Influence of Calcium, Magnesium, or Potassium Ions on the Formation and Stability of Emulsions Prepared Using Highly Hydrolyzed Whey Proteins

C. Ramkumar,† Harjinder Singh,*,† Peter A. Munro,† and Anne M. Singh‡

Institute of Food, Nutrition and Human Health, Massey University, and New Zealand Dairy Research Institute, Palmerston North, New Zealand

Oil-in-water emulsions (4 wt % soy oil) containing 4 wt % whey protein hydrolysate (WPH) (27% degree of hydrolysis) and different levels of calcium, magnesium, or potassium chloride were prepared in a two-stage homogenizer. Other emulsions containing 4 wt % WPH but including 0.35 wt % hydroxylated lecithin and different levels of the above minerals were similarly prepared. The formation and stability of these emulsions were determined by measuring oil droplet size distributions using laser light scattering and by confocal scanning laser microscopy and a gravity creaming test. Both lecithin-free and lecithin-containing emulsions showed no change in droplet size distributions with increasing concentration of potassium in the range 0−37.5 mM. In contrast, the diameter of emulsion droplets increased with increasing calcium or magnesium concentration >12.5 mM. Emulsions containing hydroxylated lecithin were more sensitive to the addition of calcium or magnesium than the lecithin-free emulsions. Storage of emulsions at 20 °C for 24 h further increased the diameter of droplets and resulted in extensive creaming in emulsions containing >25 mM calcium or magnesium. It appears that both flocculation and coalescence processes were involved in the destabilization of emulsions induced by the addition of divalent cations.

Keywords: *Emulsions; creaming; droplet sizes; lecithin; hydrolysate*

INTRODUCTION

Milk protein hydrolysates have been used extensively in infant and specialized adult nutritional formulations (Mahmood, 1994). Extensively hydrolyzed proteins, which have substantially reduced immunological reactivities, are used in hypoallergenic infant formula. These formulations are essentially multicomponent emulsion systems, and therefore the emulsifying properties of protein hydrolysates are important. Because extensively hydrolyzed proteins comprise mainly short peptides and free amino acids, they have poor ability to form and stabilize emulsions (Mahmood, 1994; Schmidl et al., 1994). Consequently, these formulations require the addition of emulsifiers and stabilizers to facilitate the formation of a stable emulsion. Most commercial infant and enteral formulations are fortified with various minerals such as calcium, magnesium, potassium, phosphorus, and chloride at levels ranging between 0.04 and 0.12% to meet a specific nutritional profile and then heat treated to improve shelf life. Therefore, the stability of these emulsion systems in the presence of these minerals is of particular interest. There have been very few studies on the stability of emulsions formed with extensively hydrolyzed proteins (Chobert et al., 1988; Turgeon et al., 1992; Agboola et al., 1998a,b), and the effects of external factors, for example, mineral, emulsifier, and stabilizer additions, have not been established.

Agboola et al. (1998a) showed that fairly stable oil-in-water emulsions could be formed using highly hydrolyzed whey proteins (WPH) as the sole emulsifier using appropriate WPH concentration and homogenization conditions. Emulsions (4% soy oil) prepared using 4 wt % WPH with two-stage homogenization, at pressures of 20.6 and 3.4 MPa in the first and second stages, respectively, were found to be stable to creaming and coalescence at 20 °C for more than a week. However, retorting (121 °C for 16 min) of the above emulsions resulted in extensive coalescence (Agboola et al., 1998b). Attempts were then made to improve the stability to retorting with the addition of lecithin (Agboola et al., 1998b). Addition of hydroxylated lecithin at 0.25 wt % was found to markedly improve the creaming stability after retorting and prevented coalescence.

The objective of this study was to extend the findings of Agboola et al. (1998a,b) and study the changes in stability behavior when mineral ions were introduced into the emulsion systems. It was also of interest to determine the effect of a combined presence of minerals and lecithins in these systems. The effects of addition of calcium, magnesium, or potassium at concentrations ranging from 2.5 to 37.5 mM, which are typically found in commercial enteral formulations, on the properties of emulsions formed with commercial whey protein hydrolysate in the absence or presence of 0.25 wt % hydroxylated lecithin were investigated.

MATERIALS AND METHODS

Materials. Whey protein hydrolysate powder (WPH 931, with 27% degree of hydrolysis; containing 90.5% protein, 4.5%

* Address correspondence to this author at the Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand (telephone 64 6 350 4401; fax 64 6 350 5655; e-mail H.Singh@massey.ac.nz).

† Massey University.

‡ New Zealand Dairy Research Institute.

10.1021/jf990792k CCC: $19.00
Published on Web 04/07/2000

moisture, 2.8% ash, 0.1% fat, and 0.2% lactose) was supplied by the New Zealand Dairy Board, Wellington. Soy oil was purchased from Davis Trading Co., Palmerston North, New Zealand. All chemicals and reagents used were of AR grade and were obtained from BDH Chemicals Ltd., Poole, U.K.

Preparation of Emulsions. Emulsions were prepared according to the procedure described by Agboola et al. (1998a) with some modifications. WPH (4 wt %) was dissolved in Milli-Q water at room temperature (20 ± 2 °C). Appropriate quantities of calcium, magnesium, or potassium chloride solutions (to give concentrations of 12.5, 25.0, 30.0, or 37.5 mM calcium, magnesium, or potassium in the emulsion) were added to the WPH solution, and the pH was adjusted to 6.80. Soy oil (4 wt %) was then added to the solution. In some trials, soy oil heated to 60 °C and containing dispersed hydroxylated lecithin (2.5 g/L of emulsion) was added to the WPH solution. The two were then combined by a single pass through the homogenizer at atmospheric pressure and homogenized using a Rannie homogenizer (Albertslund, Denmark) at first- and second-stage pressures of 20.6 and 3.4 MPa, respectively. The emulsions were homogenized twice for more effective mixing of the oil phase.

Measurement of Particle Sizes. The droplet size distribution, the volume-average particle diameter (d_{32}), and the weight-average particle diameter (d_{43}) were measured by light scattering using a Mastersizer E (Malvern Instruments Ltd., Worcestershire, U.K.). The presentation factor was 2NAD (i.e., refractive index and absorption of emulsion particles of 1.456 and 0, respectively), and a polydisperse model was chosen for the size distribution. Emulsion droplets were sized using distilled water as the dispersant. To determine the incidence of flocculation, the droplets were also dispersed in a 1:1 mixture of 0.1 M EDTA and 1% SDS solution 30 min prior to measurement. All measurements were carried out at 20 °C.

Determination of Creaming Stability. The method described by Agboola et al. (1998a) was followed to determine the amount of cream separation. About 15 mL of freshly prepared emulsion was poured into specially constructed "stability tubes". The tubes were graduated with each division equal to 0.1 mL. The level of cream separation was read after 24 h of storage at 20 °C.

Determination of Surface Peptide Concentration. The method described by Agboola et al. (1998a) was used to determine the concentration of peptides adsorbed at the emulsion droplets (mg/m^2).

Confocal Microscopic Examination of Emulsions. Nile blue (a fluorescent dye to stain the fat phase) at 0.1 wt % concentration was added to the emulsion samples and mounted on a Leica TCS 4D confocal scanning laser microscope (Leica Laser Technik, GmbH, Heidelberg, Germany). The laser source was Ar/Kr and was used at an excitation wavelength of 488 nm. Samples were observed under oil immersion using the H100 objective lens.

RESULTS

Formation of Emulsions. The effects of adding calcium, magnesium, or potassium chloride prior to emulsion formation on the average diameters (d_{43}) of emulsion droplets made with 4 wt % WPH with or without 0.25 wt % hydroxylated lecithin are shown in Figure 1. For both lecithin-free and lecithin-containing emulsions, the addition of potassium up to 37.5 mM did not affect d_{43}. In contrast, the d_{43} of emulsion droplets increased with increasing calcium or magnesium concentrations >12.5 mM (Figure 1a); the d_{43} values of magnesium-containing emulsions were greater than those of emulsions containing calcium at the same levels of addition. Emulsions containing hydroxylated lecithin were more sensitive to the addition of calcium or magnesium; the d_{43} values of emulsion droplets formed in the presence of ≥25 mM calcium or magnesium were twice those of droplets formed in lecithin-free emulsions

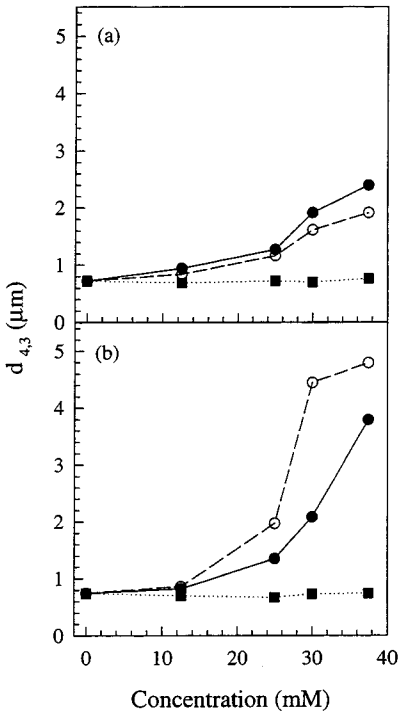

Figure 1. Weight-average particle diameter (d_{43}) of emulsions containing no lecithin (a) or 0.25% hydroxylated lecithin (b). Emulsions were prepared with added calcium (●), magnesium (○), or potassium (■) at various millimolar concentrations.

(Figure 1b). The effect of magnesium on d_{43} in the presence of lecithin was more pronounced than that of calcium addition.

The size distributions of lecithin-free emulsions remained monomodal on addition of calcium (Figure 2a) or magnesium (Figure 2c) at concentrations up to 25 mM, but the distribution became fairly broad at higher concentrations, with a considerable increase in the proportion of larger particles between 1 and 10 μm. Higher values of d_{43} (Figure 1a,c) resulted from these higher proportions of larger particles in the emulsions. When these emulsions, containing >25 mM calcium or magnesium, were dispersed in a mixture of EDTA and SDS solutions, a shift in the distribution toward a smaller size was observed (Figure 2b,d). This indicates the dissociation of flocs of particles, consequently leading to an increased proportion of smaller sized particles. However, some droplets larger (2−4 μm) than those present in the control emulsion were still present at higher concentrations of calcium or magnesium in the emulsions dispersed in EDTA/SDS. This may indicate that the presence of calcium and magnesium at high concentrations probably induced coalescence or irreversible aggregation of emulsion droplets. On the other hand, there was very little variation in the droplet size distributions of emulsions containing different levels of potassium (Figure 2e,f).

Emulsions containing hydroxylated lecithin showed a higher proportion of particles in the range of 1−20 μm (Figure 3) than did lecithin-free emulsions (Figure 2), particularly at high concentrations of calcium and magnesium. The proportion of particles in this range increased with increasing concentrations of calcium (Figure 3a) and magnesium (Figure 3c). This was also reflected in the high values of d_{43} observed (Figure 1b,d). The distributions of droplets in emulsions containing up to 12.5 mM calcium or magnesium were monomodal

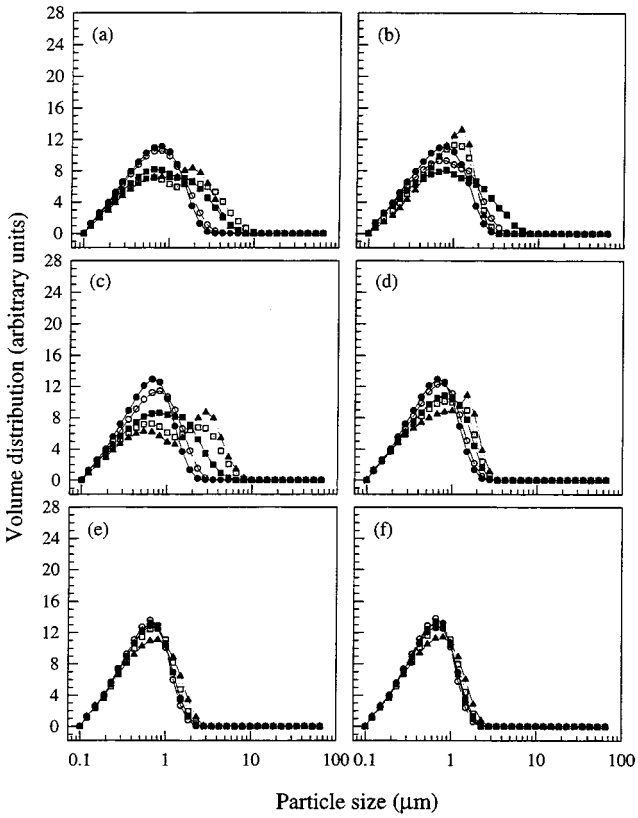

Figure 2. Size distributions of emulsions prepared without added lecithin and dispersed in water (a, c, e) or in a mixture of EDTA and SDS solutions (b, d, f). Emulsions were prepared with added calcium (a, b), magnesium (c, d), or potassium (e, f) at various concentrations: 0 (●), 12.5 (○), 25.0 (■), 30.0 (□), and 37.5 mM (▲).

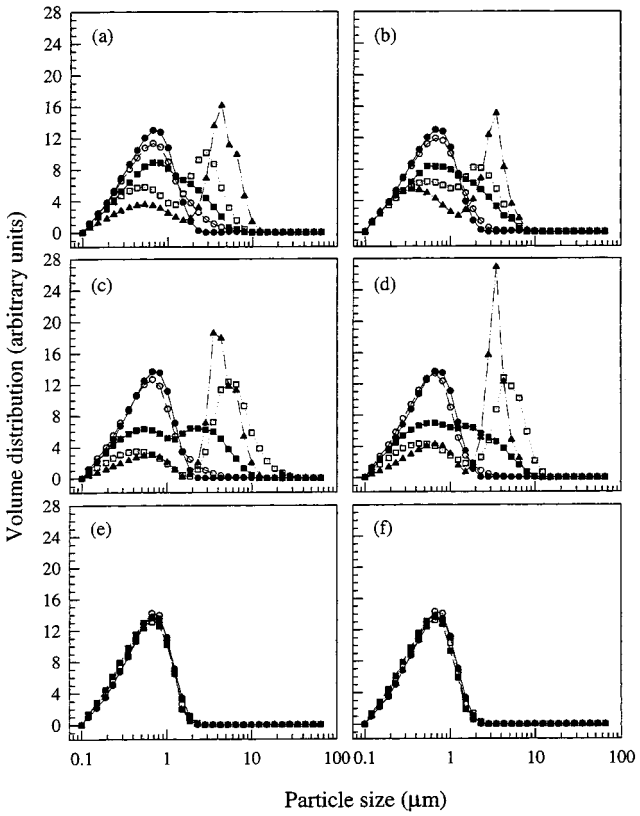

Figure 3. Size distributions of emulsions prepared with 0.25% hydroxylated lecithin and dispersed in water (a, c, e) or in a mixture of EDTA and SDS solutions (b, d, f). Emulsions were prepared with added calcium (a, b), magnesium (c, d), or potassium (e, f) at various concentrations: 0 (●), 12.5 (○), 25.0 (■), 30.0 (□), and 37.5 mM (▲).

and became very broad and bimodal at higher concentrations. Dispersion of emulsion droplets in EDTA/SDS resulted in a slight shift in the size distribution toward smaller particles. The extent of dissociation of flocculated particles by SDS/EDTA was much less in lecithin-containing emulsions as compared with lecithin-free emulsions. This indicates greater incidence of coalescence or irreversible aggregation in lecithin-containing emulsions in the presence of these minerals. As was observed for lecithin-free emulsions, addition of potassium at different levels had no effect on the droplet size distributions (Figure 3e,f).

Confocal micrographs of the lecithin-free emulsion prepared without added calcium, magnesium, or potassium (Figure 4a) showed an even distribution of droplets of up to ~1 μm in size. In the emulsions containing 30 mM calcium (Figure 4c,d) or 37.5 mM magnesium (Figure 4e,f), the size of droplets tended to be larger (in the range 1−10 μm). Several flocculated droplets were also visible in these emulsions. Hydroxylated lecithin-containing emulsions (Figure 4d,f) with added calcium or magnesium contained droplets in a much larger size range (Figure 4c,e). The bimodal nature of these size distributions was also evident from the micrographs. Addition of 30 mM potassium to lecithin-free or hydroxylated lecithin-containing emulsions did not alter the size and appearance of droplets (Figure 4b). Overall, the microscopic observations confirmed droplet size distribution results.

Surface Peptide Concentrations. Figure 5 shows the influence of the presence of calcium in lecithin-free or hydroxylated lecithin-containing emulsions. Addition of calcium up to 25 mM had no major effect on the values of surface peptide concentration (mg/m^2), but these values increased considerably at high levels of calcium addition.

Emulsion Stability after Storage. Emulsions were also examined for particle size distributions and creaming stability after 24 h of storage at 20 °C. The stability of emulsions formed in the presence of calcium or magnesium at concentrations ≥25 mM was adversely affected, as evidenced by an increase in d_{43} (Figure 6) and development of bimodal size distribution (Figure 7). The d_{43} of lecithin-free emulsions (Figure 6a) and hydroxylated lecithin-containing emulsions (Figure 6b) increased with increasing concentrations of calcium or magnesium between 12.5 and 37.5 mM. The d_{43} values of emulsions made in the presence of 30 and 37.5 mM calcium or magnesium after storage were considerably higher than that of the fresh emulsions (Figure 1a,b), suggesting that flocculation and possibly coalescence of droplets occurred during storage.

Confocal microscopic examination of emulsions containing calcium or magnesium at concentrations of 25 mM or higher showed even larger droplets and more pronounced flocculation than the corresponding emulsions before storage. Lecithin-free (Figure 8a) and hydroxylated lecithin-containing (Figure 8b) emulsions prepared with the addition of 30 mM calcium are shown as typical examples. An increase in the extent of flocculation in comparison with the emulsions before storage (Figure 4c,d) was apparent from the micrographs. High d_{43} values observed in these emulsions (Figure 6) were likely to be a result of this increased flocculation of droplets.

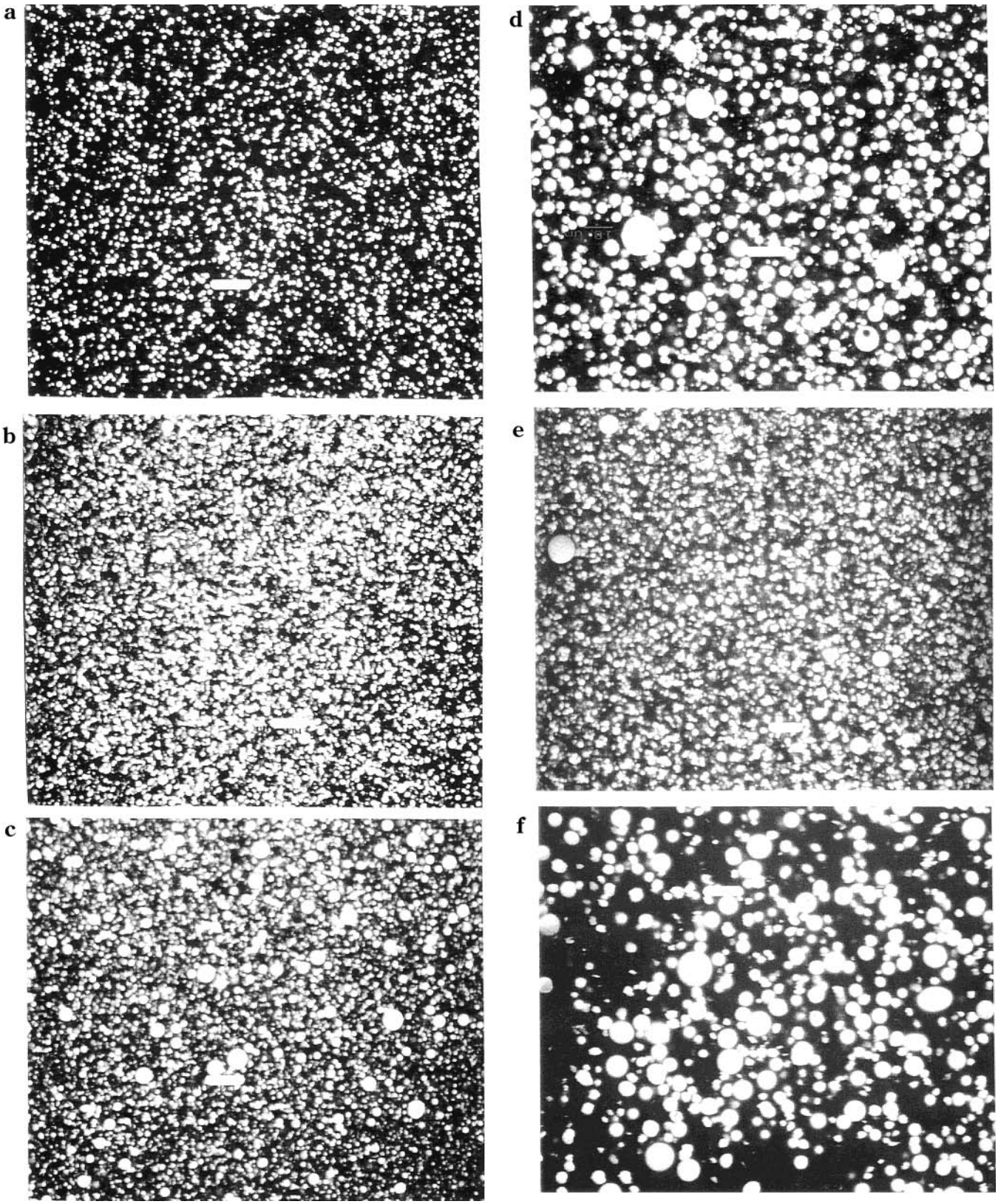

Figure 4. Typical confocal micrographs of emulsions containing no lecithin, calcium, magnesium, or potassium (a), 30.0 mM potassium with 0.25% hydroxylated lecithin (b), 30.0 mM calcium (c), 30.0 mM calcium with 0.25% hydroxylated lecithin (d), 37.5 mM magnesium (e), and 37.5 mM magnesium with 0.25% hydroxylated lecithin. (f) Bar = 10 μm.

The extent of creaming in lecithin-free emulsions over a 24 h storage period is shown in Figure 9a. Although no creaming was observed in emulsions made in the presence of potassium, the extent of creaming in emulsions in the presence of calcium and magnesium increased with increasing concentrations of calcium and magnesium, especially ≥25 mM. Similar results were observed for lecithin-free and hydroxylated lecithin-containing emulsions (Figure 9b). The increased creaming at these concentrations was expected because of the presence of larger sized droplets in these emulsions (Figures 1−5).

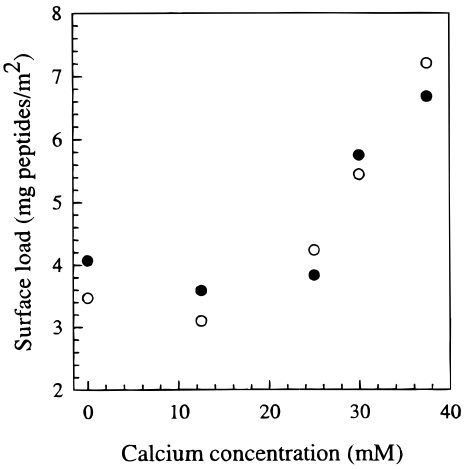

Figure 5. Surface peptide load of emulsions containing no lecithin (●) and 0.25% hydroxylated lecithin (○). Emulsions were prepared with added calcium at various millimolar concentrations.

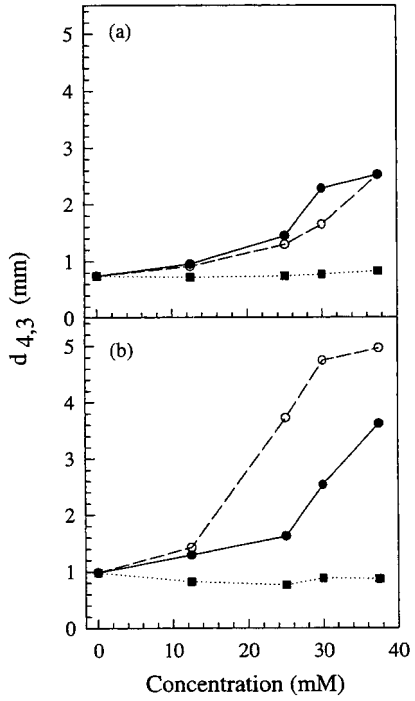

Figure 6. Weight-average particle diameter (d_{43}) of emulsions containing no lecithin (a) and 0.25% hydroxylated lecithin (b). Emulsions were prepared with added calcium (●), magnesium (○), or potassium (■) at various millimolar concentrations. The samples were analyzed after 24 h of storage at 20 °C.

DISCUSSION

During the process of emulsification, proteins or peptides become rapidly adsorbed at the oil−water interface and form an interfacial layer around the newly formed oil droplets (Chobert and Haertlé, 1997). This interfacial layer can sterically stabilize the droplets because the adsorbed protein molecules protrude some distance from the droplet surface. Because charged amino acids are present at the droplet surface, the interfacial layer is generally charged. Thus, the electrostatic repulsion between droplets also contributes to the stability of the emulsions. In some cases, formation of a strong viscoelastic layer can protect the droplets against immediate recoalescence (Dickinson and Stainsby, 1982).

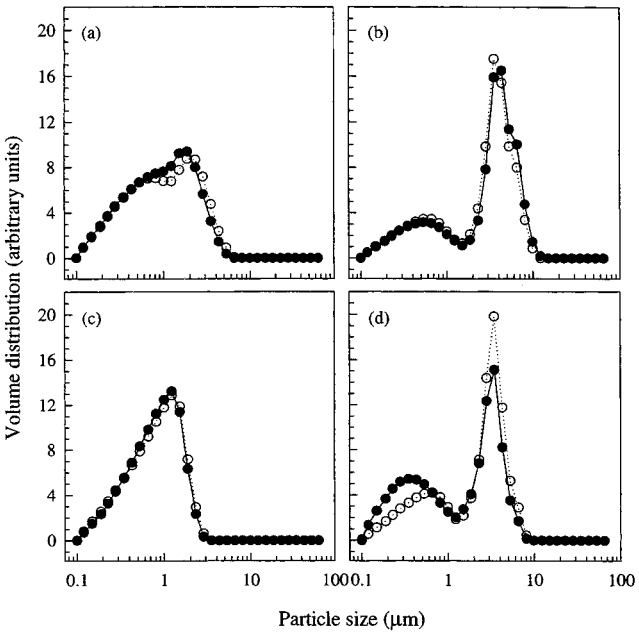

Figure 7. Size distributions of emulsions containing 37.5 mM calcium prepared without added lecithin (a, c) or with 0.25% hydroxylated lecithin (b, d). Emulsions were dispersed either in water (a, b) or in a mixture of EDTA and SDS solutions (c, d) and analyzed after homogenization (●) or after 24 h of storage at 20 °C (○).

The addition of calcium or magnesium ≥25 mM reduced emulsion stability. An increase in average particle size (Figures 1 and 5), a shift in particle size distributions toward a higher size range (Figure 2), and the presence of flocculated particles (Figure 4c,e) were observed at these ion concentrations. This instability probably arises from the interactions of calcium or magnesium with the peptides in solution and at the droplet surface. The binding of Ca^{2+} or Mg^{2+} to the adsorbed peptides could lead to a reduction in charge density at the droplet surface, which would reduce the interdroplet electrostatic repulsion and therefore enhance the likelihood of droplet flocculation. The steric stabilization provided by the adsorbed peptides may be reduced because of the changes in their conformation brought about by the binding of these ions (Horne and Leaver, 1995) arising from a partial collapse of the adsorbed peptide layer (Dalgleish, 1997). The resulting reduction in steric stabilization would promote flocculation. In addition, the conformational changes may expose some of the hydrophobic sites (Shimizu et al., 1981) promoting aggregation of droplets.

Calcium ions also influence protein−protein interactions by forming calcium bridges (Lupano et al., 1992). Formation of calcium bridges between peptides present on two different emulsion droplets would enhance flocculation. Examination of confocal micrographs (Figures 4c,e and 8a) showed the presence of individual droplets within the aggregates, confirming the occurrence of bridging flocculation. Formation of large clusters with a strong association between droplets was also shown by the bimodal particle size distributions at higher calcium and magnesium concentrations. A shift in the particle size distribution toward a smaller size range was observed when these emulsions were dispersed in EDTA/SDS solution (Figure 2b,d), which clearly demonstrated the dissociation of flocculated particles.

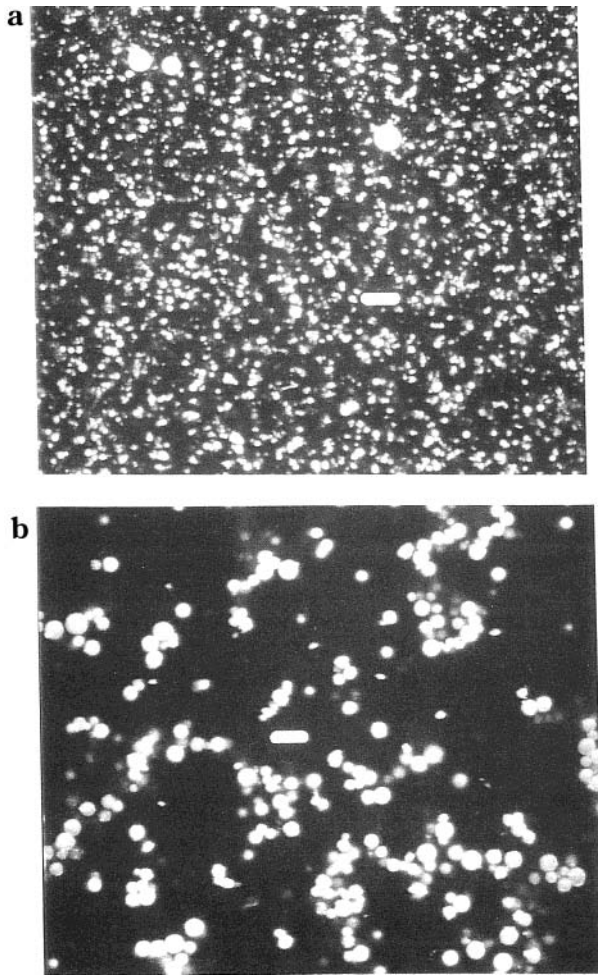

Figure 8. Typical confocal micrographs of emulsions containing 30 mM calcium (a) and 30 mM calcium with 0.25% hydroxylated lecithin (b). Samples were observed after 24 h of storage at 20 °C. Bar = 10 μm.

In these emulsions, the formation of some very large droplets, apparently formed by coalescence, was also observed in the presence of calcium and magnesium. Agboola et al. (1998a) showed that the concentration of peptides with suitable emulsification properties in the WPH solutions is fairly limited, and consequently droplet coalescence occurs under some conditions. It is likely that the binding of calcium ions to the negatively charged peptides in WPH solution causes aggregation of larger, more surface active peptides. This situation would reduce the effective concentration of "suitable" peptides, resulting in insufficient amounts of peptides available to cover the interface completely and provide stability against coalescence. Increase in surface peptide concentration with increasing concentrations of calcium in the emulsions (Figure 5) indicates involvement of cation-cross-linked peptide aggregates in adsorption.

The behaviors of calcium and magnesium were essentially similar in the emulsion systems studied. The greater destabilization of emulsions by calcium or magnesium in comparison with potassium is due to the higher binding affinity of the divalent ions to specific binding sites (e.g., COO^-) resulting in higher interpeptide bridging (Foegeding et al., 1986). In addition, electrostatic screening is also more effective for multivalent ions.

The average droplet size was found to increase much more at higher levels of calcium or magnesium in the

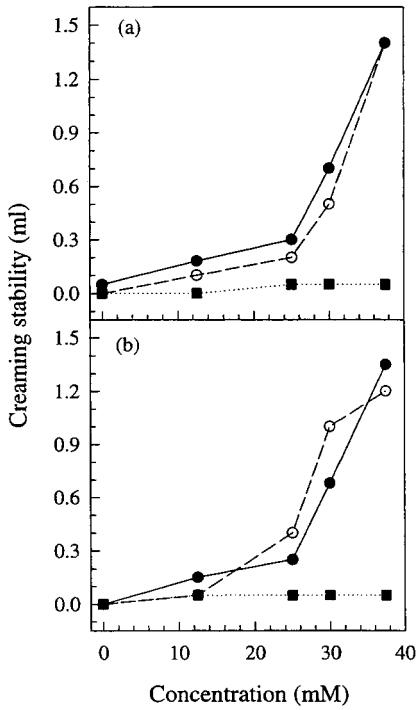

Figure 9. Creaming stability of emulsions containing no lecithin (a) and 0.25% hydroxylated lecithin (b). Emulsions were prepared with added calcium (●), magnesium (○), or potassium (■) at various millimolar concentrations.

emulsions prepared with hydroxylated lecithin (Figures 1b and 4d,f). Although the presence of surface phospholipid was not detected in emulsions prepared without calcium addition (Agboola et al., 1998b), some complexing between hydroxylated lecithin and peptides was expected. Acidic phospholipids (phosphatidic acid, phophatidylinositol, etc.) are negatively charged and interact with positively charged peptide molecules (Yamamoto and Araki, 1997). Interactions between β-lactoglobulin and phospholipids have been reported to occur through lysyl and arginyl residues of the protein and negatively charged groups of phospholipids (Brown et al., 1988; Cornell and Patterson, 1989). The greater sensitivity of hydroxylated lecithin-containing emulsions to divalent cations may be attributed to possible binding of these ions to negatively charged phospholipid components; this may affect their ability to interact with WPH peptides, as well as their adsorption behavior.

The emulsions containing ≥25 mM calcium or magnesium showed poorer stability against creaming (Figure 9). The enhanced creaming in these emulsions was primarily due to the presence of a high proportion of large-sized droplets (Figures 2 and 3) as the rate of creaming is directly proportional to the square of droplet radius. Formation of large particles at the time of emulsion formation as well as subsequent flocculation contributed to the greater creaming observed for these emulsions. An irreversible bridging flocculation during storage may also result from the formation of covalent disulfide bonds between adsorbed peptides on adjacent droplets (Dickinson, 1997). Increase in the average particle size after storage (Figure 5) was a consequence of all such effects.

ACKNOWLEDGMENT

We thank the New Zealand Dairy Research Institute for providing facilities for particle size measurements.

LITERATURE CITED

Agboola, S. O.; Singh, H.; Munro, P. A.; Dalgleish, D. G.; Singh, A. M. Destabilization of oil-in-water emulsions formed using highly hydrolyzed whey proteins. *J. Agric. Food Chem*. **1998a**, *46*, 84−90.

Agboola, S. O.; Singh, H.; Munro, P. A.; Dalgleish, D. G.; Singh, A. M. Stability of emulsions formed using whey protein hydrolysate: effects of lecithin addition and retorting. *J. Agric. Food Chem*. **1998b**, *46*, 1814−1819.

Brown, E. M.; Pfeffer, P. E.; Kumosinsky, T. F.; Greenberg, R. Accessibility and mobility of lysine residues in β-lactoglobulin. *Biochemistry* **1988**, *27*, 5601−5610.

Chobert, J.-M.; Haertlé, T. Protein−lipid and protein-flavour interactions. In *Food Proteins and Their Applications*; Damodaran, S., Paraf, A., Eds.; Dekker: New York, 1997; pp 143−170.

Chobert, J.-M.; Bertrand-hard, C.; Nicolas, M. Solubility and emulsifying properties of caseins and whey proteins modified by trypsin. *J. Agric. Food Chem*. **1988**, *36*, 883−892.

Cornell, D. G.; Patterson, D. L. Interactions of phospholipids in monolayer with β-lactoglobulin adsorbed from solution. *J. Agric. Food Chem*. **1989**, *37*, 1455−1459.

Dalgleish, D. G. Adsorption of protein and the stability of emulsions. *Trends Food Sci. Technol.* **1997**, *8*, 1−6.

Dickinson, E. Properties of emulsions stabilized with milk proteins: Overview of some recent developments. *J. Dairy Sci*. **1997**, *80*, 2607−2619.

Dickinson, E.; Stainsby, G. *Colloids in Food*; Applied Science: London, U.K., 1982.

Foegeding, E. A.; Dayton, W. R.; Allen, C. E. Effect of heating rate on thermally formed myosin, fibrinogen and albumin gels. *J. Food Sci*. **1986**, *51*, 104−108.

Horne, D. S.; Leaver, J. Milk proteins on surfaces. *Food Hydrocolloids* **1995**, *9*, 91−95.

Lupano, C. E.; Dumay, E.; Cheftel, J.-C. Gelling properties of whey protein isolate: influence of calcium removal by dialysis or diafiltration at acid or neutral pH. *Int. J. Food Sci. Technol*. **1992**, *27*, 615−628.

Mahmoud, I. M. Physicochemical and functional properties of protein hydrolyzate in nutritional products. *Food Technol*. **1994**, *48*, 89−95.

Schmidl, M. K.; Taylor, S. L.; Nordlee, J. A. Use of hydrolysate-based products in special medical diets. *Food Technol*. **1994**, *48*, 77−85.

Shimizu, M.; Kamiya, T.; Yamauchi, K. The adsorption of whey proteins on the surface of emulsified fat. *Agric. Biol. Chem*. **1981**, *45*, 2491−2496.

Turgeon, S. L.; Gauthier, S. F.; Paquin, P. Interfacial and emulsifying properties of whey peptide fractions obtained with a two-step ultrafiltration process. *J. Agric. Food Chem*. **1991**, *39*, 673−676.

Yamamoto, Y.; Araki, M. Effects of lecithin addition in oil or water phase on the stability of emulsions made with whey proteins. *Biosci., Biotechnol., Biochem*. **1997**, *61*, 1791−1795.

Received for review July 19, 1999. Revised manuscript received February 3, 2000. Accepted February 3, 2000. We thank the New Zealand Dairy Board, Wellington New Zealand, for financial support of this project.

JF990792K