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Formation of Whey Protein Isolate (WPI)−Dextran Conjugates in Aqueous Solutions

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The conjugation reaction between whey protein isolate (WPI) and dextran in aqueous solutions via the initial stage of the Maillard reaction was studied. The covalent attachment of dextran to WPI was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) with both protein and carbohydrate staining. The formation of WPI−dextran conjugates was monitored by a maximum absorbance peak at ~304 nm using difference UV spectroscopy. The impact of various processing conditions on the formation of WPI−dextran conjugates was investigated. The conjugation reaction was promoted by raising the temperature from 40 to 60 °C, the WPI concentration from 2.5 to 10%, and the dextran concentration from 10 to 30% and lowering the pH from 8.5 to 6.5. The optimal conjugation conditions chosen from the experiments were 10% WPI−30% dextran and pH 6.5 at 60 °C for 24 h. WPI−dextran conjugates were stable under the conditions studied.

KEYWORDS: Whey protein isolates; dextran; conjugates; functionality; Maillard reaction

INTRODUCTION

Many efforts have been made to develop new food ingredients with improved functional properties by utilizing the Maillard reaction—a naturally produced nonenzymatic glycosylation between amino acids or protein and reducing sugars (*1–5*). Compared with mono- or disaccharides, conjugation of polysaccharides to proteins led to significant improvement in the physical or chemical properties of proteins, such as thermal stability, emulsification, and antioxidant properties (*1–6*).

However, the conjugation of protein−polysaccharides has been usually carried out by dry-heating. The dry-heating process involves having both protein and polysaccharide materials dried before the reaction and the maintenance of constant temperature and relative humidity during the reaction. It takes a long time to complete the reaction, for example, 1−3 weeks at 60 °C; when a high temperature (80 °C) is used, the reaction takes only 2 h. It is not easy to control the extent of the reaction. The products are usually a mixture of intermediate and/or advanced Maillard reaction products that have a light yellow or brown color (see, e.g., ref *5*). Presumably, after the reaction, the powders should be dissolved and the conjugates purified by some technique. From an industrial viewpoint, this dry-heating processing is not attractive, and no commercially manufactured conjugate ingredients have been made, to our knowledge. A milder heat treatment and shorter processing time are economically desirable. The challenges in our study were to create protein−polysaccharide conjugates in aqueous solutions; to limit the Maillard reaction to the very initial stage, for example, the formation of the Schiff base; and to obtain a product with white color.

The Maillard reaction occurs in aqueous solutions under mild heating conditions (e.g., 60 °C) if proteins and simple sugars are used (see, e.g., refs *7* and *8*). No conjugation between protein and polysaccharides in aqueous solution has previously been reported, to our knowledge.

For the Maillard reaction in aqueous solutions, a likely adverse reaction is protein denaturation and polymerization at elevated temperatures. Increasing the concentration of reactants could increase the glycosylation yield, but it could also result in the greater polymerization/aggregation of the protein. We proposed to use the macromolecular crowding effect to help solve these problems. When macromolecular reactions are conducted in the presence of high concentrations of macromolecules, excluded volume theory (*9*) predicts that the reaction will be shifted in the direction of species that have the smaller excluded volume. For proteins, this should encourage compact folded or native structures over less compact denatured species. Using the crowding effect, the native protein structure could be stabilized during processing and the extent of protein denaturation and polymerization could potentially be minimized if we use harsher process conditions to increase the yield of conjugate. Because the Maillard reaction between proteins and polysaccharides in aqueous solution is slow (*10*), it is feasible to terminate the Maillard reaction at the initial stage by rapidly decreasing the temperature to ~0 °C. It is expected that when the ϵ-amino groups of lysine or the N-terminal amino group of a protein is adducted to the carbonyl group on bulky polysaccharides, the Schiff base formed would be stable as a result of steric factors (*11*).

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Whey protein isolate (WPI), a byproduct from the cheese-making industry, and dextran, a neutral polymer, were chosen as the protein and polysaccharide sources, respectively, for this study. The proteins in WPI mainly consist of β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), immunoglobulins (IgG), and lactoferrin (LF). Dextran is composed of a linear chain of glucosyl residues all linked via α(1→6) glucosidic bonds and several α(1→2), α(1→3), or α(1→4) branched linkages (*12*). Due to the high percentage of α(1→6) glucosidic linkages, dextran is a very flexible polysaccharide with a high solubility and low solution viscosity and is unable to form gels. As a reactant, uncharged dextran avoids the complication resulting from the formation of electrostatic complexes, which often occurs in mixtures of proteins with anionic polysaccharides (*2*). In addition, dextran is a good macromolecular crowding agent (*9*).

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%). Before use, WPI was dissolved in Milli-Q water and thoroughly dialyzed against Milli-Q water (dialysis membrane tubing had a molecular mass cutoff of 6000−8000 Da) for 3 days at 5 °C with changes in water every 6 h to remove lactose. After lyophilization, purified WPI was stored at 5 °C prior to its use.

Dextran (batch 115k0686, CAS Registry No. 9004-54-0) from *Leuconostoc mesenteroides*, with a molecular mass of 8500−11500 (11 kDa), was obtained from Sigma-Aldrich (St. Louis, MO). Before use, dextran was dissolved in Milli-Q water and dialyzed against Milli-Q water (dialysis membrane tubing had a molecular mass cutoff of 3000 Da; Fisher Scientific) for 2 days at 5 °C with changes in water every 6 h. After lyophilization, purified dextran was stored at 5 °C.

Prestained SDS-PAGE molecular mass standards (broad range) were purchased from Bio-Rad Laboratories (Hercules, CA). The GelCode glycoprotein staining kit was purchased from Pierce Biotechnology (Rockford, IL).

Preparation of Conjugates by Heat Treatment. Mixtures of WPI−dextran in various ratios (by weight) were dissolved in 10 mM sodium phosphate buffer (NaPi) solution (pH 6.8). Sodium azide (0.02%, w/w) was added to prevent bacterial growth. The sample solutions were stirred on a magnetic stirrer at room temperature (∼22 °C) for 2 h to completely dissolve the mixture. The pH values of the solutions were adjusted by carefully adding 0.1 N HCl or 0.1 N NaOH to the desired pH. Solutions were gently stirred overnight at 5 °C to ensure the complete hydration of the macromolecules. Aliquots of the solutions (1.0 mL, dispensed into 1.5 mL Eppendorf tubes) were placed in a water bath heated at various temperatures for 24 h. Samples were then taken out of the water bath and immediately cooled in an ice−water bath. Triplicates were carried out for each experiment.

Time Course of the Reaction. Using the same heat treatment for the sample preparation, a number of sample solutions (10% WPI−30% dextran, pH 6.5) were incubated at 60 °C for different time periods. At each time point, three sample tubes were taken out from the water bath and immediately cooled in an ice−water bath before analysis. Triplicate experiments were performed.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed on a Mini-Protean 3Cell (Bio-Rad Laboratories) according to the method of Laemmli (*13*). Nonreducing and reducing SDS-PAGE analyses were carried out on a ready gel (Tris-HCl Gel, 4−20% linear gradient, 15 wells, 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 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 Coomassie blue G-250 and the other one was stained for carbohydrate by the GelCode Glycoprotein staining kit (Pierce Biotechnology), respectively. The protein stain was destained with 10% acetic acid (v/v) containing 30% methanol (v/v).

Difference UV Spectroscopy (DUV). DUV measurements were carried out 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 samples were 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 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 (as reference). The extent of conjugation was assessed by the DUV absorbance peak value at ∼304 nm (*14, 15*).

RESULTS AND DISCUSSION

Appearance of WPI−Dextran Conjugates. When solutions of 10% WPI−30% dextran (pH 6.5) were incubated at 60 °C for 24 h, the solutions in the Eppendorf tube appeared white and opaque. There was no obvious aroma or smell, nor was any significant change in viscosity observed within the 24 h incubation period. When the sample was centrifuged at 16000*g* for 30 min, the supernatant appeared clear and translucent, and the precipitate was white. The supernatant, where the conjugate resided (it was confirmed by SDS-PAGE, as described later), was used for all analyses in this study.

To determine if the precipitate was protein or dextran, aliquots of the precipitate were subjected to several tests. The precipitate hardly dissolved in 8 M urea or 10% SDS either in the absence or in the presence of 2-mercaptoethanol. These observations made it unlikely that the precipitate was denatured WPI proteins. It has been reported that dextran self-associates in sufficiently concentrated solutions, and these dextran associates can be dissolved when resuspended in distilled water and autoclaved for 30 min (*16*). When the white precipitate was resuspended in Milli-Q water (4−10%), the precipitate apparently dissolved when heated at 100 °C for 30 min. This suggested that the white precipitate was due to the self-association of dextran.

With prolonged incubation time (>24 h), a strong aroma was noted and a yellow color appeared. The reaction was stopped after 24 h of incubation by rapidly cooling the samples in an ice−water bath.

WPI−Dextran Conjugate Interaction Studied by SDS-PAGE. The reaction between WPI and dextran was investigated by SDS-PAGE. As shown in **Figure 1** (protein stain), under nonreducing conditions (lanes 1−4 in **Figure 1A**), unheated WPI (native WPI, lane 1, **Figure 1A**) and the mixture of 10% WPI−30% dextran (5 °C for 24 h, lane 2, **Figure 1A**) had identical and characteristic bands of WPI as identified in **Figure 1**: two major bands were assigned to monomers of α-La and β-Lg, respectively; two minor bands were attributed to dimers of β-Lg and BSA; four faint bands were LF, IgG, IgL, and IgH. This indicated that no polymerization occurred in WPI in the presence of 30% dextran without heat treatment of the mixture. For the heat-treated 10% WPI alone (lane 3, **Figure 1A**), the α-La, β-Lg dimer, BSA, and IgG bands were significantly diminished; the intensity of the β-Lg monomer band was reduced; meanwhile, a dense band appeared on the top of the gel. This indicated that proteins in WPI had associated into large molecular polymers that could not migrate into the separating gel. In the case of a heat-treated mixture of 10% WPI−30% dextran (lane 4, **Figure 1A**), a new diffuse band that had a molecular mass distribution of 26−98 kDa appeared in the separating gel, indicating the formation of new protein species, which migrated into the separating gel. In addition, a thin band of large molecular mass polymers was observed on the top of the gel similar to the heated 10% WPI sample. Compared to the heated 10% WPI sample alone (lane 3, **Figure 1A**), the

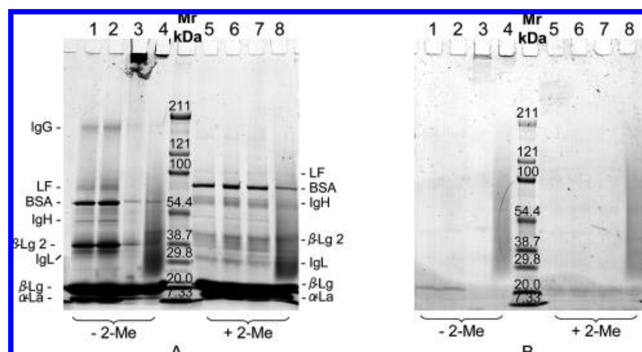

Figure 1. SDS-PAGE of WPI−dextran conjugate in the absence (lanes 1−4) and presence (lanes 5−8) of 5% of 2-mercaptoethanol: (**A**) protein stain (Coomassie blue); (**B**) carbohydrate stain (periodic acid). Mr, molecular mass standards; lanes 1 and 5, 10% WPI (unheated); lanes 2 and 6, mixture of 10% WPI−30% dextran (unheated, pH 6.5, 5 °C, 24 h); lanes 3 and 7, 10% WPI (pH 6.5, 60 °C, 24 h); lanes 4 and 8, mixture of 10% WPI−30% dextran (pH 6.5, 60 °C, 24 h).

intensity of the band with large molecular mass polymers was less intense in the heated mixture of 10% WPI−30% dextran (lane 4, **Figure 1A**). This indicated that the polymerization of WPI was greatly inhibited in the presence of 30% dextran. A similar phenomenon was reported by Schmitt et al. (*17*), who found that the polymerization of β-Lg was greatly reduced in the presence of acacia gum.

Because non-covalent interactions were dispersed during SDS-PAGE, the new protein species and the large molecular mass polymers on the top of the gel in lane 4 (**Figure 1A**) were linked by some types of covalent bonds. Under reducing conditions, for lanes 7 and 8 (**Figure 1A**), the large molecular mass polymers that resided at the tops of lanes 3 and 4 (**Figure 1A**) completely disappeared, indicating that these large polymers were linked by disulfide bonds. The heated 10% WPI under reducing conditions (lane 7, **Figure 1A**) had identical bands compared to native WPI (unheated, lanes 5 and 6, **Figure 1A**), whereas the heated mixture of 10% WPI−30% dextran still had the diffuse band with the molecular mass distribution of 28−100 kDa (lane 8, **Figure 1A**) that appeared at the same location as the new band in lane 4 (**Figure 1A**). Because the new protein species were observed in both lanes 4 and 8 (**Figure 1A**), this indicated that these species were linked by covalent bonding other than disulfide.

To further investigate the nature of the new protein-containing species, SDS-PAGE was performed with carbohydrate staining using the periodic acid−Schiff reagent (PAS) (**Figure 1B**). No bands were observed for lanes 1−3 and 5−7. This is because protein bands are not be stained by PAS. Being a neutral molecule, dextran could not migrate into the separating gel. Similar to **Figure 1A**, a faint high molecular mass band was observed at the top of the gel in lane 4 (**Figure 1B**) and it disappeared in lane 8 (**Figure 1B**), indicating that the polymers (lane 4) were reduced into small molecules in the presence of 2-mercaptoethanol (lane 8). The same broad diffuse bands as in lanes 4 and 8 (**Figure 1A**) were also observed in the separating gel in lanes 4 and 8 of **Figure 1B**, corresponding to molecular mass distributions of ∼26−110 kDa (lanes 4, **Figure 1B**) and ∼26−130 kDa (lanes 8, **Figure 1B**), respectively. The above results confirmed that glycosylated protein species, that is, WPI−dextran conjugates, were formed in the mixture of 10% WPI−30% dextran as a result of heating at 60 °C for 24 h. The molecular mass distribution of the conjugates was ∼26−110 kDa. The polymers that were observed on the tops of lanes 4 and 8 in both **Figure 1A** and **Figure 1B** disappeared, indicating some disulfide-linked proteins were also involved in conjugation.

The broad molecular mass distribution of the conjugate bands was due to the nature of the components of the two reactants.

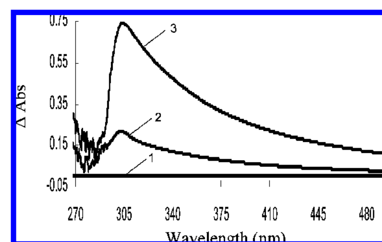

Figure 2. Difference UV absorbance spectra (DUV) of Schiff base of WPI−dextran conjugates: 1, 30% dextran (60 °C for 24 h); 2, 10% WPI (60 °C for 24 h); 3, mixture of 10% WPI−30% dextran (60 °C for 24 h). The DUV measurements were done after 33.3-fold dilution.

WPI consists of several protein types, such as α-La (∼18%), β-Lg (∼52%), BSA, and IgG (5%) (*18*). Each protein contains multiple potential reactive sites. For example, β-Lg contains 16 potential reactive primary amino groups, including 1 α-NH_2 group at the N terminus and 15 ϵ-NH_2 lysine residues. From the intensity of the remaining bands of WPI proteins (**Figure 1A**), it appeared that each of the WPI proteins was partly involved in the conjugation of WPI−dextran to different extents. The molecular mass of dextran varies from 8500 to 11500 Da as indicated by the supplier (Sigma). The polydispersity of dextran also led to a broad molecular mass distribution of the WPI−dextran conjugates.

Conjugation Reaction As Determined by DUV. To further clarify the nature of the covalent bonding in WPI−dextran conjugates, DUV spectra were recorded by scanning wavelengths between 270 and 500 nm (*14, 15*). As shown in **Figure 2**, for the heated mixture of 10% WPI−30% dextran solution, the interaction between WPI and dextran resulted in a DUV spectrum that was characterized by a maximum at 304 nm. This DUV absorbance peak shifted to 307 or 310 nm when the WPI−dextran mixture solution was incubated for 48 or 72 h; meanwhile, the appearance of the sample solution became light yellow (48 h) and deep yellow or light brown. This suggested that Amadori products or intermediate stage or final stage products were forming with a longer incubation time. The peak at 304 nm was presumably assigned to the Schiff base formation. Previously, it has been reported that the reaction of 2,4-dihydroxybenzaldehyde (DHBA) with lysine resulted in a DUV spectrum with an absorption maximum at 296 nm (*15*). When this reaction was performed on the lysine residues of aldolase, the DUV spectrum showed an absorption maximum at 311 nm (*15*). This red shift in the DUV absorption maximum was interpreted as being due to the difference in the environment of the Schiff base (*15*). The 304 nm DUV absorption maximum observed in this study might be the result of the unique

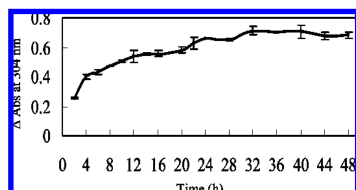

Figure 3. Time course of WPI−dextran Schiff base formation as indicated by the DUV peak at 304 nm (10% WPI−30% dextran, pH 6.5 for 60 °C, $n = 3$). Measurements were performed after 33.3-fold dilution of the original samples.

environment of the Schiff base in the WPI−dextran conjugate, which is neither as polar as that in the case of DHBA−lysine conjugate nor as nonpolar as in the case of DHBA−aldolase conjugate. Further confirmation of the Schiff base is under investigation. No change in absorbance was observed for a heated 30% dextran solution over all of the scanned wavelengths. A small absorbance peak at 304 nm was observed for the heated 10% WPI alone. This was probably caused by some low concentration of residual lactose in WPI (even though it was extensively dialyzed) forming a Schiff base product during heating.

As indicated in **Figure 2**, the DUV absorbance peak of the WPI−dextran Schiff base was asymmetric and tailed into the visible wavelengths. It was possible that the simultaneous formation of many chromophores of Maillard breakdown products in small amounts led to the tailing of the peak as reported in other studies (*19*). On the basis of the predominance of the DUV peak at 304 nm, we think that the WPI−dextran reaction mixture mainly consisted of the Schiff base, and thus the DUV absorbance value at 304 nm can be used as an estimate of the extent of the conjugation.

Storage of the Schiff base (33.3-fold diluted solution at 5 °C for at least 1 week) did not result in any change in absorbance value at 304 nm, indicating that the Schiff base of WPI−dextran conjugates was stable. This stability was probably due to the water-restricted environment of the Schiff base C═N bond, thus preventing a H_2O molecule from hydrolyzing the C═N bond.

It is worth mentioning that with prolonged incubation of mixtures of 10% WPI−30% dextran (60 °C for 48 h), the absorbance peak slightly red-shifted to ∼310 nm; meanwhile, a small new shoulder appeared around 355 nm (data not shown). The resultant solution was light yellow. This implied some WPI−dextran conjugates had slowly developed into the intermediate stage of the Maillard reaction.

Time Course of the Conjugation Reaction. The formation of conjugates from a WPI−dextran mixture was studied as a function of time. As shown in **Figure 3**, the conjugation reaction was time-dependent. The formation of conjugates gradually increased with time during the first 24 h when incubated at 60 °C. There was little further increase between 24 and 48 h of incubation at 60 °C (**Figure 3**). SDS-PAGE under reducing conditions and with carbohydrate staining indicated that the molecular mass distribution of the conjugates increased with time from 28 to 36 kDa (2 h at 60 °C); from 28 to 52 kDa (4 h at 60 °C); from 28 to 70 kDa (6 h at 60 °C); from 28 to 82 kDa (8 h at 60 °C); from 28 to 95 kDa (12 h at 60 °C); and from 28 to 100 kDa (24 h at 60 °C) (data not shown). The intensity of the bands, particularly the lower molecular mass species, increased with time.

An optimum reaction time of 24 h at 60 °C was chosen, under our experimental conditions, to allow the reaction to proceed with the highest DUV peak derived from conjugate formation but with low levels of intermediate and advanced Maillard products.

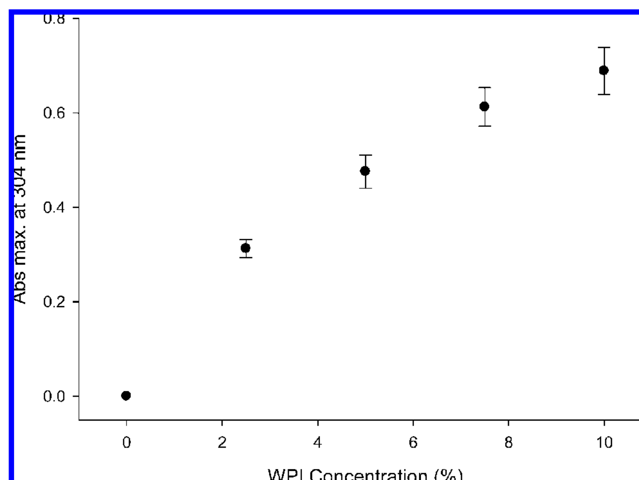

Figure 4. Effect of WPI concentration on the WPI−dextran Schiff base formation (30% dextran, pH 6.5, 60 °C for 24 h, $n = 3$). The measurements were performed after 33.3-fold dilution of the original samples.

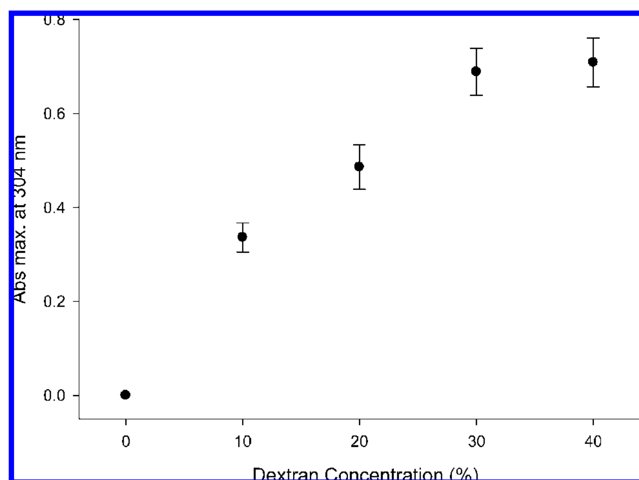

Figure 5. Effect of dextran concentration on the WPI−dextran Schiff base formation (10% WPI, pH 6.5, 60 °C for 24 h, $n = 3$). Measurements were performed after 33.3-fold dilution of the original samples.

Effect of WPI and Dextran Concentration on Schiff Base Formation. The effect of WPI or dextran concentration on the conjugation of WPI−dextran was studied. As shown in **Figure 4**, the formation of WPI−dextran conjugates increased with increasing WPI concentration from 2.5 to 10% in a mixture with 30% dextran. Further increases in the WPI concentration above 10% led to the gelation of WPI during heating at 60 °C. Each protein in WPI has multiple potential reactive amine groups. However, at pH 6.5, most of the ϵ-amino groups (>99%) are in the protonated form, which are not reactive. The effective concentration of $-NH_2$ groups in the system is lower than the concentration of carbonyl groups in a 30% dextran solution. Therefore, the conjugation increases with WPI concentration. In **Figure 5**, the formation of WPI−dextran Schiff base increased with the increase in dextran concentration from 10 to 40%, in mixtures with 10% WPI. As each polysaccharide molecule (dextran) has only one reducing group capable of reacting with amine groups in proteins (*2*), it is reasonable that the extent of conjugate formation increased with an increase in the proportion of polysaccharide. This is also true for protein amino groups at pH 6.5 because only the N-terminal amino groups of the proteins are in the nucleophilic reactive form and >99% of the ϵ-amino groups of lysine residues are in the

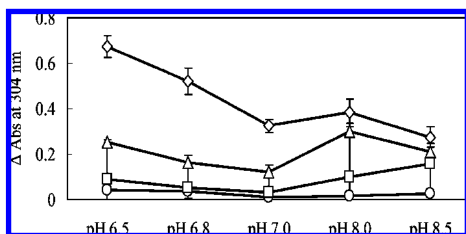

Figure 6. Effect of temperature [40 °C (○); 50 °C (□); 56 °C (△); 60 °C (◇)] and pH on the WPI−dextran Schiff base formation (10% WPI−30% dextran, 60 °C for 24 h, $n = 3$). Measurements were performed after 33.3-fold dilution of the original samples.

nonreactive NH_3^+ form. The bulky structure of dextran may restrict access to the reactive sites by the amino groups on proteins and vice versa.

Increasing the dextran concentration from 30 to 40% did not result in significant increase in the WPI−dextran Schiff base formation (**Figure 5**), but a marked increase in viscosity was observed. It is also possible that the increase in viscosity of the system, which will decrease molecular mobility and thereby the reaction rate, might be responsible for the nonlinear response in the extent of product formation with increase of reactant concentration. On the basis of these results 10% WPI and 30% dextran were chosen as standard conditions for conjugate formation.

An excess of polysaccharide compared to protein in the mixture of reactants (1:3−1:9, by weight) is often used in preparing Maillard reaction products by dry-heating (see, e.g., ref *2*). There is a lack of detailed explanation for this ratio. One plausible explanation was that each polysaccharide molecule has only one reducing group capable of reacting with amine groups in the proteins; thus, the extent of conjugate formation would increase with increasing proportion of polysaccharide (*2*). However, for the reaction in aqueous solutions, the mixing ratio of reactants did not mean it was the binding ratio of protein to polysaccharides. For the conjugates formed at 10%WPI−30% dextran (pH 6.5, 60 °C for 24 h), our preliminary analysis suggested that we had a yield of <5% (results not shown).

Effect of Temperature and pH. Heating a mixture of WPI and dextran solution promotes two competitive reactions—aggregation of WPI proteins and conjugation between WPI and dextran. High temperature favors the conjugation reaction (as chemical reactions are favored by an increase in temperature); it also promotes WPI denaturation/aggregation, thus leading to the loss of reactant for the conjugation of WPI−dextran. The critical structural changes in β-Lg occur at 63 °C and pH 7.0 [from circular dichroism spectroscopy (*20*)]; the denaturation temperature of α-La is 64 °C at pH 7.0 [from differential scanning calorimetry (*21*)] and that of BSA is 62.2 °C at pH 6.7 (*22*). Therefore, the effect of temperature on the conjugation of WPI−dextran was investigated at ≤60 °C to minimize denaturation/aggregation of WPI proteins. Dextran is stable at ambient temperatures over the range of pH 4−10 (http://www.dextran.net/dextran_stability.html). Any structural change in dextran during heat processing was ignored in this study. As shown in **Figure 6**, the Schiff base was hardly formed at low temperatures (40 or 50 °C) over the pH range studied. Regardless of pH, the formation of Schiff base was favored by raising the temperature.

As far as pH was concerned, as seen in **Figure 6**, the effect of pH on Schiff base formation was negligible at lower temperatures (40 and 50 °C). At 60 °C, the formation of Schiff base was enhanced by reducing the pH from 7.0 to 6.5. There was no significant difference in the reaction in the pH range from 7.0 to 8.5. The generation of Schiff base was greatly enhanced at pH 6.5. This is because the reactivity of the electronegative carbonyl group and the acid-catalyzed condensation reaction are favored at lower pH (*23*). The conditions pH 6.5 and 60 °C were used to maximize the extent of formation of Schiff base in the study.

In conclusion, WPI−dextran conjugates were formed in mixtures of 10% WPI−30% dextran during incubation at pH 6.5 and 60 °C for 24 h. The conjugation of WPI and dextran was confirmed by SDS-PAGE with both protein staining and the periodic acid−Schiff reagent (conjugate) staining. The WPI−dextran conjugate was assumed to be mainly composed of Schiff base, which was characterized by maximum absorbance peak values at 304 nm by DUV. Dextran acted not only as a reactant taking part in the conjugation but also as a protective reagent (via macromolecular crowding) in preventing excessive WPI denaturation and aggregation.

LITERATURE CITED

(1) Kato, A.; Sato, T.; Kobayashi, K. Emulsifying properties of protein−polysaccharide complexes and hybrids. *Agric. Biol. Chem.* **1989**, *53*, 2147–2152.
(2) Dickinson, E.; Semenova, M. G. Emulsifying properties of covalent protein-dextran hybrids. *Colloids Surf.* **1992**, *64*, 299–310.
(3) Hattori, M. Functional improvements in food proteins in multiple aspects by conjugation with saccharides: case studies of β-lactoglobulin−acidic polysaccharides conjugates. *Food Sci. Technol. Res.* **2002**, *8*, 291–299.
(4) Dunlap, C. A.; Côté, G. L. β-Lactoglobulin−dextran conjugates: effect of polysaccharide size on emulsion stability. *J. Agric. Food Chem.* **2005**, *53*, 419–423.
(5) Akhtar, M.; Dickinson, E. Emulsifying properties of whey protein−dextran conjugates at low pH and different salt concentrations. *Colloids Surf. B* **2003**, *31*, 125–132.
(6) Nakamura, S.; Kato, A.; Kobayashi, K. Enhanced antioxidative effect of ovalbumin due to covalent binding of polysaccharides. *J. Agric. Food Chem.* **1992**, *40*, 2033–2037.
(7) Nacka, F.; Chobert, J.-M.; Burova, T.; Léonil, J.; Haertlé, T. Induction of new physicochemical and functional properties by the glycosylation of whey proteins. *J. Protein Chem.* **1998**, *17*, 495–503.
(8) Groubet, R.; Chobert, J.-M.; Haertlé, T. Functional properties of milk proteins glycated in mild conditions. *Sci. Aliment.* **1999**, *19*, 423–438.
(9) Hall, D.; Minton, A. P. Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges. *Biochim. Biophys. Acta* **2003**, *1649*, 127–139.
(10) Morgan, F.; Bouhallab, S.; Molle, D.; Henry, G.; Maubois, J.-L.; Léonil, J. Lactolation of β-lactoglobulin monitored by electrospray ionization mass spectrometry. *Int. Dairy J.* **1998**, *8*, 95–98.
(11) March, J. *Advanced Organic Chemistry Reactions, Mechanisms and Structure*, 3rd ed.; Wiley: New York, 1985.
(12) Seymour, F. R.; Knapp, R. D. Structural analysis of dextrans from strains of *Leuconostoc* and related genera, that contain 3-*O*-α-D-glucosylated-D-glucopyranosyl residues at the branched points or in consecutive linear position. *Carbohydr. Res.* **1980**, *81*, 105–129.
(13) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(14) Heinert, D.; Martell, A. E. Pyridoxine and pyridoxal analogs. VI. Electronic absorption spectra of Schiff bases. *J. Am. Chem. Soc.* **1963**, *85*, 183–188.
(15) Blonski, C.; De Moissac, D.; Perie, J.; Sygusch, J. Inhibition of rabbit muscle aldolase phosphorylated aromatic compounds. *Biochem. J.* **1997**, *323*, 71–77.

(16) Cadwallader, D. E.; Becker, C. H.; Winters, J. H.; Marcus, D. A note on particulate matter encountered in some dextran injections. *J. Am. Pharm. Assoc. (Sci. Ed.)* **1958**, *47*, 894–895.
(17) Schmitt, C.; Bovay, C.; Frossard, P. Kinetics of formation and functional properties of conjugates prepared by dry-state incubation of β-lactoglobulin/acacia gum electrostatic complexes. *J. Agric. Food Chem.* **2005**, *53*, 9089–9099.
(18) Wang, T.; Lucey, J. A. Use of multi-angle laser light scattering and size-exclusion chromatography to characterize the molecular weight and types of aggregates present in commercial whey protein products. *J. Dairy Sci.* **2003**, *86*, 3091–3101.
(19) Hofmann, T. Studies on the relationship between molecular weight and the color potency of fractions obtained by thermal treatment of glucose/amino acid and glucose/protein solutions by using ultracentrifugation and color dilution techniques. *J. Agric. Food Chem.* **1998**, *46*, 3891–3895.
(20) Prabakaran, S.; Damodaran, S. Thermal unfolding of β-lactoglobulin: characterization of initial unfolding events responsible for heat-induced aggregation. *J. Agric. Food Chem.* **1997**, *45*, 4303–4308.
(21) McGuffey, M. K.; Epting, K. L.; Kelly, R. M.; Foegeding, E. A. Denaturation and aggregation of three α-lactalbumin preparations at neutral pH. *J. Agric. Food Chem.* **2005**, *53*, 3182–3190.
(22) Mulvihill, D. M.; Donovan, M. Whey proteins and their thermal denaturation—a review. *Ir. J. Food Sci. Technol.* **1987**, *11*, 43–75.
(23) Feeney, R. E.; Blankenhorn, G.; Dixon, H. B. F. Carbonyl—amine reactions in protein chemistry. *Adv. Protein Chem.* **1975**, *29*, 135–203.

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