

Interactions of Whey Proteins during Heat Treatment of Oil-in-Water Emulsions Formed with Whey Protein Isolate and Hydroxylated Lecithin

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The interactions of proteins during the heat treatment of whey-protein-isolate (WPI)-based oil-in-water emulsions with and without added hydroxylated lecithin were studied by examining the changes in droplet size distribution and the quantity and type of adsorbed and unadsorbed proteins. Heat treatment at 90 °C of WPI emulsions resulted in an increase in total adsorbed protein; unadsorbed β -lactoglobulin (β -lg) was the main protein interacting with the adsorbed proteins during the first 10 min of heating, but after this time, unadsorbed α -lactalbumin (α -la) also associated with the adsorbed protein. In emulsions containing hydroxylated lecithin, the increase in total adsorbed protein during heat treatment was much lower and the unadsorbed β -lg did not appear to interact with the adsorbed proteins during heating. However, the behavior of α -la during heat treatment of these emulsions was similar to that observed in the emulsions containing no hydroxylated lecithin. In the presence of NaCl, the particle size of the emulsion droplets and the quantities of adsorbed protein increased markedly during heating. Emulsions containing hydroxylated lecithin were less sensitive to the addition of NaCl. These results suggest that the binding of hydroxylated lecithin to unfolded monomers or intermediate products of β -lg reduces the extent of heat-induced aggregation of β -lg and consequently decreases the interactions between unadsorbed β -lg and adsorbed protein. This was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of heated whey protein and hydroxylated lecithin solutions.

KEYWORDS: Whey protein; hydroxylated lecithin; emulsion; heat treatment

INTRODUCTION

Whey proteins are widely used as food ingredients because of their good functional and nutritional properties (1). An important functional property of whey proteins is their ability to facilitate the formation and stability of oil-in-water emulsions (2). Whey proteins, which consist principally of β -lactoglobulin (β -lg), α -lactalbumin (α -la), and bovine serum albumin (BSA), have globular structures; upon heat treatment above 70 °C, these proteins unfold and aggregate (3, 4). In whey-protein-stabilized emulsions, heat treatment may lead to aggregation of the emulsion droplets (2, 5–9). The stability of whey-protein-stabilized emulsions is affected by many factors, including pH, ionic strength, and the presence of emulsifiers such as lecithin. The heat stability of the emulsion decreases with increasing ionic strength in the emulsion (5–7).

Hunt and Dalgleish (5) and Kulmyrzaev et al. (7) reported that emulsions made with whey proteins were stable against

heating at low and neutral pH and at low ionic strength. Monahan et al. (10) and Sliwinski et al. (8) reported that heating at 75–80 °C caused significant droplet aggregation but that the effect decreased at higher heating temperatures. The authors explained this trend by the predominance of interdroplet interactions, leading to aggregation of the emulsion droplets, on heating in the temperature range of 75–80 °C, whereas intradroplet protein–protein interactions were favored at higher temperatures. In contrast, Euston et al. (11) reported that heat-induced aggregation of droplets in emulsions formed when whey protein concentrate (WPC) was extensive and proceeded more rapidly as the concentration of whey proteins in the emulsion was increased. Removal of nonadsorbed protein or replacement of the serum with a solution of caseinate decreased the rate of aggregation severalfold (11). It was suggested that the nonadsorbed protein would act as a “glue”, holding the aggregated emulsion droplets together (5).

Lecithins are important ingredients in the commercial manufacture of emulsions. Many studies have reported their surface-active properties (12–15), competition with proteins at oil/water interfaces (16–18), and interactions with proteins (19, 20). Hydroxylated lecithin, a commercially available modified

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lecithin, is produced by the introduction of hydroxyl groups to the fatty acid double bonds of concentrated and purified soya bean lecithin (21). This reaction leads to increased water dispersibility and enhanced oil-in-water emulsifying properties. Agboola et al. (22) found that the stability of the emulsion (4% soya oil) formed with hydrolyzed whey proteins to retort treatment at 121 °C for 16 min was improved markedly by the addition of hydroxylated lecithin. However, the mechanism by which hydroxylated lecithin stabilized the emulsions was unclear. It was suggested that the adsorption of hydroxylated lecithin to the droplet surface after retorting increased the overall charge and hydration at the droplet surface and/or that there were some interactions between nonadsorbed peptides and hydroxylated lecithin.

The objective of this work was to study the adsorption behavior and aggregation of whey proteins in emulsions made with whey protein isolate (WPI) with and without hydroxylated lecithin under a range of ionic strengths. The implications of the behavior of proteins in the stability of the emulsions are also discussed.

MATERIALS AND METHODS

Materials. WPI (containing 95% protein and 0.06% Na) was supplied by the Fonterra Co-operative Group Limited, New Zealand. Hydroxylated lecithin (PRECEPT 8120) was supplied by Central Soya Co. Inc., IN; its phospholipid content was 95% (as acetone insolubles). Soya oil was purchased from Davis Trading Co., Palmerston North, New Zealand.

All of the chemicals used were of analytical grade and were obtained from either BDH Chemicals (BDH Ltd., Poole, England) or Sigma Chemical Co. (St. Louis, MO) unless specified otherwise.

Preparation of Emulsions. WPI emulsions were prepared according to the procedure described by Singh et al. (23). WPI solutions were prepared by adding the WPI powder to Milli-Q water (water purified by treatment with a Milli-Q apparatus; Millipore Corp., Bedford, MA) and then stirring for 60 min at room temperature to ensure complete dispersion. Hydroxylated lecithin (0.1 wt % in the final emulsion) was dispersed in WPI solution with stirring for 60 min at room temperature. The pH of these solutions was adjusted in the range of 6.8–7.0. Appropriate quantities of soya oil were then mixed with the protein-hydroxylated lecithin solutions to give 20% oil in the final emulsion. The mixture of WPI solution and corn oil was heated to 55 °C and then homogenized in a two-stage homogenizer (APV 2000, Albertslund, Denmark) at a first-stage pressure of 20 MPa and a second-stage pressure of 4 MPa. The emulsions were homogenized twice for more effective mixing of the oil phase. Emulsions were prepared in duplicate.

Heating of Emulsions. The emulsions were heated to 90 °C within 2 min in a boiling water bath. They were then held at 90 °C for 0–30 min in a water bath and cooled immediately in an ice bath to room temperature.

Determination of Average Droplet Size. A Malvern MasterSizer MSE (Malvern Instruments Ltd., Malvern, Worcestershire, U.K.) was used to determine the average diameter (d_{43}) of the emulsion droplets. The parameters that were used to analyze the droplet size distribution were defined by the presentation code 2NAD. The relative refractive index (N), i.e., the ratio of the refractive index of the emulsion droplets (1.456) to that of the dispersion medium (1.33), was 1.095. The absorbance value of the emulsion droplets was 0.001. Droplet size measurements are reported as sauter-average diameters, d_{32} . The d_{32} is defined as $\sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of particles with diameter d_i . Mean particle diameters were calculated as the average of duplicate measurements.

Determination of Surface Protein Concentration and Composition. The emulsions were centrifuged at 45000g for 40 min at 20 °C in a temperature-controlled centrifuge (Sorvall RC5C, DuPont Co., Wilmington, DE). The subnatants were carefully removed using a syringe. The cream layer was dispersed in deionized water and recentrifuged at 45000g for 40 min. The subnatant was filtered

sequentially through 0.45 and 0.22 μm filters (Millipore Corp., Bedford, MA). The filtrates were analyzed separately for total protein using the Kjeldahl method (1026 Distilling Unit and 1007 Digestor Block, Tecator AB, Hoganas, Sweden). The surface protein concentration (mg/m^2) was calculated from the surface area of the oil droplets, determined by MasterSizer, and the difference in the amount of protein used to prepare the emulsion and that measured in the subnatant and sediment after centrifugation.

$$\text{adsorbed protein (g)} = \text{total protein (g) taken for making an emulsion} - [\text{protein (g) present in the subnatant} + \text{protein (g) present in the sediment}]$$

$$\text{total protein coverage (mg}/\text{m}^2) = \text{total protein adsorbed (mg)}/\text{total fat surface area (m}^2)$$

The composition of the adsorbed protein at the surface of the emulsion droplets and of the nonadsorbed protein in the aqueous phase was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as described by Ye and Singh (24). A certain amount of cream was spread on a filter paper and a known amount of dried cream was mixed with SDS buffer (0.5 M Tris, 2% SDS, and 0.05% mercaptoethanol at pH 6.8). A portion (5 μL) of this dispersion or the subnatant (after filtration) was applied to the SDS gels previously prepared on a Miniprotein II system (Bio-Rad Laboratories, Richmond, CA). After destaining, the gels were scanned on a laser densitometer (LKB Ultrascan XL, LKB Produkter AB, Bromma, Sweden). The percentage composition of each sample was determined by scanning the areas for α -la and β -lg and expressing the individual protein peaks as a fraction of the sum total.

Confocal Laser Scanning Microscopy. A Leica (Heidelberg, Germany) confocal laser scanning microscope with a chosen objective lens and an Ar/Kr laser with an excitation line of 488 nm (in such a way that only the fluorescent wavelength band could reach the detector system) was used to determine the microstructure of the emulsions. Emulsions were made as described above; approximately 3 mL of sample was taken in a test tube, Nile Blue (fluorescent dye) was mixed through, and then the mixture was placed on a microscope slide. The slide was covered with a coverslip and observed under the microscope.

Analysis of six separate emulsions, made with 1.0% WPI and 20% soya oil, showed that the variations were $\pm 0.02 \mu\text{m}$ for d_{32} , $\sim 4\%$ for surface protein concentration, $\sim 4\%$ for α -la in the serum, and $\sim 4\%$ for β -lg in the serum.

Statistical Analysis. The results were analyzed statistically using the Minitab 12 for Windows package. Differences were considered to be significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Surface Protein Coverage and Composition. No differences were observed in the average droplet size ($d_{32} = \sim 0.5 \mu\text{m}$) of whey-protein (1 wt % WPI)-stabilized oil-in-water emulsions containing 0 and 0.1% hydroxylated lecithin, indicating that the formation of the emulsions was not affected by the presence of hydroxylated lecithin. Heating these emulsions at 90 °C for up to 30 min did not cause a significant change in d_{32} (data not shown). These results, which are in agreement with the reports of others (5–7), indicate that no aggregation occurred between the proteins adsorbed on different droplets under the conditions used in this study. The lack of interactions between the proteins adsorbed on different droplets was probably due to strong electrostatic repulsions between the droplets, preventing their close approach.

However, heat treatment of control emulsions (no hydroxylated lecithin) resulted in a slight increase in the amount of adsorbed protein (Figure 1), with the surface protein coverage increasing from ~ 1.2 to $\sim 1.4 \text{ mg}/\text{m}^2$ within the first 10 min at 90 °C. Further heating resulted in a decrease in the surface

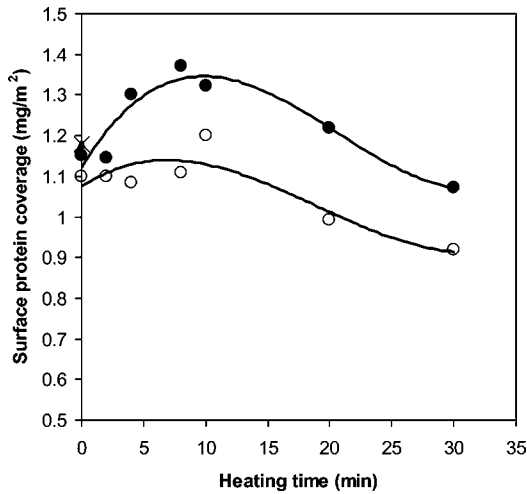


Figure 1. Changes in total surface protein coverage (mg/m²) of emulsions made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (●) and 0.1 wt % hydroxylated lecithin (○) as a function of heating time (min) at 90 °C. The total surface protein coverage (mg/m²) of unheated emulsions containing no hydroxylated lecithin (▲) and 0.1 wt % hydroxylated lecithin (×) is also shown. Each data point is the average of determinations on two separate emulsions.

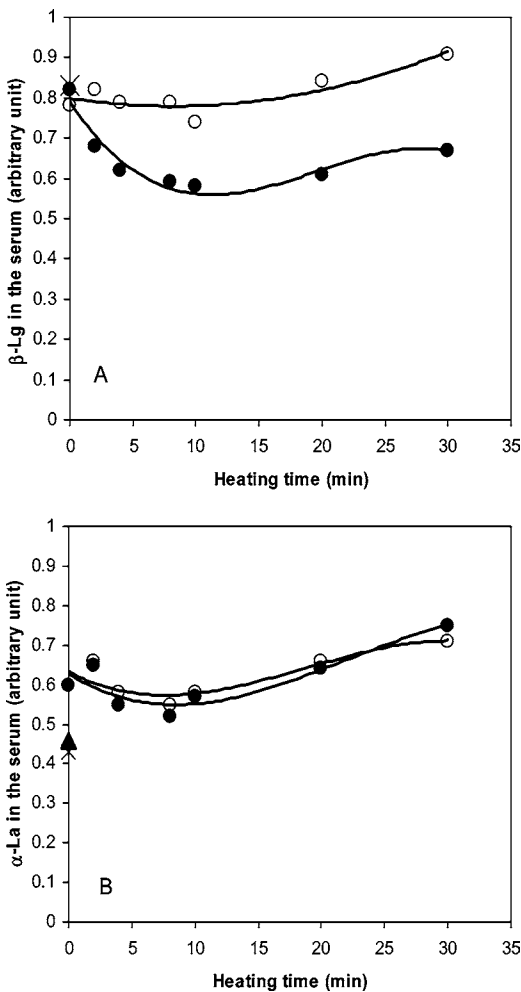


Figure 2. β-Lg (A) and α-la (B) in the aqueous phase of emulsions (unadsorbed protein) made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (●) and 0.1 wt % hydroxylated lecithin (○) after heating at 90 °C for up to 30 min. The unadsorbed β-lg (A) and α-la (B) in unheated emulsions containing no hydroxylated lecithin (▲) and 0.1 wt % hydroxylated lecithin (×) are also shown.

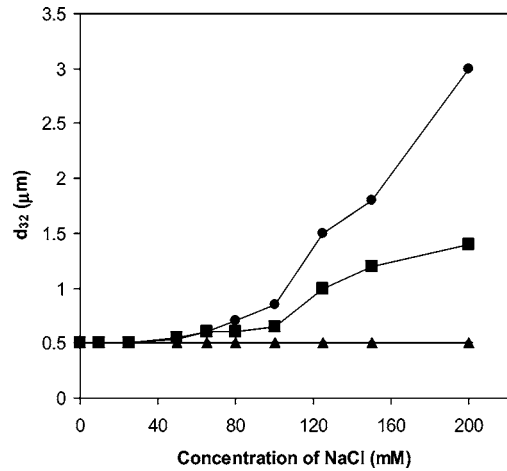


Figure 3. Changes in average particle size (d_{32}) of emulsions made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (●) and 0.1 wt % hydroxylated lecithin (■) after heating at 90 °C for 20 min as a function of added NaCl (mM). d_{32} of unheated emulsions (▲).

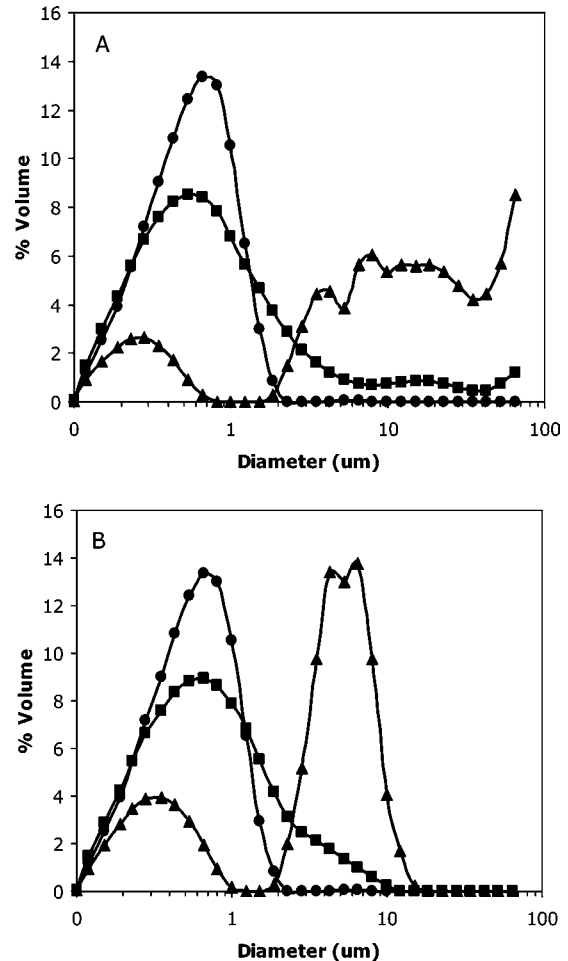


Figure 4. Droplet size distributions of emulsions made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (A) and 0.1 wt % hydroxylated lecithin (B) after heating at 90 °C for 20 min in the presence of 0 mM (●), 75 mM (■), and 125 mM (▲) NaCl.

protein coverage. The surface protein coverage in the emulsions containing hydroxylated lecithin was lower than in the control emulsions and showed a slight decrease during heating.

The amounts of β-lg and α-la in the aqueous phase of the emulsions (unadsorbed proteins) during heating at 90 °C are

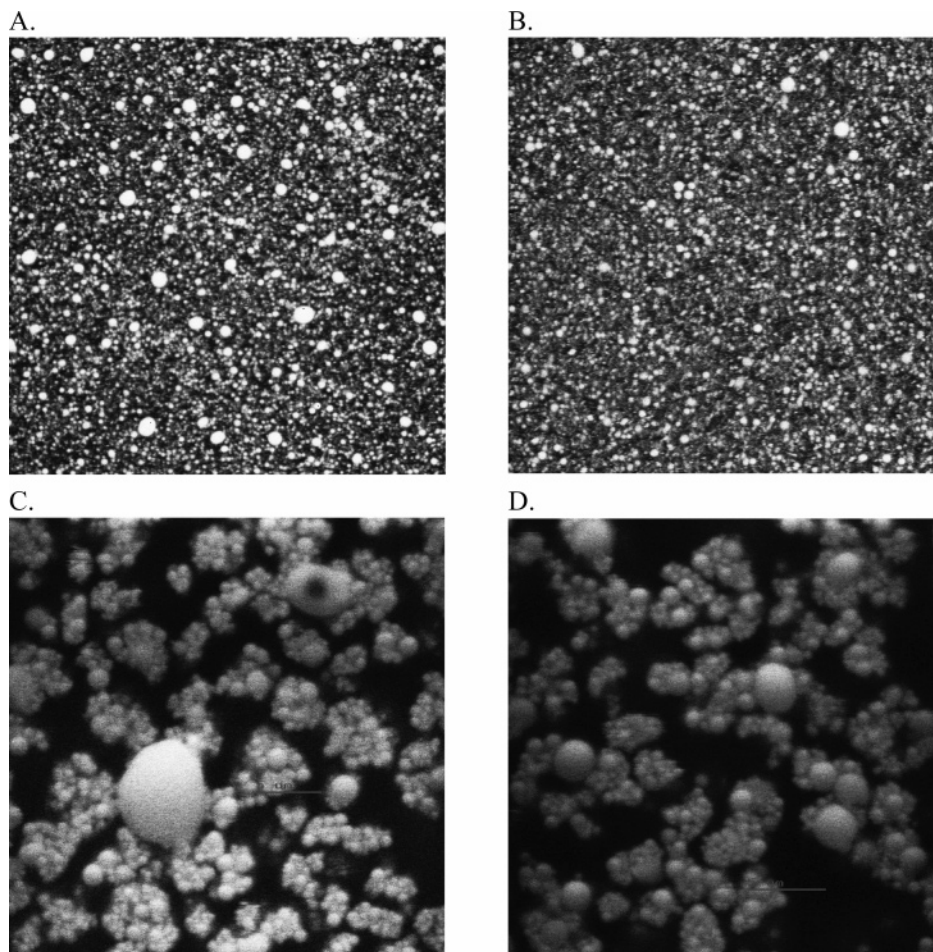


Figure 5. Confocal micrographs of emulsions made with 20% soya oil at pH 7.0 and 1 wt % WPI containing 50 mM NaCl (A and B) and 125 mM NaCl (C and D) after heating at 90 °C for 20 min in the absence of hydroxylated lecithin (A and C) and in the presence of 0.1 wt % hydroxylated lecithin (B and D).

shown in **Figure 2**. In the control emulsions, the amount of unadsorbed β -lg decreased within the first 10 min of heating and then slightly increased with further heating. In contrast, the amount of unadsorbed α -la appeared to increase after heating for ≥ 10 min at 90 °C (**Figure 2B**). In emulsions containing hydroxylated lecithin, the amount of unadsorbed β -lg did not change significantly during heating, whereas the amount of unadsorbed α -la showed a considerable increase, similar to that observed in the emulsions containing no hydroxylated lecithin. These results suggest that β -lg molecules from the aqueous phase associated with the surface of the emulsion droplets during heating, whereas some of the adsorbed α -la dissociated from the droplet surface during heating. This association of unadsorbed β -lg with the proteins adsorbed at the droplet surface was markedly reduced by the presence of hydroxylated lecithin. However, the dissociation of α -la from the droplet surface appeared to be unaffected by the presence of hydroxylated lecithin.

The results obtained for the total surface protein coverage of whey-protein-stabilized emulsions containing no hydroxylated lecithin during heating are generally in agreement with the reports of previous workers (8, 10). They showed that the amount of total adsorbed protein increased within the first few minutes of heating at 90 °C and then reached a plateau value during further heating. However, they did not observe a decrease in the adsorbed protein at longer heating times. Our results on the amounts of individual proteins at the droplet surface suggest that the increase in the total surface protein coverage during

heating was essentially caused by the association of unadsorbed β -lg with the adsorbed proteins. The interactions of unadsorbed β -lg with hydroxylated lecithin appeared to prevent its association with the adsorbed protein. The decrease in total surface protein coverage at heating times above 10 min was probably caused by the loss of α -la from the droplet surface, although the reason for the dissociation of α -la from the droplet surface is not clear. One possibility is that α -la was displaced from the surface by β -lg at high temperature, because β -lg may be more surface active at high temperatures (25, 26).

Effect of NaCl. The average size (d_{32}) of the emulsion particles was unaffected by the addition of NaCl (**Figure 3**). However, when the emulsions were heated at 90 °C for 20 min, the d_{32} started to increase at ~ 50 mM added NaCl. At > 50 mM NaCl, the d_{32} of the emulsions containing hydroxylated lecithin was considerably smaller than that of the control emulsions. The size distribution of the emulsions showed that some large particles ($> 50 \mu\text{m}$) were formed in the emulsions containing no hydroxylated lecithin, whereas the sizes of all droplets in the emulsions containing hydroxylated lecithin were smaller than $20 \mu\text{m}$ (**Figure 4**).

Confocal micrographs showed some aggregation of the emulsion droplets in the heated emulsions containing 50 mM NaCl (**Figure 5A**). In the presence of hydroxylated lecithin, this aggregation of droplets appeared to occur to a lesser extent at the same NaCl concentration (**Figure 5B**). At a high NaCl concentration (125 mM), many emulsion droplets aggregated to form large particles ($\sim 5 \mu\text{m}$); these large particles subse-

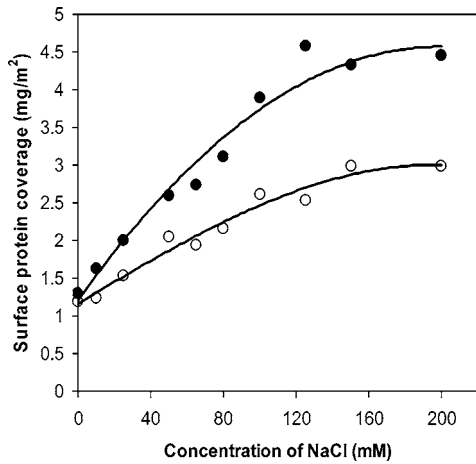


Figure 6. Changes in total surface protein coverage (mg/m^2) of emulsions heated at 90°C for 20 min made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (●) and 0.1 wt % hydroxylated lecithin (○) as a function of added NaCl (mM). NaCl was added prior to emulsification. Each data point is the average of determinations on two separate emulsions.

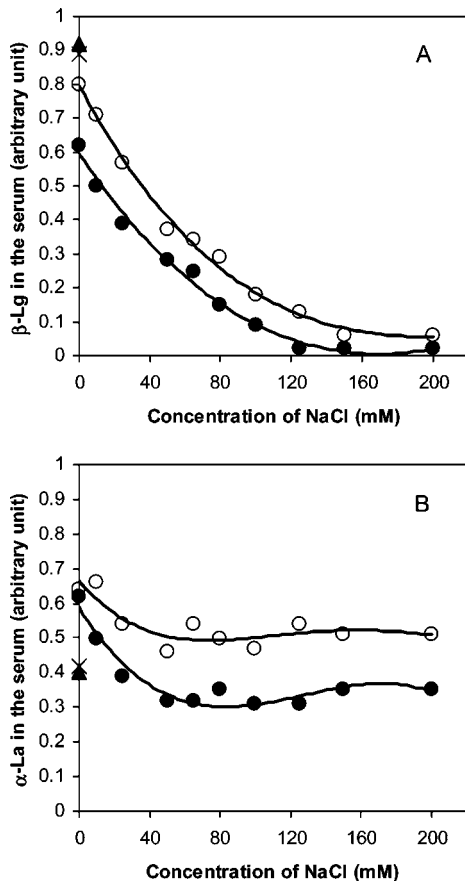


Figure 7. Changes in β -Lg (A) and α -La (B) in the aqueous phase of emulsions heated at 90°C for 20 min made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (●) and 0.1 wt % hydroxylated lecithin (○) as a function of added NaCl (mM). The unadsorbed β -Lg (A) and α -La (B) in unheated emulsions containing no hydroxylated lecithin (▲) and 0.1 wt % hydroxylated lecithin (x), in the absence of NaCl, are also shown.

quently linked together to form larger structures (Figure 5C) in the heated emulsion. Similar to the above, addition of 125 mM NaCl to the hydroxylated-lecithin-containing emulsion caused most of the droplets to aggregate, but the particles formed

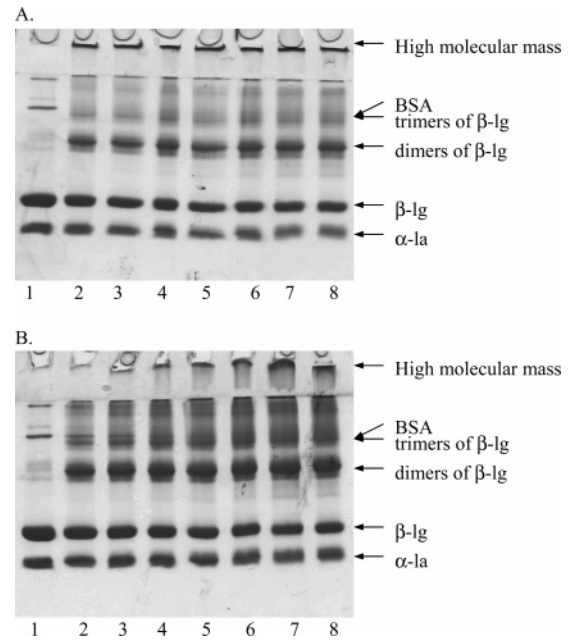


Figure 8. SDS-PAGE patterns (15% acrylamide gel), under reducing conditions, of 1 wt % WPI solutions containing no hydroxylated lecithin (A) and 0.1 wt % hydroxylated lecithin (B) heated at 90°C . Lane 1, unheated WPI solution; lane 2, 0 min; lane 3, 2 min; lane 4, 5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 20 min; and lane 8, 30 min.

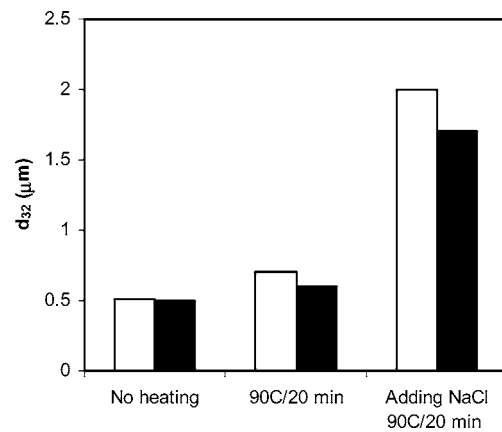


Figure 9. Average particle size (d_{32}) of emulsions made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (white bar) and 0.1 wt % hydroxylated lecithin (black bar).

were smaller than in the control emulsion. These micrographs confirmed the observations on the changes in the droplet size distributions (Figure 4).

The total surface protein coverage in the heated emulsion increased markedly with an increase in the concentration of added NaCl from 5 to 125 mM (Figure 6). The total surface protein coverage of the control emulsions was substantially greater than that of the emulsions containing hydroxylated lecithin.

The increase in the total surface protein coverage can be attributed to a corresponding increase in the amount of β -Lg adsorbed at the surface after heating in the presence of NaCl, as shown by the decrease in the amount of β -Lg in the aqueous phase of the emulsions with an increasing concentration of NaCl (Figure 7A). At a given concentration of added NaCl, the amount of β -Lg in the aqueous phase of the emulsions containing hydroxylated lecithin was higher than that of the control emulsions, indicating that a smaller amount of β -Lg associated

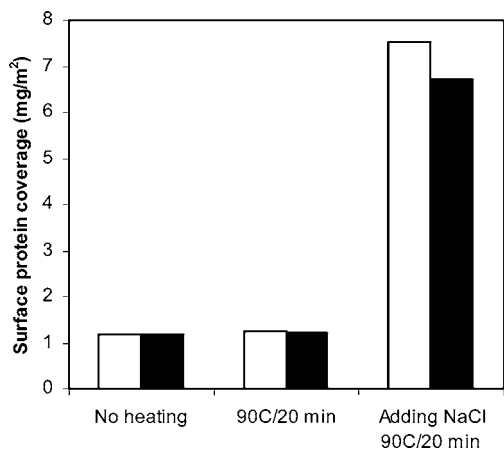


Figure 10. Total surface protein coverage (mg/m^2) of emulsions made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (white bar) and 0.1 wt % hydroxylated lecithin (black bar).

with the surface of the emulsion droplets in the presence of hydroxylated lecithin. At high concentrations of NaCl (>100 mM), almost all of the β -lg associated with the surface of the droplets during heating (Figure 7A).

The changes in α -la in the aqueous phase of the emulsions with NaCl were different from those observed for β -lg. The amount of unadsorbed α -la decreased with an increasing NaCl concentration up to 50 mM but remained unchanged at higher concentrations of NaCl. The amount of unadsorbed α -la in the emulsions containing hydroxylated lecithin was higher than that in the control emulsions, although the overall trends as a function of the NaCl concentration were similar. This indicates that adsorbed α -la was removed from the surface of the droplets during heating in the absence of NaCl but that addition of NaCl resulted in some of the unadsorbed α -la associating with the droplet surface during heating. The presence of hydroxylated lecithin appeared to impede this interaction.

These results suggest that, when WPI-based emulsions are heated well above the denaturation temperature, interactions between adsorbed and unadsorbed β -lg are promoted, leading to a greater association of β -lg at the droplet surface. In the presence of NaCl, these interactions also lead to aggregation of the droplets, because of screening of the electrostatic repulsions between droplets.

β -Lg has been shown to have the ability to bind small hydrophobic molecules, such as retinol and fatty acids, within a central cavity (27, 28). This binding of fatty acids to β -lg has been reported to increase its resistance to unfolding in urea and by heat treatment (29). The relatively large size of hydroxylated lecithin would probably preclude its entry into the central cavity of native β -lg. However, it is possible that, once β -lg is heated, hydrophobic sites exposed to the surface would be available for interactions with hydrophobic parts of hydroxylated lecithin. It is also likely that β -lg adsorbed at the droplet surface, with exposed hydrophobic groups, is able to interact with hydroxylated lecithin in a similar way. This interaction could probably increase the overall charges on the droplet surface, thus reducing the extent of droplet aggregation in the presence of NaCl.

To determine the effect of hydroxylated lecithin on the whey protein aggregation in aqueous solution during heating, WPI solutions with and without added hydroxylated lecithin were heated at 90 °C for 30 min. SDS-PAGE of the heated protein solutions under nonreducing conditions is shown in Figure 8. As expected, the intensity of both β -lg and α -la decreased with increasing heating time, with corresponding increases in the

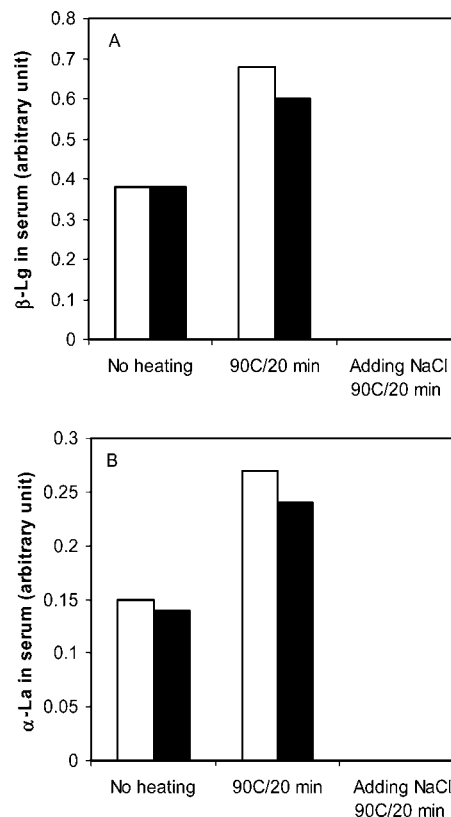


Figure 11. Amounts of nonadsorbed β -lg (A) and α -la (B) in the serum of emulsions made with 20% soya oil at pH 7.0 and 1 wt % WPI containing no hydroxylated lecithin (white bar) and 0.1 wt % hydroxylated lecithin (black bar).

intensities of dimers, trimers, and high molecular weight material that accumulated on top of the gels. The quantity of high molecular weight material was higher in the heated whey protein solution containing no hydroxylated lecithin, even after 2 min of heating at 90 °C. In contrast, there were more dimers and trimers, intermediate products, in the heated whey protein solution containing hydroxylated lecithin but relatively less high molecular weight material. It therefore appears that the hydroxylated lecithin may interact with the intermediate products, e.g., unfolded monomers, dimers, and trimers, of β -lg generated during heating, preventing their further aggregation to form high molecular weight species. This could explain the results observed in the emulsions containing hydroxylated lecithin after heat treatment.

Heat Treatment of WPI Solution Before Emulsification.

Heat treatment of WPI (1 wt %) solution at 90 °C for 20 min prior to emulsification slightly increased the d_{32} value of the resulting emulsion. However, when the WPI solutions were heated in the presence of 100 mM NaCl, a dramatic increase in the d_{32} of the final emulsion was observed. Emulsions containing hydroxylated lecithin had lower d_{32} values for both heated emulsions (Figure 9).

No change in the total surface protein coverage was observed in the emulsions made with WPI and heated WPI. Hydroxylated lecithin did not change the total surface protein coverage in either WPI or heated WPI emulsions (Figure 10). In contrast, the total surface protein coverage markedly increased in the emulsions made with heated WPI solution containing NaCl (Figure 10).

Analysis of the protein in the aqueous phase of the emulsion showed the greater amount of both unadsorbed β -lg and unadsorbed α -la in the heated WPI emulsions (Figure 11). In

comparison with the heated WPI emulsions, the amounts of unadsorbed β -Ig and α -la in the heated WPI emulsions containing hydroxylated lecithin were lower. Almost no protein remained in the aqueous phase of the heated WPI emulsions containing NaCl (Figure 11). This indicates that smaller amounts of proteins (β -Ig and α -la) adsorbed on the surface when the WPI solution was heated before emulsification, although the total surface protein coverage was similar in both unheated and heated WPI emulsions, because the heated protein produced a smaller surface area of emulsion droplets (larger d_{32}). Similar to the above result, larger amounts of proteins (β -Ig and α -la) were adsorbed on the droplet surface when the WPI solution containing hydroxylated lecithin was heated compared with heated whey protein alone. The aggregation of whey protein caused by heat treatment may be detrimental to the surface activity of proteins and reduce their adsorption on the droplet surface. An increase in the adsorption of the heated whey protein in the presence of hydroxylated lecithin is likely because hydroxylated lecithin inhibited the heat-induced aggregation of whey proteins to some extent and resulted in the formation of more smaller, intermediate, stable protein products, as shown in Figure 8.

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