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Influence of Sucrose on Droplet Flocculation in Hexadecane Oil-in-Water Emulsions Stabilized by β-Lactoglobulin

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The influence of sucrose on the flocculation stability of hydrocarbon oil-in-water emulsions stabilized by a globular protein was examined using laser diffraction. Salt (150 mM NaCl) and sucrose (0−40 wt %) were added to *n*-hexadecane oil-in-water emulsions stabilized by β-lactoglobulin (β-Lg, pH 7.0) either before or after isothermal heat treatment (30−95 °C for 20 min). When salt was added to emulsions before heat treatment, appreciable droplet flocculation was observed below the thermal denaturation temperature of the adsorbed β-Lg ($T_m \sim 70$ °C), and more extensive flocculation was observed above T_m. On the other hand, when salt was added to emulsions after heat treatment, appreciable droplet flocculation still occurred below T_m, but little flocculation was observed above T_m. Addition of sucrose to the emulsions increased T_m and either promoted or suppressed droplet flocculation depending on whether it was added before or after heat treatment. These results are interpreted in terms of the influence of sucrose on protein conformational stability, protein−protein interactions, and the physiochemical properties of aqueous solutions. This study has important implications for the formulation and production of protein stabilized oil-in-water emulsions.

KEYWORDS: Emulsions; β-lactoglobulin; sucrose; surface denaturation; thermal denaturation; droplet flocculation; preferential interactions

INTRODUCTION

Globular proteins are often used as emulsifiers because of their ability to facilitate the formation and improve the stability of oil-in-water emulsions (*1*−*6*). To be an effective emulsifier, a globular protein must rapidly adsorb to the surfaces of the oil droplets formed by mechanical agitation of an oil−water−protein mixture. Protein adsorption lowers the interfacial tension, which facilitates droplet disruption by reducing the amount of energy required to generate small droplets. The formation of a protein film around a droplet helps to prevent it from coalescing with other droplets during the homogenization process. After homogenization, the adsorbed protein film must be capable of imparting long-term stability to food emulsions against droplet coalescence and flocculation. The stability of an emulsion to droplet aggregation largely depends on the relative magnitudes of the attractive and repulsive interactions between the droplets, for example, van der Waals, steric, electrostatic, hydrophobic, and depletion forces (*7*−*9*). Droplets tend to aggregate when the attractive forces dominate but remain as separate entities when the repulsive forces dominate. Physiochemical properties of emulsions, such as rheology, appearance, and stability, are strongly influenced by droplet aggregation, and so it is important from a technological standpoint to understand the factors that determine droplet aggregation (*9, 10*).

Materials based on emulsions are usually compositionally complex and must undergo a variety of different processing operations before they are packaged and distributed. It is therefore particularly important to identify the influence of molecular environment and processing conditions on the functionality of globular proteins in emulsions. In this study, we focus on the influence of salt (NaCl), sugar (sucrose), and heating (30−95 °C) on the susceptibility of oil-in-water emulsions stabilized by β-lactoglobulin (β-Lg) to droplet aggregation. The incorporation of NaCl into a protein-stabilized emulsion would be expected to alter its aggregation stability by modulating the electrostatic interactions between the oil droplets (*9*−*13*). On the other hand, the incorporation of sucrose would be expected to alter the aggregation stability of protein-stabilized emulsions by modifying the conformational stability and intermolecular interactions of the globular proteins, as well as the kinetics of droplet−droplet collisions (*14*−*16*). Heating emulsions stabilized by globular proteins above their thermal denaturation temperature (T_m) causes the adsorbed proteins to undergo conformational changes (*17, 18*). These conformational changes can increase the attractive forces (hydrophobic attraction and disulfide bonds) between emulsion droplets, which leads to droplet aggregation provided the repulsive interactions (electrostatic repulsion) are not too large (*19*−*23*).

The main objectives of this study are to examine the combined influence of an ionic (NaCl) and a neutral (sucrose) cosolvent on the thermal stability of globular protein stabilized emulsions

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and to examine the influence of the order of ingredient addition relative to the thermal treatment. The results of this study will have important implications for the formulation and manufacture of protein-stabilized oil-in-water emulsions.

MATERIALS AND METHODS

Materials. Analytical grade sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), sucrose, and sodium azide (NaN_3) were purchased from Sigma Chemical Co. (St. Louis, MO). Powdered β-Lg was obtained from Davisco Foods International (lot JE 001-1-922, Le Sueur, MN). As stated by the manufacturer, the β-Lg content of the powder determined by electrophoresis was 98% (the remainder being mostly globulins). The decrease in mass of the protein powder upon drying was 2.6%, and the nitrogen content of the powder was 15.6%. Distilled and deionized water was used for the preparation of all solutions.

Solution Preparation. Emulsifier solutions were prepared by dispersing 1 wt % of powdered β-Lg into deionized and distilled water containing 0.04 wt % NaN_3 (as an antimicrobial agent) and stirring for at least 2 h to ensure complete dispersion. Solutions containing different NaCl and sucrose concentrations were prepared by dispersing weighed amounts of the powdered material into 5 mM phosphate buffer (pH 7.0).

Emulsion Preparation. An oil-in-water emulsion was prepared from 10 wt % *n*-hexadecane oil and 90 wt % emulsifier solution (1 wt % β-Lg in distilled water) at room temperature. The oil and emulsifier solution was blended using a high-speed blender for 2 min (model 33BL79, Waring Inc., New Hartford, CT) and then passed through a high-pressure valve homogenizer five times at 7500 psi (Rannie High Pressure, APV-Gaulin, model Mini-Lab 8.30H, Wilmington, MA). The pH of this emulsion was adjusted to 7.0 using HCl solution (pH meter 320, Corning Inc., Corning, NY). The emulsions were then diluted with phosphate buffer (5 mM, pH 7) containing salt and/or sucrose, either before or after heat treatment, to give a series of emulsions containing 5 wt % oil, 2.5 mM phosphate buffer, 0−150 mM NaCl, and 0−40 wt % sucrose. Addition of the salt/sucrose solutions to the emulsions for the "before heat treatment" samples was carried out soon after homogenization (within 30 min). The heat treatment of the emulsions was carried out ~3 h after homogenization. Heat treatment involved placing emulsions in glass test tubes and heating isothermally at a fixed temperature ranging from 30 to 95 °C for 20 min. Addition of the salt/sucrose solutions to the emulsions for the "after heat treatment" samples was carried out soon after the heat treatment (within 30 min), which was about 4−5 h after homogenization. Emulsions were then stored in a temperature-controlled water bath at 30 °C for 24 h.

Particle Size Determination. The particle size distribution of the emulsions was measured using a laser diffraction instrument (LS230, Coulter Corp., Miami, FL). This instrument measures the angular dependence of the intensity of light scattered from a stirred dilute emulsion and then indicates the particle size distribution that gives the closest fit between theoretical calculations and experimental measurements. The manufacturer reports that the instrument can detect particles with diameters between 0.04 and 2000 μm. A refractive index ratio of 1.08 was used in the particle size calculations. To avoid multiple scattering effects, the emulsions were diluted with pH-adjusted distilled water (pH 7) prior to making the measurements. The emulsions were stirred continuously throughout the measurements to ensure the samples were homogeneous. Dilution and stirring may have partially disrupted weakly flocculated droplets, although it is unlikely that they will have disrupted any strongly flocculated droplets. The theory used to calculate the particle size distribution assumes that the particles are spherical and homogeneous, and therefore the data obtained on emulsions that contained flocs should be treated with caution because they are nonspherical and nonhomogeneous. Particle size measurements are reported as weight-average mean diameters, d_{43} ($=\Sigma n_i d_i^4/\Sigma n_i d_i^3$, where n_i is the number of particles with diameter d_i). The d_{43} of each individual sample was calculated from the average of three measurements on the diluted emulsion.

ζ-Potential Measurements. The electrical charge (ζ-potential) on the particles was measured using a particle electrophoresis instrument (ZEM5003, Zetamaster, Malvern Instruments, Worcs., U.K.). Ten weight percent *n*-hexadecane oil-in-water emulsions were diluted 500-fold with a 150 mM NaCl solution adjusted to pH 7 prior to measurements. The diluted emulsion was mixed thoroughly and then injected into the measurement chamber of the instrument. The ζ-potential of each individual sample was calculated from the average of five measurements on the diluted emulsion.

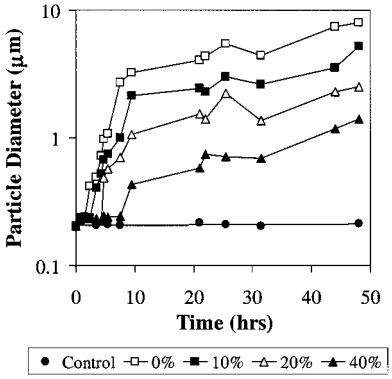

Figure 1. Time dependence of the mean particle diameter (d_{43}) of 5 wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % β-Lg, 150 mM NaCl, pH 7.0) stored at 30 °C containing different sucrose concentrations (0–40 wt %). The control emulsion contained no added sugar or salt.

Statistical Analysis. Experiments were performed twice using freshly prepared samples. Averages and standard deviations were calculated from these duplicate measurements. The standard deviation of the particle diameter measurements was <10% of the mean for nonflocculated emulsions and <20% of the mean for flocculated emulsions. The standard deviation of the ζ-potential measurements was <8%. Statistical differences between samples were calculated using Students's *t* test for independent samples.

RESULTS AND DISCUSSION

Influence of Sucrose on Isothermal Droplet Aggregation. Initially, we measured the time dependence of the mean particle diameter (d_{43}) of 5 wt % *n*-hexadecane oil-in-water emulsions stored at 30 °C to which salt (0 or 150 mM NaCl) and sugar (0, 10, 20, 30, or 40 wt % sucrose) were added immediately after homogenization (**Figure 1**). There was no significant change ($P < 0.05$) in the mean particle diameter of a control emulsion (0 mM NaCl, 0 wt % sucrose) during the measurement period (**Figure 1**). The droplets in this emulsion had a relatively high negative ζ-potential (−35.0 ± 0.5 mV); hence, droplet aggregation was probably prevented because of the relatively strong electrostatic repulsion between the droplets (*23*). The addition of salt to the emulsions led to qualitatively different kinetic behavior, presumably because of its ability to screen the electrostatic interactions between the droplets. When salt was present (150 mM NaCl, 0 wt % sucrose), there was a lag period (~2 h) during which the particle diameter did not change significantly ($P < 0.05$), followed by a rapid growth period (~2−10 h) during which there was a steep and significant ($P < 0.05$) rise in d_{43}, followed by a relatively slow growth period (~10−50 h) when d_{43} increased more slowly but still significantly ($P < 0.05$). As observed in previous studies (*23*), the increase in mean particle diameter was accompanied by a change in the particle size distribution from monomodal to bimodal, with one population containing particles with a diameter similar to that of the original droplets and another population containing particles with much larger diameters (data not shown).

Previous studies have established that particle growth in whey protein stabilized emulsions is due to droplet flocculation caused by progressive surface denaturation of β-Lg molecules after

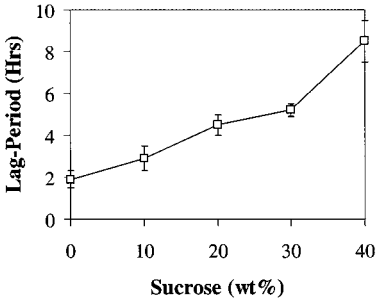

Figure 2. Dependence of the lag period of 5 wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % β-Lg, 150 mM NaCl, pH 7.0) stored at 30 °C on sucrose concentration.

adsorption to oil droplet surfaces (*17*, *18*, *23*−*25*). These conformation changes lead to an increase in the number of nonpolar and sulfhydryl amino acids exposed to the aqueous phase, which increases the hydrophobic attraction and disulfide bond formation between protein molecules adsorbed on different droplets (*23*, *26*, *27*). The existence of a lag period suggests that a certain amount of protein surface denaturation had to occur before the emulsion droplet surfaces became "reactive" enough to promote droplet aggregation. In other words, the increase in droplet surface hydrophobicity and free thiol content due to surface denaturation of the proteins had to reach a certain level before the attractive interactions between the droplets were sufficiently large to overcome the repulsive interactions. The length of the lag period should therefore depend on the height of the energy barrier that normally prevents the droplets in protein-stabilized emulsions from aggregating (*9*). The major contribution to the height of this energy barrier is the electrostatic repulsion between the droplets (*23*). Hence, the lag period should be particularly sensitive to the NaCl concentration in the emulsions. At low salt concentrations, the electrostatic repulsion may be so large that the increase in attractive forces between the droplets due to protein surface denaturation is not sufficient to promote aggregation. On the other hand, at high salt concentrations, the energy barrier may be very low or may not be present, so that the flocculation kinetics is determined mainly by the droplet−droplet encounter frequency. We propose that protein surface denaturation plays an important role only in marginally stable emulsions, that is, those in which the energy barrier is just high enough to prevent rapid flocculation. In these systems, the increase in the attractive forces between droplets due to surface denaturation may decrease the height of the energy barrier sufficiently to promote flocculation. Nevertheless, further work needs to be performed to confirm or refute this hypothesis.

The presence of sucrose in the aqueous phase of the emulsions had a strong impact on the kinetics of droplet aggregation, with the length of the lag period increasing and the rate of particle growth after the lag period decreasing as the sucrose concentration increased (**Figure 1**). The lag period of an emulsion was defined empirically as being the time when the particle diameter first exceeded 0.3 μm, that is, 50% larger than the original droplet size (**Figure 2**). There was a significant ($P < 0.05$) increase (~4.5-fold) in the lag period when the sucrose concentration was increased from 0 to 40 wt %. Unfortunately, we could not accurately quantify the change in particle growth rate with sucrose concentration because of the lack of an appropriate mathematical model to describe the change in particle diameter with time. By assuming that the change in particle diameter with time was linear in the rapid growth period, we calculated approximate particle growth rates of ~0.5 and ~0.01 μm/h for the emulsions containing 0 and 40 wt % sucrose, respectively. Hence, high levels of sucrose were extremely effective at retarding droplet flocculation.

The ability of sucrose to retard droplet aggregation in the emulsions may be due to a number of different physicochemical phenomena. First, the viscosity of the aqueous solution surrounding the emulsion droplets increases as the sucrose concentration increases, which would reduce the frequency of droplet−droplet collisions. At 25 °C, aqueous sucrose solutions have viscosities of 1.0, 1.3, 1.9, 3.2, and 6.2 mPa s at sucrose concentrations of 0, 10, 20, 30, and 40 wt %, respectively (*CRC Handbook of Chemistry and Physics*). The collision frequency in a colloidal suspension containing monodisperse spherical particles is inversely proportional to the viscosity of the continuous phase (*28*). Consequently, the decrease in the droplet−droplet collision frequency would be expected to decrease the rate of droplet aggregation 6.2-fold when the sucrose concentration was increased from 0 to 40 wt %. Second, the dielectric constant, refractive index, and interfacial tension of the aqueous solution surrounding the emulsion droplets are changed by the presence of sucrose, which would influence the strength of the attractive van der Waals and hydrophobic interactions and the repulsive electrostatic interactions between the droplets (*9*). Sucrose may therefore have altered the delicate balance of attractive and repulsive interactions between the droplets, thus changing their propensity to aggregate. Third, sucrose may have altered the rate and extent of conformational changes of protein molecules adsorbed to the droplet surfaces. Previous studies have shown that sucrose is capable of stabilizing molecular conformations of globular proteins that are more compact relative to those that are less compact through a preferential interaction mechanism (*14*, *15*). At room temperature, the molecular origin of this effect has been attributed primarily to steric exclusion of sucrose molecules (relative to water molecules) from the immediate vicinity of globular proteins (*29*, *30*). Steric exclusion leads to a concentration gradient between the sucrose-rich bulk solution and the sucrose-depleted region surrounding the protein surface, which generates an osmotic stress that favors a decrease in contact area between the protein surface and the surrounding solution (*14*). The presence of sucrose therefore favors more compact molecular conformations that have lower contact areas (*15*). The magnitude of the osmotic stress increases as the concentration of sucrose in the aqueous phase increases, and therefore higher sugar concentrations are more effective at retarding conformation changes of adsorbed proteins. Sucrose may therefore have increased the conformational stability of the adsorbed proteins, reducing the number of reactive exposed amino acids capable of promoting droplet−droplet interactions through hydrophobic attraction and disulfide bond formation (*23*). Fourth, the strength of protein−protein interactions increases in the presence of sucrose for the same reason the conformational stability of the proteins increases; that is, preferential interactions favor more compact states of the protein system (*14*). Consequently, this interaction increases the strength of the attractive interactions between the droplets relative to the repulsive interactions, which may promote droplet flocculation or increase the strength of the bonds between droplets within flocs.

It is not possible from the laser diffraction experiments alone to quantify the relative contribution of each of these different mechanisms to droplet aggregation in protein-stabilized emulsions. Even so, our results suggest that the increase in the laf period with increasing sucrose concentration is primarily caused by the ability of sucrose to stabilize more compact conformations of the adsorbed proteins, that is, to retard surface denaturation.

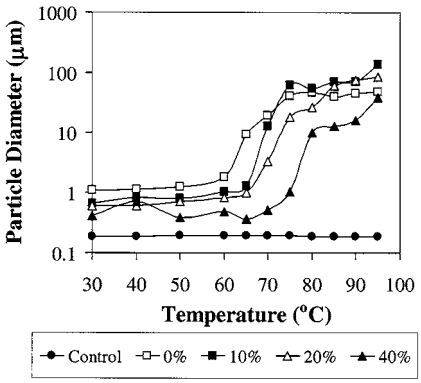

Figure 3. Influence of sucrose concentration on the formation of aggregates in 5 wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % β-Lg, 150 mM NaCl, pH 7.0) during isothermal heat treatment (30–95 °C, 20 min). The control emulsion contained no added sugar or salt.

Otherwise, we would have expected the lag period to be relatively independent of sucrose concentration but the subsequent particle growth rate to decrease with increasing sucrose concentration. After the lag period, we believe that the slower increase in droplet diameter with time is due to a combination of the ability of sucrose to suppress protein unfolding and to increase the viscosity of the continuous phase and therefore slow droplet−droplet encounters.

Influence of Sucrose on Thermally Induced Droplet Aggregation. From a practical standpoint, it is important to understand the influence of sugars on the thermal stability of oil-in-water emulsions stabilized by globular proteins. We therefore investigated the influence of sucrose on heat-induced droplet aggregation in 5 wt % *n*-hexadecane oil-in-water emulsions stabilized by β-Lg. Emulsions containing 150 mM NaCl and different amounts of sucrose (0−40 wt %) were held at fixed temperatures between 30 and 95 °C for 20 min, cooled to room temperature, and stored for 24 h. The degree of droplet aggregation in the emulsions was then determined by measuring the increase in particle diameter using laser diffraction (**Figure 3**). A control emulsion (0 mM NaCl, 0 wt % sucrose) subjected to the same heat treatments showed no change in mean particle diameter at any temperature, which has been attributed to the strong electrostatic repulsion between the droplets at low salt concentrations (*23*). On the other hand, droplet aggregation was observed in emulsions containing 150 mM NaCl at certain temperatures. In the absence of sucrose, a significant ($P < 0.05$) increase in mean particle diameter (compared to the control emulsion) was observed for emulsions that had been held at temperatures below 65 °C, which can be attributed to flocculation caused by surface denaturation of the adsorbed proteins (see previous section). There was a further significant ($P < 0.05$) increase in particle diameter in emulsions that had been held at or above temperatures of 65 °C, indicating that droplet flocculation became more extensive at these elevated temperatures. When adsorbed β-Lg is heated above its thermal denaturation temperature ($T_m = 71-73$ °C), it undergoes a conformational change, which exposes nonpolar and sulfhydryl-containing amino acid groups normally buried in the hydrophobic protein interior (*12*, *26*, *31*). Thermal denaturation of adsorbed β-Lg causes more extensive conformational changes than surface denaturation (*18*). Hence, one would expect stronger hydrophobic attraction and disulfide bond formation to occur between thermally denatured proteins adsorbed to different emulsion droplets than between surface-denatured proteins, which would explain the greater amount of droplet aggregation observed at elevated temperatures (*16*, *31*).

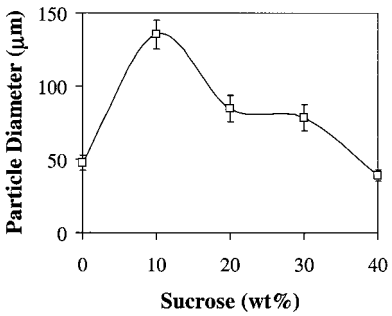

Figure 4. Dependence of the mean particle diameter of 5 wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % β-Lg, 150 mM NaCl, pH 7.0) heated to 95 °C for 20 min on sucrose concentration.

The temperature at which extensive droplet flocculation was first observed increased with increasing sugar concentration, being around 65, 70, 75, 75, and 80 °C for 0, 10, 20, 30, and 40 wt % sucrose, respectively. The most likely reason for this significant ($P < 0.05$) increase in flocculation temperature is the ability of sucrose to increase the thermal stability of globular proteins through a preferential interaction mechanism (*14*, *32*). The extent of droplet flocculation at temperatures below the thermal denaturation temperature of the adsorbed proteins ($T < T_m$) appeared to be suppressed by increasing levels of sucrose, which was discussed in the previous section. Sucrose also altered the extent of droplet aggregation in emulsions heated above T_m, for example, heated to 95 °C (**Figure 4**). There was initially a significant ($P < 0.05$) increase in aggregate size from 0 to 10 wt % sucrose, followed by a significant ($P < 0.05$) decrease when the sucrose concentration was increased further. In a previous study of the influence of sucrose on the thermal stability of emulsion droplets stabilized by whey protein isolate we observed a similar effect (*16*). The maximum degree of droplet aggregation at temperatures above the thermal denaturation temperature of the proteins increased when the sucrose concentration was increased from 0 to 10 wt % but decreased when the sucrose concentration was increased further (*16*). It was proposed that sucrose influenced the extent of droplet aggregation in the emulsions through two opposing mechanisms. First, sucrose increases the strength of protein−protein interactions through a preferential interaction mechanism, which increases the tendency for droplets to come into close proximity and favors droplet aggregation. Second, sucrose increases the thermal stability of the adsorbed proteins, which reduces their tendency to unfold and expose reactive amino acids to the aqueous phase. Consequently, the hydrophobicity and free thiol content of the emulsion droplet surfaces are decreased in the presence of sucrose, which reduces the strength of the attractive forces between droplets and opposes droplet aggregation. At relatively low sucrose concentrations the first mechanism dominates because the majority of adsorbed proteins have unfolded into an active state, but at higher concentrations the second mechanism dominates because many of the adsorbed proteins have not unfolded into an active state (or the extent of unfolding is less).

Influence of Ingredient Order of Addition. In a previous study, we showed that the susceptibility of globular protein-stabilized emulsions to droplet aggregation during thermal processing was strongly dependent on the order of addition of NaCl relative to the heat treatment (*31*). This phenomenon could be practically utilized as a method of improving the thermal stability of emulsions. In the current study, we examine the combined influence of salt and sucrose order of addition relative to thermal treatment on the heat stability of emulsions.

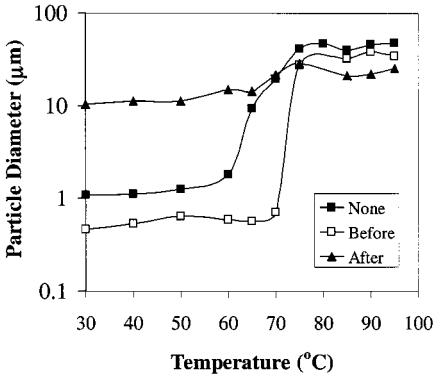

Figure 5. Influence of cosolvents on the stability of 5 wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % β-Lg, 150 mM NaCl, pH 7.0) to droplet flocculation during thermal processing. Salt was added to the emulsions *before* heating. The addition of sugar to the emulsions is indicated in the annotation box: none, no added sugar; before, 40 wt % sucrose added before heating; after, 40 wt % sucrose added after heating. Nonflocculated emulsions had a mean diameter of 0.19 μm.

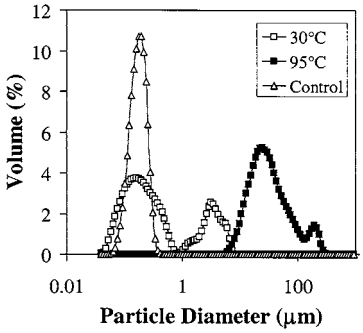

Figure 6. Influence of cosolvents on the particle size distribution of 5 wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % β-Lg, 150 mM NaCl, pH 7.0) after thermal processing. Salt was added to the emulsions *before* heating. Heating temperatures are shown in the annotation box. The control emulsion contained no salt and no sugar.

NaCl Added before Heating. The mean particle diameters of heat-treated β-Lg stabilized oil-in-water emulsions to which 150 mM NaCl was added before thermal treatment are shown in **Figure 5**. The influence of temperature on the particle size distributions of selected emulsions containing no sucrose is shown in **Figure 6**. Under these conditions the electrostatic repulsion between the emulsion droplets is relatively weak, and so the droplets can come into close proximity during thermal processing if the attractive interactions between them increase slightly. In the absence of sucrose, a significant ($P < 0.05$) amount of droplet flocculation was observed when the emulsions were held at temperatures from 30 to 65 °C, due to the surface denaturation of β-Lg mentioned above (**Figure 5**). Flocculation at these lower temperatures (e.g., 30 °C) led to the formation of a bimodal particle size distribution, with one population of particles having a mean diameter similar to that of the control emulsion and the other population having a much larger diameter (**Figure 6**). At higher temperatures (70−95 °C), there was a further significant ($P < 0.05$) increase in particle diameter, which indicated that the extent of droplet flocculation in the emulsions increased (**Figure 5**). This increase was attributed to intermolecular interactions between heat-denatured protein molecules adsorbed to the surface of different emulsion droplets (see previous section). Flocculation at these elevated temperatures (e.g., 95 °C) led to the formation of a monomodal particle size distribution, containing particles with mean diameters much greater than those of either the control emulsion or the surface-denatured flocculated emulsion (**Figure 6**).

When sucrose was added to the emulsions prior to thermal treatment, there was a significant ($P < 0.05$) decrease in the extent of droplet flocculation at lower temperatures (30−65 °C). As mentioned earlier, the influence of sucrose could be attributed to a number of different physiochemical processes, including stabilization of protein conformation, alteration of droplet−droplet interactions, and reduction of droplet−droplet collision frequency. Adding sucrose prior to heating also caused a significant ($P < 0.05$) increase in the temperature at which extensive flocculation was first observed (**Figure 5**), which was attributed to the ability of sucrose to increase the thermal denaturation temperature of the adsorbed proteins (*32*). Nevertheless, addition of sucrose prior to heating was unable to prevent extensive droplet flocculation above T_m.

When sucrose was added to the emulsions after thermal treatment, there was a significant ($P < 0.05$) increase in the extent of droplet flocculation below T_m, which was in contrast to the significant ($P < 0.05$) decrease found when sucrose was added before thermal treatment (**Figure 5**). The most likely explanation for this effect is the ability of sucrose to increase both the conformational stability of globular proteins and the strength of protein−protein interactions through a preferential interaction mechanism (*32*). When sucrose was added to emulsions immediately after homogenization, surface denaturation of the adsorbed protein molecules was suppressed. Consequently, the hydrophobic attraction between the droplets was reduced, which decreased their susceptibility to aggregation. In addition, the presence of the sucrose would have reduced the droplet−droplet collision frequency; hence, intradroplet protein−protein interactions may have become more favorable than interdroplet protein−protein interactions. On the other hand, when sucrose was added to emulsions sometime after homogenization, extensive surface denaturation of the proteins had already occurred. In this case, sucrose promoted droplet flocculation because it increased the strength of the attractive protein−protein interactions. The fact that sucrose suppressed droplet flocculation when added before heating, but enhanced flocculation when added after heating (at $T < T_m$), suggests that it mainly affects emulsion stability through its influence on the conformational stability and interactions of adsorbed proteins, rather than on its ability to alter the magnitude of droplet−droplet interactions (e.g., van der Waals and electrostatic) by changing the physiochemical properties of the continuous phase. Otherwise, one would have expected that the influence of sucrose on droplet flocculation below T_m would be independent of the order of its addition. Adding sucrose after heating could not prevent extensive droplet flocculation from occurring above T_m.

NaCl Added after Heating. The mean particle diameters of heat-treated β-Lg stabilized oil-in-water emulsions to which 150 mM NaCl was added after thermal treatment are shown in **Figure 7**. The influence of temperature on the particle size distributions of selected emulsions containing no sucrose is shown in **Figure 8**. Under these conditions there is a relatively strong electrostatic repulsion between the emulsion droplets during thermal processing, so that the droplets are not in close proximity during the thermal denaturation of the adsorbed protein molecules. In the absence of sucrose, a significant ($P < 0.05$) amount of droplet flocculation was still observed when the emulsions were heated at temperatures from 30 to 65 °C ($T < T_m$) (**Figures 7** and **8**), which was presumably caused by surface denaturation of β-Lg after adsorption to the droplet

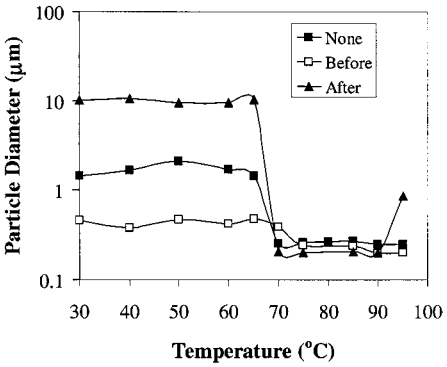

Figure 7. Influence of cosolvents on the stability of 5 wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % β-Lg, 150 mM NaCl, pH 7.0) to droplet flocculation during thermal processing. Salt was added to the emulsions *after* heating. The addition of sugar to the emulsions is indicated in the annotation box: none, no added sugar; before, 40 wt % sucrose added before heating; after, 40 wt % sucrose added after heating. Nonflocculated emulsions had a mean diameter of 0.19 μm.

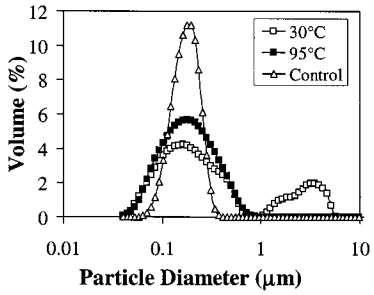

Figure 8. Influence of cosolvents on the particle size distribution of 5 wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % β-Lg, 150 mM NaCl, pH 7.0) after thermal processing. Salt was added to the emulsions *after* heating. Heating temperatures are shown in the annotation box. The control emulsion contained no salt and no sugar.

surfaces (see above). There was little evidence of droplet flocculation when the emulsions were heated to temperatures above T_m (70−95 °C), with the mean particle diameter being similar to the control emulsion (**Figure 7**) and the particle size distribution broadening but not moving in position (**Figure 8**). This result is in dramatic contrast to the results for the emulsions to which salt was added before heating (**Figures 5** and **6**). The most likely explanation of this phenomenon is that protein−protein interactions between thermally denatured β-Lg molecules adsorbed to the same droplet are promoted when there is a strong electrostatic repulsion between the droplets during heating, rather than those between β-Lg molecules adsorbed to different droplets (*31*).

At temperatures below T_m, there was a significant ($P < 0.05$) decrease in the extent of droplet flocculation when sucrose was added to the emulsions prior to thermal treatment, but a significant ($P < 0.05$) increase when it was added after. These results are similar to those discussed above for the emulsions to which NaCl was added prior to heating (**Figure 5**). The presence of sucrose had little effect on droplet flocculation in most of the emulsions at temperatures above T_m, with the mean particle diameters being close to those of the control emulsion. Nevertheless, there was some evidence of droplet flocculation in the emulsion to which sugar was added after heating to 95 °C. This instability may have occurred because the droplets heated to this temperature in the absence of sucrose may have had a slightly more hydrophobic character than those heated to lower temperatures (but still above T_m). Hence, increasing the strength of the protein−protein interactions by adding sucrose was sufficient to promote flocculation in this marginally stable emulsion.

Conclusions. This study has shown that cosolvents, such as sucrose and salt, have a pronounced influence on the thermal stability of oil-in-water emulsions stabilized by globular proteins. These cosolvents may either increase or decrease the stability of an emulsion to flocculation during heat treatment depending on their nature and their order of addition. Our study has important consequences for the formulation of many food, cosmetic, and pharmaceutical products based on protein-stabilized emulsions. Nevertheless, further work is needed to clarify the precise molecular origin of the influence of sucrose on the flocculation stability of globular protein-stabilized emulsions.

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