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Calcium Effects on the Functionality of a Modified Whey Protein Ingredient

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The primary objective for this study addressed the effects of supplemental calcium on the functional properties of a modified whey protein ingredient (mWPC), prepared by acidification to pH 3.35, followed by extended heat treatment, gelation, and spray drying. In the presence of added calcium (mWPC–Ca^{2+}), protein solutions showed increased thickening capacity, especially under refrigeration temperatures, compared to dispersions made with mWPC alone. A rheological assessment included the determination of (i) power law parameters, (ii) viscoelastic properties, and (iii) the effects of heating and cooling on these protein systems. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) banding profile suggested that various disulfide-linked molecular forms of β-lactoglobulin, bovine serum albumin, and immunoglobulin were likely formed during manufacturing of the mWPC ingredient based on the patterns obtained when electrophoresis was performed in the absence of β-mercaptoethanol compared to those observed with commercial WPC samples. An enhanced water-holding capacity was measured in mWPC–Ca^{2+} dispersions. Differential scanning calorimetry established that the addition of calcium salts caused a 2-fold increase in the amount of bound or unfreezeable water compared to mWPC controls. The physical appearance of the network structure varied significantly upon visualization with scanning electron microscopy, in which case the formation of large, rounded, spherical structures was noted in mWPC–Ca^{2+} samples, ascribed to an increased surface tension caused by the higher salt content. Ultimately, such attributes may afford distinct advantages for whey-based ingredients intended for application within food systems, especially under cold processing conditions.

INTRODUCTION

Whey protein concentrates provide an excellent food resource because of their relatively high protein concentration, excellent nutritional quality, and exceptional functional characteristics. In previous work, Hudson et al. devised a process to modify a whey protein isolate (mWPI), forming a new dairy ingredient with improved water-holding capacity and expanded gelation characteristics when compared to other commercial WPI products. This bioprocessing method involved a pH adjustment and thermal treatment (*1*). After gelation, the materials were frozen, freeze-dried, and ground into a powder. Later, Resch et al. and Firebaugh et al. extended these approaches using less costly whey protein concentrates (mWPC) and discovered that these alternative whey protein fractions also afforded instantaneous thickening capacity and cold-set gel functionality and stability (*2*, *3*). Apparently, these expanded rheological characteristics can be directly attributed to the unique methodologies used during manufacture (*4*).

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The added effects of mono- or divalent salts on the physical/functional attributes of whey protein solutions have been well-documented (*5*–*15*). However, in many cases, prior studies were accomplished using whey-based solutions, prepared at more neutral pH values with a higher protein content. Although monovalent salts, such as sodium chloride, can alter viscoelastic parameters, such as gelation times and/or temperatures, gel strength, viscosity, etc., divalent cations, including barium and most notably calcium, appear to have a more significant impact on whey protein systems (*5*–*7*, *9*, *10*, *12*–*15*). Calcium-induced aggregation presumably occurs as a result of three major events, in which case (1) electrostatic interactions are diminished as a result of charge neutralization, (2) ion-specific hydrophobic interactions are induced, and (3) Ca^{2+}–protein bridges are formed, a process resulting in the cross-linking of adjacent anionic groups, such as glutamic and/or aspartic acid residues (*16*).

Herein, the functional changes that occurred upon the addition of supplemental calcium chloride to mWPC dispersions were systematically evaluated. Initially, an optimal ratio of [mWPC (protein)/salt] was defined to maintain a stable liquid suspension at room temperature. Afterward, this formulation was analyzed

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using standard instrumental techniques to evaluate functional attributes, including power law constants and viscoelastic properties. In earlier reports, Resch et al. (*2*) identified the cold gelling characteristics associated with mWPC solutions; therefore, a storage study was performed with mWPC–Ca^{2+} dispersions, held at 4 °C, to monitor changes in viscoelasticity over time. Also, the general effects of heating and cooling on this system were investigated.

Previously, Resch et al. observed an ~8-fold enhancement in the water-holding capacity of mWPC dispersions compared to those prepared with commercial WPC ingredients (*2*). Because the properties of water molecules can play such an important role in protein solution chemistry, the potential effects of supplemental calcium chloride on the water-binding properties of mWPC–Ca^{2+} dispersions were addressed as well. Ultimately, the results obtained from these studies may provide new insight for designing improved whey protein ingredients with expanded functionality for their use within the food industry.

MATERIALS AND METHODS

Materials. Both the mWPC and commercial ULTRA 8000 (WPC) used for these studies were prepared by Grande Cheese Incorporated (Lomira, WI), according to published procedures, with slight modification (*1*,*4*). In this work, the heated whey protein solutions were maintained at 80 °C for 90 min. Calcium chloride was purchased from Fisher Scientific (Fair Lawn, New Jersey). All other reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Rheological Instrumentation. A StressTech controlled stress rheometer (ATS Rheosystems, Bordentown, NJ) equipped with a 25 mm serrated couette assembly was used for all tests. Prior to analysis, each sample was covered with a thin layer of mineral oil to minimize dehydration. Most dispersions were presheared for 30 at 15 s^{-1} to establish a baseline shear history, unless indicated otherwise.

Preparation of Whey Protein Solutions. Protein solutions were hydrated with distilled water to a final concentration of 3, 5.6, or 7% (w/v) using ULTRA 8000 (WPC) or the mWPC ingredient for 1–2 h at room temperature prior to adding supplemental calcium chloride to a final concentration of 0, 25, 50, 75, or 100 mM, if required. The pH was adjusted to ~3.5 with phosphoric acid, if necessary. In some experiments, the pH was raised to pH 6.5 using 1 N sodium hydroxide. All hydrated test fractions were stirred, again, for a minimum of 3 h at room temperature and then stored overnight at 4 °C prior to analysis unless otherwise noted.

Apparent Viscosity Measurements. The apparent viscosity of mWPC, mWPC-Ca^{2+}, and control WPC solutions was determined at 25 °C. All test samples were presheared at 15 s^{-1} for 30 s prior to measurement. Duplicate samples were subjected to a shear rate ranging from 0.1 to 500/s over 900 s. The apparent viscosity data were modeled according to the power law equation (*17*)

$$\eta = K\dot{\gamma}^{n-1}$$

Dynamic Testing. After the linear viscoelastic regions were determined, mWPC dispersions and mWPC dispersions containing 100 mM calcium chloride (5.6% protein, pH 3.5) were analyzed at a stress setting of 0.7 Pa as the frequency was increased from 0.01 to 1 Hz. All test samples were presheared at 15 s^{-1} for 30 s prior to measurement. Under these experimental conditions, the storage (G') modulus and loss (G'') modulus were recorded at both 25 and 4 °C.

Rheological Measurements of Calcium Supplemented mWPC during Cold Storage. To simulate cold storage conditions, control mWPC (5.6% protein, pH 3.5) and calcium-supplemented mWPC protein solutions (5.6% protein, 100 mM $CaCl_2$, pH 3.5) were prepared as previously described, loaded into the rheometer at a stress setting of 1 Pa with a frequency of 0.1 Hz, presheared at 10 s^{-1} for 1 min, and maintained under refrigeration temperatures (4 °C) for a period of 14 h.

Heating and Cooling Temperature Ramps. The mWPC dispersions were prepared at 5.6% protein (w/v) in deionized water, in the presence and absence of 100 mM calcium chloride, and stirred for several hours at room temperature with extended hydration overnight at 4 °C. All test solutions were analyzed at a stress level of 1 Pa and a frequency of 0.1 Hz and presheared at 15 s^{-1} for 30 s prior to measurement. Heating and cooling cycles were accomplished according to the following regime: (1) heat treatment from 25 to 80 °C, (2) holding at 80 °C for 5 min, (3) cooling to 4 °C and holding for 30 min, and then (4) reheating to 25 °C. Heating and cooling rates were set at 1 °C/min.

Electrophoresis Methodology. All WPC and mWPC protein samples were appropriately diluted with distilled water, and mixed 1:1 (v/v) with 8% sodium dodecyl sulfate (SDS) and 0.9 M Tris-Tricine sample buffer (InVitrogen, Inc., Carlsbad, CA). In identified cases, β-mercaptoethanol was added to 5% in the sample buffer. All samples were then heated at 100 °C for 10 min prior to loading onto 10–20% Tris-Tricine-gradient polyacrylamide gels (InVitrogen, Inc.). After electrophoresis, the samples were stained directly for visualization of protein bands using a colloidal Coomassie Blue staining reagent (InVitrogen, Inc.).

Water-Holding Capacity. The water-holding capacity was determined according to the method described by Resch et al. (*2*).

Measurement of Bound Water Using Differential Scanning Calorimetry (DSC). The percentage of bound or unfreezeable water in mWPC test samples at pH 3.5, containing 8% solids (5.6% protein) and prepared in the presence and absence of supplemental calcium chloride (100 mM), were measured with a DSC7 differential scanning calorimeter (Perkin-Elmer Instruments, LLC, Norwalk, CT). The DSC was calibrated with indium (melting onset temperature = 156.6 °C; enthalpy = 28.45 J/g) and mercury (melting onset temperature = −38.8 °C). The reference, an empty stainless-steel pan, weighed within 0.3 mg of the sample pan. Nitrogen, the purging gas, was maintained at a constant flow rate of 30 cc/min.

The stainless-steel calorimeter pans were filled with mWPC test fractions (~60 mg), sealed with a press, and loaded into the DSC. The dispersions were then cooled to −50 °C at 10 °C/min, held for 5 min at −50 °C, and heated to 30 °C at 5 °C/min. Deionized water (~60 mg) was also analyzed in this manner. The heat of fusion for water was calculated using the peak analysis function of the Pyris software program (version 5.0, Perkin-Elmer Instruments, LLC, Norwalk, CT) and found to be within 1% of the known value (ΔH = 335 J/g).

The phase transition of water in the mWPC sample during heating was recorded as the endothermic peak. The fraction of the freezable and unfreezable water in the sample was determined using the following equation (*18*), which assumes that the heat of fusion of free water in the mWPC sample was the same as that for the distilled water/ice:

$$X_{BW} = X_{TW} - \left(\frac{Q_{endo}}{Q_f}\right) \quad (2)$$

For these calculations, X_{TW} represents the total water fraction in mWPC, X_{BW} represents the bound water, Q_{endo} represents the heat of fusion for freezable water as obtained from the DSC thermogram (J/g), and Q_f represents the heat of fusion measured for distilled water. X_{TW} was estimated by assuming that the total amount of water in each dispersion was 92% of the sample weight (100–8% solids).

Fixation of mWPC Samples for Scanning Electron Microscopy. Experimental mWPC dispersions were prepared at 5.6% protein (w/v) in the presence and absence of 100 mM calcium chloride at pH 3.5. In all cases, ~0.5 mL of each sample was placed in a 78 μm microporous capsule (Structure Probe, Inc., West Chester, PA). The capsule was left in 0.1 M sodium acetate buffer at pH 3.5 containing 2 mL of cold 3% glutaraldehyde for 24 h. After 24 h, the capsule was transferred to a Petri dish containing 0.1 M sodium acetate buffer. The whey sample formed a solid mass that was cut into 2–3 mm^3 pieces, washed 3 times with 0.1 M sodium acetate buffer for 20 min at 4 °C, and dehydrated using an ethanol series of 30, 50, 70, 95, and 100% (20 min each) at room temperature. The final dehydration step was accomplished in 100% ethanol for 20 min at 4 °C. Critical point drying was achieved with a Samdri-795 dryer (Tousimis, Rockville, MD). Afterward, a 25–30 nm coating of gold/palladium was applied onto each test sample using an Anatech Hummer 6.2 sputter coater (Anatech Ltd., Denver, NC).

Table 1. Viscoelastic Properties of Whey Protein Dispersions, WPC at pH 6.5 versus mWPC at pH 3.5, Prepared in the Presence and Absence of Supplemental $CaCl_2$[a]

sample 25 °C	0 mM $CaCl_2$	25 mM $CaCl_2$	50 mM $CaCl_2$	75 mM $CaCl_2$	100 mM $CaCl_2$
3%WPC pH 6.5	$K = 0.0023$ $n = 0.98$ $r^2 = 0.920$	$K = 0.0023$ $n = 0.98$ $r^2 = 0.920$	$K = 0.0023$ $n = 0.98$ $r^2 = 0.920$	$K = 0.0023$ $n = 0.98$ $r^2 = 0.920$	$K = 0.0023$ $n = 0.98$ $r^2 = 0.920$
5.6% WPC pH 6.5	$K = 0.0051$ $n = 0.86$ $r^2 = 0.996$	$K = 0.0044$ $n = 0.88$ $r^2 = 0.995$	$K = 0.0041$ $n = 0.89$ $r^2 = 0.997$	$K = 0.0042$ $n = 0.89$ $r^2 = 0.997$	$K = 0.0044$ $n = 0.87$ $r^2 = 0.996$
7.0% WPC pH 6.5	$K = 0.0051$ $n = 0.87$ $r^2 = 0.987$	$K = 0.0043$ $n = 0.88$ $r^2 = 0.985$	$K = 0.0042$ $n = 0.90$ $r^2 = 0.988$	$K = 0.0039$ $n = 0.92$ $r^2 = 0.990$	$K = 0.0052$ $n = 0.86$ $r^2 = 0.985$
3% mWPC pH 3.5	$K = 0.0167$ $n = 0.86$ $r^2 = 0.999$	$K = 0.0183$ $n = 0.86$ $r^2 = 0.999$	$K = 0.0117$ $n = 0.88$ $r^2 = 0.998$	$K = 0.0054$ $n = 0.87$ $r^2 = 0.986$	$K = 0.0045$ $n = 0.86$ $r^2 = 0.978$
5.6% mWPC pH 3.5	$K = 0.1799$ $n = 0.75$ $r^2 = 0.999$	$K = 1.1174$ $n = 0.47$ $r^2 = 0.993$	$K = 6.004$ $n = 0.26$ $r^2 = 0.991$	$K = 14.584$ $n = 0.17$ $r^2 = 0.954$	$K = 10.545$ $n = 0.14$ $r^2 = 0.873$
7% mWPC pH 3.5	$K = 28.508$ $n = 0.24$ $r^2 = 0.968$	$K = 55.743$ $n = 0.07$ $r^2 = 0.578$	$K = 57.22$ $n = 0.05$ $r^2 = 0.392$	$K = 13.608$ $n = 0.20$ $r^2 = 0.359$	$K = 22.699$ $n = 0.09$ $r^2 = 0.237$

[a] Whey protein dispersions were prepared at the indicated protein concentrations using a commercial WPC at pH 6.5 and a modified WPC (mWPC) at pH 3.5. Supplemental calcium chloride was added as specified. On the basis of a log plot of shear stress versus shear rate, the K value (Pa s^n) or consistency coefficient was calculated as the y intercept while the n value (dimensionless) or flow index behavior was determined from the slope. The correlation coefficient is reflected by r^2.

Table 2. Effect of Heat and pH on the Viscoelastic Properties of Whey Protein Dispersions Containing 5.6% Protein and Tested in the Presence and Absence of Supplemental $CaCl_2$[a]

test sample	0 mM $CaCl_2$	25 mM $CaCl_2$	50 mM $CaCl_2$	75 mM $CaCl_2$	100 mM $CaCl_2$
5.6% WPC pH 6.5	$K = 0.0051$ $n = 0.86$ $r^2 = 0.996$	$K = 0.0044$ $n = 0.88$ $r^2 = 0.995$	$K = 0.0041$ $n = 0.89$ $r^2 = 0.997$	$K = 0.0042$ $n = 0.89$ $r^2 = 0.997$	$K = 0.0044$ $n = 0.87$ $r^2 =0.993$
5.6% WPC, ~80 °C 1.5 h, pH 6.5 effect of heat	$K = 1.623$ $n = 0.37$ $r^2 = 0.993$	$K = 3.465$ $n = 0.28$ $r^2 = 0.954$	$K = 3.296$ $n = 0.34$ $r^2 = 0.995$	$K = 3.30$ $n = 0.34$ $r^2 = 0.997$	$K = 2.696$ $n = 0.36$ $r^2 = 0.993$
5.6% WPC pH 3.5 effect of pH	$K = 0.0967$ $n = 0.75$ $r^2 = 0.999$	$K = 0.0986$ $n = 0.74$ $r^2 = 0.999$	$K = 0.0989$ $n = 0.74$ $r^2 = 0.999$	$K = 0.0963$ $n = 0.75$ $r^2 = 0.999$	$K = 0.0998$ $n = 0.74$ $r^2 = 0.999$
5.6% mWPC pH 3.5	$K = 0.1799$ $n = 0.75$ $r^2 = 0.999$	$K = 1.1174$ $n = 0.47$ $r^2 = 0.993$	$K = 6.004$ $n = 0.26$ $r^2 = 0.991$	$K = 14.584$ $n = 0.17$ $r^2 = 0.954$	$K = 10.545$ $n = 0.14$ $r^2 = 0.873$

[a] Whey protein dispersions were prepared at 5.6% protein concentrations using a commercial WPC at pH 6.5 and a modified WPC (mWPC) at pH 3.5. The addition of supplemental calcium chloride was added as specified. On the basis of a log plot of shear stress versus shear rate, the K value (Pa s^n) or consistency coefficient was calculated as the y intercept, while the n value (dimensionless) or flow index behavior was determined from the slope. The correlation coefficient is reflected by r^2.

RESULTS AND DISCUSSION

Effect of pH Adjustment and Heat Treatment on the Viscoelastic Properties of Commercial WPC versus mWPC Dispersions in the Absence of Supplemental Calcium. To compare the viscoelastic properties of WPC at pH 6.5 versus mWPC dispersions at pH 3.5, the consistency coefficient, K, and flow behavior index, n, were determined. Instrumental readings for K and n power law parameters were calculated as the y intercept and slope, respectively, obtained from a logarithmic plot of shear stress versus shear rate (*17*). Other solution parameters, such as viscosity, for example, simply report instrumental readings collected at a single shearing speed and may not be representative of the total changes that occur.

The flow characteristics of mWPC versus commercial WPC protein dispersions differed significantly, even in the absence of supplemental calcium. In mWPC samples, the K value was amplified with an increasing protein concentration, while the flow behavior indexes (n) were lowered (**Table 1**). In contrast, these parameters were only slightly affected using equivalent amounts of protein for WPC dispersions. Notably, the K readings for mWPC dispersions (5.6% protein, pH 3.5) were considerably higher than equivalent WPC protein solutions at pH 6.5, likely caused, at least in part, by the additional heating steps used in the manufacture of the mWPC ingredient itself.

The effects of heating, alone, were examined when WPC dispersions (5.6% protein) at pH 6.5 were thermally treated at ~80 °C for 90 min, a slight modification from the original method (*2*). Upon cooling to ambient temperatures, the consistency coefficient exhibited a significant fold enhancement compared to nonheated controls prepared at equivalent pH values (**Table 2**).

The effect of simply dropping the pH of commercial WPC protein solutions (5.6%) from pH 6.5 to 3.5 caused an ~20-fold increase in the consistency coefficient (**Table 2**). Also, the apparent viscosity of 5.6% mWPC protein solutions at pH 3.5 measured ~69 mPa s compared to readings of 2.8 mPa s at 50 s^{-1} for control WPC samples at pH 6.5 (**Figure 1**). Rattray and Jelen and Jelen et al. made a similar observation when they

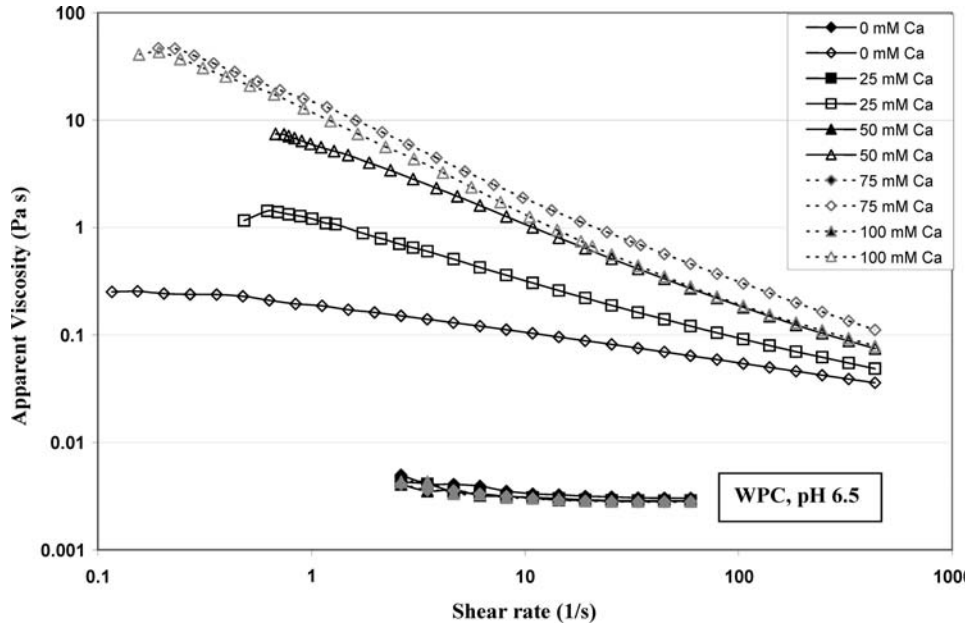

Figure 1. Effect of $CaCl_2$ on the apparent viscosity of commercial WPC versus modified whey protein concentratre (mWPC) dispersions. WPC protein solutions [closed symbols, 5.6% protein (*w/v*), pH 6.5] and mWPC dispersions [open symbols, 5.6% protein (*w/v*), pH 3.5] were prepared at 25 °C in the presence and absence of supplemental $CaCl_2$. The apparent viscosity was measured at 25 °C over the shear rates indicated.

demonstrated that the viscosity of WPC dispersions, prepared at pH values less than 4.0, were significantly higher than those prepared at pH 6.8 (*10, 19*). In addition, mWPC dispersions exhibited shear thinning behavior evidenced by decreasing viscosity with increasing shear rate, a pattern not seen in equivalent, nonheated, whey protein dispersions prepared with commercial WPC products (**Figure 1**).

Effect of Salt Addition on WPC versus mWPC Dispersions. In earlier work, Hudson et al. examined the effect of sodium chloride on several functional parameters of modified whey protein isolates (mWPI); however, in their experiments, the protein concentrations were significantly higher, ~10%, while the sodium chloride content ranged from 0 to 50 mM (*1*). Under these reaction conditions, a fine-stranded gel was formed, exhibiting increased strain and decreased stress at fracture, which correlated with lower amounts of the monovalent salt.

The effect of divalent cations on heated whey protein suspensions has also been well-documented and reportedly causes even more dramatic changes with respect to certain functional attributes (*5–7, 9, 10, 12–15*). In one example, Kuhn and Foegeding described a generalized effect of divalent cations with respect to increased shear stresses and shear strains at failure using WPI gels containing supplemental calcium chloride, magnesium chloride, or barium chloride compared to those changes brought about by the inclusion of monovalent salts, such as Na, Li, K, Rb, and Cs (*5*). Their work clearly demonstrated that the addition of divalent salts to heated whey protein solutions promoted the formation of molecular cross-links, resulting in an increased firmness of the gel.

Hence, the next series of experiments were designed to characterize the rheological changes elicited by the addition of calcium salts over a protein concentration ranging from 3 to 7% (w/v), while supplemental calcium chloride concentrations varied between 0 and 100 mM. A summary of these results is presented in **Table 1** and **Figure 1**.

Calcium effects were mostly negligible in all WPC dispersions prepared at pH 6.5, in which case thermal exposure was limited to the pasteurization process itself (**Figure 1**). Furthermore, additional heating (~80 °C, 90 min) produced a ~320-fold rise in the consistency coefficient of these dispersions (pH 6.5) compared to nonheated control solutions. The added impact of 25 mM supplemental calcium increased this *K* value only 2× more and remained relatively constant over the entire range of salt tested (0–100 mM; **Table 2**). By comparison, the *K* readings for equivalent mWPC samples (5.6% protein) at pH 3.5 were ~35-fold higher than comparable WPC solutions at pH 6.5; however, the impact of calcium addition was much more dramatic over the entire concentration range (0–100 mM). In fact, 5.6% mWPC solutions containing added 75 mM calcium exhibited a ~3500-fold increase in the *K* reading compared to analogous whey protein samples at pH 6.5. Moreover, an ~80-fold rise was observed in mWPC dispersions (5.6% protein) at pH 3.5 that were supplemented with 75 mM calcium salt compared to the mWPC control. Overall, the viscosity of mWPC dispersions at pH 3.5 was higher than equivalently prepared WPC solutions at pH 6.5, and the additional effects of supplemental calcium were even still more evident (**Figure 1**). Equivalent experiments were also performed using sodium chloride (NaCl) added to the same ionic strength. Although the apparent viscosity was increased, the data trends were not identical to those observed with mWPC–Ca^{2+} dispersions (data not shown). Thus, these patterns were not solely causes by the effects of increased ionic strength.

Previously, Sherwin and Foegeding (*7*) noted that the impact of calcium on the aggregation of whey protein isolates occurred when the ratio of $CaCl_2$ (mM) to protein (%, w/v) fell between 3.3 and 23.3. In a similar manner, the values obtained during these experiments ranged from ~4 to 18 using mWPC dispersions containing 5.6% protein and 25–100 mM $CaCl_2$. Similarily, Ju and Kilara examined turbidity changes upon varying the amount of calcium chloride added to whey protein solutions (w/w) and also observed that the highest aggregational state was achieved at optimal ratios (*12*). Likewise, these same investigators reported that specific proportions of protein/calcium impacted gel formation and gel hardness using WPI solutions (*13*). When these findings are taken together, they may be explained, at least in part, on the basis of thermal denaturation

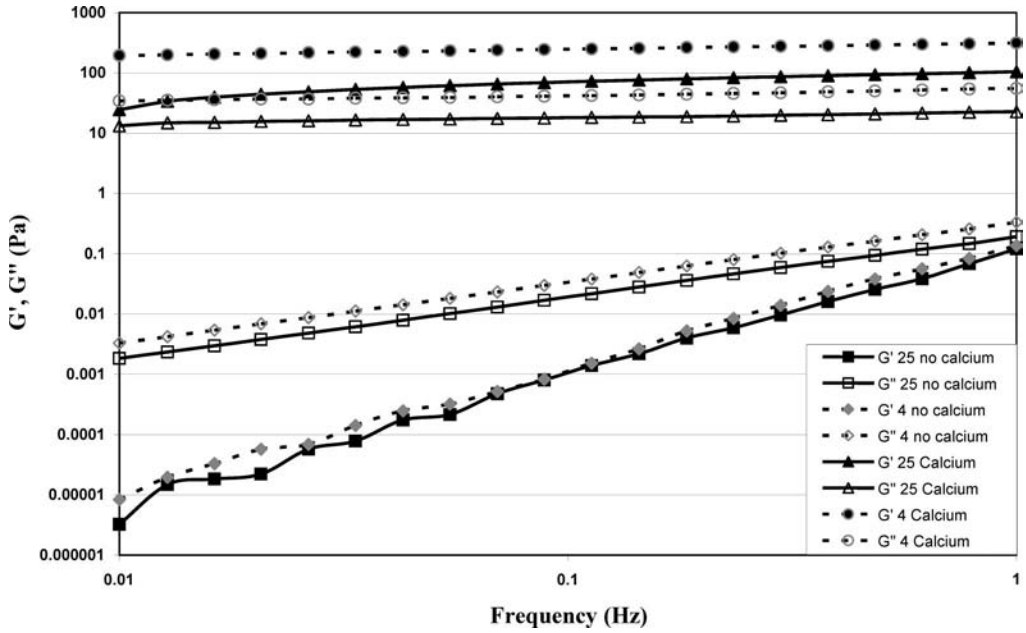

Figure 2. Effect of supplemental calcium chloride (100 mM $CaCl_2$) on the storage (G') and loss (G'') modulus of modified whey protein concentrate dispersions [mWPC, 5.6% protein (*w/v*), pH 3.5] at 4 and 25 °C.

of the whey protein constituents, a process that may cause increased exposure of partially buried aspartic/glutamic acid residues, promoting an enhanced calcium-binding capacity, especially with respect to β-lactoglobulin (*20*).

Not surprisingly, mWPC dispersions manifested the classical rheological characteristics of a thickened fluid; in that, data curves revealed a zero shear plateau at low shear rates and exhibited shear thinning behavior at moderate speeds (**Figure 1**). This trend was observed in all mWPC test samples containing 5.6% protein, plus and minus supplemental calcium, at 25 and 4 °C (data not shown for 4 °C).

At lower protein concentrations (3%), a phase separation occurred in dispersions prepared with either the WPC or mWPC ingredient, attributed to the fact that the amount of protein was below the critical concentration required for network formation. At higher amounts, such as 7% protein, the r^2 values (mWPC) did not evidence a linear instrumental response to increasing shear rates, likely caused by the dramatic thickening of the solution itself. In fact, upon the addition of 25 mM calcium salts, there was a significant deviation from linearity, $r^2 = 0.578$, a finding that could be attributed to increased electrostatic repulsion caused by a higher protein content. In contrast, test dispersions prepared with commercial WPC powders to 7% protein at pH 6.5 were extremely fluid even in the presence of calcium.

Effect of Calcium Addition on Rheological Attributes. In early experiments, it was observed that 5.6% protein solutions, containing a minimum of 75 mM calcium chloride, afforded maximal viscosity with optimal solution stability. In fact, inclusion of 100 mM calcium ion resulted in a highly thickened dispersion that was very resistant to flow even at ambient temperatures (**Table 1** and **Figure 1**). When these dispersions were refrigerated overnight at ~4 °C, a significant hardening of the gel was noted; therefore, mWPC dispersions, prepared at 5.6% protein, with 100 mM added calcium chloride were selected for further study because a primary goal for this work was focused on developing a cold gelling agent.

A series of small strain oscillatory experiments were performed to evaluate the response of this test system to varying harmonic oscillations caused by mechanically imparted stress. The data were then used to evaluate the elasticity and/or complex viscosity of mWPC test dispersions over a range of different frequencies. As illustrated in **Figure 2**, protein dispersions, devoid of added salt, exhibited more "liquid-like" characteristics evidenced by the fact that G'' (loss modulus) dominated G' (storage modulus) at both 4 and 25 °C. Furthermore, [$G'_{4\ °C}$ and $G''_{4\ °C}$] moduli data were slightly elevated compared to those values observed at 25 °C, indicative of increased thickening at colder temperature settings even in the absence of supplemental calcium. However, upon inclusion of 100 mM $CaCl_2$ into the system, a predominant "gel-like" characteristic of the mWPC protein solution was observed, in which case $G' > G''$ at both 25 and 4 °C. Although mWPC dispersions containing 100 mM calcium chloride were essentially gelled at room temperature, the gel strength was significantly higher (~10×) for chilled samples. The differences between the (G') values of mWPC–Ca^{2+} dispersions compared to mWPC control solutions lacking additional calcium were especially notable at low shear rates (**Figure 2**).

Effect of Supplemental Calcium on Apparent Viscosity during Long-Term Storage at 4 °C. One of the more striking results revealed that the gel strength, reflected by G' readings of mWPC–Ca^{2+} dispersions at pH 3.5, was dramatically higher than that of equivalent control solutions, especially at 4 °C (**Figure 3**). Likely, this finding can be at least partially attributed to the exposure of protein constituents to an acidic solution environment coupled with thermal unfolding of globular whey proteins, such as β-lactoglobulin, which occurred during processing (*2*). At these pH values, the addition of calcium chloride may reduce electrostatic repulsion as a result of charge neutralization effects, attributable to both ionic species. Concomitantly, hydrophobic forces may play a more minor role at lower temperatures, which in turn, could decrease internal attraction forces, resulting in the swelling of the protein aggregates and increased gel strength. Ultimately, it is this combination of multiple molecular interactions that results in network formation creating a more "solid-like" protein matrix especially under refrigeration temperatures.

Because the increased thickness of the calcium-supplemented mWPC sample was so pronounced at lower temperatures, these

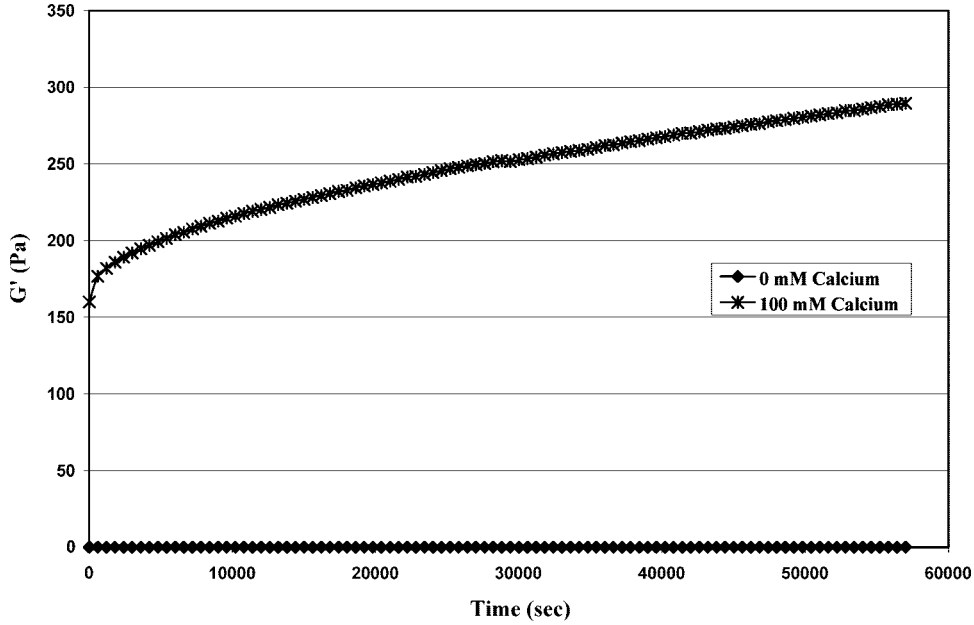

Figure 3. Gel strengthening at 4 °C during storage of modified whey protein concentrate dispersions [mWPC, 5.6% protein (*w/v*), pH 3.5] containing supplemental $CaCl_2$ (100 mM) as measured by an increased storage modulus (*G*′).

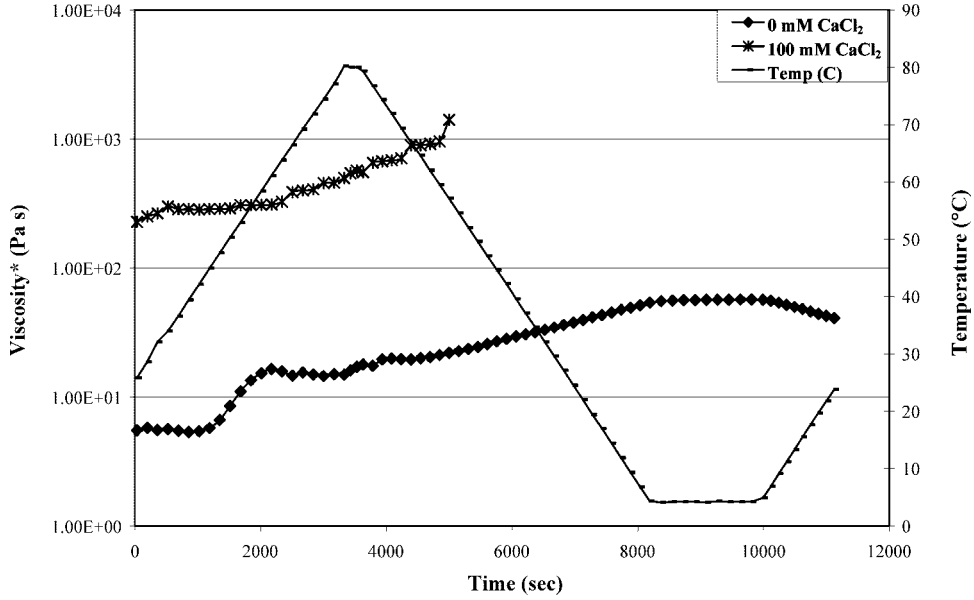

Figure 4. Effect of heating and cooling on the complex viscosity of modified whey protein concentrate dispersions [mWPC, 5.6% protein (*w/v*), pH 3.5] prepared in the presence and absence of supplemental $CaCl_2$ (100 mM). Heating and cooling cycles were performed according to the following regime: (1) heat treatment from 25 to 80 °C, (2) holding at 80 °C for 5 min, (3) cooling and holding at 4 °C for 30 min, then (4) reheating to 25 °C. Heating and cooling rates were accomplished at 1 °C/min.

changes were also investigated during long-term cold storage of the dispersion (~4 °C). Maintenance of the protein solution under refrigeration temperatures showed a steady climb in the viscosity over a 14 h interval time (**Figure 3**). Apparently, the strength of the gelling network continued to progress, albeit at a slow rate, upon holding at 4 °C, suggesting that the elastic rigidity of the protein/salt suspension was enhanced during cold storage.

Effects of Heating and Cooling on the Rheological Attributes of mWPC Protein Dispersions. The effect of heating and cooling on these systems was examined, and the data revealed that the complex viscosity was slightly increased in control mWPC samples (5.6% protein) lacking supplemental Ca^{2+}, upon heating from ~47 to 61 °C (**Figure 4**). Furthermore, these values continued to climb, albeit at a slower rate, for the remainder of the cycle. This rise might be attributed to additional unfolding and further denaturation of mWPC starting materials, resulting in a larger hydrodynamic radius for various protein components. In addition, hydrophobic associations may be enhanced under these experimental conditions.

Added Effects of Calcium Salt on Heating and Cooling on the Complex Viscosity Profile. The addition of calcium into this system had a significant effect, especially during the heating phase (step 1 in **Figure 4**). Initially, the complex viscosity was much higher in mWPC–Ca^{2+} dispersions as noted before. Also, the viscosity rose during the heating cycle, although the temperature at which this rise occurred was significantly higher than in those dispersions lacking additional calcium (~72 versus 47 °C). With continued thermal exposure to ~75–80 °C, followed by subsequent cooling, the viscosity

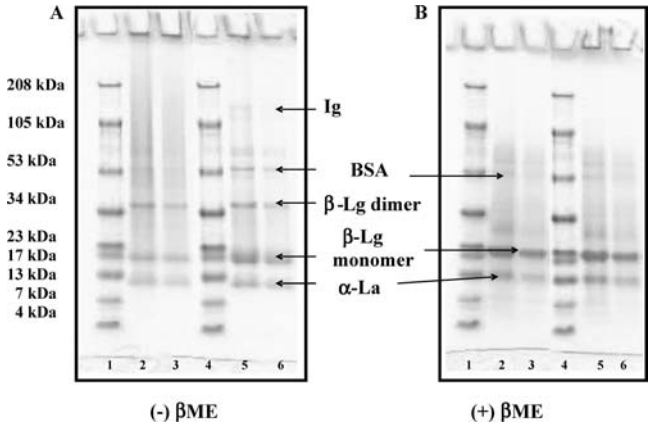

Figure 5. SDS–PAGE banding profile of WPC, pH 6.5, and mWPC protein dispersions pH 3.5, prepared at 5.6% protein (*w/v*) in the absence (A) and presence (B) of β-mercaptoethanol. Panel A: Lane [1]: Marker, [2]: mWPC, 40 μg, [3] mWPC, 30 μg, [4] Marker, [5] WPC, 40 μg, [6] WPC, 30 μg. The samples seen in panel B were loaded in an identical manner.

continued to increase; however, upon removal of the sample from the cup, a water layer appeared to have formed on the top of the sample, attributed to a phase separation. Ultimately, such changes prevented a collection of reliable rheological instrumental data during cycles 3 and 4 (data not shown). Apparently, mWPC–Ca^{2+} dispersions were less stable to these heating/cooling regimens compared to mWPC dispersions, such that syneresis occurred.

Protein Banding Profile of WPC versus mWPC Whey Protein Dispersions (SDS–PAGE). Because the rheological profile of mWPC dispersions differed so significantly from commercial WPC protein solutions, the SDS–PAGE banding patterns of the two were investigated (**Figure 5**). The manufacturing process used for preparing either of the dried powders involved high-temperature short-time (HTST) pasteurization of raw milk, followed by a second HTST pasteurization of the whey, itself (Grande cheese, personal communication). However, mWPC was further heated after a pH adjustment to ~3.35 (*1, 4*). On the basis of the electropherograms, obtained under reducing and nonreducing conditions, the results revealed that protein aggregation and disulfide bond formation likely occurred during the manufacture of the mWPC ingredient. These effects were especially notable with respect to β-lactoglobulin (β-Lg), bovine serum albumin (BSA), and the immunoglobulin (Ig) fraction. In the absence of βME, the staining intensity of monomeric β-Lg was somewhat diminished in mWPC samples compared to those prepared with commercial WPC powders, while BSA and Ig bands were also much less distinct. Instead, these banding patterns evidenced a broad "smearing configuration", likely indicative of the formation of higher molecular-weight aggregates, some of which were likely disulfide-linked, including β-Lg polymers, BSA aggregates, and/or hybrids: β-Lg–BSA, BSA–Ig, β-Lg–Ig, and so forth, all of which migrated with increased molecular size.

Water-Holding Capacity. Whey proteins can undergo irreversible denaturation upon heating, and oftentimes, gel formation occurs. If the gel is later dried, the protein displays increased water-holding capacity (WHC) attributable to enhanced capillary action within the insoluble protein network (*21*). In previous work, the WHC of the mWPC ingredient was determined by Resch et al. using centrifugal methods (*2*), who reported that 1 g of a mWPC powder held ~8 g of water. Herein, a similar finding was made, although the water-holding

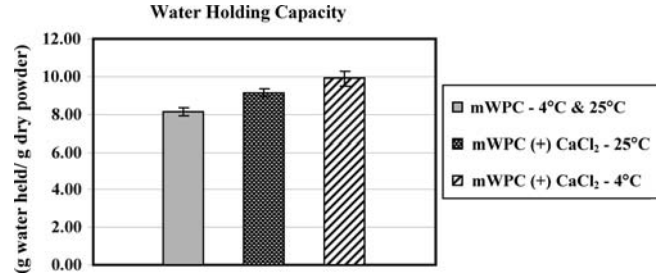

Figure 6. Changes in the water holding capacity of modified whey protein concentrate dispersions [mWPC, 5.6% protein (*w/v*), pH 3.5] upon addition of supplemental $CaCl_2$ (100 mM) at 25 and 4 °C.

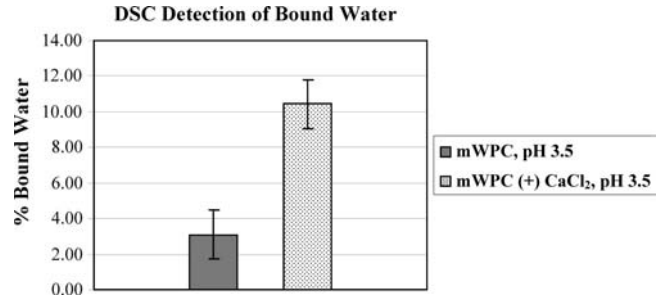

Figure 7. Differential scanning calorimetry analysis of non-freezable (bound) water in modified whey protein concentrate dispersions [mWPC, 5.6% protein (*w/v*), pH 3.5] prepared in the presence and absence of supplemental $CaCl_2$ (100 mM).

capacity of mWPC dispersions containing added calcium was even slightly higher, especially at 4 °C (**Figure 6**). In a similar manner, Barbut and Foegeding demonstrated that salt-induced WPI gels exhibited an improved water-holding capacity (*6*), while Hongsprabhas and Barbut also reported an increased WHC, especially at lower temperatures, using whey protein–calcium test systems (*22*).

DSC: Bound or Nonfreezable Water. Bound water, defined as nonfreezable water at −40 °C, exhibits different properties from that of "bulk" water molecules (*23*). It is often detected by DSC (*24*), in which case the fusion of ice in the sample is measured as an endothermic peak and the area under the curve is assumed to be proportional to the amount of freezable water. Others have reported that such measurements are independent of the initial moisture content if calculated from enthalpy values (*25*). Consequently, in these experiments, the amount of nonfreezable water was calculated as a percentage of the total water in the sample.

Herein, we observed that the amount of bound water in mWPC–Ca^{2+} samples was at least 2 times higher than the amount detected in mWPC dispersions (**Figure 7**). Presumably, calcium ions hold water more tightly, resulting in a decreased mobility of bound water molecules, an attribute that could be due to the strength of the electric field that was generated (*26*).

Scanning Electron Microscopy. Because calcium ions bind water more tightly, these effects can increase hydrogen bonding between surrounding water molecules. In turn, this type of solution configuration may represent an unfavorable thermodynamic state attributable to decreased entropy within the system. Therefore, to minimize such unstable molecular interactions, hydrophobic groups quite often associate. Harwalker and Kalab found that whey protein solutions prepared at low pH and high ionic strength lowered the ionic forces and enhanced hydrophobic interactions (*27*). Also, Shimizu et al. noted that the relative hydrophobicity of β-lactoglobulin, a major constituent of whey protein fractions, was increased ~200-fold simply by adjusting the pH from 7.0 to 3.0 (*28*).

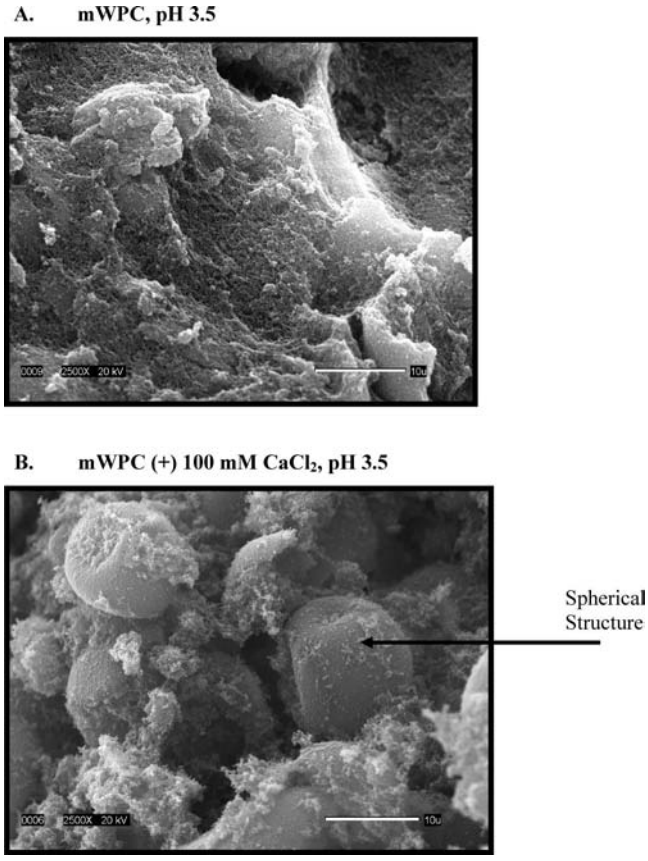

Figure 8. Scanning electron micrographs of modified whey protein concentrate dispersions [mWPC, 5.6% (*w/v*), pH 3.5] prepared in the absence (A) and presence (B) of supplemental $CaCl_2$ (100 mM). The magnification is 2500×.

As shown in **Figure 8**, scanning electron micrographs clearly depicted whey protein aggregates, which appeared as large, rounded, "ball-like" structures in mWPC–Ca^{2+} dispersions. Perhaps, this particular configuration was created as a result of hydrophobic effects, which minimized surface contact between neighboring water molecules associated with protein hydrophobic side chains, leading to a more thermodynamically stable state. Also, in one previous report, Ohki and Zschornig suggested that hydrophobic associations could be promoted as a result of the increased surface tensions caused by the addition of divalent salts (*29*).

Ju and Kilara showed that the size of the aggregate formed during calcium-induced gel formation of whey protein isolates was larger than those of noncalcium-supplemented samples and ascribed these findings to hydration effects caused by the increased salt content (*12*). In a separate paper, Britten and Giroux suggested that spherical polymers prepared with whey protein solutions containing 4 mM calcium at pH 6.5 appeared to be extensively aggregated and formed softer gels (*30*).

CONCLUSIONS

A process was developed for creating whey protein formulations that exhibited gel-like characteristics at both ambient and refrigeration temperatures. The amount of protein required to form such a network was somewhat less in the presence of 75 mM supplemental calcium, i.e., 5.6% mWPC protein, compared to the report of Tang et al., who suggested that a minimum of 6% protein was required to form a gelling matrix using commercial WPC powders (*31*).

Ultimately, formulations can be manipulated to yield whey protein ingredients that deliver specific functional attributes under well-defined processing conditions. Therefore, to design products that deliver consistent characteristics for specific food applications, the details regarding the raw materials, "preprocessing" and storage conditions, and numerous experimental variables, such as ionic strength, temperature, and pH, must be identified.

When these results are taken together, they established that the inclusion of calcium into protein solutions prepared with mWPC powders enhanced the functionality of the final whey protein dispersion, delivering superior performance characteristics when compared to other commercial whey-based ingredients on the market.

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LITERATURE CITED

(1) Hudson, H. M.; Daubert, C. R.; Foegeding, E. A. Rheological and physical properties of derivitized whey protein isolate powders. *J. Agric. Food Chem.* **2000**, *48*, 3112–3119.

(2) Resch, J. J.; Daubert, C. R. Rheological and physiochemical properties of derivatized whey protein concentrate powders. *Int. J. Food Prop.* **2002**, *5*, 419–434.

(3) Firebaugh, J. D.; Daubert, C. R. Emulsifying and foaming properties of a derivatized whey protein ingredient. *Int. J. Food Prop.* **2005**, *8*, 243–253.

(4) Hudson, H. M.; Daubert, C. R.; Foegeding, E. A. Thermal and pH stable protein thickening agent and method of making the same. U.S. Patent 6,261,624, 2001.

(5) Kuhn, P. R.; Foegeding, E. A. Mineral salt effects of whey protein gelation. *J. Agric. Food Chem.* **1991**, *39*, 1013–1016.

(6) Barbut, S.; Foegeding, E. A. Calcium-induced gelation of preheated whey protein isolate. *J. Food Sci.* **1993**, *58*, 867–871.

(7) Sherwin, C. P.; Foegeding, E. A. The effects of calcium chloride on aggregation of whey proteins. *Milchwissenschaft* **1997**, *52*, 93–96.

(8) Vardhanabhuti, B.; Foegeding, E. A.; McGuffey, M. K.; Daubert, C. R.; Swaisgood, H. E. Gelation properties of dispersions containing polymerized and native whey protein isolate. *Food Hydrocolloids* **2001**, *15*, 165–175.

(9) Caussin, F.; Famelart, M.; Maubois, J.; Bouhallab, S. Mineral modulation of thermal aggregation and gelation of whey proteins: From β-lactoglobulin model system to whey protein isolate. *Lait* **2003**, *83*, 1–12.

(10) Rattay, W.; Jelen, P. Viscous behavior of whey protein concentrate dispersions. *Int. Dairy J.* **1995**, *5*, 673–684.

(11) Tang, Q. N.; Munro, P. A.; McCarthy, O. J. Rheology of whey–protein concentrate solutions as a function of concentration, temperature, pH, and salt concentration. *J. Dairy Res.* **1993**, *60*, 349–361.

(12) Ju, Z. Y.; Kilara, A. Aggregation induced by calcium chloride and subsequent thermal gelation of whey protein isolate. *J. Dairy Sci.* **1998**, *81*, 925–931.

(13) Ju, Z. Y.; Kilara, A. Gelation of pH-aggregated whey protein isolate solution induced by heat, protease, calcium salt, and acidulant. *J. Agric. Food Chem.* **1998**, *46*, 1830–1835.

(14) Fujino, H.; Muguruma, M.; Ito, T.; Ohashi, T. Effects of pH, sodium chloride and calcium chloride on heat aggregation of whey protein concentrate and whey protein isolate. *Nippon Shokuhin*

Kagaku Kogaku Kaishi **1993**, *40*, 309–315.
(15) Fujino, H.; Muguruma, M.; Oniki, H.; Ito, T.; Ohashi, T. Role of calcium on heat aggregation of whey–protein isolate—Studies on available utilization of whey proteins. *Nippon Shokuhin Kagaku KogaKu Kaishi* **1995**, *42*, 769–775.
(16) Kinsella, J. E.; Whitehead, D. M.; Brady, J.; Bringe, N. A. Milk proteins: Possible relationships of structure and function. In *Development in Dairy Chemistry*; Fox, P. F., Ed.; Appl. Sci. Publ.: London, U.K., 1989; Chapter 4: Functional Milk Proteins, pp 53–93.
(17) Steffe, J. F.; Daubert, C. R. Rheological properties of biological fluids. In *Bioprocessing Pipelines: Rheology and Analysis*; Freeman Press: East Lansing, MI, 2006; p 5.
(18) Wang, T.; Gunasekaran, S. State of water in chitosan–PVA hydrogel. *J. Appl. Polym. Sci.* **2006**, *101*, 3227–3232.
(19) Jelen, P.; Hostin, S.; Jeschke, J.; Kerr, G. Effects of low temperature on viscosity and gelling properties of concentrated whey protein solutions. Proceedings International Dairy Federation, IDF Seminar, Munich, Germany, 1992.
(20) Pappas, C. P.; Rothwell, J. The effect of heating, alone or in the presence of calcium or lactose on calcium binding to milk proteins. *Food Chem.* **1991**, *42*, 183–201.
(21) Fennema, O. R. Amino acids, peptides, and proteins. In *Food Chemistry*, 2nd ed.; Tannenbaum, S. R., Walstra, P., Eds.; Marcel Dekker, Inc.: New York, 1985; p 286.
(22) Hongsprabhas, P.; Barbut, P. Effect of temperature on Ca^{2+}-induced gelation of whey protein isolate. *Lebensm.-Wiss. Technol.* **1997**, *30*, 45–49.
(23) Fennema, O. Water and ice. In *Food Chemistry*, 2nd ed.; Fennema O. R., Ed.; Marcel Dekker, Inc.: New York, 1985; p 37.
(24) Duckworth, R. B. Differential thermal analysis of frozen food systems. I. The determination of unfreezable water. *J. Food Technol.* **1971**, *6*, 317–327.
(25) Roos, Y. H. Phase transitions and unfreezable water content of carrots, reindeer meat and white bread studied using differential scanning calorimetry. *J. Food Sci.* **1986**, *51*, 684–687.
(26) Fennema, O. Water and ice. In *Food Chemistry*, 2nd ed.; Fennema, O. R., Ed.; Marcel Dekker, Inc.: New York, 1985; p 42.
(27) Harwalker, V.; Kalab, M. Thermal denaturation and aggregation of β-lactoglobulin at pH 2.5. Effect of ionic strength and protein concentration. *Milchwissenchaft* **1985**, *40*, 31–34.
(28) Shimizu, M.; Saito, M.; Yamauchi, K. Emulsifying and structural properties of β-lactoglobulin at different pHs. *Agric. Biol. Chem. (Tokyo)* **1985**, *49*, 189–194.
(29) Ohki, Z.; Zschornig, O. Ion-induced fusion of phosphatidic acid vesicles and correlation between surface hydrophobicity and membrane fusion. *Chem. Phys. Lipids* **1993**, *65*, 193–204.
(30) Britten, M.; Giroux, H. Acid-induced gelation of whey protein polymers: Effects of pH and calcium concentration during polymerization. *Food Hydrocolloids* **2001**, *15*, 609–617.
(31) Tang, Q.; McCarthy, O. J.; Munro, P. A. Oscillatory rheological study of the gelation mechanism of whey protein concentrate solutions: Effects of physiochemical variables on gel formation. *J. Dairy Res.* **1993**, *60*, 543–555.

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