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ARTICLE

Zinc-Induced Precipitation of Milk Fat Globule Membranes: A Simple Method for the Preparation of Fat-Free Whey Protein Isolate

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A simple method to isolate milk fat globule membrane (MFGM) from cheese whey has been developed. The method was based on the premise that divalent cations should be able to form insoluble complexes with MFGM under certain solution conditions; this ability, however, is dependent on their coordination chemistry. Incubation of cheese whey with 0−50 m*m* $CaCl_2$ or $MgCl_2$ at 30 °C did not cause precipitation of MFGM, whereas incubation with $Zn(Ac)_2$ under similar conditions induced selective precipitation of MFGM in a concentration-dependent manner at pH 5.2, with complete precipitation occurring above 20 m*m* $Zn(Ac)_2$. The whey proteins remained soluble in the supernatant under these conditions. The ability or inability of a cation to induce precipitation of MFGM is related to the ionic radius as well as its coordination geometry. Calcium and magnesium ions have a strong tendency to form hexa-coordinated ($n = 6$) complexes in a regular octahedral geometry, whereas zinc prefers to form a tetra-coordinated complex in a tetrahedral geometry with MFGM phosphate groups. It is proposed that the tetrahedral geometry of zinc coordination in the zinc−MFGM complex permits hydrophobic interaction between MFGM particles, resulting in precipitation at 30 °C. Further processing of the supernatant using membrane ultrafiltration/diafiltration resulted in a fat-free whey protein isolate.

KEYWORDS: Whey proteins; milk fat globule membranes; zinc−membrane complex; fat-free WPC

INTRODUCTION

The annual production of cheese whey in the United States was about 80 billion pounds in 2006 (*1*). This byproduct of the cheese industry, which was once regarded as a waste, has enormous potential not only as a source of nutritionally exceptional proteins but also as a rich source of pharmacological, immunological, antibacterial, and bioactive agents.

With the advent of ultrafiltration, a significant portion of the whey is being converted into whey protein concentrates and isolates. Whey protein concentrate 80 (WPC80) has become a popular ingredient as it contains highly nutritive proteins with good functional properties, such as gelling and water-binding characteristics. It is used in dairy, bakery, meats, snacks, and confectionary products and in infant formulas. It has the potential for use in sports nutrition, protein bars, low-carbohydrate diet formulations, yogurt, ice cream, and dry mixes.

Commercial WPC80 typically contains 80−82% protein, 4−8% lactose, 4−8% fat, 3−4% ash, and 3.5−4.5% moisture. Although most WPC80 products are generally tasteless immediately after production, they develop a typical stale oxidized off-flavor and brown discoloration during storage due to a series of complex and inter-related chemical reactions that include lipid oxidation and Maillard browning (*2*). This often limits extensive use of WPC80 in various food products (*3*).

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Much of the quality defects of commercial WPC80 can be traced back to its high lipid content, which is typically in the range of 6−8%. The lipid fraction in WPC80 stems mainly from milk fat globule membrane (MFGM) fragments and very tiny intact fat globules (*4*), which partition into the whey phase during cheesemaking. These stable colloidal particles remain in the whey after clarification and are retained in the protein retentate during manufacture of WPC80. In the final product, they impart turbidity when the WPC80 powder is reconstituted into a solution, which is not a desirable quality attribute in protein beverage-type applications. They also impair the foaming and emulsifying properties (*5*). It is evident that to improve the flavor and color stability of WPC80, it is imperative to develop an industrially applicable process to remove MFGM from cheese whey.

Although the presence of MFGM in cheese whey adversely affects the quality of WPC80, it has been shown that MFGM contains several bioactive lipids and protein components (*6*, *7*). For instance, the fatty acid binding protein (FABP) found in MFGM has been shown to inhibit the growth of some breast cancer cell lines at extremely low concentration (*6*, *8*). Butyrophilin is a transmembrane protein in MFGM, and it constitutes about 40% of total proteins in MFGM. The extra-membrane N-terminal domain of butyrophilin belongs to the immunoglobulin (Ig) superfamily (*9*), and this Ig(V)-like domain suppresses multiple sclerosis (*10*). Other protein components of MFGM, especially the glycoproteins, have been shown to inhibit *Helicobacter pylori* infection of the stomach mucosa in BALB/cA mice (*11*). Xanthine

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oxidase, which constitutes 13% of the total proteins of MFGM, is a potent antimicrobial agent.

In addition to these bioactive proteins and enzymes, MFGM contains several bioactive phospholipids. The phospholipids fraction of MFGM is composed of about 24% sphingomyelin, 27% phosphatidylcholine, 25% phosphatidylethanolamine, and 12% phosphatidylserine on a weight basis (*12*, *13*). Several recent studies have indicated that consumption of phospholipids, especially phosphatidlyserine, derived from milk improves cell growth and development, memory, stress, suppression of Alzheimer's disease, and brain development and cognitive function in infants (*7*, *14*−*16*). An emerging body of knowledge suggests that sphingomyelin and its hydrolysis products ceramide and sphingosine are involved in a pathway known as "sphingomyelin cycle" or "ceramide signaling", which controls a multitude of cell regulation processes, ranging from cell proliferation/differentiation (*17*, *18*) to inhibition of cell growth (*19*) and apoptosis (*7*, *20*−*23*). When sphingomyelin isolated from milk powder was included in a diet, it inhibited 1,2-dimethylhydrazine-induced colon cancer in CFI mice (*24*, *25*).

It is evident from the above discussion that while the presence of MFGM in cheese whey may create problems during both manufacture and storage of WPC80, MFGM is an excellent source of several bioactive lipids and proteins, which can potentially be used as a functional food (*13*). Thus, there is a need to develop a simple economical process that can simultaneously accomplish separation of MFGM with intact bioactive components and a clear whey stream that can be efficiently processed into a fat-free WPC80 with good flavor and color stability and functional properties.

Several methods have been reported in the past to remove lipids from cheese whey (*26*−*30*), and the efficacy of these methods has been reviewed (*2*). However, most of these methods denature and insolubilize whey proteins and impair their functional properties.

To address the longstanding issue of quality defects caused by MFGM lipids in WPC80, we had previously developed a simple process to flocculate MFGM. We had shown that chitosan, which is a polyglucosamine polymer derived from chitin, could cause selective precipitation of MFGM fragments under certain conditions (*31*). Although the chitosan process is simple and produces highly functional fat-free WPC and WPI, whey processors are unable to use this technology because chitosan has only self-affirmed "generally regarded as safe" (GRAS) status in the United States.

In the present study, we show that zinc salts can selectively precipitate MFGM from cheese whey under certain conditions at low concentrations.

MATERIALS AND METHODS

Clarified and pasteurized Cheddar cheese whey was obtained from the Dairy Plant in the Department of Food Science at the University of Wisconsin—Madison. The pH of the whey was 6.4. $ZnCl_2$, $MgCl_2$, $CaCl_2$, and molecular weight markers for SDS-PAGE were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals were of reagent grade.

Selective Precipitation of MFGM with Divalent Salts. To an aliquot of clarified and pasteurized cheese whey was added increasing amounts of divalent salt so that the final salt concentration was in the range of 0−0.05 *m*. The pH of the whey solution was adjusted to a specified experimental pH using 1 M HCl and incubated for 30 min in a water bath maintained at 30 °C. After incubation, the solutions were centrifuged at 3000 rpm in a Sorvall centrifuge using a S-34 rotor. The turbidity of the supernatant was measured at 500 nm in a Shimadzu model UV-1601PC spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). A control, containing no added salt, was performed under identical conditions. The difference in turbidity values between the control and the treated whey samples represented the extent of removal of MFGM from the whey. All experiments were carried out in duplicate. All statistical analyses were done using Microsoft Office Excel 2007 software (Microsoft Corp., Redmond, WA).

Analytical Methods. SDS-PAGE was performed using 12% slab gels as described in ref *32*. 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.

To determine the lipid content, the whey samples were dialyzed exhaustively to remove lactose and minerals against water and then lyophilized. The lipid content was determined according to the Mojonnier method (*33*). The protein content of the lyophilized samples was determined by the biuret method.

Ultrafiltration of cheese whey was performed using a benchtop Millipore ProFlux M12 Tangential Flow Filtration System (Millipore Corp., Billerica, MA) using a spiral wound 10 kDa molecular weight cutoff polysulfone membrane.

RESULTS

Figure 1 shows the effect of zinc acetate concentration on precipitation and removal of MFGM in single-strength Cheddar cheese whey at 30 °C. The initial pH of the whey as received was about 6.4. Addition of zinc acetate in the concentration range shown in **Figure 1** caused the pH to drop from 6.4 to the range of 5.5−5.2. No further adjustment of the pH to a final value was made for the data shown in **Figure 1**. The turbidity of the supernatant, obtained after incubation with zinc acetate for 30 min at 30 °C and centrifugation at 3000 rpm for 5 min, decreased with increasing zinc acetate concentration and reached a minimum value at 0.025 *m*, indicating that the turbidity causing MFGM particles were effectively removed from whey by the treatment. Similar results were also obtained with $ZnCl_2$ (data not shown). The supernatants of the samples treated with 0.025 *m* $Zn(Ac)_2$ or higher were very clear (**Figure 2**), indicating almost total precipitation of MFGM. It should be noted that treatment of the whey with other divalent salts, such as $MgCl_2$ and $CaCl_2$, under similar conditions, in the pH range of 5.2−5.5, did not cause precipitation of MFGM (**Figure 1**). Thus, the Zn^{2+}-induced precipitation phenomenon appears not to be due to nonspecific charge neutralization effects, but due to ion-specific interaction of Zn^{2+} with MFGM.

To determine if the MFGM−Zn^{2+} complex formation and precipitation were reversible, the MFGM−Zn^{2+} precipitate was resuspended in water at 30 °C, stirred for 10 min, and centrifuged. These steps were repeated two times. Even after repeated washing to dissociate and remove Zn^{2+} from the MFGM−Zn^{2+} complex, the washed MFGM−Zn^{2+} particles sedimented upon centrifugation, indicating that the MFGM−Zn^{2+} complex formation was essentially irreversible.

Figure 3 shows SDS-PAGE of control whey and the supernatants of $Zn(Ac)_2$-treated whey samples. The number of protein bands and the intensities of these bands, especially the β-lactoglobulin (β-lg) and α-lactalbumin (α-la), IgG subunits, and serum albumin (BSA), were similar in both the control and treated whey samples, indicating that almost all of the soluble whey proteins remained in the supernatant after Zn^{2+}-induced precipitation of MFGM. The protein content of the treated and untreated whey, as determined by the biuret method, were almost the same (∼6 mg/mL), with < 5% protein loss above 0.025 *m* zinc acetate concentration. This minor loss might be related to loss of membrane-bound proteins and/or coprecipitation and removal of some of the denatured whey proteins along with MFGM.

As the Zn^{2+}-induced precipitation of MFGM appears to be due to electrostatic interaction and/or formation of a coordination complex between MFGM and Zn^{2+}, the efficiency of

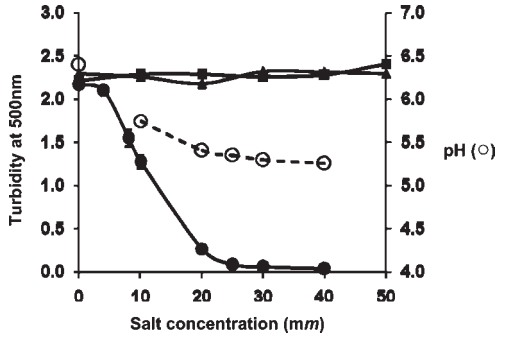

Figure 1. Effect of zinc acetate (●), $CaCl_2$ (▲), and $MgCl_2$ (■) on precipitation of MFGM in single-strength cheese whey at 30 °C. The decrease in turbidity (left *y*-axis) of the supernatant of the treated samples represents the extent of removal of MFGM. The dotted line represents the drop in pH (right *y*-axis) of the solution upon addition of zinc acetate.

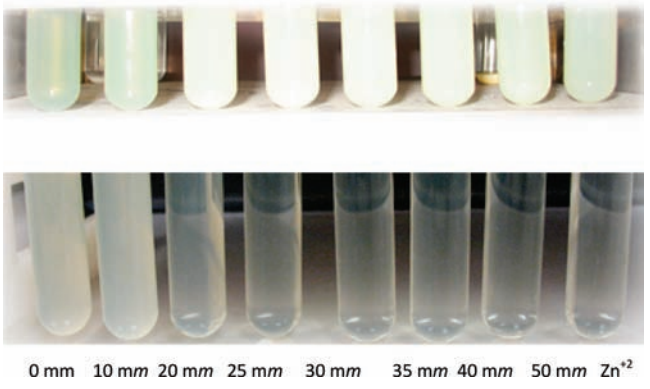

Figure 2. Clarity of zinc acetate-treated single-strength cheese whey before (top) and after (bottom) centrifugation at 1744*g*.

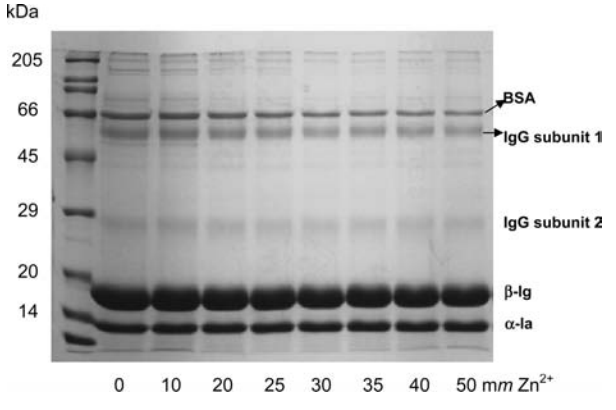

Figure 3. SDS-PAGE profiles of protein in the control whey and the supernatants of the zinc acetate-treated whey samples. Columns (from left to right): 1, molecular weight markers; 2, control whey without zinc acetate treatment; 3−9, supernatants of whey samples treated with 10, 20, 25, 30, 35, 40, and 50 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.

precipitation would be expected to be a function of the pH of the whey. The effect of pH on the efficiency of removal of MFGM in the presence of 0.025 M zinc acetate is shown in **Figure 4**. The pH−turbidity profile of the supernatant exhibited a minimum at pH 5.2−5.6 and a maximum at about pH 4.0, indicating that net charge of the MFGM−Zn^{2+} electrostatic complex was close to

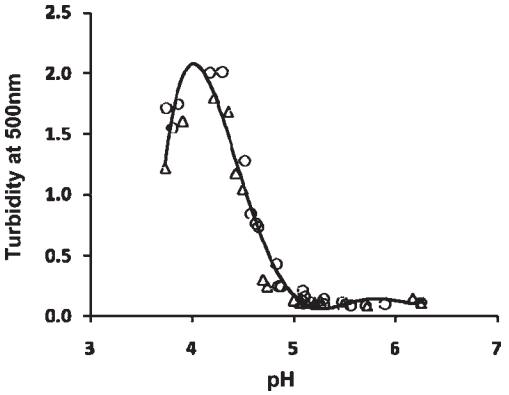

Figure 4. Effect of pH on the turbidity of the whey supernatant after treatment with 25 m*m* zinc acetate at 30 °C for 30 min followed by centrifugation at 1744*g* for 5 min. The triangles and circles represent replicate experiments.

0 at pH 5.2 and the complex precipitated as a result of hydrophobic interaction between membrane proteins. The rise in turbidity with lowering of the pH below 5.2 might be due to a progressive decrease in the charge of the phosphate groups of membrane phospholipids as the pH approaches their pK_a ($pK_a \sim 2-5$), which may cause partial dissociation of the Zn^{2+}−MFGM complex. It has been reported that the pK_1 and pK_2 of phosphatidic acid in self-organized monolayers, which is representative of the environment in a biological membrane, were about 4 and 8, respectively (*34*). Because membrane phospholipids, for example, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, contain only one ionizable proton corresponding to pK_1 on the phosphate moiety, it is reasonable to assume that the pK_1 of MFGM phospholipids might be close to 4. This might explain dissociation of the Zn^{2+}−MFGM complex below pH 5 as the pH approached pK_1. Charge variations on membrane proteins as a function of pH also would contribute to total net charge of the MFGM and its interaction with Zn^{2+}.

Figure 5 shows the effect of temperature on Zn^{2+}-induced precipitation of MFGM. Above 30 °C, temperature had no significant effect ($P > 0.05$) on Zn^{2+}-induced precipitation of MFGM. However, at temperatures < 30 °C (e.g., at 15 °C), the efficiency of Zn^{2+}-induced precipitation of MFGM was lower than that at higher temperatures at all zinc acetate concentrations (**Figure 5**). This suggested that flocculation/precipitation the MFGM−Zn^{2+} complex was partly driven by hydrophobic interaction between membrane-bound proteins, as such interactions are stronger at higher temperatures than at lower temperatures (*5*, *35*).

The effect of preconcentration of cheese whey on the efficiency of Zn^{2+}-induced precipitation of MFGM is shown in **Figure 6**. Because the 3-fold concentrated cheese whey was prepared by using a 10 kDa ultrafiltration membrane, the ionic strength and the lactose content of the concentrated whey were the same as that of single-strength whey, but the MFGM and whey protein concentrations were 3 times that of the single-strength whey. The data in **Figure 6** show that whereas MFGM in single-strength whey was effectively precipitated by 25 m*m* $Zn(Ac)_2$, a proportionately higher concentration of $Zn(Ac)_2$ was needed to cause precipitation of MFGM in 3-fold concentrated whey. This tentatively indicated that complex formation between MFGM and Zn^{2+} followed a stoichiometric ratio.

The clear whey supernatant obtained after removal of MFGM was further concentrated and 3 times diafiltered with water using a 10 kDa ultrafiltration membrane. The final retentate was lyophilized. The fat content of the whey protein isolate

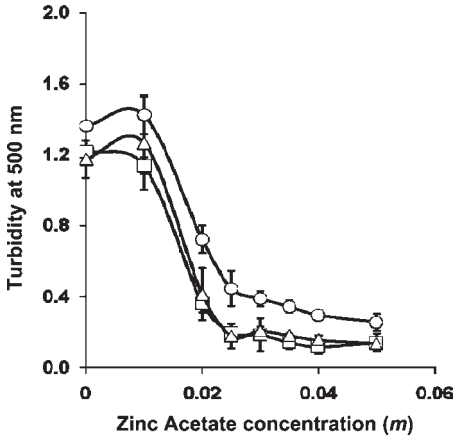

Figure 5. Effect of temperature on zinc acetate-induced precipitation of MFGM at incubation temperatures of 15 °C (circles), 30 °C (squares), and 45 °C (triangles).

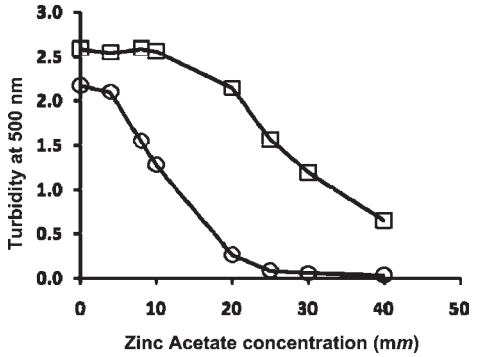

Figure 6. Effect of preconcentration of cheese whey on zinc acetate-induced precipitation of MFGM: (○) single-strength whey; (□) 3-fold concentrated whey.

(95% protein) thus prepared contained < 0.2% total lipids on dry weight basis, as determined by the Mojonnier method.

DISCUSSION

It is known that the MFGM particles in cheese whey remain in a stable dispersed state over a wide range of pH due to electrostatic repulsion arising from the negatively charged phosphate groups of membrane phospholipids. Although in theory an increase of ionic strength should effectively neutralize electrostatic charges on the membrane surface and cause precipitation of MFGM fragments via hydrophobic interaction between membrane proteins (e.g., butryophilin), this does not occur when monovalent salts, such as NaCl, are used. Monovalent ions form a diffuse electrical double layer around the MFGM surface, and if the thickness of this electrical double layer is large, which depends on the strength of electrostatic interaction between mobile cations and the fixed negative charges on the MFGM surface, it may not be able to lower electrostatic repulsion sufficiently enough to facilitate flocculation of the MFGM fragments via hydrophobic interaction between membrane-bound proteins. In contrast, polycationic polymers, such as chitosan (polyglucosamine polymer), which contain multiple fixed positive charges, can engage in strong multiple electrostatic interactions with negatively charged MFGM fragments and thus facilitate flocculation and precipitation of MFGM fragments (*31*).

The fact that Zn^{2+}-induced precipitation of MFGM was not readily reversible upon dilution with water indicated that the mechanism was not related to the salting-out phenomenon (*36*), but due to other ion-specific interaction. Theoretically, divalent cations, such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, should be able to cross-link MFGM fragments via interaction with phosphate groups of MFGM phospholipids, and the charge-neutralized MFGM particles should be able to flocculate and precipitate via hydrophobic interaction between MFGM membrane proteins under certain solution conditions.

The basic premise of this investigation was that the effectiveness of cross-linking and precipitation of the MFGM fragments by divalent cations is dependent upon the ionic radius as well as their coordination geometry. For instance, Mg^{2+} has an ionic radius of 0.65 Å and has a strong tendency to form hexacoordinated ($n = 6$) complexes in a regular octahedral geometry (*37*,*38*). On the other hand, Ca^{2+} has an ionic radius of 0.99 Å and has a strong tendency to form hexacoordinated ($n = 6$) complexes with regular octahedral geometry (*39*). In contrast, Zn^{2+}, with an ionic radius of 0.71 Å, intermediate between those of Mg^{2+} and Ca^{2+}, exhibits a coordination number of 4 or 6 with no preference in terms of energy penalty (*37*, *39*). However, in about 50% of known biological molecules, Zn^{2+} exists as a tetracoordinated complex in a tetrahedral geometry (*37*). Evidence indicates that while Zn^{2+} prefers hexacoordination in aqueous solution, it mostly prefers tetracoordination in a tetrahedral geometry when it binds to proteins and membranes and other glassy systems (*40*−*42*). Thus, it is mostly likely that Zn^{2+} exists in a tetracoordinated state in the MFGM−Zn^{2+} complex.

The differences in the coordination number and geometry preferences of divalent cations would influence the mode of their interaction with MFGM and consequently the colloidal stability of the MFGM−ion complexes. For instance, hexacoordination of Ca^{2+} in an octahedral geometry with phosphate groups of two MFGM sheets would not be possible for steric reasons; even if it occurs, it would inevitably cause membrane−membrane repulsion and therefore would not be thermodynamically stable. As a result, when Ca^{2+} cross-links two MFGM fragments, four phosphate groups (two from each membrane) would occupy the four coordinates of the square plane of the regular octahedron and, because the fifth and sixth positions cannot be occupied by the membrane phosphate groups for steric reasons, it is most likely that water molecules would occupy these positions (see the schematics shown in **Figure 7A**). The average Ca−O bond length in hexacoordinated Ca^{2+}−phospholipid complexes is about 2.45 Å (*43*,*44*). On the basis of regular octahedral geometry, this would indicate that the closest distance between MFGM particles in the MFGM−Ca^{2+} complex would be about 3.43 Å, as shown in **Figure 7A**. 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+}. We hypothesize that the spatial geometry of this interaction and the presence of water at the fifth and sixth positions would make this complex unstable and would not permit precipitation of the complex via hydrophobic interaction between the membrane-bound proteins, as depicted in **Figure 7A**.

On the other hand, it has been shown that Zn^{2+} forms a tetracoordinated complex with membrane phospholipids in a tetrahedral geometry (*41*, *42*) and that the Zn−O bond length in such complexes is about 1.54 Å (*40*). The tetrahedral geometry of this complex would imply that three of the coordinate bonds will involve phosphate groups of one membrane and the fourth coordinate bond will be with the phosphate group of another membrane particle, as shown in **Figure 7B**. In this tetrahedral geometry, the closest distance between MFGM sheets would be about 2.04 Å, which is smaller than the diameter of a water molecule (~2.8 Å). As a result, diffusion of water into the

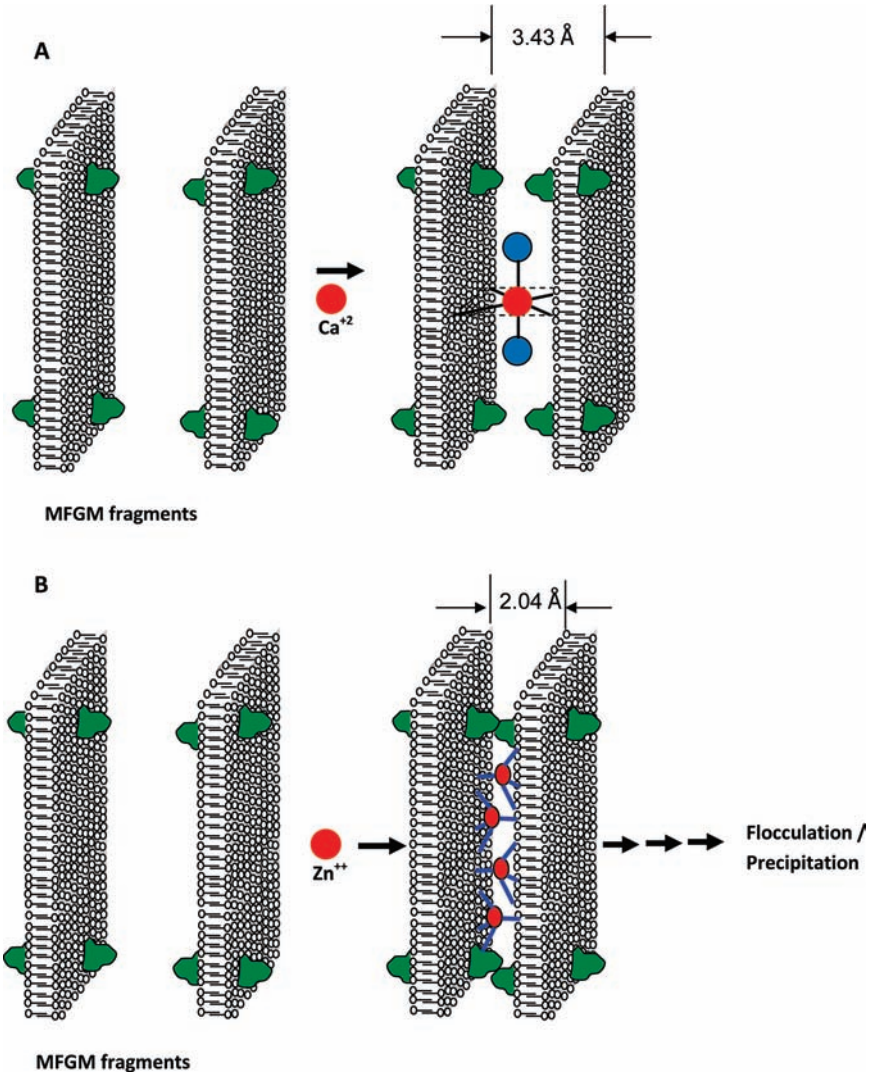

Figure 7. Schematic representation of the proposed (**A**) hexacoordination of Ca^{2+} in a octahedradal geometry and (**B**) tetracoordination of Zn^{2+} in a tetrahedral geometry with MFGM particles.

interstices of the MFGM−Zn^{2+} complex would not be possible, which may provide stability to the complex. Additionally, nonpolar interaction between membrane proteins, facilitated by the close proximity of the membranes in this complex, may further stabilize the complex. A series of such cross-linking interactions seems to be responsible for the precipitation of the complex at pH 5.2 (**Figure 7B**).

The results of the present study provide a simple method for removing MFGM fragments from cheese whey, and this should facilitate manufacture of fat-free whey protein isolate with good flavor and color stability. The isolated MFGM−Zn^{2+} complex can be used as a source of neutraceutical lipids as well as Zn in food formulations.

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