins. However, the dry-heating method is not feasible for large-scale production. No commercially manufactured conjugate ingredients are available (*10*). Previously, we reported a novel processing method to prepare conjugates in a mixture of highly concentrated whey protein isolate (10% WPI) and dextran (30% DX) solution by using the macromolecular crowding effect (*10*). Crowding occurs in systems with high concentrations of macromolecules as this reduces the volume of solvent available for other molecules; by limiting the excluded volume available for unfolding, crowding helps stabilize the native protein structure (*23*). We monitored the formation of a Schiff base, which is the initial product of the Maillard reaction, and confirmed that DX was covalently attached to WPI. This method greatly simplifies the process of conjugation.

In this study, we selected WPI and DX with a molecular weight of ∼440 kDa as the source of proteins and polysaccharide, respectively. Whey proteins are widely used as ingredients due to their excellent emulsification properties. Whey proteins consist of several types of globular proteins, α-lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA), and immunoglobulins (IG). Both α-la and β-lg adsorb to oil−water interfaces and are capable of stabilizing emulsions (*11*). However, for whey proteins, stable emulsions were not obtained at protein concentrations below 2%. Increasing the protein concentration resulted in the adsorption of additional protein to the interface, which produces more stable emulsions (*12*). The stability of emulsions made with whey proteins decreased at pH values near the isoelectric point (p$I$ ∼ 4.8) and at higher salt concentrations (0−100 mM NaCl), leading to flocculation (*13*). In addition, whey proteins are prone to heat-induced denaturation. Heating of whey proteins prior to or after preparation of emulsions resulted in the aggregation of the emulsion droplets (*13*). Therefore, for WPI, any improvement in the physicochemical and emulsifying properties under the conditions of high salt concentration, high heat temperature, and acidic pH range (which are often encountered in the food industry) has been a challenge for food scientists. DX was chosen because it is a neutral molecule, avoiding the complication resulting from the formation of electrostatic complexes, which often occurs in mixtures of proteins with anionic polysaccharides (*3*); DX also has high solubility and low solution viscosity. DX with an average molecular weight of 440 kDa was

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selected because it has been reported that larger molecular weight polysaccharides impart better emulsion stabilization than lower molecular weight ones (*6*).

Our objectives were to produce a WPI−DX conjugate in aqueous solution by heating the mixture of 10% WPI and 30% DX at 60 °C for 48 h on the basis of the method we previously described (*10*) and investigate the physicochemical and emulsifying properties of the resultant purified WPI−DX conjugates. The higher the molecular weight of the DX, the slower of WPI−DX conjugation reaction rate is (Schiff base formation) (unpublished data). Thus, a reaction time of 48 h was chosen for the conjugation of 10% WPI and 30% DX (440 kDa) to produce a sufficient amount of Schiff base for functionality testing (*10*).

## MATERIALS AND METHODS

**Materials.** WPI was kindly provided by Davisco Foods International, Inc. The total amount of protein in the dry powder was >95%, and the lactose was low (<1%). DX from *Leuconostoc mesenteroides* with an average molecular weight of 440 kDa and soybean oil were obtained from Sigma-Aldrich (St. Louis, MO). Both WPI and DX were used directly without further purification in this study. The molecular weight standard was purchased from Fisher Scientific (Fisher BioReagents *EZ-RUN* prestained Rec protein ladder, Pittsburgh, PA). Milli-Q water (Millipore) was used for all of the experiments.

**Preparation of WPI−DX Conjugates.** On the basis of the previously reported method (*10*), the WPI, DX, and 10 mM sodium phosphate buffer solution (PBS) (pH 6.5, 0.02% (w/w) sodium azide to prevent bacterial growth) were mixed to make a dispersion in which the concentration of WPI was 10% (w/w) and DX was 30% (w/w). The mixture was stirred on a stirring plate for 2 h at room temperature (~22 °C), to dissolve the mixture. The pH of the mixture was adjusted to 6.5 by carefully adding 0.1 N HCl. Subsequently, the mixture was transferred to 5 °C with gentle stirring overnight to allow for the complete hydration of both macromolecules. The following morning, the hydrated mixture of 10% WPI and 30% DX was placed in a water bath (60 °C). The conjugation reaction (Schiff base formation) was monitored by measuring the difference UV (DUV) absorbance value at ~305 nm (see below). The reacted solution (crude conjugate) was then removed from the water bath and immediately cooled in an ice−water bath.

**Difference UV Spectroscopy (DUV).** DUV measurements were carried out as described by Zhu et al. (*10*). The DUV was performed on a UV−visible spectrophotometer (Shimadzu UV-1601 PC, Shimadzu Corp., Kyoto, Japan) in a 1 cm quartz cell at a constant temperature of 20 °C. The crude conjugate was diluted 33.3-fold, followed by centrifugation at 16000*g* for 10 min in an Eppendorf centrifuge (model 5414, Brinkmann Instruments Inc., Westbury, NY) at room temperature. The supernatant solution was used for a wavelength scan from 270 to 500 nm. The difference absorption spectra of conjugates after processing were recorded against an unreacted sample solution (as reference).

**Purification of WPI−DX Conjugate.** The crude WPI−DX conjugate mixture was diluted 3-fold with 0.5 M PBS (pH 6.5, containing 0.02% $NaN_3$) and subsequently centrifuged at 10000*g* for 30 min. The precipitate was associated DX, which could be disassociated and redissolved in the PBS by autoclaving at 121 °C for 15 min. The precipitate was not used for purification because it was DX. The supernatant solution was separated with an anion-exchange column (diethylaminoethyl cellulose, DE 53, Whatman International Ltd., Maidstone, U.K.). The column (5.5 × 60 cm) was washed with 10 mM PBS buffer and eluted with a linear gradient of 0−0.5 M NaCl in PBS. The fractions were monitored by the absorbance at 280 nm for protein content and the absorbance at 550 nm on a microplate reader for carbohydrate content by using a Glycoprotein Carbohydrate Estimation kit (Pierce, Rockford, IL). The unreacted DX and most of the unreacted WPI were removed. The fractions that contained the residual unreacted WPI and conjugate were pooled. The DE-purified conjugates were dialyzed against water in a dialysis membrane tube with a molecular mass cutoff of 3500 Da and lyophilized. The lyophilized DE-purified conjugates (also containing unreacted WPI) were dissolved in water (~1%, w/v) and further separated on an affinity column (concanavalin A Sepharose 4B, Amersham Biosciences, GE Healthcare Life Sciences). The column (3 × 23 cm) was washed with a solution containing 0.5 M NaCl, 1 mM $Mn^{2+}$, and 1 mM $Ca^{2+}$ and eluted with a linear gradient of 0−0.1 M methyl-α-D-mannopyranoside containing 0.5 M NaCl, 1 mM $Mn^{2+}$, and 1 mM $Ca^{2+}$. The fractions were monitored for their absorbances at 280 and 550 nm, for protein and carbohydrate contents, respectively, as described above for the DE separation procedure. The unreacted WPI was removed by this method. The fractions containing conjugate were pooled and dialyzed against water in a dialysis membrane tube with a molecular mass cutoff of 50000 Da and then lyophilized. This lyophilized, purified WPI−DX conjugate was used in all of the investigations in this study, unless otherwise specified.

**Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE).** SDS-PAGE was performed on a Mini-Protean 3 cell (Bio-Rad Laboratories, Hercules, CA) according to the method of Laemmli (*14*). Nonreducing and reducing SDS-PAGE analyses were carried out on a Ready Gel (Tris-HCl gel, 4−20% linear gradient, 15 well, Bio-Rad Laboratories). Sample solution (15 μL corresponding to 40 μg of whey protein) was loaded into each well. Electrophoresis was run for 35 min at 200 V in a 0.025 M Tris-HCl buffer solution (pH 8.3, including 0.192 M glycine and 0.1% SDS, w/w) at room temperature. Two gels were run at the same time. After electrophoresis, one gel was stained for protein by a Coomassie blue stain kit (Biosafe, Bio-Rad Laboratories Inc.). The other gel was stained for carbohydrate by the GelCode Glycoprotein staining kit (Pierce Biotechnology, Rockford, IL), respectively.

**Determination of the Molecular Weight by Size Exclusion Chromatography with Multiangle Laser Light Scattering (SEC-MALLS).** The purified WPI−DX obtained above was dissolved in 10 mM PBS. The sample solution was filtered through a 0.45 μm filter before injection into the SEC column. WPI−DX conjugate (1%) was applied to a SEC-MALLS system, using a tandem array of a Superose 6HR 10/30 and Superose 12HR 10/30 columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at a flow rate 0.3 mL/min. The SEC was connected to a Waters 600 HPLC system. Sample injection volume was 100 μL. Ten millimolar PBS was used as an eluent. Elution from columns was monitored at 280 nm with a photodiode array (PDA) detector (Waters Corp., Milford, MA; model 996) and with a differential refractive index (DRI) detector (Waters Corp.; model 2410) and a MALLS photometer (DAWN-EOS, Wyatt Technology, Santa Barbara, CA) (fitted with a helium−neon laser ($\lambda$ = 690 nm) and a K5-flow cell). A value of 0.151 mL/g was used for the refractive index increment (d$n$/d$c$), as this is the reported value for DX (*15*). Data collected by the PDA, DRI detectors, and MALLS photometer were processed by the software ASTRA (version 4.73 04, Wyatt Technology) to calculate the weight-average molecular weight of the WPI−DX conjugate according to the method of Lucey et al. (*16*). The SEC-MALLS measurement was carried out at ~22 °C.

**Determination of Molar Ratio of WPI to DX in Conjugate.** The purified WPI−DX conjugate was dissolved in 10 mM PBS. The total protein content was assayed using a BCA protein assay kit (Pierce), and total carbohydrate content was assayed by a glycoprotein carbohydrate estimation kit (Pierce). The purity of the WPI−DX conjugate was further examined using SDS-PAGE by overloading (60 μg of sample/lane). The unreacted WPI was quantified by the densitograms of SDS-PAGE, which is based on the band intensity, and SEC-MALLS analysis, which is based on the corresponding eluted peak area.

**Solubility and Heat Stability at Various pH Values and Ionic Strengths.** The solubility of WPI−DX conjugates was assessed by turbidimetry of the protein solution at various pH values and ionic strengths (*1*). The turbidity of samples is expressed as the absorbance at 500 nm as determined on a UV−visible spectrophotometer (Shimadzu UV-1601 PC, Shimadzu Corp., Kyoto, Japan) in a 1 cm cuvette at 20 °C.

The effect of pH on the solubility of WPI−DX conjugate at low ionic strength was determined as follows: purified WPI−DX conjugates and native WPI were dissolved in Milli-Q water. The final protein concentration was 1.0 mg/mL (i.e., protein portion of the conjugate). The pH of the sample solutions was adjusted to the required value by adding 1 N NaOH or 1 N HCl dropwise with gentle stirring. Additional water was added to make all sample volumes consistent, and the final pH values of all solutions were determined. The solutions at each pH were divided into two parts (1.5 mL for each): one was submitted for heating at 80 °C for 30 min in a thermostatically controlled water bath, then rapidly cooled under tap water; the other portion was left at room temperature (22 °C). The absorbance of all samples was measured at 500 nm.

To evaluate the effect of ionic strength on the solubility of WPI−DX conjugate, the following procedure was used. The purified WPI−DX conjugate and native WPI were dissolved in 10 mM PBS, and the pH was adjusted to 4.5. The final protein concentration was 1.0 mg/mL (protein portion for the conjugate). A given volume of NaCl (3.6 M), diluted if necessary, was added to reach the desired ionic strengths. The sample solutions were allowed to stand for ~2 h at room temperature. Then the sample solutions with different ionic strengths were divided into two parts (1.5 mL for each): one was submitted to heating at 80 °C for 30 min in a thermostatically control water bath and then rapidly cooled with tap water; the other fraction was left at room temperature (22 °C). The absorbance of all samples was measured at 500 nm.

**Thermal Analysis by Differential Scanning Calorimetry (DSC).** The native WPI (10% w/w), DX (10% w/w), mixture of 4% WPI and 36% DX (w/w) without heat treatment, and purified WPI−DX conjugate (10% w/w) were dissolved in 10 mM PBS. A 620 mg sample solution was sealed in a stainless steel crucible with an O-ring lid. The reference crucible contained 10 mM PBS. DSC curves were recorded on a DSC instrument (Micro DSC VII, Setaram Inc., Scientific and Industrial Equipment, Pennsauken, NJ), which was programmed at a heating rate of 1 °C/min for the temperature range from 20 to 120 °C.

**Microstructure by Transmission Electron Microscopy (TEM).** A drop of the sample (5 $\mu$L of a 0.1% solution) was spotted onto a Pioloform (Ted Pella Inc., Redding, CA) carbon-coated copper 200 grid for 1 min. After five washings with distilled water, the grid was treated with Nano-W (containing methylamine tungstate, Nanoprobes Inc., Yaphank, NY) for staining. The grid was air-dried and examined with a JEOL 200CX TEM (JEOL USA, Inc., Peabody, MA) at an accelerating voltage of 200 kV at the Materials Science Center of the University of Wisconsin-Madison.

**Emulsion Preparation.** The various materials were dissolved in 10 mM PBS (pH 6.5, 0.02% sodium azide) to make 2% (w/v, total solids) sample stock solutions with gentle stirring at 22 °C. The pre-emulsions (2.0 mL) were formed by mixing the soybean oil into the protein solutions (finally containing 20% (v/v) soybean oil and 0.5% (w/v) conjugate sample) and vortexed at 3000 rpm for 2 min by using a vortex mixer. O/W emulsions were prepared by homogenizing the pre-emulsions through a high-pressure homogenizer (EmulsiFlex-B3, Avestin, Inc., Ottawa, Canada) at an operating hydraulic pressure of 152 MPa with three passes. The discharged emulsion for each pass was received in a glass tube, which was kept in a water/ice bath to cool the emulsion. Emulsions were stored at 22 °C, and the particle sizes were determined.

**Particle Size Determination.** Emulsion droplet size distributions were measured by a static laser light scattering analyzer (Malvern MasterSizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, U.K.). The relative refractive index of the disperse phase was 1.473; the dispersion medium was 1.330. The value of weight-average mean diameter ($d_{43}$) was used to monitor changes in droplet size distribution on storage (*4*). The mean diameter $d_{43}$ was defined as

$$d_{43} = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3}$$

where $n_i$ is the number of droplets of diameter $d_i$.

## RESULTS

**DUV Spectrum.** The WPI−DX conjugate was confirmed to be a Schiff base by DUV. The DUV spectrum of the WPI−DX conjugate was recorded by scanning between 270 and 500 nm. As shown in **Figure 1**, the curve was characterized by a maximum difference absorbance peak at 305 nm, an indication of Schiff base formation (*10*).

**Confirmation of WPI−DX Conjugate by SDS-PAGE.** To further confirm the covalent coupling of DX to WPI, SDS-PAGE was performed as shown in **Figure 2**. On the top of the gel, a broad dense band was observed for the WPI−DX conjugate and the band diffused into the gel under protein staining (lane 2, **Figure 2A**). The same band was also observed under carbohydrate staining (lane 5, **Figure 2A**), indicating that DX was covalently bonded with WPI because most noncovalent interactions are generally disrupted in SDS-PAGE and the uncharged DX does not migrate in gel

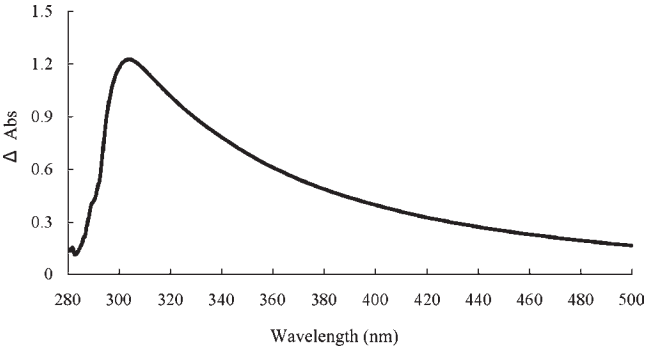


**Figure 1.** Formation of Schiff base made of 10% WPI−30% DX (60 °C for 48 h) by DUV spectroscopy (33.3-fold dilution).

electrophoresis. In the presence of 2-ME, the broad dense band also appeared on the top of the gel in lane 2 (protein staining) and lane 5 (carbohydrate staining) in **Figure 2B**. This indicated that the covalent bond in the WPI−DX conjugate, which could not be disrupted by 2-ME, was not caused by a disulfide bond but was likely Schiff base formation (i.e., C═N bond). The broad diffusive nature of the band is due to the polydispersity of DX. No such broad diffusive bands near the top of the lanes were observed for the mixture at 0 h (lanes 3 and 6, **Figure 2**) and native WPI (lanes 4 and 7, **Figure 2**). A faint diffuse band, stained by both protein and carbohydrate, was observed at the tops of lanes 3 and 6, respectively, for the WPI + DX mixture at 0 h (**Figure 2**). This could be some WPI−DX conjugate formed on mixing WPI (10%) with DX (30%) because the mixture 0 h sample was treated by dialysis and then freeze-drying as per the heated samples. Other bands that had migrated into the gels were identified as whey proteins: α-la, $\beta$-lg, $\beta$-lg dimer, and BSA in the absence and presence of 2-Me and assigned as shown in **Figure 2**. The intensity of those bands was almost the same as for the mixture at 0 h (lane 3) and native WPI (lane 4) but considerably reduced for WPI−DX conjugate (lanes 2) in **Figure 2**, suggesting that the whey proteins reacted with DX and formed the conjugate that appeared on the tops of the gels (lanes 2, **Figure 2**).

**Characterization of WPI−DX Conjugate.** The purified WPI−DX conjugate was a white lyophilized powder. The total protein content and total carbohydrate content in the WPI−DX conjugate was determined. SDS-PAGE under sample overloading conditions (60 $\mu$g/lane) showed that some unreacted WPI was still present in the purified WPI−DX conjugate (data not shown). Further analysis by SEC-MALLS showed that the WPI−DX conjugate consisted of ~2.2% unreacted WPI, ~7% WPI in conjugate, and ~91% DX (by weight) in conjugate. The weight-average molecular weights ($M_w$) were 531 and 440 kDa for the WPI−DX conjugate and DX, respectively, by SEC-MALLS. The radii of gyration ($R_g$) were 30 and 22 nm for the WPI−DX conjugate and DX, respectively. The plot of molecular weight of WPI−DX conjugate as a function of elution volume is shown in **Figure 3**. The size of DX was in agreement with published reports. For example, DX with a $M_w$ of 506 kDa was reported to have a $R_g$ of 21 nm (*15*), and DX with a $M_w$ of 440 kDa had a hydrodynamic diameter of 18 nm (*17*). The average molecular weight of WPI was reported as 30400 Da (*18*). By calculation, the mole ratio of WPI to DX in conjugate was ~1. WPI has an average value of ~15 amine groups (N-terminal and lysine residues) per mole available for Maillard reaction (*19*). DX has only one reducing group per mole. Despite this high amine/carbonyl group ratio in the reaction mixture, the product yield was only about 10% based on WPI content used in the reaction mixture. This low yield might be due to steric hindrance imposed by the large DX chain on potential interaction with the carbonyl group of WPI.

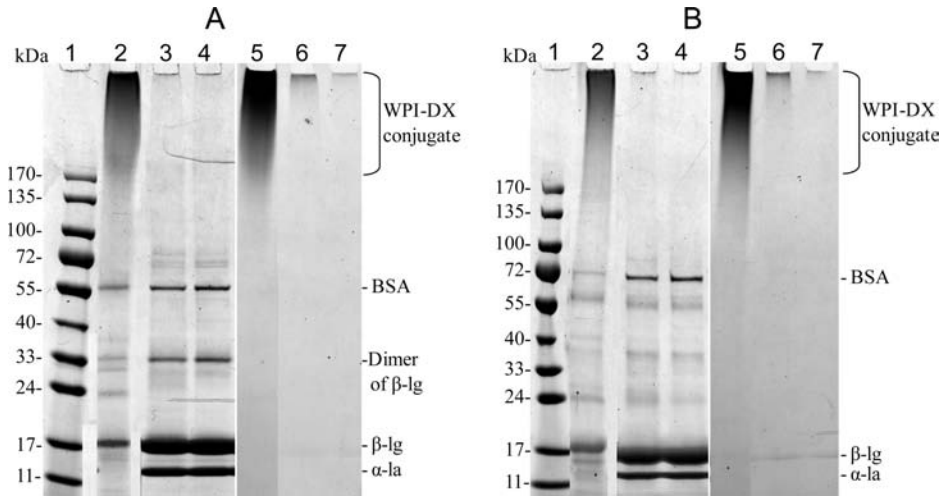


**Figure 2.** SDS-PAGE of WPI−DX conjugate (60 °C for 48 h) in the absence (**A**) and presence (**B**) of 2-Me. Lanes: 1, molecular weight marker; 2, 3, and 4, stained for protein; 5, 6, and 7, stained for carbohydrate; 2 and 5, WPI−DX conjugate; 3 and 6, mixture at 0 h of 10% WPI−30% DX (without any heat treatment, but the mixture was dialyzed in water and freeze-dried as conjugate was); 4 and 7, native 10% WPI without treatment. Loading = 5 $\mu$g of protein/lane.

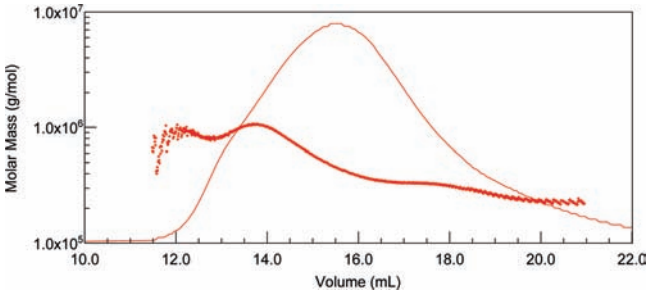


**Figure 3.** Molecular weight as a function of elution volume for 1% (w/v) WPI−DX conjugate. The elution profile is overlaid with the calculated molar mass (◆) and MALLS at the 90° angle (—).

**Micrographs of Purified WPI−DX Conjugate Observed by TEM.** The images of the native WPI, DX, and WPI−DX conjugate were observed by TEM (**Figure 4**). WPI appears as very tiny particles with a size of ~3−5 nm (**Figure 4A**). It has been reported that $\beta$-lg monomer and whey protein concentrate (WPC 80) are 2.5 nm by atomic force microscopy (*20*). DX appears to have phase-separated, with spherical-like structures (see arrow a) or a continuous zone (see arrow b) (**Figure 4B**). Considerable variation in size was observed. For example, the DX indicated by arrow b was ~850 nm in diameter. This is quite different from the result obtained by SEC-MALLS, where DX had a $R_g$ of 22 nm. It is considered that the image of DX is the assembly of many molecules, which is probably caused by the incompatibility between very hydrophilic DX and film on the grid during the specimen preparation for TEM observation. WPI−DX conjugate appears as particles with sizes of ~15−30 nm (**Figure 4C**), which is similar to the results obtained by SEC-MALLS.

**Effect of pH, Ionic Strength, and High Temperature on the Solubility of WPI−DX Conjugate.** The solubility was estimated by measuring the turbidity of sample solutions at 500 nm. The higher the turbidity, the lower is the solubility of the protein or the conjugate. The heat treatment used was 80 °C for 30 min because WPI was denatured under that condition. As shown in **Figure 5A**, heated WPI had higher absorbance in the pH range from 3.5 to 6 with a maximum absorbance peak at pH ~4.8; that is, there was a significant decrease of solubility in this pH region. This means that WPI was heat unstable. Native WPI, WPI−DX conjugate, and heated WPI−DX conjugate (that was heated at 80 °C for 30 min) had very low absorbances, indicating that they had good solubility over the pH range from 3.2 to 7.5. In **Figure 5B**, which shows a magnified absorbance axis, there was an increase in absorbance for native WPI at pH 4−5.5 and a maximum absorbance peak at pH ~4.8. The decrease in solubility for native WPI at pH 3.5−6 was due to the neutralization of charge on the WPI molecules because the isoelectric point (p*I*) of WPI was 4.8−5.4 (*21*). In contrast, for both unheated and heated WPI−DX conjugates, there was no change in absorbance over the range of pH 3−7.5, which indicated that the WPI−DX conjugate was both heat and pH stable. The addition of salt (0.05−0.2 M NaCl, pH 4.5, 22 °C) resulted in the same changes in the absorbance for either WPI or WPI−DX conjugate no matter whether it was heated or not. Regardless of ionic strength, the changes in absorbance at 500 nm were in the following order: WPI−DX conjugate (Abs ~0.009) ≤ heated WPI−DX conjugate (Abs ~0.013) < native WPI (Abs ~0.044) ≪ heated WPI (Abs ~0.300). This suggested that the solubilities of both the WPI−DX conjugate and heated WPI−DX conjugate were higher than that of native WPI and much higher than that of heated WPI at ionic strengths 0.05−0.2 M. A small increase in the absorbance for the heated WPI−DX conjugate compared with the untreated WPI−DX conjugate was probably due to the presence of a low amount of unreacted WPI in the conjugate.

**Thermal Behavior of WPI−DX Conjugate by DSC.** To further understand the heat stability of the WPI−DX conjugate, the thermal behavior was studied by DSC. As seen from the DSC thermogram in **Figure 6**, 10% WPI showed a typical endothermic denaturation profile with a peak centered at ~74 °C ($\beta$-lg) and a shoulder at ~66 °C (α-la) (curve 1), and similar results were observed by de Wit and Klarenbeek (*22*). For 10% DX, it was almost a flat line (curve 2), indicating that DX was thermally stable in the temperature range investigated. For the mixture of 4% WPI and 36% DX, the endothermic denaturation profile was similar to that of WPI, but the denaturation peak shifted to ~71 °C ($\beta$-lg) and the shoulder shifted to ~64 °C (α-la) (curve 4), suggesting a slight decrease in the heat stability of native WPI in the presence of a high concentration of DX. For the 10% (total w/w) WPI−DX conjugate, like 10% (w/v) DX, it was almost a flat line (curve 3). This suggested that WPI in the WPI−DX conjugate was probably in an unfolded state. Hattori et al. (*24*)

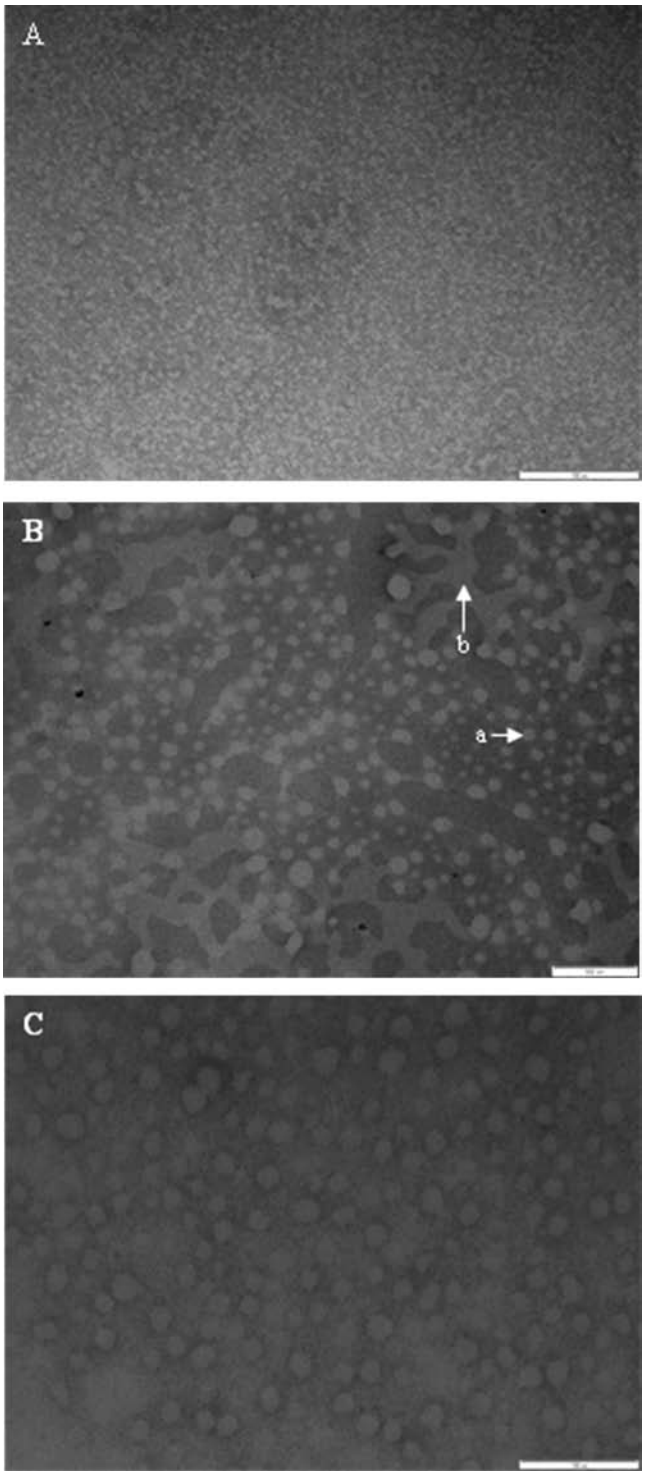


**Figure 4.** Images of native WPI (**A**), DX (**B**), and WPI−DX conjugate (**C**) by TEM. The bar represents 100 nm (**A** and **C**) or 500 nm (**B**).

observed that the $\Delta H$ for the conjugate of $\beta$-lg and carboxymethyl dextran decreased to ~40% of the value for $\beta$-lg alone. They explained that this was due to a decrease in the secondary structure content, such as α-helix, in $\beta$-lg as a result of conjugation with carboxymethyl. Chevalier et al. (*25*) thought that $\beta$-lg underwent extensive unfolding when it was glycated with ribose or arabinose because they did not observe any thermal transition in DSC thermograms of the conjugates.

**Emulsifying Properties of WPI−DX Conjugate.** Emulsifying ability and emulsion stability of the oil droplets stabilized by various materials were evaluated by measuring the droplet size $d_{43}$

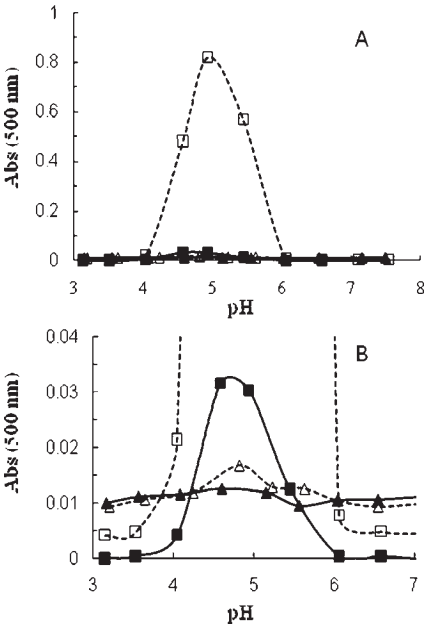


**Figure 5.** Solubility of WPI−DX conjugate and WPI as a function of pH: (■) WPI at 22 °C; (□) heated WPI (80 °C for 30 min); (▲) WPI−DX conjugate at 22 °C; (△) heated WPI−DX conjugate (80 °C for 30 min). Protein concentration is 1.0 mg/mL. **B** is the same as **A**, but with scales expanded.

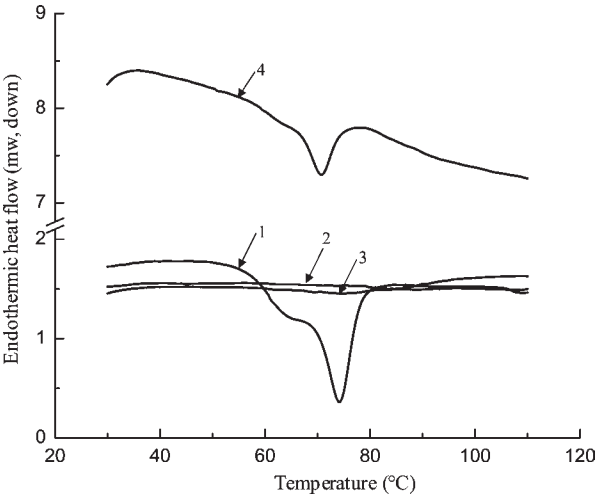


**Figure 6.** DSC thermograms of 10% WPI (curve 1), 10% DX (curve 2), 10% WPI−DX conjugate (curve 3), and the mixture of 4% WPI−36% DX (curve 4) at a scan rate 1 °C/min.

and the visual observation of coalescence. As shown in **Figure 7**, both the WPI−DX conjugate and the heated WPI−DX conjugate (80 °C for 30 min) had better emulsifying ability than other materials. The emulsifying ability of the materials was in the order WPI−DX conjugate ($d_{43}$ ~ 0.94 $\mu$m) > heated WPI−DX conjugate ($d_{43}$ ~ 1.2 $\mu$m) > native WPI ($d_{43}$ ~ 1.6 $\mu$m) > gum arabic ($d_{43}$ ~ 2.2 $\mu$m) > mixture of 10% WPI and 30% DX (0 h) ($d_{43}$ ~ 7.3 $\mu$m) > heated WPI (60 °C for 48 h) ($d_{43}$ ~ 13 $\mu$m). Although WPI alone showed good emulsifying ability, it should be noted that the protein content in WPI alone is almost 10 times higher than that in the conjugate. The heated WPI could not form a very stable emulsion. After 4 weeks of storage at room temperature, the oil droplet sizes ($d_{43}$) increased to ~1.4 $\mu$m for the conjugate, ~1.6 $\mu$m for the heated conjugate, and > 2 $\mu$m for all other materials. After 8 weeks of storage at room temperature,

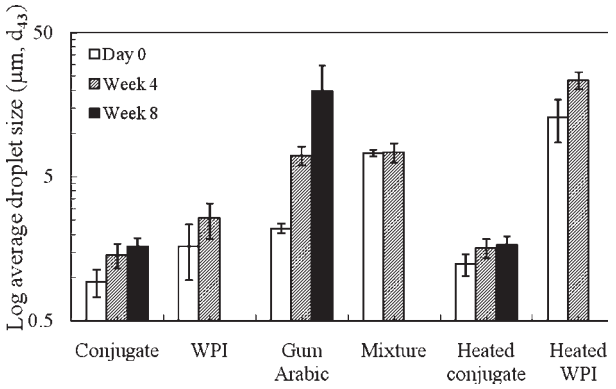


**Figure 7.** Comparison of average droplet sizes $d_{43}$ for emulsions containing 20% (v/v) soybean oil and 0.5% (w/v) emulsifier stabilized by WPI−DX conjugate, WPI, gum arabic, mixture of 10% WPI and 30% DX at 0 h (without any heat treatment but the mixture was dialyzed in water and freeze-dried as conjugate was), heated WPI−DX conjugate (80 °C for 30 min prior to emulsion), and heated WPI (60 °C for 48 h prior to making emulsion at day 0, week 4 and week 8). Samples were stored at 22 °C. Replication $n = 3$.

emulsions stabilized by WPI, heated WPI, and 10% WPI + 30% DX mixture completely coalesced. Therefore, no particle size data were available. Gum arabic-stabilized emulsion also coalesced with a $d_{43}$ of ~20 μm. A slight flocculation was observed for conjugate and heated conjugated-stabilized emulsions. However, after gentle shaking by hand, they redispersed. These results suggested that the WPI−DX conjugate produced by our aqueous reaction method has improved emulsion stability compared to the WPI alone or gum arabic. The improved emulsifying property of the WPI−DX conjugate made in aqueous solution is in agreement with the whey protein−DX conjugates obtained via dry-heating method (*3*, *4*, *6*). Heating of the WPI−DX conjugate prior to preparation of the emulsion did not affect its emulsifying properties, whereas heating WPI prior to making the emulsion resulted in greatly impaired emulsification properties. This could be due to the superior heat stability of the WPI−DX conjugate compared to WPI. The mixture of 10% WPI and 30% DX (weight ratio of WPI to DX would be 1:3 in dry powder) had inferior emulsifying properties compared to WPI alone. This could be explained by the relatively low content of WPI in the mixture compared with the WPI alone sample and the presence of DX.

## DISCUSSION

It is well-known that the improved solubility and heat stability of the protein polysaccharide conjugates are attributed to the attachment of the bulky highly hydrophilic polysaccharides to proteins (*1*, *9*). The covalent bonds produced between proteins and polysaccharides in Maillard-type conjugates are very stable against changes in pH, temperature, and ionic strength (*27*). The WPI−DX conjugate exhibited high solubility over the pH range from 3.2 to 7.5 and ionic strengths from 0.05 to 0.2 M and good heat stability regardless of pH and ionic strength compared to WPI. We assumed that these findings could be ascribed to the molecular structure of the WPI−DX conjugate. The WPI−DX conjugate consisted of 7% WPI and 93% DX (by weight). For the WPI−DX conjugate, the charged WPI portion was relatively small in mass compared to the neutral DX portion. It was possible that the DX portion dominated the physicochemical properties of the conjugate so that the influence of pH and ionic strength on the WPI portion could not cause the insolubility of the whole conjugate molecule.

WPI tended to be adsorbed at the emulsion interface and covered the oil droplets, thus stabilizing the droplets from flocculation, creaming, and coalescence. Ideally, if there was sufficient protein available, it could completely cover and stabilize the oil droplets. Once WPI is adsorbed on oil droplets, the molecules become unfolded. Droplet aggregation resulted from noncovalent interactions between unfolded protein molecules adsorbed on the different droplets, and the interactions can be strengthened by disulfide bonds (*26*). Furthermore, nonadsorbed protein acts as "glue" and holds the aggregated droplets together, leading to creaming (*13*). For the WPI−DX conjugate, we considered its improved emulsifying properties to be due to the following:

(*1*) *Thick Steric Barrier*. In WPI−DX conjugate, the hydrophobic moiety on the WPI portion firmly adsorbed at the interface of the oil droplets, and the hydrophilic DX portion protruded into the aqueous medium and formed a protective layer, conferring improved emulsion stability. In addition, from the viewpoint of emulsifier size, the WPI−DX conjugate had a ($R_g$) size of ~30 nm, whereas the WPI was ~3−5 nm. This means that the adsorbed film on oil droplets stabilized by the WPI−DX conjugate would be ~6 times thicker than droplets stabilized by WPI, ignoring other factors. Dalgleish et al. (*28*) stated that whey proteins formed a thin (2−3 nm), dense interface in emulsions with very little protein protruding into the bulk. In the WPI−DX conjugate, the large random coil-like DX molecules would protrude into the liquid phase. Wooster and Augustin (*17*) reported that the adsorbed layer thickness was 3 nm for β-lg alone; but this increased to 5 nm for the conjugate with low molecular weight DX ($M_w$ of 18.5 kDa), 20 nm for that with high molecular weight DX ($M_w$ of 440 kDa), and 23 nm for DX ($M_w$ of 500 kDa). Dunlap and Côté (*6*) also reported that a larger, bulkier polysaccharide should produce a thicker polymeric layer and provide greater stability to the emulsion.

(*2*) *Increased Oil Droplet Surface Hydrophilicity because of DX*. In the WPI−DX conjugate, the hydrophilic DX portion protruded into the aqueous medium, leading to the establishment of a highly solvated layer near the interface, which enhanced steric repulsion forces between neighboring oil droplets and retarded the creaming and coalescence processes (*3*).

(*3*) *Possible Increased Adsorptive Ability of Conjugates on the Surface of Oil Droplets*. The WPI−DX conjugate was unfolded as shown by DSC (**Figure 6**). Kato et al. (*2*) showed that the unfolded structure of proteins enhanced the emulsifying and foaming properties. However, denatured proteins are usually insoluble in aqueous solutions. In the case of the WPI−DX conjugate, it was unfolded but had high solubility regardless of pH, ionic strength, and heating.

(*4*) *Decreased Oil Droplet Aggregation Caused by Noncovalent Interaction and SH−SS Interchange Reaction*. For the emulsions stabilized by the WPI−DX conjugate, the DX portion surrounded the WPI portion and protruded into the liquid phase. This protective layer was ~6 times thicker than WPI alone as discussed above, and it was highly hydrophilic. This thick, hydrophilic protective layer provided almost no chance for WPI to become exposed to the liquid phase. Thus, the noncovalent interaction and SH−SS interchange reaction on the WPI portion were less likely to occur among the oil droplets as they did for the emulsion stabilized by WPI.

In conclusion, the purified WPI−DX conjugate, produced in aqueous solution at mild conditions (reacted at 60 °C for 48 h), had good solubility over the pH range from 3.2 to 7.5 and at high ionic strengths from 0.05 to 0.2 M and had high heat stability compared to native WPI. The emulsifying ability and stability of emulsions made with the WPI−DX conjugate were greatly improved compared with the native WPI or natural commercial glycoprotein emulsifier, such as gum arabic. At some point, WPI−DX conjugates could become an alternative to gum arabic as a food ingredient in emulsions. The improved heat and pH

stability of protein conjugates suggest that these ingredients could be useful to fortify acid beverages that are heat processed.

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