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Influence of EDTA and Citrate on Physicochemical Properties of Whey Protein-Stabilized Oil-in-Water Emulsions Containing $CaCl_2$

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The influence of chelating agents (disodium ethylenediaminetetraacetate (EDTA) and sodium citrate) on the physicochemical properties of whey protein isolate (WPI)-stabilized oil-in-water emulsions containing calcium chloride was determined. The calcium-binding characteristics of EDTA and citrate at 30 °C were characterized in aqueous solutions (20 mM Tris buffer, pH 7.0) by isothermal titration calorimetry (ITC). EDTA and citrate both bound calcium ions in a 1:1 ratio, but EDTA had a much higher binding constant. Oil-in-water emulsions (pH 7.0) were prepared containing 6.94% (w/v) soybean oil, 0.35% (w/v) WPI, 0.02% (w/v) sodium azide, 20 mM Tris buffer, 10 mM $CaCl_2$, and 0−40 mM chelating agent. The particle size, apparent viscosity, creaming stability, free calcium concentration, and particle surface potential of the emulsions were measured. The chelating agents reduced or prevented droplet aggregation in the emulsions. When they were present above a certain concentration (>3.5 mM EDTA or >5 mM citrate), droplet aggregation was prevented. The reduction of aggregation was indicated by decreases in particle size, shear-thinning behavior, apparent viscosity, and creaming. Emulsions containing chelating agents had lower free calcium concentrations and more negatively charged droplets, indicating that the chelating agents improved emulsion stability by binding calcium ions. EDTA could be used at lower concentrations than citrate because of its higher calcium ion binding constant.

KEYWORDS: Emulsion; stability; whey protein; chelating agents; binding; EDTA; citrate; calcium

INTRODUCTION

Whey proteins can be used as emulsifiers in foods because of their ability to adsorb to the surface of oil droplets and form protective membranes that stabilize the droplets against aggregation (*1*−*5*). The stability of oil-in-water emulsions stabilized by whey proteins is mainly governed by the balance of attractive and repulsive colloidal interactions between the oil droplets, e.g., van der Waals, electrostatic, steric, hydrophobic, and hydration forces (*1*, *6*−*8*). Repulsive electrostatic interactions play a major role in preventing whey protein-coated oil droplets from aggregating, and so the overall stability of whey protein-stabilized emulsions is particularly sensitive to pH, electrolyte concentration, and ion type (*8*). This has important practical implications for the application of whey proteins as emulsifiers in food products fortified with minerals, e.g., infant formula, athletic beverages, and meal replacement drinks (*9*, *10*).

In this article, we focus on the influence of calcium ions and chelating agents on the stability and physicochemical properties of a model nutritional beverage: a 7 wt % soybean oil-in-water emulsion stabilized by whey protein isolate at pH 7.0, with a mean droplet diameter between 0.6 and 0.7 μm. The composition and microstructure of the model emulsion were chosen to be representative of commercial nutritional beverages (*11*). Calcium ions were used as counterions in the model emulsion because they are the major source of multivalent ions present in commercial nutritional beverages (*11*). Ethylenediaminetetraacetate (EDTA) and citrate were used as chelating agents in the model emulsion because they are two of the most commonly used food ingredients for sequestering multivalent ions (*12*).

Relatively low levels of multivalent ions (<10 mM) can dramatically reduce the aggregation stability of whey protein-stabilized oil droplets through two mechanisms. First, they reduce the electrostatic repulsion between droplets through electrostatic screening (*13*−*15*). Second, they reduce the electrostatic repulsion by binding to oppositely charged groups on the surface of the emulsion droplets, thereby decreasing the magnitude of their ζ potential (*13*, *16*). A number of studies have examined the influence of calcium ions on the stability and rheology of oil-in-water emulsions stabilized by whey protein (*17*−*24*). These studies have shown that protein-stabilized droplets aggregate when the multivalent ion concen-

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tration exceeds a critical level because the electrostatic repulsion between the droplets is no longer sufficient to overcome the attractive interactions (*8*). At neutral pH, the critical calcium concentration has been reported to range between 3 and 10 mM, depending on solution conditions, e.g., protein concentration. This means that it is not possible to make stable nutritional beverages fortified with calcium ions above this critical concentration, unless some procedure is used to prevent electrostatic screening and ion binding effects. The most widely used method of overcoming physical and chemical instability problems caused by multivalent ions in foods involves the use of chelating agents to sequester them, e.g., EDTA or citrate (*12*, *25*, *26*). The primary objective of the current study is to investigate the ability of EDTA and citrate to improve the physical stability of whey protein-stabilized emulsions containing a relatively high level of calcium ions.

It should be mentioned that legal limits have been established concerning the maximum concentration of many chelating agents used in foods because of concerns about their ability to reduce mineral bioavailability. Consequently, these chelating agents have only limited application for improving the stability of food emulsions containing minerals. Nevertheless, a more rational understanding of the impact of chelating agents on emulsion stability is important because it could lead to the development of novel strategies for improving food emulsion properties. In future work, we intend to use the knowledge gained from this study to help interpret the influence of natural biopolymers capable of sequestering calcium on the stability of emulsions with high mineral contents.

MATERIALS AND METHODS

Materials. Powdered whey protein isolate (WPI) was obtained from Davisco International (Eden Prairie, MN) and used without further purification. The manufacturer's analysis of the product was 96.2% protein (dry weight basis), 1.54% sulfated ash, and 5.0% moisture. NaOH, HCl, $CaCl_2$, disodium EDTA, sodium citrate, Tris buffer ($C_4H_{11}NO_3$), and sodium azide were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium azide was used as an antimicrobial agent (its use is not permitted in foods because of its toxicity). The standard calcium solution and ionic strength adjuster (4 M KCl) for free calcium concentration measurements by ion-selective electrode were purchased from Orion (Beverly, MA). Soybean oil was purchased from a local supermarket and used without further purification. Distilled and deionized water was used to prepare all aqueous solutions. A stock buffer solution was prepared that consisted of 20 mM Tris dissolved in distilled water and adjusted to pH 7.0.

Calcium-Binding Measurements by Isothermal Titration Calorimetry. The calcium-binding characteristics of EDTA and citrate were determined by isothermal titration calorimetry (ITC) (VP-ITC microcalorimeter, MicroCal, Inc., Northampton, MA) (*27*). $CaCl_2$ solutions, citrate solutions, and EDTA solutions were prepared by dissolving appropriate amounts of powdered material into stock buffer solution. The $CaCl_2$ solution was placed in the injector (0.3 mL), and the chelating agent solution was placed in the reaction cell (1.45 mL). The system was then allowed to come to thermal equilibrium (at 30.0 °C) while the contents of the reaction cell were continuously stirred (315 rpm). The heat change resulting from injection of 29 aliquots, 10 μL each, of $CaCl_2$ solution (260 s apart) into the reaction cell containing the chelating agent solution was then measured. The heat change from each injection peak was integrated after subtracting the heat of dilution obtained from control experiments, in which the same $CaCl_2$ solution was injected into buffer in the absence of chelating agent. The integrated data were fitted to equilibrium binding equations using a nonlinear least-squares algorithm to obtain the binding constant (K), number of binding sites per molecule (N), and binding enthalpy (ΔH) (*28*). The measurements were carried out in duplicate. Typical standard deviations were <5%.

For the EDTA experiments, 5 mM $CaCl_2$ was placed in the injector, and 0.5 mM EDTA was placed in the reaction cell. For the citrate experiments, 50 mM $CaCl_2$ was placed in the injector, and 5 mM citrate was placed in the reaction cell. The reason that much lower concentrations were needed for the EDTA experiments was that enthalpy changes resulting from EDTA−calcium interactions were much larger than those resulting from citrate−calcium interactions.

Emulsion Preparation. An emulsion containing stock buffer solution (85.37% w/v), soybean oil (13.9% w/v), WPI (0.69% w/v), and sodium azide (0.04% w/v) was prepared by homogenizing aqueous emulsifier solution with soybean oil. The aqueous emulsifier solution was prepared by dispersing powdered WPI and sodium azide into stock buffer solution and stirring for at least 3 h. A coarse emulsion was prepared by mixing the aqueous emulsifier solution and the soybean oil using a high-speed blender (model 33BL79, Waring, New Hartford, CT). A fine emulsion (mean diameter ~0.6 μm) was prepared by passing the coarse emulsion through a two-stage high-pressure valve homogenizer (APV-Rainie 1000, Wilmington, MA) three times at pressures of 34 MPa during the first stage and 4 MPa during the second stage. This emulsion was then stored at 30 °C for 24 h. Various amounts of buffer solution, 100 mM $CaCl_2$ in buffer solution, and 100 mM chelating agent in buffer solution were mixed together and adjusted to pH 7 (because interaction between $CaCl_2$ and the chelating agents caused a significant pH change) using HCl (0.1 or 1 N) or NaOH (0.1 or 1 N). The emulsion was then added in order to obtain final emulsions (pH 7.0) containing 6.94% (w/v) soybean oil, 0.35% (w/v) WPI, 0.02% (w/v) sodium azide, 20 mM Tris buffer, 10 mM $CaCl_2$, and 0−40 mM chelating agent. The emulsions were kept at room temperature for about 24 h before further measurements were begun.

Particle Size Measurements. The particle size distribution of the emulsions was measured using a laser light-scattering instrument (LA-9000, Horiba, Irvine, CA). The instrument measured the intensity of laser light scattered from a dilute emulsion and then reported the particle size distribution that gave the closest fit between theoretical calculations (Mie theory) and experimental measurements of intensity versus scattering angle. A refractive index ratio (refractive index of oil/refractive index of aqueous phase) of 1.08 was used in the calculations of the particle size. Particle size measurements are reported either as full particle size distributions or as volume−surface mean diameters, d_{32} ($=\Sigma n_i d_i^3/\Sigma n_i d_i^2$, where n_i is the number of particles with diameter d_i). The measurements were carried out in duplicate, with standard deviations typically being <8%.

The particle size distribution of flocculated emulsions determined by light scattering should be considered as approximate. The theory used to calculate the size distribution assumes that the particles are isolated homogeneous spheres. In flocculated emulsions, the droplets aggregate into fairly loose nonspherical and nonhomogeneous *particles*. Thus, the particle size determined by light scattering in a flocculated emulsion should be taken to be only an approximate indication of the actual size of the flocs. In addition, the emulsions must be diluted in distilled water to a droplet concentration <0.04% (to eliminate multiple scattering effects) and must be stirred (to ensure they are homogeneous) prior to measurement. Dilution and stirring could disrupt any weakly flocculated droplets but leave strongly flocculated droplets intact.

Particle Surface Charge Measurements. The emulsion particle surface charge (ζ potential) was measured using a particle microelectrophoresis instrument (Zetamaster, ZEM 5003, Malvern Instruments, Worcester, UK). A dilute emulsion was injected into an electrophoresis cell that had electrodes at either end. An electrical field was applied to the sample, which caused the electrically charged particles to move toward the oppositely charged electrode. The velocity of the particles was determined by measuring the frequency of the scattered light when the droplets moved through the crossing point of two focused laser beams. The ζ potential was then calculated from knowledge of the applied field strength and the particle velocity using the Henry equation (*16*). In our study, emulsions were diluted with their own aqueous phase so as not to change the ionic environment of the droplets. The aqueous phase of an emulsion was obtained by centrifuging the emulsion at 20000g for 30 min to separate it into an upper creamed layer and a lower serum layer. The serum layer was then collected and used to dilute the emulsions to a concentration where the instrument count rates

were in the optimum measurement range (500K−3500K count/s) specified by the manufacturer. The results are reported as the average of at least three measurements. Typical standard deviations were <10%.

Rheology Measurements. The rheological properties of the emulsions were measured at 30 °C using a controlled stress shear rheometer with a concentric cylinder measurement cell (CS-10, Bohlin Instruments, Cranbury, NJ). Before each measurement, a shear rate of 5.0 s^{-1} was applied to the emulsions for 10 s, and the emulsions were allowed to stand for 90 s in the absence of stirring. The shear rate was then recorded as the shear stress was increased. The measurements were carried out in duplicate, with standard deviations typically being <10%.

Creaming Stability Measurements. Ten grams of each emulsion was placed in a glass test tube (internal diameter 16 mm and height 100 mm) and then stored at 30 °C. After storage, a number of emulsions separated into a creamed layer at the top and a transparent serum layer at the bottom. The total height of the emulsions (H_e) and the height of the serum (H_s) were measured. The extent of creaming was reported as *creaming index* = 100 × (H_s/H_e). The measurements were carried out in duplicate, with standard deviations typically being <7%.

Free Calcium Ion Concentration Measurements. Aqueous phases of emulsions that were highly unstable to gravitational separation were collected directly using a syringe. Aqueous phases of emulsions that were relatively stable to gravitational separation were collected using a syringe after the cream had been separated by centrifugation of the emulsions at 20000*g* for 30 min. The concentration of free calcium ions in the aqueous phase was determined by a calcium ion-selective electrode (ISE) (97-20 Ionplus, Orion). Initially, a standard curve was prepared by measuring the millivolt response of the electrode when it was dipped in a series of calcium chloride solutions of known concentration (10^{-3}−10 mM). The free calcium ion concentration of the aqueous solutions was then determined from the millivolt response of the electrode using the standard curve. If necessary, samples were diluted with buffer solution prior to analysis to ensure that the calcium ion concentration was in the range covered by the calibration curve. The millivolt response of the electrode was measured at constant ionic strength (80 mM KCl), which was achieved by adding ionic strength adjuster (4 M KCl) in a volume ratio of 1:50 to each standard calcium solution and sample prior to analysis by ISE. Free calcium concentrations were calculated from standard curves and dilution factors. The measurements were carried out in duplicate. Typical standard deviations were <5%.

RESULTS AND DISCUSSION

Calcium-Binding Characteristics of Chelating Agents Determined by Isothermal Titration Calorimetry. Before examining the influence of chelating agents on the physicochemical characteristics of protein-stabilized emulsions containing calcium ions, we carried out a series of experiments to establish the calcium-binding characteristics of EDTA and citrate under the environmental conditions used in our experiments. The change in the power required to keep a zero temperature difference between the reference and sample cells was measured using ITC as calcium solution was injected into EDTA solution at 30 °C (**Figure 1**, top). The enthalpy change per mole of $CaCl_2$ injected into the sample cell (ΔH_{mix}) versus the molar ratio of calcium ions to EDTA in the sample cell after each injection (R = $CaCl_2$:EDTA) was calculated (**Figure 1**, bottom) by integrating the areas under each injection peak shown in the top of **Figure 1** (*28*). The binding interaction between EDTA and calcium ions was exothermic (ΔH_{mix} < 0). When $CaCl_2$ was injected into the sample cell at R < 1, the mixing enthalpy was approximately −13 kcal/mol, which was associated with the $CaCl_2$−EDTA binding interaction. At $R \approx 1$, the mixing enthalpy decreased abruptly, and at R > 1 the mixing enthalpy was approximately zero, indicating that the binding sites on the EDTA had become saturated with calcium ions. A one-site binding model was fitted to the experimental data, and the thermodynamic binding parameters that gave the best fit to the model were as follow: binding enthalpy, $\Delta H_{bind} = -12.73 \pm 0.04$ kcal/mol; number of calcium-binding sites per EDTA molecule, $N = 0.94 \pm 0.01$; and binding constant, $K = (1.9 \pm 0.4) \times 10^7$ M^{-1}. These values are in good agreement with the binding parameters measured for EDTA and calcium using the same technique but at pH 7.5 (*29*): $\Delta H_{bind} = -11.97 \pm 0.15$ kcal/mol, $N = 1.0$, and $K = (2.0 \pm 0.6) \times 10^8$ M^{-1}. The fact that N was nearly 1.0 indicated that the molecular binding ratio between EDTA and calcium was about 1:1, which was in good agreement with the equation (*30*)

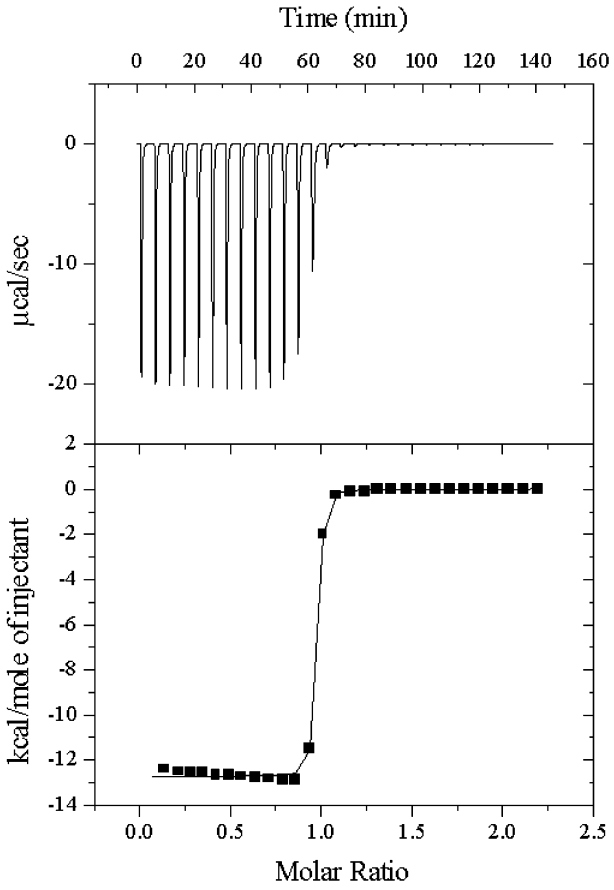

Figure 1. Calorimetric titration isotherm of the interaction between EDTA and calcium ions in 20 mM Tris buffer at 30 °C as 5 mM $CaCl_2$ was injected into 0.5 mM EDTA: (top) heat flow versus time and (bottom) mixing enthalpy versus molar ratio ($CaCl_2$:EDTA).

$$Ca^{2+} + EDTA^{2-} \leftrightarrow (Ca{-}EDTA)^{2-} + 2H^{+} \qquad (1)$$

However, the binding constant measured by ITC was appreciably lower than values reported in the literature, i.e., $10^{10.7}$ M^{-1} (*30*). This is because determination of the binding constant using ITC involves measurement of the slope of the ΔH versus R curve at the point where the binding sites are half filled (*31*). This slope is extremely steep in the case of very strong binding and therefore cannot be measured accurately (**Figure 1**, bottom). It has been reported that ITC techniques can accurately measure binding constants only up to about 10^8 M^{-1} (*27*).

The enthalpy change per mole of $CaCl_2$ injected into the reaction cell (ΔH_{mix}) versus the molar ratio of calcium ions to citrate (R = $CaCl_2$:citrate) in the reaction cell after each injection is shown in **Figure 2**. The binding interaction between citrate and calcium was also exothermic; however, the mixing enthalpy was considerably smaller than for calcium−EDTA interactions (compare **Figures 1** and **2**). The experimental data could be modeled much better by using a two-site binding model than

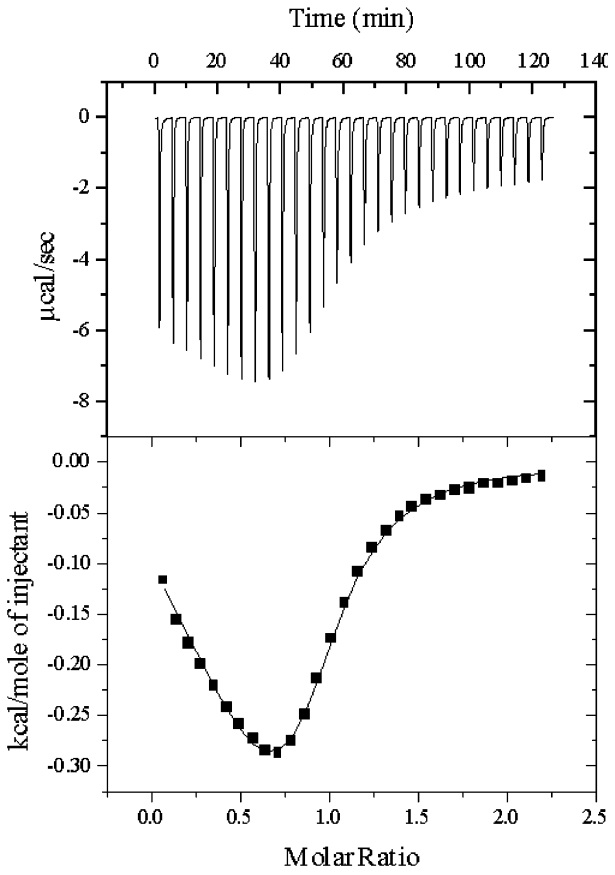

Figure 2. Calorimetric titration isotherm of the interaction between citrate and calcium ions in 20 mM Tris buffer at 30 °C as 50 mM $CaCl_2$ was injected into 5 mM citrate: (top) heat flow versus time and (bottom) mixing enthalpy versus molar ratio ($CaCl_2$:citrate).

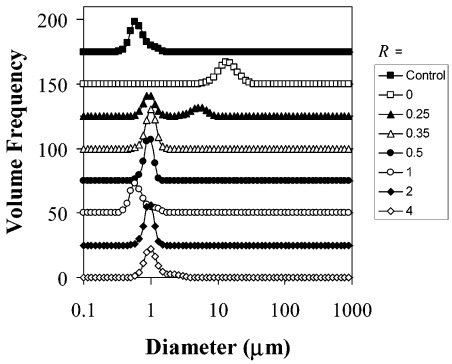

Figure 3. Effect of EDTA-to-calcium ion ratio (R) on the particle size distributions of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$.

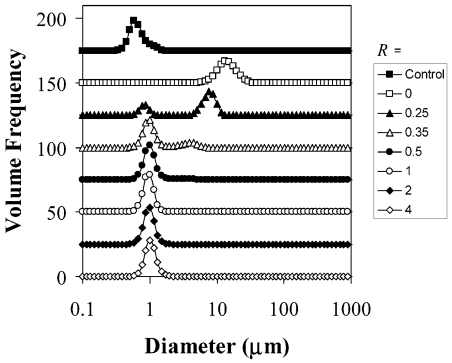

Figure 4. Effect of citrate-to-calcium ion ratio (R) on the particle size distributions of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$.

by using a one-site binding model. The thermodynamic binding parameters for the two kinds of binding sites that gave the best fit to the two-site binding model were as follow: $\Delta H_{bind,1} = -60 \pm 270$ cal/mol and $\Delta H_{bind,2} = -270 \pm 60$ cal/mol; $N_1 = 0.19 \pm 0.15$ and $N_2 = 0.75 \pm 0.10$; and $K_1 = (3.9 \pm 6.0) \times 10^4$ M^{-1} and $K_2 = (7.4 \pm 1.7) \times 10^3$ M^{-1}. The overall binding constant between citrate and calcium ions, $10^{3.5}$ M^{-1} (*32*), is close to the two binding constants determined here. The two different calcium-binding sites determined by ITC can be attributed to the two different forms of dissociated citrate that occur in aqueous solutions at pH 7: $citrate^{2-}$ and $citrate^{3-}$, with the latter being the more prevalent (*33*). The sum of N_1 and N_2 was close to 1.0, indicating that the overall molar binding ratio of calcium to citrate was about 1:1, which is in good agreement with the equations (*33*)

$$Ca^{2+} + citrate^{2-} \leftrightarrow (Ca-citrate) \quad (2a)$$

$$Ca^{2+} + citrate^{3-} \leftrightarrow (Ca-citrate)^{-} \quad (2b)$$

The shape of the mixing enthalpy versus molar ratio curve for the calcium−citrate interaction (**Figure 2**) can be described in terms of the differences in the binding parameters of the two binding sites. Initially, when $CaCl_2$ was injected into the citrate solution, the calcium ions bound to the citrate predominantly at the binding sites with the higher binding constant (K_1). These binding sites have a less exothermic binding enthalpy ($|\Delta H_{bind,1}| < |\Delta H_{bind,2}|$), so the mixing enthalpy at the beginning of the titration was relatively small. As more $CaCl_2$ was added to the reaction cell, the high-affinity binding sites became saturated with calcium, and so the additional calcium bound to the lower affinity binding sites (K_2). These binding sites have a more exothermic binding enthalpy, and so there was an increase in the exothermic mixing enthalpy associated with the interaction. When the molar ratio was close to 1.0, the binding (and therefore the mixing enthalpy) started to decrease because free citrate was being depleted. Adding $CaCl_2$ to the reaction cell at $R > 1$ led to a small constant exothermic mixing enthalpy, which was associated with dilution of the calcium salt in the reaction cell.

Effect of Chelating Agents on Emulsion Particle Size. The influence of chelating agents on the particle size of WPI-stabilized oil-in-water emulsions containing calcium was determined by light scattering. The control emulsion (containing no $CaCl_2$ or chelating agent) had a mean particle diameter of 0.65 μm and a monomodal particle size distribution (**Figure 3**). The mean particle diameters and particle size distributions of emulsions containing 10 mM $CaCl_2$ and 0−40 mM EDTA or citrate ($R = 0-4$) were measured (**Figures 3−5**). An increase in mean particle diameter and a shift of particle size distribution to larger sizes indicate droplet aggregation in an emulsion (*34*, *35*). At $R = 0$ (no EDTA or citrate), the mean particle diameter was about 13.5 μm (**Figure 5**), and the particle size distribution was monomodal and shifted to larger particle sizes (**Figure 3**), indicating that all of the original droplets had extensively aggregated. The most likely explanation for droplet aggregation in the presence of 10 mM $CaCl_2$ is ion binding and electrostatic screening effects, which reduce the electrostatic repulsion between the droplets (*8*). Multivalent counterions, such as Ca^{2+}, are known to be particularly effective at binding to oppositely charged surfaces and screening electrostatic interactions (*14*−*16*). It should be noted that the addition of calcium ions to the emulsions may have also modified the steric repulsive interac-

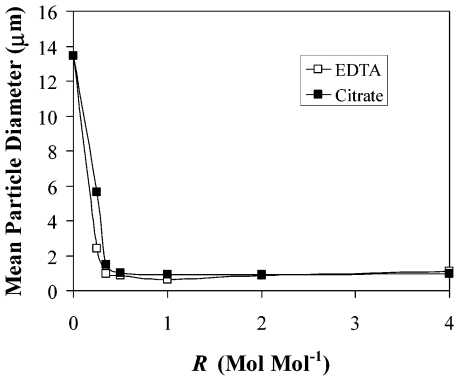

Figure 5. Effect of chelating agent-to-calcium ion ratio (*R*) on mean particle diameter of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$.

tions between the droplets by changing the thickness, composition, or structure of the interfacial membrane. For example, hydrated calcium ions may have adsorbed to the surface of the proteins, or the presence of calcium in the aqueous phase may have altered the interfacial conformation of the proteins. Nevertheless, these short-range steric interactions are normally more important in determining the stability of globular protein-stabilized emulsions to coalescence than to flocculation (*8*).

Droplet aggregation in the emulsions containing calcium could be decreased appreciably by adding chelating agents (**Figure 5**). For EDTA at $R = 0.25$, the mean particle diameter was 2.4 μm and the particle size distribution was bimodal, indicating that sequestering free calcium with EDTA prevented some of the droplets from aggregating and retarded the formation of larger flocs. At $R = 0.35$ and 0.5, the mean particle diameters were about 0.9 μm, and the particle size distributions were monomodal but slightly shifted to larger sizes than the control emulsion. This suggested that the formation of large flocs was completely prevented, but that some of the droplets were still aggregated into small flocs. At $R = 1$, the mean particle diameter decreased to 0.65 μm, and the particle size distribution was similar to that of the control emulsion, which suggested that none of the droplets were flocculated. Presumably, all of the free calcium ions had been sequestered by the EDTA, and the calcium−EDTA complex formed was incapable of electrostatic screening or ion binding effects. Raising the EDTA concentration to $R = 2$ or 4 resulted in an increase in the mean particle diameter to 1.0 μm and a shift in the monomodal particle size distribution peak to higher sizes, indicating a small degree of aggregation. The most likely cause of an increase in droplet flocculation for $R > 1$ is the ability of free Na^+ and $EDTA^{2-}$ ions (from the disodium EDTA) to increase the ionic strength of the aqueous phase, thereby reducing the electrostatic repulsion between droplets.

The ability of citrate to improve the flocculation stability of the WPI-stabilized emulsions containing calcium followed a trend similar to that of EDTA (**Figure 5**). Even so, the citrate was somewhat less effective than EDTA at preventing flocculation at low concentrations; i.e., at $R = 0.25$ and 0.35, the measured particle sizes were larger for emulsions containing citrate than for those containing EDTA (**Figure 5**). The reason for the greater effectiveness of EDTA is that it can bind calcium ions more strongly than citrate (i.e., it has a higher binding affinity), so the free calcium concentration available for promoting emulsion instability is less at the same *R* value (see below). It should also be noted that for both chelating agents the emulsions were stable to droplet flocculation for $R \geq 0.5$, which indicates that it is not necessary to chelate all of the free calcium

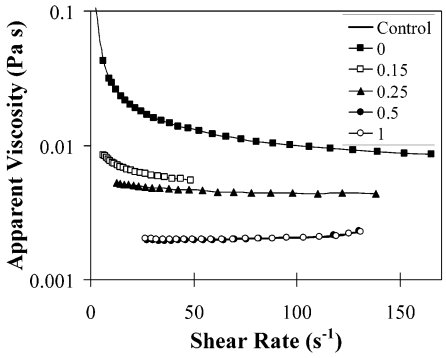

Figure 6. Effect of EDTA-to-calcium ion ratio (*R*) on the shear dependence of the apparent viscosity of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$. The viscosities of the control emulsion and emulsions with $R \geq 1$ were fairly similar.

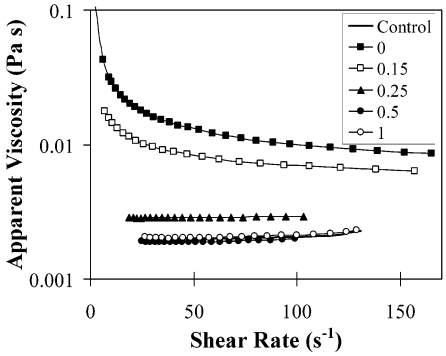

Figure 7. Effect of citrate-to-calcium ion ratio (*R*) on the shear dependence of the apparent viscosity of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$.

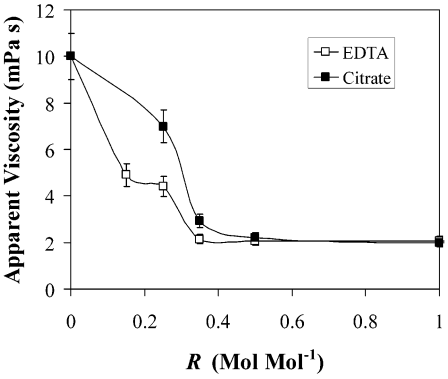

Figure 8. Effect of chelating agent-to-calcium ion ratio (*R*) on the apparent viscosity (at 100 s^{-1}) of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$. The viscosities of the control emulsion and emulsions with $R \geq 1$ were fairly similar.

in the aqueous phase to prevent droplet flocculation. This finding supports previous studies using whey protein-stabilized emulsions at neutral pH that have shown that between 3 and 10 mM $CaCl_2$ is required to promote droplet flocculation (*17*−*21*, *24*).

Effect of Chelating Agents on Emulsion Rheology. The apparent viscosity of the control emulsion and of emulsions that contained 10 mM $CaCl_2$ and 0−40 mM EDTA or citrate ($R = 0-4$) were measured with increasing shear rate (**Figures 6** and **7**). The influence of *R* on the apparent viscosity of emulsions at a fixed shear rate of 100 s^{-1} is also reported (**Figure 8**). The control emulsion exhibited Newtonian behavior; i.e., the apparent viscosity was independent of shear rate (**Figures 6** and **7**). The calcium-containing emulsion without chelating agent

($R = 0$) had an apparent viscosity that was orders of magnitude greater than that of the control emulsion and exhibited strong shear-thinning behavior (**Figures 6** and **7**). The increase in apparent viscosity and the shear-thinning behavior of these emulsions can be attributed to extensive calcium-induced droplet aggregation. Flocculated emulsions are more viscous than nonflocculated ones because the effective volume fraction of the particles in the system is increased due to the water molecules trapped inside the flocs (*36*). Strong shear-thinning behavior is observed in flocculated emulsions because flocs are progressively deformed or partially disrupted in the shear field as the shear stress is increased (*34*, *35*, *37*). When the concentration of chelating agents in the emulsions was increased from $R = 0.0$ to 0.35, there was a dramatic decrease in emulsion viscosity and shear-thinning behavior, indicating that the extent of droplet aggregation decreased with increasing chelating agent concentration. At higher chelating agent concentrations ($R = 0.5-4$), the rheological properties of the emulsions containing calcium were similar to those of the control emulsion, which suggested that floc formation was hindered. The EDTA was more effective than the citrate at reducing the apparent viscosity at low molar ratios ($R < 0.5$), presumably because its higher binding affinity means that it sequesters a greater concentration of free calcium (see below) and therefore causes less droplet flocculation.

Effect of Chelating Agents on Emulsion Creaming Index. The creaming index (CI) of the control emulsion and of emulsions that contained 10 mM $CaCl_2$ and 0−40 mM EDTA or citrate ($R = 0-4$) were measured after 1 week of storage at room temperature. The control emulsion was stable to creaming throughout the storage period; i.e., no separation of the emulsion into creamed and serum layers was observed. The calcium-containing emulsion without chelating agent ($R = 0$) was highly unstable to creaming (CI = 28%), which was presumably because of the increase in particle size in the emulsion due to flocculation. The creaming velocity of emulsions is proportional to the square of the droplet radius, provided that the droplet concentration is sufficiently low to prevent the formation of a three-dimensional network of aggregated droplets (8). When the concentration of chelating agents in the emulsions was increased, there was a dramatic decrease in creaming index: CI = 11% and 0% for $R = 0.25$ and 0.35 EDTA; CI = 22% and 17% for $R = 0.25$ and 0.35 citrate. This decrease indicated that the extent of droplet aggregation decreased with increasing chelating agent concentration. At higher chelating agent concentrations ($R = 0.5-4$), the creaming stability of the emulsions containing calcium was similar to that of the control emulsion (CI = 0%), which suggested that floc formation was hindered. The EDTA was more effective than the citrate at improving creaming stability at low molar ratios ($R < 0.5$), presumably because its higher binding affinity means that it sequesters a greater concentration of free calcium and therefore causes less droplet flocculation. It should be noted that creaming measurements are carried out on undiluted emulsions, whereas particle size measurements are carried out on highly diluted emulsions. Consequently, light-scattering measurements are sensitive only to the presence of flocs that are held together by relatively strong droplet−droplet attractive forces, whereas creaming measurements are sensitive to the presence of flocs held together by both weak and strong attractive forces (*8*). This probably explains the fact that the creaming stability of the emulsion containing citrate was much worse than that of the one containing EDTA at $R = 0.35$, even though the measured particle size was only slightly bigger (**Figure 5**). The flocs in the marginally stable emulsion containing citrate at $R = 0.35$ may have been partially disrupted during the dilution process in the light-scattering instrument.

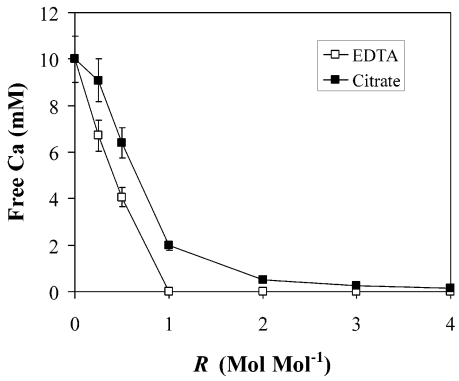

Figure 9. Effect of chelating agent-to-calcium ion ratio (R) on the free calcium concentration of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$.

Effect of Chelating Agents on Emulsion Free Calcium Ion Concentration. The control emulsion had a free calcium ion concentration of about 0 mM (not detectable) in the aqueous phase. The WPI, therefore, was not a significant source of free calcium ions in the emulsion, and so the control emulsion was very stable to calcium-induced aggregation, as demonstrated by the particle size, rheology, and creaming measurements reported above. The free calcium concentration in the aqueous phase of emulsions containing 10 mM $CaCl_2$ and 0−40 mM EDTA or citrate was measured (**Figure 9**). In the absence of chelating agents ($R = 0$), the free calcium concentration in the aqueous phase of the emulsion was 10 mM. This indicates that almost all of the $CaCl_2$ added to the emulsion had dissociated into free ions and that there was little binding of calcium ions to WPI or other components within the emulsion (but see the next section). The large concentration of nonsequestered calcium in the emulsion was the cause of the extensive droplet aggregation revealed by the previous measurements. Free electrolyte ions such as calcium can induce droplet aggregation in an emulsion by reducing electrostatic repulsion between droplets through electrostatic screening (*14*, *15*) and by binding with oppositely charged groups on the droplet surface (*16*). As expected, when chelating agents were added to the emulsion, there was an appreciable decrease in the concentration of free calcium ions (**Figure 9**). At relatively low chelating agent concentrations ($R \leq 2$), the EDTA was more effective at reducing the free calcium concentration than the citrate because it bound the mineral ions more strongly; i.e., it has a higher calcium ion binding constant (see above).

The droplet size, rheology, and creaming stability measurements showed that droplet aggregation was eliminated in the emulsions at $R \geq 0.35$ for EDTA and $R \geq 0.5$ for citrate, which corresponds to a free calcium concentration of about 6 mM (**Figure 9**). Thus, the emulsions became unstable toward droplet flocculation when the free calcium concentration exceeded this level. This shows that it is not necessary to sequester all of the free calcium ions in order to make a stable emulsion.

Effect of Chelating Agents on Particle Charge. Particle surface potential (ζ potential) measurements were carried out to obtain information about possible binding of calcium ions to emulsion droplet surfaces. The ζ potential of the control emulsion was measured to be −35 mV. The particles in this emulsion had a negative charge because the adsorbed proteins were above their isoelectric point at pH 7 (*38*). The negative

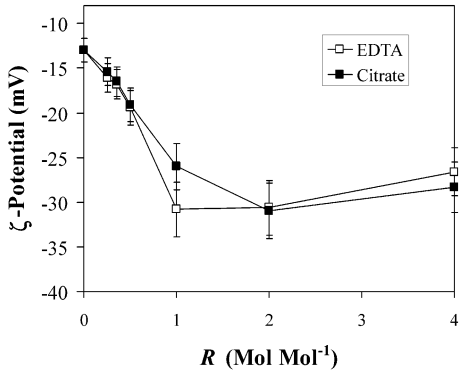

Figure 10. Effect of chelating agent-to-calcium ion ratio (*R*) on the droplet surface potential of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$.

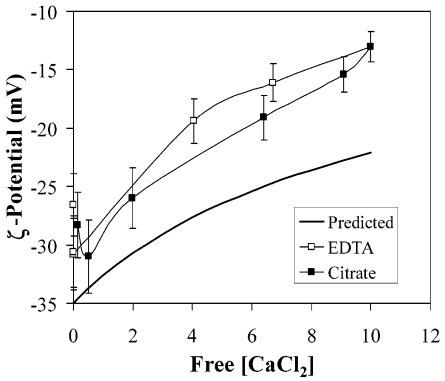

Figure 11. Influence of the free calcium concentration in the aqueous phase on the droplet surface potential of WPI-stabilized oil-in-water emulsions containing 10 mM $CaCl_2$ and chelating agents (0–40 mM). The experimentally measured surface potentials are less negative than those predicted theoretically (assuming no ion binding), which suggests that some calcium ions bound to the droplet surfaces.

surface charge generates an electrostatic repulsion between the droplets, which helps to prevent them from aggregating (*8*). The stability of the control emulsion to droplet flocculation can therefore be attributed to the fact that the electrostatic repulsive forces between the droplets were sufficiently large to dominate any attractive forces (e.g., van der Waals and hydrophobic).

The particle surface charges of emulsions containing 10 mM $CaCl_2$ and 0−40 mM EDTA or citrate ($R = 0-4$) were measured (**Figure 10**). In the absence of chelating agent ($R = 0$), the calcium caused the ζ potential of the emulsion droplets to become much less negative (−13 mV) than that of the droplets in the control emulsion (−35 mV). This reduction in surface charge could have occurred through electrostatic screening and/or ion binding effects (*13*, *20*, *21*). The positively charged multivalent calcium ions are counterions to the negatively charged emulsion droplets and so would have effectively screened the electrostatic repulsion between the droplets (*8*). It has been reported that calcium ions can bind with β-lactoglobulin and α-lactalbumin in aqueous solution through free carboxylic groups of aspartic and glutamic acids (*39*−*41*). Thus, it is possible that some of the negative charge on the emulsion droplets was lost because of positively charged calcium ions binding to negatively charged $-COO^-$ surface groups on the absorbed whey proteins. The decrease in magnitude of the surface negative charge reduced the electrostatic repulsion between the emulsion droplets, allowing them to come closer and aggregate.

The surface charge of the droplets became increasingly negative as the concentration of chelating agents in the emulsions was increased from $R = 0$ to 1 (**Figure 10**), which is presumably because the chelating agents bound calcium ions, thus reducing the magnitude of the ion binding and electrostatic screening effects. It is likely that the chelating agents overcome the adsorbed proteins in competing to bind calcium because the chelating agents have much higher binding constants than the proteins (*30*, *32*, *41*−*43*). The calcium−EDTA and calcium−citrate complexes are not positively charged like free calcium ions, so they do not act as counterions. The increase in the magnitude of the negative particle surface charge increased the electrostatic repulsion between the droplets, making the emulsions less prone to aggregation, which supports our light-scattering, rheology, and creaming measurements. At relatively low concentrations of chelating agent ($R < 2$), the EDTA was more effective at increasing the negativity of the droplets than the citrate, presumably because it bound the calcium ions more strongly due to its higher binding affinity.

To establish the relative magnitude of ion binding and electrostatic screening effects on emulsion instability, we calculated the dependence of the droplet surface potential on calcium ion concentration, assuming that the surface charge density (σ) of the emulsions containing calcium was the same as that of the control emulsion, i.e., that no calcium binding occurred (**Figure 11**). The surface charge density of the control emulsion was calculated using the relationship $\sigma = \epsilon_0\epsilon_R\kappa\zeta$, where ϵ_0 is the dielectric constant of a vacuum, ϵ_R is the relative dielectric constant of water, κ is the inverse Debye screening length, and ζ is the measured ζ potential of the droplets (*8*). The inverse Debye screening length was calculated using the relationship $\kappa = 3.2I^{0.5}$ nm^{-1}, where I is the ionic strength of the aqueous phase (*44*). The ionic strength of the aqueous phase was calculated from the Tris (20 mM) + free $CaCl_2$ (0−10 mM) concentration. We then assumed that the surface charge density of the emulsions remained constant, and we used the same equation to calculate the dependence of the ζ potential on the free calcium chloride concentration: $\zeta = \sigma/\epsilon_0\epsilon_R\kappa$. As the calcium chloride concentration increased, the inverse Debye screening length increased; thus, the droplet ζ potential decreased (**Figure 11**). These calculations were compared with experimental measurements of the dependence of the droplet ζ potential (**Figure 10**) on the free aqueous phase calcium concentration (**Figure 9**) for emulsions containing EDTA and citrate (**Figure 11**). The emulsions containing high levels of free calcium have a less negative surface potential than theoretically predicted assuming constant surface charge density, which indicates that some calcium ions bound to the surface of the emulsion droplets. In addition, the surface potential is less negative at low free calcium concentrations ($CaCl_2 < 1$ mM) in the emulsions containing chelating agents ($R > 1$) than the surface potential of the control emulsion because of the increased ionic strength of the solution associated with the presence of the chelating agent.

It should be noted that a significant decrease in the free calcium ion concentration in emulsions containing no chelating agents ($R = 0$) was not observed (**Figure 9**), even though calcium ions were bound to the droplet surfaces (**Figure 11**). The reason for this apparent anomaly is that only a very small fraction of the calcium ions in the aqueous phase bind to the droplet surfaces, but this amount is still sufficient to greatly reduce the droplet surface potential. The concentration of adsorbed ions (in millimoles per liter) required to reduce the ζ potential in an emulsion by $\Delta\zeta$ can be calculated from the following equation (*45*):

$$C_{\text{adsorbed}} = \frac{\Delta\zeta}{N_A \epsilon_0 \epsilon_R \kappa \nu e} \frac{6\phi(1-\phi)}{d_{32}} \quad (3)$$

where N_A is Avogadro's number ($=6.0 \times 10^{23}$ mol^{-1}), ν is the ion valency, e is the elementary charge ($=1.6 \times 10^{-19}$ C), ϕ is the disperse phase volume fraction, and d_{32} is the surface−volume mean diameter. We used this equation to calculate the concentration of calcium ions that would have to be adsorbed to the droplets to reduce their ζ potential from −35 to −13 mV when 10 mM calcium chloride was added to the emulsions in the absence of chelating agents ($\Delta\zeta = +0.022$ V, $\nu = +2$, $\phi = 0.05$, $d_{32} = 0.65$ μm). The calculated value of C_{adsorbed} was <0.1 mM, which explains why we saw an appreciable reduction in the magnitude of the droplet ζ potential (**Figure 11**), even though we did not see an appreciable change in the concentration of free calcium in the aqueous phase (**Figure 9**).

It should also be noted that the reason that the chelating agents were so effective at pulling the calcium ions off the droplet surfaces is that they have higher calcium ion binding constants than the whey proteins and that they were present in much higher concentrations than the whey proteins. For example, the binding constant for calcium to β-lactoglobulin, the most prevailing protein in whey proteins (*38*), has been reported to be about 10^2 M^{-1} (*41*).

There are alternative possible explanations for the deviation between the predicted and experimentally measured ζ potentials of the emulsions in the presence of free calcium chloride (**Figure 11**). For example, the ζ potential could be less than predicted because of changes in the concentration of adsorbed protein upon addition of calcium ions to the emulsions. Nevertheless, the fact that the measured ζ potential was less than the predicted one would mean that some of the protein had to be desorbed upon addition of calcium, which seems unlikely since calcium ions normally favor protein aggregation.

CONCLUSIONS

We have shown that two food-grade chelating agents, EDTA and citrate, can be used to effectively improve the flocculation stability of protein-stabilized emulsions containing $CaCl_2$. The chelating agents operated by sequestering calcium ions that were either free in the aqueous phase or adsorbed to the surface of emulsion droplets, which reduced their ability to promote droplet flocculation through surface charge reduction and electrostatic screening effects. The affinity of EDTA for calcium is stronger than the affinity of citrate for calcium; hence, EDTA can be used at lower concentrations than citrate to prevent calcium-induced droplet aggregation. For example, 3.5 mM EDTA could be used to prevent droplet flocculation in WPI-stabilized emulsions containing 10 mM $CaCl_2$, whereas 5 mM citrate was required to achieve the same. The addition of chelating agents to protein-stabilized emulsions is therefore an effective way to prevent droplet aggregation, creaming instability, and thickening. Nevertheless, there are legal limits to the maximum amount of specific chelating agents that can be used in food products, which have to be taken into account when formulating a particular food product. It may be possible to overcome this problem by using combinations of two or more different chelating agents, with each one used at a concentration below its legal limit. However, it is still important to consider the effect of the presence of relatively high concentrations of chelating agents on the bioavailability of the minerals in a product.

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