Effects of Calcium and Magnesium Ions on Aggregation of Whey Protein Isolate and Its Effect on Foaming Properties

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The effects of calcium and magnesium ions on time-dependent aggregation of whey protein isolate (WPI) and the influence of the aggregates on the foaming properties of WPI have been studied. Both Mg^{2+} and Ca^{2+} induced formation of stable colloidal aggregates; the rate of aggregation was slow, and the rate and extent of aggregation were maximum at 0.02–0.04 M concentration. Both foamability and foam stability of WPI foams were affected by the concentration of the divalent ions, as well as incubation time in salt solutions before foaming: Maximum foamability and foam stability were observed when WPI was foamed immediately after the addition of the divalent salts, and they decreased progressively with incubation time, indicating that solution-phase aggregation of WPI adversely affected its foaming properties. In addition, evidence also is presented which suggests that, during initial stages, the protein films formed at the foam interface undergo contraction in the presence of Ca^{2+} and Mg^{2+} ions, resulting in a decrease in pressure at plateau borders of the foam.

INTRODUCTION

Whey protein concentrates (WPC) prepared by ultrafiltration typically contain about 0.5% calcium (Liao and Mangino, 1987; Kim et al., 1989; Patel and Kilara, 1990). Literature reports on the effect of calcium on the foaming properties of whey proteins are often contradictory. Some studies have reported that addition of calcium to whey proteins decreased the overrun (Cooney, 1974; Richert et al., 1974), whereas other studies have shown that calcium enhanced the foaming properties of whey proteins (Hansen and Black, 1972; McDonough et al., 1974). In a comparative study on the surface active properties of WPC prepared from several batches of skim milk whey, whole milk whey, and buttermilk-enriched skim milk whey a positive correlation was found between the foaming properties and calcium content of WPC samples (Patel and Kilara, 1990). However, since the calcium content of those WPC samples varied only narrowly from 0.42 to 0.51%, it is not clear whether the positive correlation observed would be true at higher and at lower calcium concentrations as well. Since calcium is a major mineral component of WPC, systematic studies based on a sound methodology are needed to elucidate the effects of calcium on the film-forming and foaming properties of whey proteins. In the present study, the effects of divalent calcium and magnesium ions on the foaming properties of whey protein isolate prepared by an ion-exchange process are presented.

MATERIALS AND METHODS

Commercial whey protein isolate (BiPRO) prepared by an ion-exchange process was obtained from Le Sueur Isolates Co., Le Sueur, MN. All of the experiments reported here were conducted on a single lot of whey protein isolate. According to the manufacturer, the typical composition of this WPI on dry basis was 95% protein, <1% ash, <1% fat, <1% lactose, and <5% moisture. The calcium content of the WPI sample was not determined but was assumed to be very low. All other chemicals used in this study were of analytical grade. Protein solutions were prepared in ultrapure water from a Milli-Q Plus water purification system and adjusted to pH 6.8 by the addition of 0.1 N HCl or NaOH. Protein concentration was measured by the biuret method using bovine serum albumin as a standard. All foaming studies were performed with 5% (w/v) whey protein isolate solutions.

Rate of Aggregation. The rate of aggregation of WPI in $CaCl_2$ and $MgCl_2$ solutions was studied as follows. WPI solutions (5%, pH 6.8) containing known amounts of the divalent salts were incubated at room temperature (24 ± 1 °C). Aliquots of the solutions were withdrawn at intervals of time and percent transmittance at 500 nm was measured using a Beckman DU-68 spectrophotometer (Beckman Instruments Co., Fullerton, CA). Water was used as the blank.

Kinetic Studies. The kinetics of foam decay was studied using the differential pressure method described by Yu and Damodaran (1991) and as outlined in the preceding paper. Foams were formed by sparging prepurified nitrogen through the protein solution at a flow rate of 40 mL/min. The total volume of foam generated in the column was fixed at 67 mL in all experiments. The data were analyzed according to the relation (Nishioka and Ross, 1981)

$$A_t = (3V/2\gamma)\,(\Delta P_\infty - \Delta P_t) \quad (1)$$

where A_t is the interfacial area of the foam at time t, V is the total volume of the foam apparatus, γ is the surface tension of the protein solution, and ΔP_t and ΔP_∞ are the net pressure change at time t and at infinite time when the foam completely collapsed, respectively. Surface tension of protein solutions was determined by the Whilhelmy plate method using an electrobalance (Cahn Instruments Co., Cerritos, CA) as described previously (Xu and Damodaran, 1992). A dimensionless fractional interfacial area at time t during foam decay was calculated by using the relation

$$A_t/A_0 = (\Delta P_\infty - \Delta P_t)/\Delta P_\infty \quad (2)$$

where A_0 is the initial interfacial area of the foam, which is given by $A_0 = 3V\Delta P_\infty/2\gamma$ (Yu and Damodaran, 1991). The foam decay curve was analyzed according to biphasic first-order kinetics (Yu and Damodaran, 1991)

$$A_t/A_0 = Q_g \exp(-k_g t) + Q_d \exp(-k_d t) \quad (3)$$

where the first exponential term refers to decay due to gravitational drainage and the second term refers to decay due to interbubble gas diffusion and disproportionation. k_g and k_d are the first-order rate constants, and Q_g and Q_d are the amplitude

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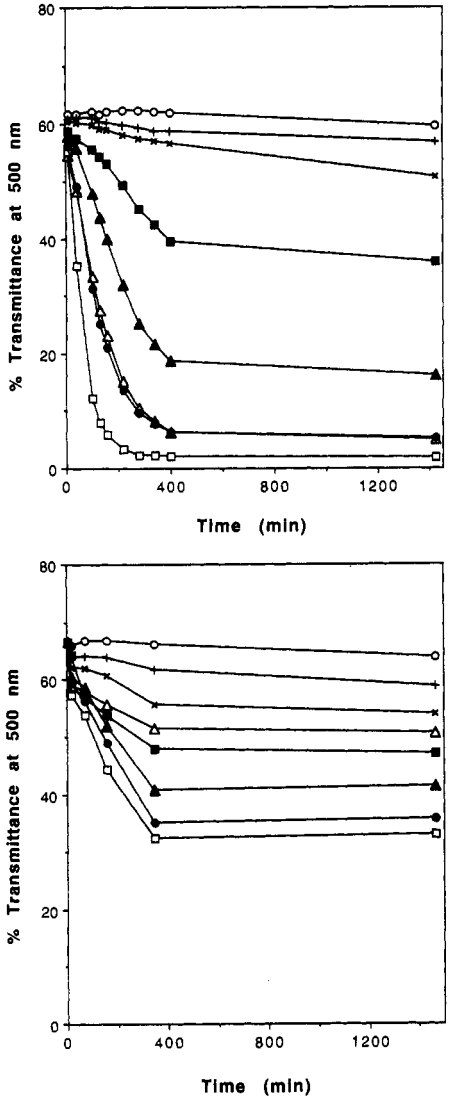

Figure 1. Effects of $CaCl_2$ (A, top) and $MgCl_2$ (B, bottom) on the rate of aggregation of WPI (5%) in water at pH 6.8: ○, no salt; △, 0.02 M; □, 0.04 M; ●, 0.06 M; ▲, 0.08 M; ■, 0.10 M; ×, 0.15 M; + 0.20 M.

parameters of the two kinetic phases. Each foaming experiment was done at least in triplicate.

RESULTS AND DISCUSSION

Effects of Divalent Cations on Aggregation of WPI. The effects of calcium and magnesium ions on the rate of aggregation of WPI are shown in Figure 1. The rate and extent of aggregation of WPI progressively increased when the concentration of either $CaCl_2$ or $MgCl_2$ was increased from 0 to 0.04 M. A greater increase in the rate and extent of aggregation was observed between 0.01 and 0.02 M in the case of $CaCl_2$ and between 0.02 and 0.04 M in the case of $MgCl_2$ (Figure 1). Above 0.04 M, both $CaCl_2$ and $MgCl_2$ progressively decreased the rate and extent of aggregation of WPI. At all salt concentrations, the rate of aggregation of WPI was slow and reached an equilibrium value only after about 200–400 min. Prolonged standing of the solutions did not cause settling of the aggregated particles at the bottom of the tube, and the solutions remained translucent in apppearance, indicating that the aggregates were stable colloidal particles. The salt concentration *vs* turbidity profiles of WPI incubated for 1400 min in $CaCl_2$ and $MgCl_2$ solutions are presented in Figure 2. In both $CaCl_2$ and $MgCl_2$ solutions maximum aggregation of WPI occurred at about 0.04 M salt concentration. However,

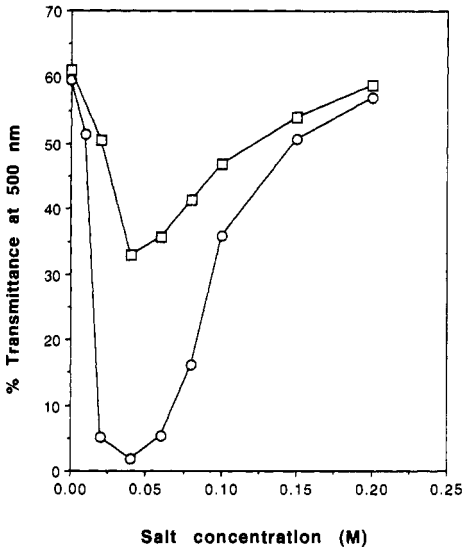

Figure 2. Salt concentration *vs* turbidity profiles of WPI (5%) in $CaCl_2$ (○) and in $MgCl_2$ (□) solutions. The transmittance at 500 nm of the protein solutions was taken after the solutions were incubated for 24 h at room temperature. All solutions contained 0.02% sodium azide.

the extent of aggregation of WPI was more in $CaCl_2$ than in $MgCl_2$ solution. This may be attributed either to formation of larger colloidal aggregates or to a greater amount of protein aggregation in $CaCl_2$ than in $MgCl_2$ solutions.

Previously, Zittle et al. (1957) reported that calcium was bound very strongly to β-lactoglobulin even below 10 mM $CaCl_2$ concentration. The amount of calcium bound was stoichiometrically equivalent to the net charge of the protein. Although several studies relating to aggregation and precipitation of heated β-lactoglobulin and WPC by calcium ions have been reported (Zittle et al., 1957; Varunsatian et al., 1983; Patocka and Jelen, 1991), the time-dependent aggregation behavior of neither native β-lactoglobulin nor WPI by calcium ion has been reported. The data presented here clearly indicate that the native whey proteins in WPI undergo time-dependent slow aggregation in divalent salt solutions, and the rate and extent of aggregation are dependent both on salt concentration and on the type of divalent cation. This aggregation phenomenon might be related to specific binding of Ca^{2+} and Mg^{2+} ions to individual whey proteins. Under equilibrium conditions, that is, at long incubation time, the salt concentration *vs* aggregation profile (Figure 2) exhibits a salting-in and salting-out solubility profile similar to those observed for other food proteins (Shen, 1981; Kumosinski, 1990). At low salt concentration (0–40 mM), binding of Ca^{2+} and Mg^{2+} ions to whey proteins causes aggregation (salting-out) of the proteins; at higher salt concentrations, however, additional binding of these divalent cations to the protein aggregates results in resolubilization (salting-in) of the aggregates. The initial aggregation at low salt concentrations might be attributed to two possible mechanisms: First, binding of divalent cations might cross-link the proteins via ionic bridges, leading to polymerization. However, Zittle et al. (1957) had shown that even at low concentrations (10 mM) the number of moles of Ca^{2+} ions bound to β-lactoglobulin was directly related to the net charge of the protein and was independent of both protein concentration and the denatured state of β-lactoglobulin. This could not be possible if Ca^{2+} was involved in formation of ionic bridges between the protein molecules. Second, binding of Ca^{2+} or Mg^{2+} ions to strong binding sites in whey proteins might

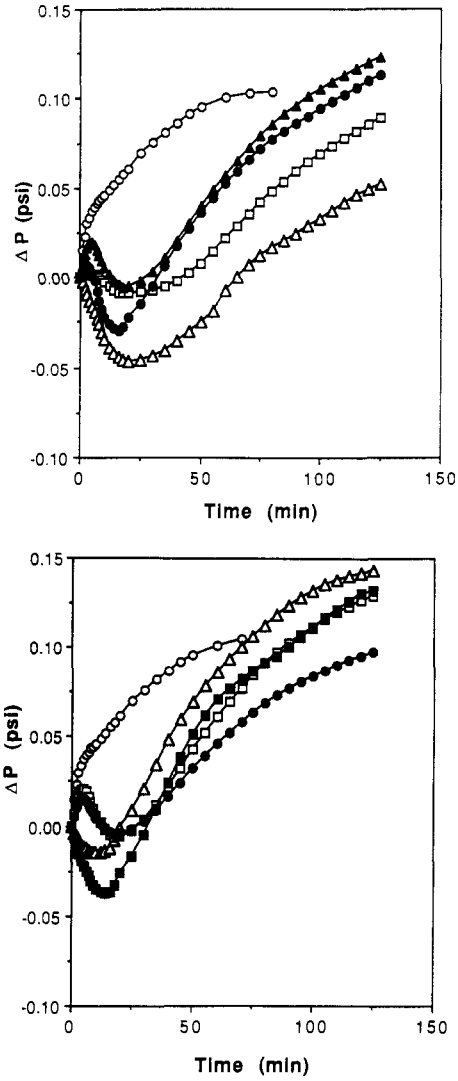

Figure 3. Changes in the pressure inside the foam apparatus as a function of time during decay of WPI foams. (A, top) WPI in $CaCl_2$: ○, no salt; △, 0.02 M; □, 0.04 M; ●, 0.10 M; ▲, 0.20 M. (B, bottom) WPI in $MgCl_2$; ○, no salt; △, 0.02 M; □, 0.04 M; ●, 0.06 M; ■, 0.10 M. WPI concentration was 5%, and the pH was 6.8.

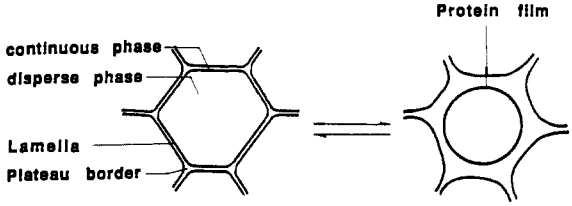

Figure 4. Schematic representation of initial contraction of the protein film and expansion of plateau borders in WPI foams in the presence of calcium and magnesium ions.

induce conformational changes in whey proteins, resulting in an increase in the hydrophobic area of the protein surface, which may facilitate protein–protein interaction and aggregation. It is not clear which of these two mechanisms is primarily responsible for the time-dependent aggregation of whey proteins. It is probable that both of these mechanisms might be operative at varying degrees. The salting-in of the aggregates observed at higher $CaCl_2$ and $MgCl_2$ concentration might be due to additional binding of salt to the whey proteins, which may increase the hydrophilicity and solvation of the proteins, resulting in dissociation and resolubilization of the aggregates.

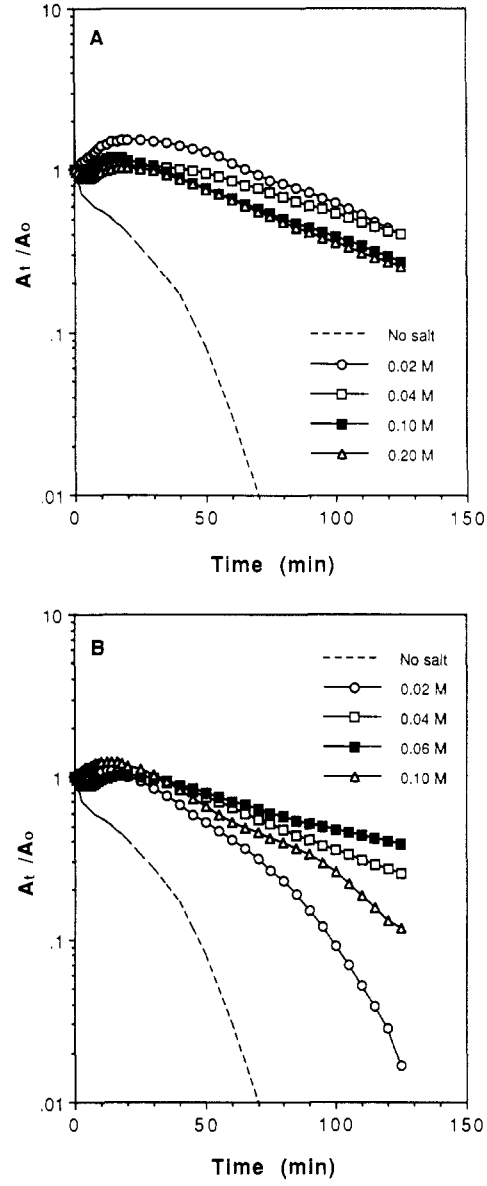

Figure 5. Interfacial area decay of WPI foams at various concentrations of $CaCl_2$ (A) and (B) $MgCl_2$. WPI concentration was 5%, and the pH of the solutions was 6.8. The average standard error (based on triplicate runs) of A_t/A_0 for all of the curves for $CaCl_2$ was 2.03×10^{-2}, and that for $MgCl_2$ was 9.17×10^{-3}.

Effects of Ca^{2+} and Mg^{2+} Ions on Foaming Properties. To elucidate the effects of divalent cation induced aggregation of whey proteins on the surface active properties of WPI, the foaming properties of WPI were studied in the presence of Ca^{2+} and Mg^{2+} ions. The changes in the pressure inside the foam column (with respect to atmospheric pressure) during decay of the foam as a function of time are shown in Figure 3. In these experiments, the foams were formed (67 mL) immediately after the required concentration of $CaCl_2$ or $MgCl_2$ ("zero" time) was added to the protein solution. In the case of the control, i.e., the foam containing no added divalent cations, the pressure inside the foam apparatus increased from the beginning, as expected, and reached a plateau after about 70 min when most of the foam bubbles in the column were collapsed. In contrast, in the case of the WPI forma formed in the presence of Ca^{2+} and Mg^{2+} ions, there was an initial decrease in the headspace pressure up to about 20–30 min, followed by an increase thereafter. The initial negative pressure development in the headspace was observed only

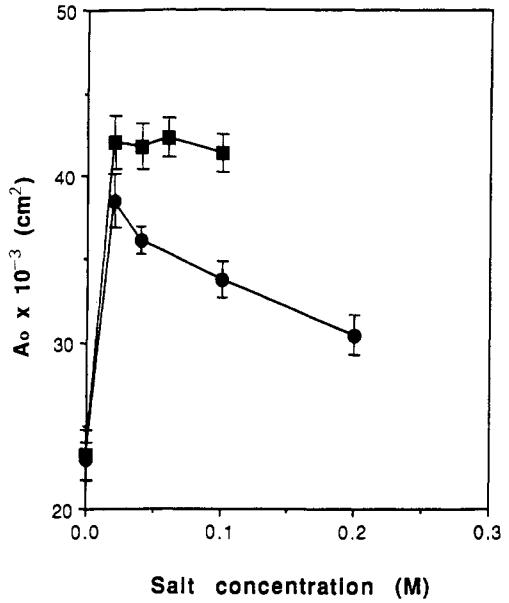

Figure 6. Effects of calcium (●) and magnesium (■) concentration on the initial interfacial area, A_0, of WPI foam. The protein concentration was 5%, pH 6.8; the total volume of the foam was 67 mL.

in WPI foams formed in the presence of Ca^{2+} and Mg^{2+} ions, and this behavior was not observed under any other foaming conditions, including in the presence of up to 0.15 M NaCl (data now shown). During this initial negative pressure development the foam was very stable, and no breakage of the foam was visually observed. Since the system was an isothermal closed system and the total volume of the system was constant, the negative pressure change at the headspace should be associated with a positive pressure change in one of the phases within the system. Careful analysis of this phenomenon suggests that since the continuous liquid phase of the foam is incompressible, the negative pressure development at the headspace could possibly occur only with a positive pressure change in the dispersed gas phase of the foam. However, since the pressure inside the foam bubbles was already greater than the headspace pressure to start with (Laplace pressure), an increase in the internal pressure of the bubbles cannot conceivably occur via transport of gas molecules from the headspace into the dispersed phase against the pressure gradient. Therefore, the most probable mechanism by which the decrease in headspace pressure could occur is via contraction of the bubble size after the foam bubbles are formed. This is shown schematically in Figure 4. The gas bubbles in a typical foam, in which the volume fraction of the gas phase is above 0.74, are in polyhedra shape (Halling, 1981). After the foam bubbles are formed, if the protein film surrounding the dispersed gas phase contracts, as shown in Figure 4, the internal pressure of the bubbles would increase with a corresponding increase in the value and a decrease in the pressure at plateau borders. Since the initial pressure at plateau borders (continuous phase) was in equilibrium with the headspace pressure, the decrease in plateau border pressure would exert a suctioning effect on the headspace, resulting in a decrease in the headspace pressure. In other words, the initial gradual decrease in the headspace pressure might be due to contraction of the protein film surrounding the gas bubbles. It is also likely that the decrease in plateau border pressure might slow the rate of lamella liquid drainage by gravity and thus partly account for the enhanced stability of the foam.

The mechanism of contraction of the protein film near the plateau border, which occurs only in the pressure of Ca^{2+} and Mg^{2+} ions and not at any other experimental conditions, must be related to binding of these divalent cations to the proteins in the film. It is likely that since binding of these divalent cations to whey proteins seems to be time-dependent (Figure 1), as the divalent ions bind to the protein film, they may cross-link the protein molecules via ionic bridges. The formation of such bridges as a function of time might cause a lateral shrinkage of the protein film, resulting in increased curvature at the plateau borders. However, since continuous contraction of the film cannot take place because of the increase in the internal pressure of the bubble, the bubble might reach a metastable state at which the force of contraction is quantitatively opposed by the net increase in the internal pressure of the bubble. After this stage, drainage of the lamella liquid by gravity and changes in the physical properties of the protein film, induced either by additional binding of cations or by conformational changes in the adsorbed proteins, might alter the balance of the contractile force of the film and the internal pressure of bubbles; this results in gradual expansion of bubbles, resulting in concomitant increase in the headspace pressure. Continuous gravitational drainage and interbubble gas diffusion causes breakage of bubbles and eventual collapse of the foam.

To understand the effects of divalent cations on the kinetics of decay of WPI foam, the data in Figure 3 were converted to dimensionless fractional interfacial area of the foam *vs* time using eq 3. In the case of calcium, maximum foam stability occurred at 0.02–0.04 M Ca^{2+} (Figure 5A) Above this concentration range, the foam stability slightly decreased, and there was no difference in foam stability between 0.1 and 0.2 M Ca^{2+} concentration. The stability of WPI foam was extremely stable at all Ca^{2+} concentrations studied compared to that of the control (zero calcium concentration). In the case of magnesium, the foam stability progressively increased as the Mg^{2+} concentration was increased up to 0.06 M and decreased thereafter (Figure 5B). Maximum foam stability occurred at 0.06 M $MgCl_2$ instead of at 0.02 M as in the case of $CaCl_2$. Furthermore, although both Ca^{2+} and Mg^{2+} significantly increased the stability of WPI foam, in relative terms the stability imparted by Ca^{2+} at any given concentration was higher than that imparted by Mg^{2+}. This difference might be probably related to greater binding affinity of Ca^{2+} than Mg^{2+} for whey proteins; this may facilitate formation of a protein film with better viscoelastic properties and mechanical strength even at low Ca^{2+} concentration in the bulk phase.

The effects of Ca^{2+} and Mg^{2+} on the initial interfacial area, A_0, of the foam (67 mL) are shown in Figure 6. In the case of Ca^{2+}, A_0 dramatically increased initially at 0.02 M and then slightly decreased at higher concentrations. However, A_0 was significantly higher at all concentrations of Ca^{2+} than that of the control. Likewise, in the case of Mg^{2+}, A_0 increased initially at 0.02 M and remained the same at higher Mg^{2+} concentrations. The data indicate that in the presence of Ca^{2+} and Mg^{2+} WPI forms highly stable foams with small bubble size and large interfacial area per unit volume of the foam. In other words, both foamability and foam stability of WPI are greatly enhanced by the addition of small amounts of Ca^{2+} and Mg^{2+} ions.

Since WPI exhibits time-dependent aggregation in the presence of Ca^{2+} and Mg^{2+} ions (Figure 1), the effects of the initial aggregation state of WPI on its foaming properties were studied. In these experiments, WPI solutions (5%) containing a known amount of added $CaCl_2$

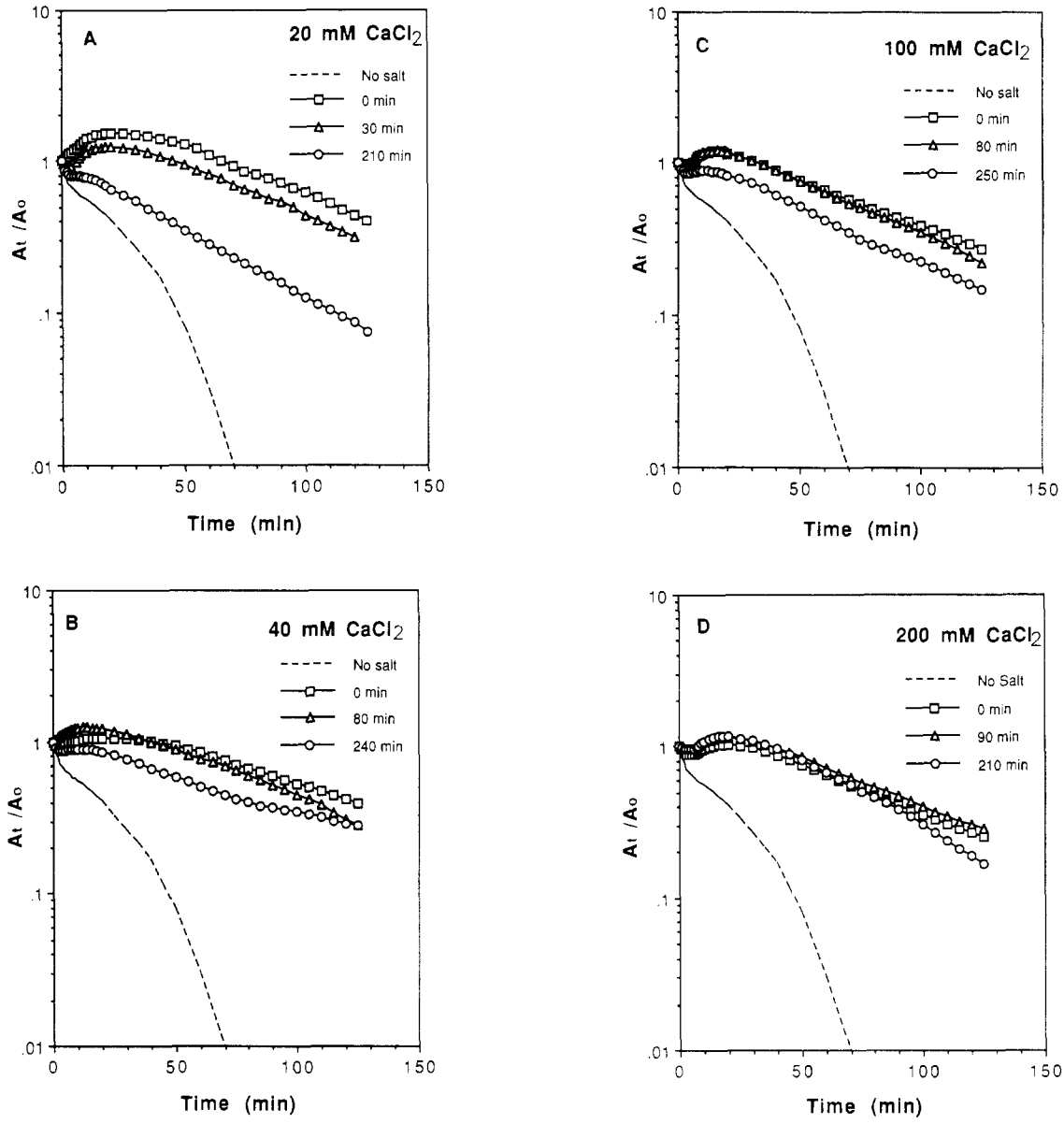

Figure 7. Effect of incubation time of WPI solutions (5%) at various $CaCl_2$ concentrations on the rate of decay of WPI foams. The solution pH in all cases was 6.8. The average standard errors of A_t/A_0 for all of the curves in each $CaCl_2$ concentration were 2.03×10^{-2} (A), 6.03×10^{-3} (B), 4.41×10^{-3} (C), and 4.68×10^{-3} (D).

or $MgCl_2$ were incubated for a specified period of time. At the end of the incubation time, the solutions were foamed (67 mL), and the rate of decay and the initial interfacial area of the foams were determined. The results are shown in Figures 7 and 8. In general, the WPI foams formed immediately after the addition of either $CaCl_2$ or $MgCl_2$ exhibited the highest stability, and solutions incubated for longer times exhibited poorer stability. This was particularly true at 0.02 and 0.04 M $CaCl_2$ concentrations and at 0.04 and 0.06 M $MgCl_2$ concentrations. At 0.1 and 0.2 M $CaCl_2$ concentrations, the effects of incubation time on foam stability were not very significant. It should be pointed out that the extent of aggregation of WPI was greater at 0.02 and 0.04 M than at any other $CaCl_2$ concentration (Figures 1 and 2). Similarly, the extend of aggregation of WPI was greater at 0.04 and 0.06 M than at any other $MgCl_2$ concentration. The results tentatively indicate that the stability of WPI foams was adversely affected by the amount of Ca^{2+}- or Mg^{2+}-induced protein aggregates present in WPI solutioins before foaming. Conditions that promoted aggregation resulted in the formation of a less stable foam. It is likely that these preformed protein aggregates may not be able to integrate themselves with other proteins in the lamella protein film and thus may impair formation of a continuous protein film network with high viscoelastic properties.

The effects of incubation time at various Ca^{2+} and Mg^{2+} concentrations on the initial interfacial area A_0 of WPI foams are presented in Tables 1 and 2, respectively. In general, similar to the behavior of foam stability, A_0 also decreased with incubation time for both Ca^{2+} and Mg^{2+}. The extent of decrease of A_0 was greater at 0.02 and 0.04 M than at other Ca^{2+} concentrations (Table 1). Similarly, for Mg^{2+}, the extent of decrease of A_0 was greater at 0.04 and 0.06 M than at other concentrations. These data, combined with the data in Figures 7 and 8, clearly indicate that divalent metal ion induced protein aggregates impair both foamability and foam stability of WPI foams.

The results of this study clearly show that calcium and magnesium ions significantly enhance foamability and stability of WPI foams even at concentrations as low as 20 mM. Since addition of NaCl to WPI causes a destabilizing effect on WPI foams (data not shown), the positive effects of $CaCl_2$ and $MgCl_2$ on WPI foams cannot be attributed to a nonspecific ionic strength effect. They should be related to ion-specific effects of these divalent

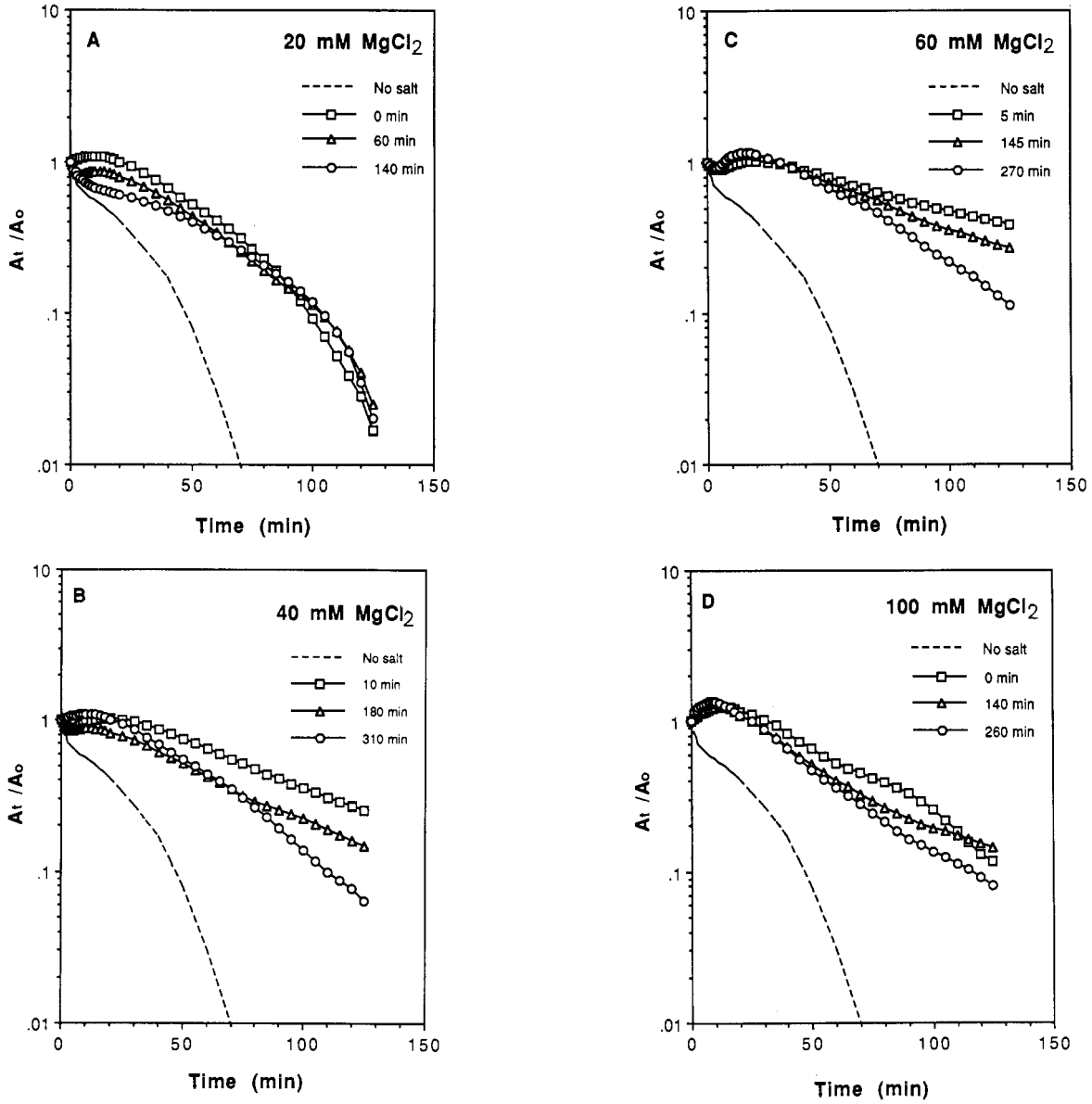

Figure 8. Effect of incubation time of WPI solution (5%) at various Mg^{2+} concentrations on the rate of decay of WPI foams. The average standard errors of A_t/A_0 for all of the curves in each $MgCl_2$ concentration were 1.1×10^{-2} (A), 9.16×10^{-3} (B), 9.7×10^{-3} (C), and 1.42×10^{-2} (D).

Table 1. Effect of Incubation Time at Various $CaCl_2$ Concentrations on the Initial Interfacial Area, A_0, of WPI Foams

	$A_0 \times 10^{-3}$ (cm^2) at $CaCl_2$ concn of				
time (min)	0.0 M	0.02 M	0.04 M	0.1 M	0.2 M
0	23.03	38.50	36.15	33.82	30.40
30		37.44			
80			36.02	32.04	
90					28.10
210		35.61			28.11
240			33.00		
250				31.14	

Table 2. Effect of Incubation Time at Various $MgCl_2$ Concentrations on the Initial Interfacial Area, A_0, of WPI Foams

	$A_0 \times 10^{-3}$ (cm^2) at $MgCl_2$ concn of				
time (min)	0.0 M	0.02 M	0.04 M	0.06 M	0.10 M
0	23.23	42.11	41.88	42.42	41.47
60		40.46			
140		38.49			38.00
145				36.42	
180			37.12		
260					36.46
270				35.86	
310			34.42		

cations. Specifically, enhancement of the foaming properties of WPI by these ions might be related to binding-induced conformational changes in whey proteins or polymerization via ionic bridges. It is interesting to note that a greater effect of these divalent salts on WPI foams is observed only when the foam is formed immediately after the salt is added, i.e., at zero incubation time. Incubation for longer time progressively decreases the effect. This suggests that the mode of action of these salts with the whey proteins that are already at the foam interface might be different from that in the solution phase. Interaction of Ca^{2+} and Mg^{2+} with the proteins at the interface might cause protein unfolding and polymerization via ionic linkages, leading to formation of a viscoelastic film that provides stability to the foam. However, prolonged incubation in the solution phase causes time-dependent aggregation and, possibly, micellization of whey proteins. These discrete protein aggregates might not be able to adsorb at the interface as easily as monomeric whey proteins, and even on adsorption these particles might not be able to form a cohesive protein film network

at the interface. In other words, while ion binding-induced conformational change and polymerization of whey proteins directly at the interface result in the formation of a better viscoelastic protein film, such interactions in the bulk phase slightly reduce their film-forming abilities.

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