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Straightforward Process for Removal of Milk Fat Globule Membranes and Production of Fat-free Whey Protein Concentrate from Cheese Whey

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ABSTRACT: A straightforward method for the separation of milk fat globule membrane (MFGM) and production of fat-free whey protein concentrate/isolate from cheese whey has been developed. Lowering of the conductivity of the whey from its initial value of about 5600 μS cm^{-1} to about 2000−500 μS cm^{-1} via diafiltration with water caused selective precipitation of MFGM when incubated for 30 min at pH 4.2 and 35 °C. The whey proteins remained soluble in the supernatant under these conditions. Experimental evidence suggested that precipitation of MFGM at pH 4.2 was not due to a nonspecific effect of lowering of the conductivity of the whey but due to the specific effect of removal of Ca^{2+} from the whey. The lipid content of whey protein isolate obtained by this process was <0.2%, and the protein loss was <14%. The method provides an industrially feasible process for the production of fat-free whey protein concentrate/isolate. The MFGM, which is reported to contain bioactive/nutraceutical lipids and proteins, is a valuable byproduct of the process.

KEYWORDS: milk fat globule membrane, cheese whey, fat-free WPC, bioactive lipids

INTRODUCTION

Whey proteins are generally considered to be highly nutritious. However, a major factor that often limits widespread use of commercial whey protein concentrates (WPC80) is the development of a stale, oxidized off-flavor and brown discoloration during storage.[1] This arises mainly from lipid oxidation and Maillard browning reactions.[2] The lipids in WPC80 originate from milk fat globule membrane (MFGM) fragments and very tiny intact fat globules, which partition into the whey phase during cheesemaking.[3,4] These charged colloidal particles in part contribute to undesirable turbidity when the product is reconstituted into a solution.[3] They also impair the functional properties, especially the foaming and emulsifying properties, of WPC80.[5] It is evident, therefore, that if an industrially economical process to remove MFGM from cheese whey is developed, it would solve many of the quality problems associated with the utilization of WPC80.

It should be recognized that although the presence of MFGM in WPC80 is undesirable, the MFGM itself is considered to be a rich source of bioactive lipids and proteins.[6−9] Several studies have suggested that consumption of phospholipids, especially phosphatidylserine, derived from MFGM affects cell growth and development, improves memory, reduces stress, suppresses Alzheimer's disease, and enhances brain development in infants,[7,10,11] albeit the veracity of such claims remains to be studied further. Nevertheless, if MFGM could be separated as a byproduct from cheese whey in an industrially feasible and economical way, it can potentially be used as a standalone functional food or as a functional food ingredient in several food products, including infant and geriatric foods. In addition, currently, egg yolk and soybean are the main sources of food-grade lecithin available to the food industry. In this respect, an alternative source of lecithin to cater to the needs of a segment of the population who are allergic to egg and soybean products would be desirable. Thus, a straightforward economical process that can simultaneously accomplish both separation of MFGM and production of fat-free WPC would be very desirable.

Several methods to remove lipids from cheese whey have been reported.[12−16] However, most of these methods cause denaturation and insolubilization of whey proteins and thereby impair their functional properties.[2] Hwang and Damodaran[3] reported a chitosan-based process for selective flocculation of MFGM, which results in the production of a highly functional fat-free WPC and whey protein isolate (WPI).

In a recent paper Zn^{2+} has been shown to induce precipitation of MFGM particles in cheese whey in a pH-dependent manner at 30−35 °C, with maximum precipitation occurring in the pH range 5.2−5.5 at 0.025 *m* zinc acetate concentration.[17] In the present study it is shown that the MFGM in cheese whey can be selectively precipitated at pH 4.2 and 30−35 °C when the conductivity of the whey is decreased to below a critical level.

MATERIALS AND METHODS

Clarified and pasteurized whey from Cheddar cheese processing was obtained from the dairy plant in the Department of Food Science at the University of Wisconsin—Madison. Preclarification of whey was performed in a normal cream separator in the dairy plant to remove the residual butterfat and para-casein particles. The pH of the whey as received was 6.4. Zinc acetate dihydrate was obtained from Fisher Scientific (Pittsburgh, PA). All chemicals used in this study were of reagent grade.

Ultrafiltration and Diafiltration. Ultrafiltration and diafiltration of cheese whey were performed using a benchtop Millipore ProFlux M12 Tangential Flow Filtration System using a spiral wound 10 kDa

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molecular weight cutoff polysulfone membrane (Millipore Corp., Billerica, MA).

Effect of Zn^{2+} on Precipitation of MFGM. In the case of experiments involving the addition of zinc acetate to induce precipitation of MFGM, 3 L of cheese whey was concentrated 3-fold (i.e., 3×) by ultrafiltration to a final volume of 1 L. To an aliquot (5 mL) of 3× concentrated whey were added increasing aliquots of a stock solution of zinc acetate (2 *m*) so that the final zinc acetate concentration was 0 (control), 4, 8, 10, 20, 25, and 30 m*m*. The pH of the solutions was then adjusted to 5.2 using 1 M HCl and incubated for 30 min in a water bath maintained at 30−35 °C. After incubation, all of the solutions, including the control sample, were centrifuged for 5 min at 1300*g* in a Sorvall centrifuge using an SS-34 rotor. The absorbance (turbidity) of the supernatant of the control and Zn^{2+}-treated samples was measured at 500 nm using a Shimadzu model UV-1601PC spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The reduction in the turbidity values as a function of added zinc acetate concentration represented the extent of removal of MFGM from the whey by zinc acetate.[17] These experiments were also carried out on 1-, 2-, and 3-fold-diafiltered 3× concentrated whey.

Effect of Conductivity on Precipitation of MFGM. A basic premise of this study is that the stability of MFGM in cheese whey against precipitation near its isoelectric point is due to the presence of mineral salts, especially Ca^{2+} and Mg^{2+}. Binding of these divalent ions alters the electrostatic properties of MFGM and prevents it from flocculation/precipitation near its isoelectric point.[17] Thus, it can be hypothesized that removal of mineral salts from cheese whey via diafiltration should facilitate precipitation of MFGM under certain pH and temperature conditions. The extent of removal of mineral salts in diafiltered whey can be followed by monitoring the conductivity of the whey.

The following protocol was used to study the effect of removal of endogenous whey mineral salts on the ability of MFGM to flocculate near its isoelectric point: Five liters of cheese whey was concentrated 5-fold (i.e., 5×) by ultrafiltration to a final volume of 1 L. The retentate was then diafiltered in a continuous mode from 0- to 3-fold with 3 L of water using a 10 kDa ultrafiltration membrane. During the continuous diafiltration step, 10 mL aliquots of the retentate samples were withdrawn at various stages of diafiltration. The conductivity of these samples was determined using a conductivity meter (Fisher Scientific). The pH of these aliquots was then adjusted to 4.2 using 1 M HCl and incubated for 30 min in a water bath maintained at 30−35 °C. After incubation, the solutions were centrifuged for 5 min at 1300*g* in a Sorvall centrifuge using an SS-34 rotor. The absorbance (turbidity) of the supernatant was measured at 500 nm. The reduction in the turbidity values of the supernatants as a function of fold diafiltration and/or conductivity represented the effect of salt removal on the precipitation and removal of MFGM from the whey. The selection of pH 4.2 for precipitating MFGM was based on preliminary experiments in which the pH of aliquots of the 5×-concentrated and 3-fold-diafiltered whey was adjusted to within the range 3.5−6.4 using 1 M HCl, followed by incubation of the sample for 30 min at 30−35 °C in a water bath and centrifugation at 1300*g* for 5 min. The absorbance at 500 nm of the supernatant was found to be lowest at pH 4.2, implying that maximum precipitation of MFGM in the diafiltered whey occurred at pH 4.2.

Analytical Methods. The effect of removal of MFGM on the protein content in the supernatant of the control and diafiltered 5× concentrated whey samples was determined according to the biuret method. In these cases, after reaction with the biuret reagent, the reaction mixture was centrifuged at 5000*g* for 5 min, and the absorbance of the clear solution was measured at 550 nm. In the case of the final lyophilized control and treated whey protein samples, the protein content was determined according to the Kjeldahl method, using 6.25 as the nitrogen factor. The lipid content of lyophilized control and treated whey protein samples was determined according to the Mojonnier method.[18]

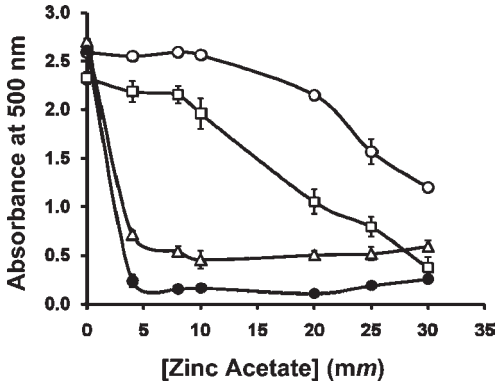

Figure 1. Effect of zinc acetate concentration on precipitation of MFGM in control 3× cheese whey and 1-, 2-, and 3-fold-diafiltered 3× whey at pH 5.2: ○, nondiafiltered 3× whey; □, 1-fold-diafiltered 3× whey; △, 2-fold-diafiltered 3× whey; ●, 3-fold-diafiltered 3× whey. The treated whey solutions were incubated at 30−35 °C for 30 min and centrifuged at 1300*g* for 5 min. Absorbance values were measured without dilution. The decrease in absorbance (turbidity) of the supernatant of the treated samples represents the extent of removal of MFGM. The bars represent standard deviation (n = 2).

The conductivity of the control and diafiltered whey samples was measured using a conductivity meter (Fisher Scientific). The conductivity probe was calibrated using 0.1 M NaCl.

The mineral contents (Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) of control and treated whey were determined by inductively coupled argon plasma (ICP) emission spectroscopy (model Varian Vista-Pro AX, Varian Australia Pty Ltd., Clayton, Victoria, Australia).

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% slab gels as described by Laemmli.[19] Samples were prepared by mixing whey solutions with 2-fold-concentrated sample buffer solution containing 2% SDS and heating the mixture in a boiling water bath for 5 min.

RESULTS AND DISCUSSION

Previously, it has been reported that the addition of Zn^{2+} to whey caused precipitation of MFGM particles at pH 5.2 with maximum precipitation occurring at an optimum zinc acetate concentration of 0.025 *m*.[17] To determine if endogenous cations in cheese whey affected the efficiency of MFGM precipitation by Zn^{2+}, the effect of diafiltration on Zn^{2+}-induced precipitation of MFGM was studied. Figure 1 shows the turbidity of the supernatants of 0-, 1-, 2-, and 3-fold-diafiltered 3×-concentrated whey as a function of zinc acetate concentration at pH 5.2. The turbidity at 500 nm of the supernatant of nondiafiltered 3× whey (control), obtained after incubation with Zn^{2+} for 30 min at 30−35 °C and centrifugation at 1300*g* for 5 min, decreased with increasing Zn^{2+} concentration, indicating that the Zn^{2+} treatment caused precipitation of the turbidity-causing MFGM particles. However, it was also apparent that the concentration of Zn^{2+} needed to induce precipitation of MFGM decreased progressively with increase of fold diafiltration. For instance, whereas 30 m*m* Zn^{2+} was needed to reduce the turbidity of 1-fold-diafiltered 3× whey from about 2.4 (at 0 m*m* Zn^{2+} concentration) to about 0.4, only about 4 m*m* Zn^{2+} was needed to reduce the turbidity of 3-fold-diafiltered 3× whey from about 2.7 (at 0 m*m* Zn^{2+} concentration) to about 0.2 (Figure 1).

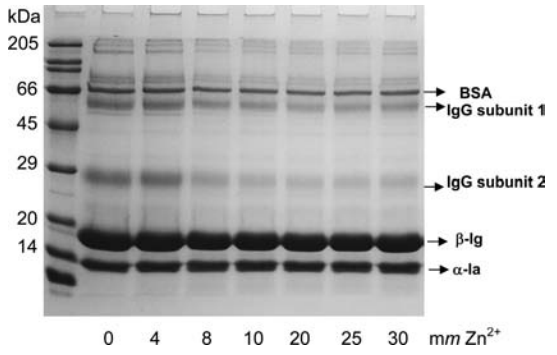

Figure 2. SDS-PAGE profiles of protein in the control whey and the supernatants of the zinc acetate-treated 3-fold-diafiltered 3× whey samples. Columns (from left to right): 1, molecular weight markers; 2, control whey without zinc acetate treatment; 3−8, supernatants of whey samples treated with 4, 8, 10, 25, and 30 m*m* zinc acetate, respectively. The protein bands corresponding to bovine serum albumin (BSA), immunoglobulin-G subunits 1 and 2 (IgG-1 and IgG-2), β-lactoglobulin, and α-lactalbumin are identified.

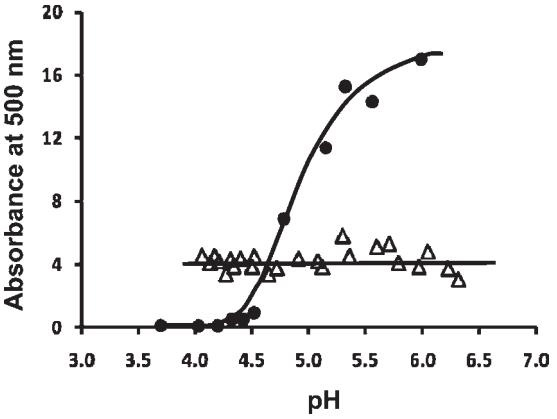

Figure 3. Effect of pH on precipitation of MFGM in 5× whey (△) and in 3-fold-diafiltered 5× whey (●). The whey sample was adjusted to various pH values using 1 N HCl and incubated at 30−35 °C for 30 min and centrifuged at 1300*g* for 5 min. The supernatants were diluted 5−11 times before the absorbance was measured and then corrected for dilution. The decrease in absorbance at 500 nm indicates the extent of removal of MFGM. (n = 1.)

The SDS-PAGE of the supernatants of the control and Zn^{2+}-treated 3-fold-diafiltered 3× whey samples is shown in Figure 2. The intensities of some protein bands, for example, BSA and IgG, in the Zn^{2+}-treated whey samples slightly decreased compared to the control whey sample. However, the extent of loss of β-lactoglobulin and α-lactalbumin was apparently minimal, and the loss might be related to precipitation and removal of some denatured fractions of these proteins along with MFGM at pH 4.2. Also, it should be noted that protein bands corresponding to caseins were not found in the SDS-PAGE, suggesting that the amount of para-casein particles (cheese fines) in the preclarified whey was negligible.

The fact that the concentration of Zn^{2+} needed to induce precipitation of MFGM at pH 5.2 decreased dramatically with increasing removal of endogenous salt ions in whey via diafiltration is noteworthy (Figure 1). A logical extrapolation of this observation would suggest that if the concentration of endogenous

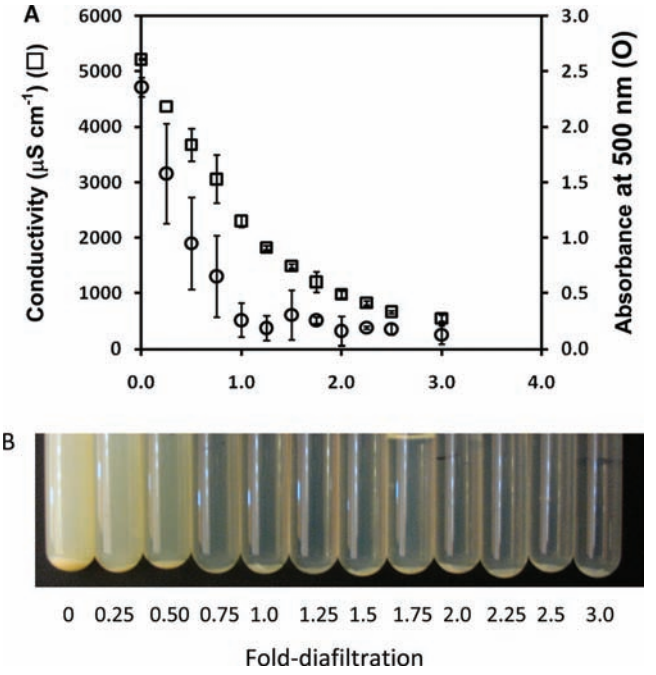

Figure 4. (A) Effect of continuous diafiltration on the conductivity of 5× whey (□) and the extent of precipitation of MFGM at pH 4.2 (○). Other experimental conditions were as described in Figure 3. Absorbance values were measured without dilution. The bars represent standard deviation (n = 2). (B) Clarity of the supernatants as a function of fold diafiltration.

ions is decreased below a threshold level, it might be possible to precipitate the MFGM by pH adjustment alone, albeit at a pH other than 5.2, without the need for the addition of Zn^{2+}.

Figure 3 shows the effect of pH on the absorbance (turbidity) at 500 nm of 3-fold-diafiltered 5× whey supernatant obtained after incubation for 30 min at 30−35 °C and centrifugation at 1300*g* for 5 min. The turbidity of the supernatant decreased as the pH was lowered from 6.4 to about 4.0. A clear supernatant with an absorbance of <0.2 at 500 nm was obtained at pH 4.2. The effect of pH on the turbidity of the supernatants of 5× whey control (no diafiltration) is also shown in Figure 3. In this case, the absorbance did not change significantly over the pH range 4−6.4. It should be noted that the turbidity of the 5× whey control was lower than that of the 3-fold-diafiltered 5× whey at pH 6.4, which indicated that removal of whey salts and lactose caused aggregation of MFGM.

These results (Figure 3) clearly indicate that the colloidal stability against flocculation/precipitation of MFGM particles in the nondiafiltered 5× whey was partly maintained by interaction of endogenous whey salt ions with MFGM particles. Addition of Zn^{2+} to whey (Figure 1) apparently disrupts and/or displaces those interactions, and the resulting MFGM−Zn^{2+} complex is able to precipitate at pH 5.2. Alternatively, physical removal of the endogenous salt ions in whey by diafiltration alters the electrostatic−hydrophobic balance of the MFGM particles and, as a consequence, the ion-depleted MFGM particles precipitate at pH 4.2 without the aid of Zn^{2+}. This also might suggest that the isoelectric pH of the ion-depleted MFGM particles might be in the neighborhood of 4.2 (Figure 3).

To understand the factors that influence flocculation/precipitation of the MFGM particles in cheese whey at pH 4.2,

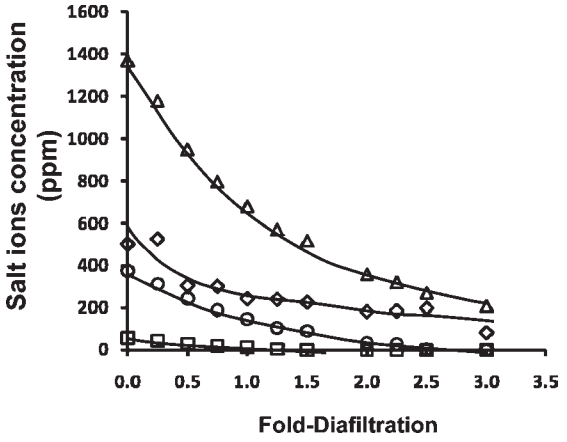

Figure 5. Decrease in the concentration (ppm) of various cations in 5× whey as a function of continuous diafiltration with water: △, K^+; ◇, Na^+; ○, Ca^{2+}; □, Mg^{2+}.

the relationship between the conductivity of various fold-diafiltered 5× whey samples and the extent of precipitation of MFGM at pH 4.2 was analyzed. These results are shown in Figure 4A. The conductivity of 5× whey progressively decreased with increase of fold diafiltration. Also shown in Figure 4A is the relationship between fold diafiltration and absorbance (turbidity) of the supernatant of the whey samples after adjustment of the pH to 4.2 followed by incubation at 30−35 °C for 30 min and centrifugation at 1300*g* for 5 min. The turbidity of the supernatant decreased, that is, the extent of precipitation and removal of MFGM increased, as the conductivity of the whey was lowered by diafiltration. Complete precipitation of MFGM (absorbance < 0.2) occurred when the conductivity of the diafiltered whey approached about 500 μS cm^{-1} at 3-fold diafiltration. It should be noted that the absorbance (turbidity) at 500 nm of the supernatant of the treated whey samples dropped to about 0.2 after 1-fold diafiltration when the conductivity was approximately 2000 μS cm^{-1}. Above 1-fold diafiltration, although the conductivity decreased with increase of diafiltration, the absorbance at 500 nm decreased only slightly. The visual clarity of these supernatants is shown in Figure 4B. Taking the standard deviation of the data shown in Figure 4A into account, it appears that lowering of the conductivity of the whey to <2000 μS cm^{-1}, but more preferably to 500 μS cm^{-1}, facilitates almost complete precipitation of MFGM at pH 4.2 and 30−35 °C.

To determine the extent of protein loss during the removal of MFGM from cheese whey, the protein content of the 5× whey control and the supernatants of the 1-, 2-, and 3-fold-diafiltered 5× whey obtained after pH 4.2/30−35 °C treatment was determined. The protein losses were 13.6, 13.6, and 14.5%, respectively. The data indicated that, on an average, about 13.9 ± 0.54% of total protein in 5× concentrated whey was lost during the removal of MFGM. It is known that the lipid-to-protein weight ratio in cell membranes is typically about 1:1,[20] and it ranges from 1.5 to 4 in some specialized membranes.[21] Thus, assuming that the lipid content of a typical commercial WPC80 is about 7−8%,[4] some of the observed protein loss (up to about 7% at the minimum) might be obligatory loss arising from the removal of MFGM-bound proteins. The rest might be due to loss of small peptides during diafiltration and/or some denatured whey protein fraction that might precipitate along with MFGM at 4.2. The fat content of the lyophilized treated whey supernatant

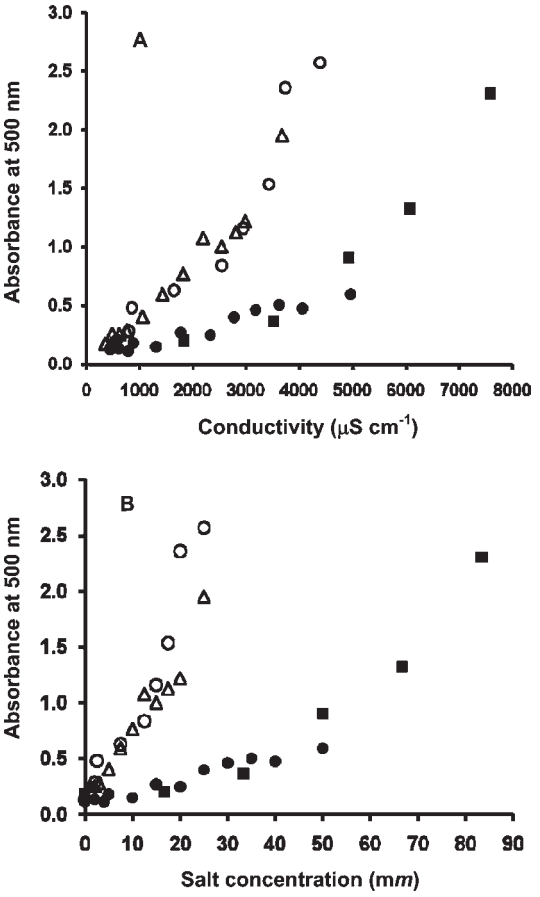

Figure 6. Effect of chloride salts of Ca^{2+} (○), Mg^{2+} (△), Na^+ (●), and K^+ (■) ions on inhibition of precipitation of MFGM in 3-fold-diafiltered 5× whey at pH 4.2. The samples were incubated for 30 min at 30−35 °C prior to centrifugation at 1300*g* for 5 min and measurement of absorbance of the supernatant at 500 nm. Absorbance measurements were made without dilution. The results are presented as conductivity (A) and salt concentration (B) versus absorbance. The data points are from three separate experiments.

was <0.2% on dry weight basis. On the other hand, the total fat content of the 5× concentrated 3-fold diafiltered whey (control) was 6.9% on dry weight basis. This indicated that the process removed >97% of lipids in cheese whey. The lipid and protein contents of the MFGM precipitate fraction were found to be about 19 and 65−70%, respectively, on a dry weight basis, indicating that much of the original lipids in cheese whey were present in the MFGM precipitate.

Several inorganic ions in the whey contribute to its conductivity. The major cationic species in whey that may interact with and affect the electrostatic properties of MFGM particles are Ca^{2+}, Mg^{2+}, Na^+, and K^+. Changes in the concentration of these ions in the 5× concentrated whey as a function of fold diafiltration are shown in Figure 5. It should be noted that the initial concentration of Mg^{2+} in nondiafiltered 5× whey (control) was very low (58 ppm or 2.4 mM) and dropped to zero concentration after 2-fold diafiltration. On the other hand, the initial concentration of Ca^{2+} was about 377 ppm (9.4 mM), which dropped to near zero concentration after 2.5-fold diafiltration. Therefore, the residual conductivity (<500 μS cm^{-1}) of the 3-fold-diafiltered whey emanates mainly from Na and K salts, proteins, and membrane phospholipids.

To elucidate if the ability of MFGM in diafiltered 5× whey to precipitate at pH 4.2 was mainly due to a nonspecific effect of lowering of the conductivity of the whey or to the specific effect of Ca^{2+} (and Mg^{2+}) removal from the whey, the following experiments were conducted: The conductivity of the 3-fold-diafiltered 5× whey (5 mL), which had a residual conductivity of ~400 μS cm^{-1}, was progressively increased by adding aliquots of 2 M stock solution of either NaCl, KCl, $CaCl_2$, or $MgCl_2$. The solution was mixed well, adjusted to pH 4.2, and incubated for 30 min at 30−35 °C. The solution was then centrifuged at 1300*g* for 5 min, and the turbidity of the supernatant was measured at 500 nm. The results are shown in Figure 6. When $CaCl_2$ or $MgCl_2$ was used to increase, that is, to re-establish, the conductivity of the 3-fold-diafiltered 5× whey from ~400 μS cm^{-1} back to 5000 μS cm^{-1}, they inhibited precipitation of MFGM at pH 4.2, whereas when either NaCl or KCl was used to increase the conductivity, they did not greatly inhibit precipitation of MFGM at pH 4.2 (Figure 6A). Figure 6B shows a plot of the above results in the form of concentration of these salts versus absorbance (turbidity) at 500 nm. The data clearly indicate that inhibition of MFGM precipitation by Ca^{2+} and Mg^{2+} was greater than that by Na^+ and K^+ ions.

The concentrations of Ca^{2+}, Mg^{2+}, Na^+, and K^+ ions in the 5× concentrated whey before diafiltration were 9.4, 2.4, 21.8, and 35 mM, respectively, and the conductivity was about 5200 μS cm^{-1}. On the basis of the data in Figure 6, it appears that the inability of MFGM to precipitate at pH 4.2 in the nondiafiltered 5× whey (control) (Figure 4) was not simply related to the conductivity, but was probably due to ion-specific interaction of Ca^{2+} (9.4 mM) and Mg^{2+} (2.4 mM) ions with MFGM. It is probable that much of the Ca^{2+} in cheese whey might be bound to MFGM and the MFGM−Ca^{2+} complex is unable to precipitate in the pH range 4−6. Removal of Ca^{2+} (and Mg^{2+}) by diafiltration renders the MFGM able to precipitate via hydrophobic interactions at pH 4.2, which appears to be close to the isoelectric pH of the MFGM particles in the absence of bound divalent cations.

Previously, it has been observed that although addition of 25 m*m* concentration of Zn^{2+} caused precipitation of MFGM at pH 5.2−5.4 (but not at pH 4.2), neither Ca^{2+} nor Mg^{2+} was able to do the same under similar conditions.[17] This is further confirmed by the data in Figures 1 and 6B. This ion-specific behavior was attributed to differences in the coordination number and geometry preferences of divalent cations for formation of complexes with MFGM.[17] For instance, Ca^{2+} prefers to form hexacoordinated complexes in a regular octahedral geometry with oxygen-containing molecules (e.g., phospholipids),[22] whereas Zn^{2+} prefers tetracoordination in a tetrahedral geometry with membrane phospholipids.[23,24] As a result, when Ca^{2+} cross-links two MFGM fragments, four phosphate groups, two from each membrane, occupy the four coordinates of the square plane of the regular octahedron; the fifth and sixth coordination positions would be occupied by water molecules as steric factors would preclude membrane phosphate group from occupying these fifth and sixth positions.[17] The average Ca−O bond length in hexacoordinated Ca^{2+}−phospholipid complexes is about 2.45 Å.[25,26] This bond length implies that the closest distance between MFGM particles in the MFGM−Ca^{2+} complex would be about 3.43 Å. Because this gap is larger than the diameter of a water molecule (~2.8 Å), water would be able to diffuse into the complex and occupy the fifth and sixth coordinate positions of Ca^{2+}. It was hypothesized[17] that the spatial geometry of MFGM−Ca^{2+} interaction and the presence of water at the fifth and sixth positions would render this complex unable to precipitate via hydrophobic interaction between membrane-bound proteins at the isoelectric pH of the complex. However, when Ca^{2+} is removed from the whey by diafiltration, the Ca^{2+}-depleted MFGM particles are able to precipitate at pH 4.2.

The results of this study showed that removal of divalent cations, especially Ca^{2+}, from cheese whey via diafiltration with water caused selective precipitation of MFGM in cheese whey at pH 4.2 and 30−35 °C. Operationally, the extent of removal of Ca^{2+} could be monitored by measuring the conductivity of the diafiltered whey: The concentration of Ca^{2+} was close to zero as the conductivity approached 500 μS cm^{-1}. Because ultrafiltration and diafiltration are standard unit operations employed in the manufacture of WPC and WPI, the method provides an industrially feasible process for the production of fat-free WPC/WPI. The precipitate obtained at pH 4.2, which contains MFGM, could be a valuable byproduct containing bioactive/functional lipids and proteins. The composition and thermotropic properties of MFGM isolated from cheese whey have been reported recently.[27] This byproduct can be used either as a standalone functional food or as a bioactive food ingredient in several food products, including infant and geriatric foods. Alternatively, it may be used as a starting material for the production of dairy lecithin.

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