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β-Lactoglobulin−Dextran Conjugates: Effect of Polysaccharide Size on Emulsion Stability

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A series of dextrans and β-lactoglobulin were covalently conjugated and screened for their ability to stabilize oil-in-water emulsions. Dextrans with the molecular mass of 19.6 kDa, 87 kDa, 150 kDa, 500 kDa, and 2000 kDa were attached to β-lactoglobulin via the Maillard reaction. The conjugates were then purfied and evaluated as emulsifiers under neutral conditions. The ability to stabilize emulsions was determined by monitoring oil droplet size over time. Adsorption of the conjugates to the droplet surface was characterized by determining the protein surface load. The results show that increasing polysaccharide size increases emulsion stability up to 150 kDa before leveling off. Conversely, surface protein density remains constant until 150 kDa before decreasing with polysaccharide size. A model is presented to interpret the results.

KEYWORDS: β-Lactoglobulin; Maillard reaction; polysaccharide conjugate; emulsifier

1. INTRODUCTION

It is well established that conjugating polysaccharides to proteins improves several of their functional properties, such as solubility (*1*), heat stability (*2*, *3*), emulsion stabilizing ability (*4*−*8*), and decreases allergenicity (*3*, *9*, *10*). The conjugates are typically formed by the Maillard reaction utilizing a dry heating method (*11*). Conjugates have also been prepared via chemical and enzymatic methods (*12*−*14*). These studies have been performed on a wide variety of protein−polysaccharide combinations (*5*, *11*, *15*−*19*) and demonstrate the general utility of the technique.

β-Lactoglobulin (β-lg), the predominant protein in whey protein isolate, is commonly used to stabilize food emulsions because of its surface active properties. These properties have lead β-lg to be the subject of many studies involving protein polysaccharide conjugates in an effort to improve its functionality for the food industry. Improving β-lg's functional properties would allow it to compete with other emulsifiers, such as gum arabic, in the food industry.

The conjugation of polysaccharides to proteins is believed to enhance their emulsifying properties through steric stabilization. This occurs because the polysaccharide introduces a bulky polymeric layer to the droplet surface, altering droplet aggregation and coalescence. While many types and sizes of polysaccharides have been tested in these applications, there has been little research to examine the effect of polysaccharide size for a given protein−polysaccharide combination (*9*). From a theoretical standpoint, a larger polysaccharide should produce a thicker polymeric layer and provide greater stability to the emulsion. From a practical standpoint, as polysaccharides and their hydrated radii become larger, their size may prevent the droplet surface from becoming saturated with protein, since the polysaccharides would occupy a much greater volume than provided when proteins are tightly packed on the droplet surface.

The objective of our study was to determine the effect of the polysaccharide size in stabilizing emulsions when part of a protein−polysaccharide conjugate.

2. MATERIALS AND METHODS

2.1. Materials. β-Lg was purified from whey protein isolate (Glanbia Nutritionals, Monroe, WI) using the method described by Mailliart and Ribadeau-Dumas (*20*). β-Lg was desalted by elution in water through a G-25 column (5 × 30 cm). Dextran polysaccharides and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The average molecular weight of the dextrans was assumed as reported by the manufacturer. Dextrans were subjected to ultrafiltration (molecular weight cutoff filters 3000 or 30000, Millipore, Bedford, MA) to remove low molecular weight oligosaccharides. Dextrans were diluted and filtered until the filtrate was free from reducing sugars. Gum arabic (approximately 250 kDa MW) was obtained from Sigma-Aldrich (St. Louis, MO) and hydrated in a 20% aqueous solution with agitation for 24 h before use. Sunflower oil (Conagra grocery, Irvine, CA) was obtained from a local grocery.

2.2. Conjugate Formation and Purification. Mixtures of β-lg and dextran (molar ratio 1:1, on the basis of av molecular weight of the dextran) were dissolved in deionized water and then freeze-dried. Samples were equilibrated under reduced pressure in the presence of saturated KBr solution for 24 h. Samples were then stored under nitrogen in a gravity convection oven at 55 °C or 75 °C (75 °C for dextrans greater than 100 kD) for a period of 1−7 days. Reaction mixtures were removed from the oven when the samples developed a

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beige tint to them. The samples were then dissolved in deionized water and loaded on an anion exchange column, 2.5 × 60 cm (Bio-Rad, Macro-prep, High Q support resin). The column was washed with 3−4 volumes of water and eluted with 75 mM NaCl solution. Fractions were monitored by UV spectroscopy and confirmed with SDS−PAGE. Fractions containing conjugates as determined by SDS−PAGE were pooled and freeze-dried. Freeze-dried samples were dissolved in a minimum volume of deionized water and loaded onto S-200 Sephacryl size exclusion column, 5 cm × 1.6 m, and eluted with deionized water. Fractions were monitored by UV spectroscopy and confirmed with SDS−PAGE. Conjugate-containing fractions were pooled and freeze-dried. Freeze-dried samples were stored at −20 °C until use. Recovered yields averaged between 10 and 30%. The molar ratio of protein: polysaccharide in the product was confirmed to be (1:1) by determining the protein content for a given mass and comparing it with the average molecular mass of the anticipated product.

2.3. Emulsion Preparation. β-Lg and β-lg conjugates were dissolved in 10 mM sodium phosphate buffer (pH 7.0) and assayed for protein content using a modified Bradford assay (*21*) (Coomassie plus protein kit, Pierce Biotechnology, Rockford, IL). Emulsions contained 20% sunflower oil (w/w) and 0.10% protein (w/w). The aqueous phase contained 10 mM sodium phosphate (pH 7.0) and 0.02% sodium azide. Pre-emulsions were formed by mixing the oil into the protein solution using a Tissue Tearor handheld homogenizer (Dremel, Rancine, WI) at 15 000 rpm for 1 min. The pre-emulsion was homogenized by five passes through an Emulsiflex C5 homogenizer (Avestin, Ottawa, Canada) at an operating pressure of 1500 bar. A minimum of two trials were performed for each condition. The emulsions were stored quiescently at 22 °C for the duration of the experiment.

2.4. Droplet Sizing and Creaming Measurements. At given time intervals, 10 μL of emulsion was diluted into 4 mL of 0.1% SDS and characterized with a NiComp 380 dynamic light scattering particle sizer (Particle sizing systems, Santa Barbara, CA). The reported diameters are the average of two trials for each time point and represent the mean droplet size from a particle volume vs diameter plot, d_v. Creaming experiments were performed in 12 × 100-mm-capped test tubes. The cream layer was determined visually and measured.

2.5. Protein Surface Load. The surface concentration of the protein, Γ, was determined by the depletion method (*22*). The aqueous phase was separated from the oil droplets by centrifuging the sample for 60 min at 16000*g*. The concentration of protein in the aqueous phase was assayed for protein content using a modified Bradford assay (*21*) (Coomassie plus protein kit, Pierce Biotechnology, Rockford, IL). This protein assay is compatible with reducing sugars (1.0 M glucose), per the manufacturer.

2.6. SDS−Polyacrylamide Gel Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (*23*). Samples were run on a 4−12% bis-tris gel (5 cm) with MES running buffer and the manufacturers' loading buffer (Invitrogen, Carlsbad, CA). The samples were run at constant voltage (200 V) for approximately 30 min. The gels were stained for protein (Coomassie blue, G-250) and carbohydrate (periodic acid stain) as previously described (*24*).

2.7. Statistical Analysis. Statistical analysis was performed using ANOVA and multiple range tests for comparing initial droplet size and protein surface load measurements. Differences are considered significant at the 95% confidence level ($p = 0.05$).

3. RESULTS

3.1. Conjugate Formation and Characterization. β-Lg was conjugated to a series of dextrans with average molecular weights of 19.6 kDa, 87 kDa, 150 kDa, 500 kDa, and 2000 kDa. Conjugates were formed by Maillard reaction of β-lg and dextran using the dry heat method as described in Materials and Methods. The reactant ratio was 1:1 on a molar basis to limit possibility of multiply glycosylated proteins. The goal was to allow the reaction to proceed until the formation of Amadori products while limiting the formation of melanoidans and advanced glycation end products (*25, 26*), which could complicate the analysis of the results. The higher molecular weight dextran samples required higher temperature (75 °C) and longer times (up to 7 days) for the reaction to proceed to an acceptable level. This is the result of the high molecular weight dextrans that have higher glass-transition temperatures (T_g). The rate of Maillard reactions in a glass state has been previously shown to be higher when the temperature is above the T_g of the reaction matrice (*27−29*).

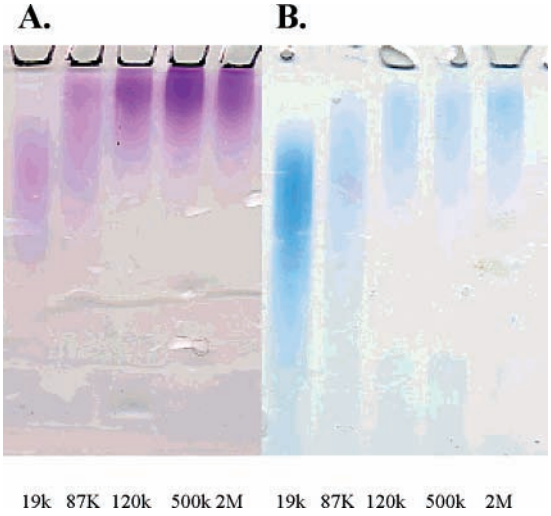

Figure 1. SDS–PAGE gel of β-lg–dextran conjugates with PAS stain (**A**) and coomassie stain (**B**). The lanes from left to right: 19.6 kDa, 87 kDa, 150 kDa, 500 kDa, 2000 kDa, dextran conjugated to β-lg.

The products were separated from the reactants with a combination of ion-exchange chromatography and size-exclusion chromatography. Anion-exchange chromatography was useful in removing the free dextran and approximately 80% of the free β-lg. It was also highly effective in removing a small amount of covalent β-lg dimer that formed during the reaction. However, performing the reaction under inert atmosphere greatly reduced the formation of this dimer. Size-exclusion chromatography was used to remove the remaining free β-lg. Formation of the conjugate was confirmed with SDS−PAGE analysis. **Figure 1** shows the SDS−PAGE gel of the conjugates stained with a protein stain (Coomassie) and a carbohydrate stain (periodic acid stain). These results confirm the formation of a stable covalently bound conjugate.

3.2. Emulsion Stability. The stability of the emulsions was evaluated by monitoring the droplet size (d_v) and the creaming of the emulsions over time. **Figure 2** shows the size of the droplets as a function of time for each emulsifier, as determined by dynamic light scattering. The results show that β-lg and the β-lg conjugates had comparable initial droplet sizes. The initial droplet sizes varied from 370 ± 18 nm for native β-lg emulsions and gradually increased with increasing polysaccharide size of the conjugate, up to 450 ± 22 nm for the β-lg 2000 kDa dextran conjugate. The initial droplet sizes are summarized in **Table 1**. In comparison with the native β-lg initial droplet size, the difference in initial droplet size becomes significant (multiple range test) with the β-lg 150 kDa dextran conjugate. This difference can be partially explained by the increased thickness of the emulsifier layer. In the β-lg 2000 kDa dextran conjugate, 2000 kDa dextran is reported to have a radius of gyration of 32 nm, which would account for 64 nm of the droplet size difference (*30*). The remaining difference could be the result of differences in diffusion or viscosity of the conjugate solutions, which influence initial droplet size (*31−34*). The gum arabic emulsion had an initial droplet size of 760 ± 25 nm and was

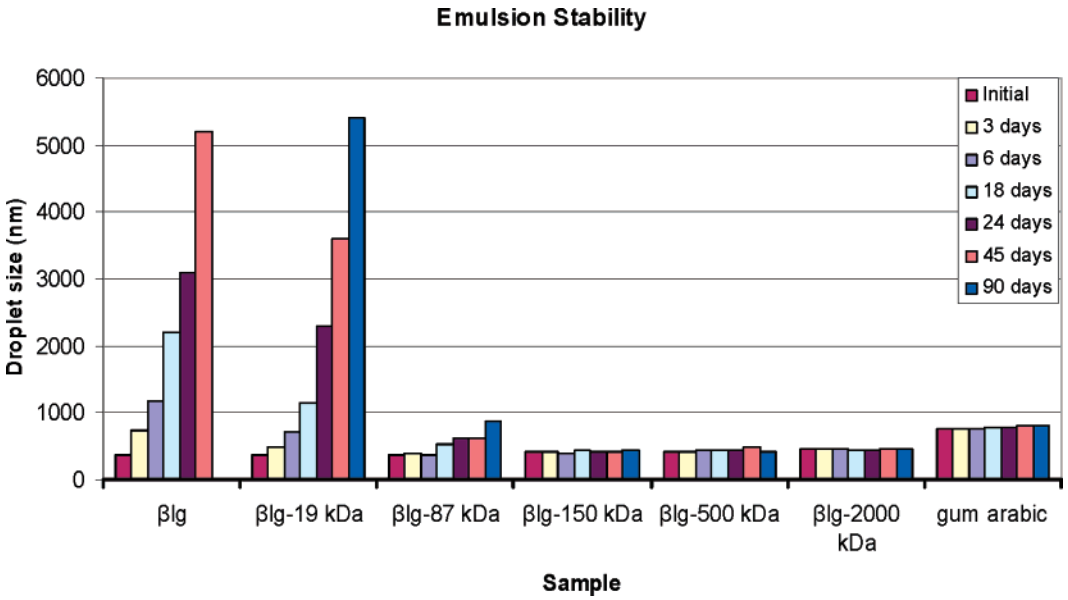

Figure 2. Emulsion droplet size of β-lg–dextran conjugates. The samples analyzed were native β-lg and 19.6 kDa, 87 kDa, 150 kDa, 500 kDa, 2000 kDa, dextran conjugated to β-lg, and 10% gum arabic. The emulsions consisted of 20% sunflower oil, 0.1% protein, 10 mM sodium phosphate (pH 7.0), and 0.02% sodium azide. The emulsion for the native β-lg sample was completely broken for the 90-day sample, so no sample was analyzed.

Table 1. Summary of Initial Droplet Size Data[a]

sample	initial droplet size (nm)
β-lg	370 ± 18
β-lg–19 kDa	371 ± 21
β-lg–87 kDa	380 ± 35
β-lg–150 kDa	407 ± 8
β-lg–500 kDa	415 ± 29
β-lg–2000 kDa	450 ± 22
gum arabic	765 ± 25

[a] Confidence intervals are reported at two standard deviations.

noticeably more viscous than the native β-lg and conjugate solutions.

The native β-lg demonstrated the least emulsion stabilty. Under these conditions, the native β-lg emulsion rapidly increased in droplet size during the experiment. The smallest β-lg conjugate (β-lg−19.6 kDa dextran) also showed poor performance under these conditions stabilizing the emulsion, which was only marginally better than native β-lg. The next larger conjugate, β-lg 87 kDa dextran, was considerably more stable than native β-lg and did not show an increase in droplet size until 18 days of storage. The droplet size for the 150 kDa, 500 kDa, and 2000 kDa dextran conjugates remained constant for the duration of the experiment. The gum arabic emulsion also remained stable for the duration of the experiment.

The creaming data (data not shown) mirrored the droplet size data. The native β-lg began forming a cream layer shortly after emulsion formation and the emulsion began breaking within a few days. The β-lg−19.6 kDa dextran conjugate formed a stable cream layer within a couple of days and began breaking near the end of the experiment (90 days). The β-lg−87 kDa dextran conjugate began forming a small cream layer near the end of the experiment. The larger polysaccharide conjugates and gum arabic showed no signs of creaming or breakage for the duration of the experiment. The larger conjugates and gum arabic can at least partially attribute their increased creaming stability to an increase in continuous phase viscosity.

3.3. Protein Surface Load. To determine if polysaccharide size had any influence on protein packing at the droplet surface, protein surface load was determined for each sample. The results

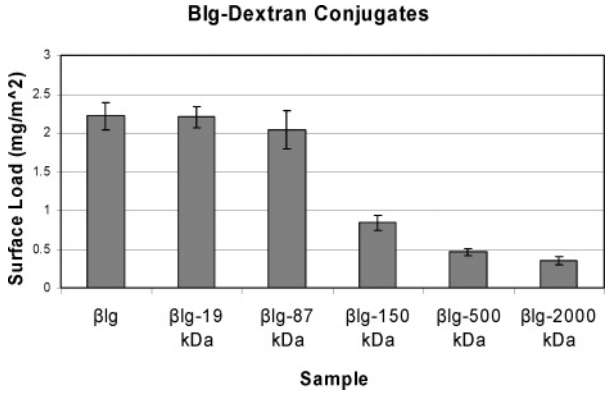

Figure 3. Protein surface load of β-lg–dextran conjugates. The samples analyzed were native β-lg and 19.6 kDa, 87 kDa, 150 kDa, 500 kDa, 2000 kDa, dextran conjugated to β-lg. The protein surface load of the droplets was determined immediately after emulsion formation. The emulsions consisted of 20% sunflower oil, 0.1% protein, 10 mM sodium phosphate (pH 7.0), and 0.02% sodium azide.

of the study are presented in **Figure 3**. The surface load for each sample was determined immediately after emulsion formation for each sample. Native β-lg was determined to have a surface load of 2.2 ± 0.2 mg/m^2, which is comparable to the previously determined surface load for β-lg under similar conditions (*35*). The results showed no significant (multiple range test) difference in protein surface density for native β-lg, β-lg−19.6 kDa dextran, and β-lg−87 kDa dextran conjugate containing emulsions. However, a significant decrease in protein surface density is observed for the β-lg−150 kDa and higher dextran conjugates, with decreasing surface density with increasing polysaccharide size. The surface protein density for native β-lg is 6 times greater than that of the β-lg−2000 kDa dextran conjugate.

4. DISCUSSION

The current study examined the effect of polysaccharide size on emulsion stability for a series of β-lg and dextran conjugates. The results show conjugation and increasing polysaccharide size

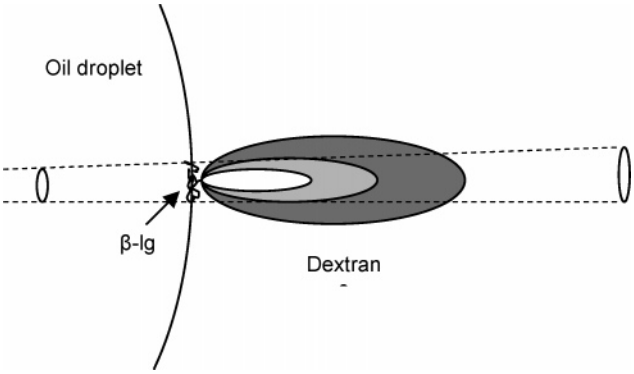

Figure 4. Schematic of a protein–polysaccharide conjugate bound to an oil droplet. The dotted lines represent a cone originating at the center of the droplet. The drawing depicts three possible scenarios. (1) When the dextran occupies a volume less than the available volume provided when the β-lg layer is saturated. (2) When the dextran occupies a volume equal to the volume available when the β-lg layer is saturated. (3) When the dextran occupies a volume greater than the volume available when the β-lg layer is saturated.

increase emulsion stability under these experimental conditions. Emulsion stability increased with polysaccharide size up to 150 kDa and maintained a stable plateau with greater molecular weights. Interestingly, the data on protein surface load shows the opposite pattern. Surface load remains nearly constant with polysaccharide size up to the 150 kDa sample and decreases thereafter. These results suggest that the greater density and thickness of the polysaccharide layer are able to compensate for the decrease in density of the protein layer. The data suggests that at around ~100 kDa the polysaccharides become tightly packed when the surface is saturated with β-lg. When conjugates containing polysaccharides greater than ~100 kDa are bound, the size of the polysaccharide prevents the protein from becoming saturated on the surface of the droplet. **Figure 4** shows a schematic of the concept. When the volume occupied by the polysaccharide is small compared to the volume available when β-lg is saturated at the droplet surface, the β-lg can form a saturated layer. When the volume occupied by the polysaccharide is equal to the volume available when β-lg is saturated, the protein and polysaccharide layers are both saturated. When the volume occupied by the polysaccharide is greater than the volume available when β-lg is saturated, only the polysaccharide layer is saturated.

To examine the feasibility of our model, we used the protein surface load results for native β-lg to calculate the area occupied on the droplet surface

$$\text{area occupied per protein molecule} = \text{Mw}\,(\Gamma N)^{-1}$$

where Γ is the protein surface load (2.2 mg(m^{-2}), Mw is the molecular weight of β-lg (18.3 kg(mol^{-1})), and N is Avogadro's number (6.02×10^{23} mol^{-1}). The calculation yields an area of 14 nm^2 per β-lg molecule on the droplet surface. If we assume β-lg occupies a circle, the circle would have a radius of 2.1 nm (area $= \pi r^2$). In **Figure 4**, this would be the circle cast by the cone at the droplet surface. The protein surface load results suggest around 100 kDa the dextran size dramatically affects the amount of β-lg absorbed to the droplet surface. The radius of gyration for a dextran polymer (assuming a spherical conformation) can be estimated with the equation $R_g = 0.66 (M_w)^{0.5}$ (*36*); a 100 kDa dextran would have an estimated R_g of 6.6 nm. At first glance, these results suggest we should observe dextran crowding at a much lower molecular weight. However, a polymer densely packed and tethered to a surface can adopt alternate conformations (nonspherical); for a review, see ref *37*. These results imply dextran must exist in an elongated conformation in the current study. For tethered polymers in a favorable solvent (such as dextran in aqueous solution), beyond a critical packing density osmotic interactions stretch polymer chains into elongated conformations (*37*). This behavior has been modeled for polymers on curved and spherical surfaces (*38*). The combination of these results suggests that molecular mass alone is insufficient in predicting what size polysaccharide (as part of a protein/polysaccharide conjugate) will limit the amount of protein absorbed to a droplet surface. Additional considerations must include system-dependent variables, such as polymer−polymer, polymer−solvent, and entropic contributions.

This model also suggests that it may be beneficial to combine a limited amount of free β-lg with the larger conjugates, so it may fill in the interstitial spaces available on the droplet surfaces. This would allow the protein layer to become saturated, while still maintaining a thick and densely packed polysaccharide layer. Future experiments are needed to determine if this indeed is the case, such as assaying with different ratios of free β-lg and β-lg conjugates. However, this supplement may not be required since the emulsions with large polysaccharides already show excellent stability even though the droplet surface is not saturated with protein. These large polysaccharide conjugates may also be more effective in stabilizing emulsions at lower molar concentrations (on a protein basis) than protein alone, which could improve their economics for use in the food industry.

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