Rheological Properties of Rennet Gels Containing Milk Protein Concentrates

M. A. Ferrer,*† A. R. Hill,† and M. Corredig†¹

*Department of Nutrition and Dietetics, University of Zulia, Maracaibo, Venezuela, 4005

†Department of Food Science, University of Guelph, Guelph, Ontario, N1G 2W1 Canada

ABSTRACT

Different milk protein concentrates (MPC), with protein concentrations of 56, 70, and 90%, were dispersed in water under different treatments (hydration, shear, heat, and overnight storage at 4°C), as well as in a combination of all the treatments in a factorial design. The particle size distribution of the dispersions was then measured to determine the optimal conditions for the dispersion. Heating at 60°C for 30 min with 5 min of shear was chosen as the best condition to dissolve MPC powders. The samples were also characterized for composition, presence of protein aggregates, and ratio of calcium to protein. The total calcium present in MPC increased with increasing concentration of protein; however, the total calcium-to-protein ratio was lower in MPC90 than in MPC56 and MPC70. The level of whey protein denaturation, the presence of κ -casein-whey protein aggregates in the supernatant after centrifugation, and the amount of caseins dissociated from the micelle increased as the protein concentration in the powder increased. The total amount of casein macropeptide released was lower in samples from powders with a higher protein concentration than for MPC56 or the skim milk control. The gelation behavior of reconstituted MPC was tested in systems dispersed in water (5% protein) as well as in systems dispersed in skim milk (6% protein). The gelation time of MPC dispersions was considerably lower and the gel modulus was higher than those of reconstituted skim milk with the same protein concentration. When MPC dispersions were dialyzed against skim milk, a significant decrease in the gelation time and modulus were shown, with a complete loss of gelling functionality in MPC90 dispersed in water. This demonstrated that the ionic equilibrium was key to the functionality of MPC.

Key words: milk protein concentrate, rennet gel, calcium equilibrium, casein

INTRODUCTION

Milk protein concentrates (MPC) have emerged over the past decade as a new category of functional ingredients and are often used in dairy formulations. Milk protein concentrates can be produced by ultrafiltration, microfiltration, diafiltration, and evaporation (Mistry and Hassan, 1991), followed by spray drying. Milk protein concentrates are usually identified by their percentage of protein content (e.g., MPC56). The use of MPC for yogurts (Guzmán-González et al., 1999), ice cream (Alvarez et al., 2005), and emulsions (Hemar et al., 2005) has been investigated, but their principal application is to increase cheese-making efficiency (Novak, 1996; Rehman et al., 2003; Zbikowska and Szafer, 2003).

Milk protein concentrate dispersions cannot be fully dissolved by stirring at 20°C because of the formation of disulfide-linked β -LG aggregates and CN complexes during processing and storage (Kameswaran and Smith, 1999; Castro-Morel and Harper, 2002, 2003; Anema et al., 2006; Havea, 2006). Kuo and Harper (2003a,b) reported that an increase in the dispersability of MPC85 improved the strength of rennet gels, and that rennet-induced gels made with MPC56 were stronger than those prepared with MPC85 tested at equivalent protein levels. Other researchers have studied the rheology of rennet gels made from reconstituted, standardized, or ultrafiltered milk (Caron et al., 1997; Pomprasirt et al., 1998; Waungana et al., 1999); however, how the changes in the CN micelle and its surroundings affect the technological properties of MPC is not fully understood.

The objective of this study was to determine the renneting behavior of selected MPC powders. For this reason, a full characterization of the MPC samples was also conducted to determine which factors most affect the renneting behavior of reconstituted MPC.

MATERIALS AND METHODS

Materials

Milk protein concentrates with protein concentrations of 56, 70, and 90% (MPC56, MPC70, and MPC90)

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¹Corresponding author: mcorredig@uoguelph.ca

Figure 1. Schematic of treatments applied during the dispersability test for milk protein concentrate.

were obtained from New Zealand Milk Proteins (Mississauga, Ontario, Canada). Instant low-heat skim milk powder (SM) was supplied by Parmalat (London, Ontario, Canada). Single-strength rennet (250 international milk-clotting units/mL) was supplied by Rhodia Inc. (Chymostar, Madison, WI). Analytical grade reagents were from Sigma-Aldrich Chemical Ltd. (St. Louis, MO). Ultrapure water (Milli-Q Ultrapure Water Purification Systems, Billerica, MA) was used to prepare all the solutions.

Characterization of MPC Powders

Dispersability. The MPC were dispersed in water (10% protein) at 25°C for 1 or 3 h. Sheared samples (20 mL) were additionally dispersed for 5 min by using a handheld homogenizer (Powergen 125, Fisher Scientific, Nepean, Canada). Some samples were heated at 60 or 40°C for 30 min in a water bath and then cooled in an ice bath to 20°C. Some samples were stored overnight at 4°C. The experimental design to determine the optimal conditions for dispersability of the MPC samples was a complete factorial, namely, 3 MPC samples (MPC56, MPC70, and MPC90), 3 applied temperatures (20, 40, or 60°C), and 2 levels (yes or no) for shear and overnight storage at 4°C (Figure 1). All samples were analyzed immediately after treatment for particle size. Particle size distribution was determined by integrated light scattering (Mastersizer 2000, Malvern Instruments, Southborough, MA). The volume-weighted mean particle size ($d_{4,3}$) was recorded. Sample drops were added into the small-dispersion unit to reach an obscuration level of 10 to 20 (dilution factor of approximately 10^{-3}) while stirring at 2,300 rpm. The refractive indexes used were 1.391 for the CN particles (Alexander et al., 2002) and 1.33 for water. Data were analyzed by ANOVA (SAS Version 8.2, SAS Institute Inc., Cary, NC). Differences were considered significant at $P < 0.05$.

Chemical Composition. Lactose was determined by the enzymatic method (AOAC, 1990; method 984.15) after TCA precipitation of proteins from the MPC. Total protein was measured by using the DC protein assay

(Bio-Rad, Mississauga, Canada). Total calcium and other ions in the powders, solutions, and centrifugal supernatants were measured by inductively coupled plasma-optical emission spectrometry (Anderson, 1996, 1999) at the Laboratory Services facilities of the University of Guelph. All analyses were duplicated.

Residual Native Whey Proteins. The amount of native whey proteins present in MPC and SM reconstituted at room temperature or at 60°C for 30 min was determined by acid-precipitation of the proteins and chromatographic analysis of the supernatants as described by others (Hoffmann et al., 1996; Vasbinder et al., 2003). The dispersion sample (0.4 g) was mixed with 0.8 g of distilled water and 40 mL of acetic acid (10%). The mixture was vortexed for 15 s. After 10 min, 40 mL of sodium acetate (1 M) and 0.72 g of distilled water were added and the mixture was vortexed again. After 1 h of standing, the mixture was centrifuged for 5 min at $3,000 \times g$ in an Eppendorf microcentrifuge (Fisher Scientific). Supernatants were filtered through 0.45- μ m filters (Millipore Corporation, Bedford, MA) and injected into a size-exclusion HPLC system consisting of a degasser, a P4000 pump, an autoinjector (AS3500), and a UV 2000 detector (Thermo Electron Corporation, San Jose, CA). The HPLC system was linked to a data acquisition and processing system (ChromQuest 4.1, Thermo Electron).

Whey protein isolate standards (mass fractions of 0.2 to 2%) were prepared by dissolving laboratory-made freeze-dried powder in water. This isolate was previously prepared by precipitating commercial whey protein isolate (New Zealand Dairy Products) at pH 4.6, adjusting the soluble fraction to pH 7, and dialyzing the protein solution against 33 vol of high-purity water before freeze-drying. Aliquots (100 μ L) of samples and standards were injected in duplicate on a Superdex 75 column (GE Healthcare, Piscataway, NJ). Elution flow rate was 1 mL/min with a 50 mM sodium phosphate buffer at pH 7.0 containing 0.15 M NaCl. The peak area was determined at a wavelength of 280 nm, and the amount of residual native whey protein was calculated by using a standard curve.

Preparative Chromatography of the Supernatants. To quantify soluble aggregates, reconstituted MPC and SM (protein fraction of 5%) were centrifuged at $84,000 \times g$ with a Beckman Coulter Optima LE-80K ultracentrifuge with rotor type 70.1 Ti (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada) for 30 min at 20°C to sediment CN micelles. The supernatant (1 mL) was injected onto a Bio-Rad Biologic Duo-Flow HPLC system (Bio-Rad Laboratories, Hercules, CA) to separate the different fractions with a Pharmacia XK 16/70 column packed with an S-500 Sephacryl high-resolution gel, with a molecular weight cutoff of 2,000 kDa (GE Healthcare) as previously described (Donato and Dalgleish, 2006). The eluting buffer (1 mL/min) was 20 mM bis-Tris-propane at pH 7.0, containing 0.02% sodium azide. Three-milliliter fractions from each peak were collected in filter tubes (Nanosep 10K), concentrated to 150 μ L in an Eppendorf microcentrifuge (Fisher Scientific), and analyzed for protein distribution by SDS-PAGE (electrophoresis unit PowerPac HC, Bio-Rad).

Gel Electrophoresis. Samples of centrifugal supernatants were diluted 1:1 with water and then at a 1:2 ratio with sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 19% glycerol, 0.05% β -mercaptoethanol, 0.01% bromophenol blue) and heated for 5 min at 95°C. To evaluate whether protein aggregates formed through disulfide linkages, SDS-PAGE under nonreducing conditions (the same sample buffer with β -mercaptoethanol replaced by water) was also carried out. Samples collected from preparative size-exclusion chromatography experiments were diluted only with sample buffer at a 1:1 ratio. Five microliters of each sample was loaded into the gels; the resolving gel contained 18% acrylamide (30% bis-acrylamide) in 0.4 M Tris-HCl at pH 8.9, and the stacking gel contained 4% acrylamide in 0.05 M Tris-HCl buffer at pH 6.7. Ammonium persulfate and tetramethylethylenediamine were used as catalysts. The gels were submerged in electrophoresis buffer (0.7 M Tris-HCl, 0.45 M Gly, pH 8.3) and run at 200 V for 40 min. The gels were then stained in a working solution [Coomassie blue (0.1%) in 50% methanol and 10% acetic acid] for 40 min and destained in 45:10:45 methanol:acetic acid:water for 2 consecutive 1-h periods. The gels were scanned for qualitative identification of α -LA, β -LG, κ -CN, and CN (β -CN and α_s -CN bands were not sufficiently distinct) in a Sharp JX-330 scanner (GE Healthcare). Whey protein isolate (0.4%) or sodium caseinate (0.3%) was used as a reference in the electrophoresis.

Renneting Properties of MPC Dispersions

Renneting of MPC Dispersions in Water. All samples were stored overnight at 4°C before renneting. The

initial pH of each reconstituted powder was recorded, and the pH was then adjusted to 6.4 with 5% lactic acid. A CaCl_2 solution was added to a 8.9 mM final concentration. Samples were equilibrated for 30 min at 30°C in a water bath before readjusting the pH and renneting. Freshly diluted 1% rennet solution was added and mixed thoroughly for 15 s. Samples were immediately transferred to the rheometer or to test tubes for the analysis of CN macropeptide (CMP) release. To optimize data collection, the final rennet concentrations were 0.00125% for the CMP release experiment and the corresponding rheology experiments, 0.0025% for the rheological experiments with MPC dispersions in water, and 0.005% for the rheological experiments with MPC added to SM. Experiments were carried out in triplicate.

CMP Release. The CMP released during renneting was determined by using reversed-phase HPLC (Thermo Electron) according to Lóz-Fandiño et al. (1993) with slight modifications. Aliquots (4 mL) of the renneted sample were transferred to test tubes and immediately placed in a water bath at 30°C. At intervals, the enzymatic reaction was stopped by adding 12% TCA to a final concentration of 2%. Preliminary experiments were carried out to determine which TCA concentration resulted in optimal CMP recoveries. One-milliliter aliquots of supernatants were transferred to microcentrifuge tubes and centrifuged at $4,500 \times g$ for 15 min at 20°C in an Eppendorf microcentrifuge (Fisher Scientific). The supernatant was filtered (0.45 μ m) and injected (100 μ L) on a reversed-phase column (μ RPC C2/C18 ST 4.6/100) with a guard column (GE Healthcare Life Sciences). Elution (flow rate constant at 1.0 mL/min) was with 0.1% (volume fraction) trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 90% acetonitrile (solvent B) as follows: 82% solvent A for 5.4 min, solvent A reduced to 61% for 35 min, solvent A to 0% in another 6 min, solvent B at 100% for 5 min, and finally back to 82% solvent A. The area under the peaks detected at 214 nm was calculated, and the total amount of CMP was expressed relative to the maximum amount (defined as the value at plateau) of CMP released from reconstituted SM at the same protein concentration.

Rheological Properties of Rennet-Induced Gels. Twenty-milliliter samples were placed in concentric cylinders (inner and outer cylinder diameters were 28 and 30 mm) in a controlled-stress rheometer AR1000 (TA Instruments, New Castle, DE) immediately after rennet addition at 30°C. The cylinder was covered to minimize evaporation. The elastic modulus (G') and the viscous modulus (G'') were recorded with Rheology Advance Data Analysis software, version 5.0.38 (TA Instruments Ltd., New Castle, DE) continuously for 3 h

in dynamic low-amplitude oscillatory mode, beginning 5 min after the addition of rennet. Oscillation frequency, shear strain, and initial stress were 0.1 Hz, 0.01, and 0.0018 Pa, respectively. The gelling point was defined as the time corresponding to the G' to G'' crossover ($\tan \delta = 1$). Rennet-induced gelation was also carried out on the same MPC dispersions (5% protein) after dialysis of the samples by using regenerated cellulose dialysis tubing with a nominal cutoff of 6 to 8 kDa (Fisher Scientific) against 33 vol of SM containing 5% protein. Samples for dialysis were dispersed as explained above and immediately transferred to the dialysis tubing, where they were kept under dialysis for 24 h at 4°C. Changes in volume were minimal but were taken into consideration in the experiments. In this way, both nondialyzed and dialyzed samples were kept at the same conditions before renneting. Analysis of variance and least squares means, as well as Duncan means, were calculated by using SAS (version 8.2, SAS Institute, Cary, NC). Significant differences ($P < 0.05$) were calculated for the values of gelation point and maximum gel stiffness (**max G'**) of 3 independent experiments. For graphical purposes, data from G' over time of each of 3 replicates was combined in a master curve: gelling points were first averaged (GP_j) and the time interval (t_x) between the individual gelling points (GP_i) and the GP_j was calculated as

$$t_x = GP_i - GP_j.$$

The variable time was then modified to fit t_x . The time values plotted were the results of the subtraction of original time values minus t_x . The G' values at selected time intervals were averaged and are represented in the curves shown in the results.

Renneting of Milk Solutions with Added MPC. Milk protein concentrate solutions were prepared by mixing MPC and SM and then adding water while stirring at room temperature. The final protein concentration was 6%, with 52% of the total protein from SM. Powders were dispersed by shearing and heating at 60°C as described above, followed by single-pass and single-stage homogenization at 500 bar (EmulsiFlex C-5, Avestin, Ottawa, Canada). A portion of the homogenized solution (30 mL) was dialyzed, as described above, against 1,000 mL of SM containing 6% protein. The viscoelastic properties of the milk samples, as well as dialyzed samples, were evaluated as previously described. Control samples were reconstituted SM (6% protein). All other conditions were kept the same as in the MPC in the water dispersion experiments. Data were analyzed statistically and plotted as described above.

Table 1. Chemical composition of skim milk powder (SM) and milk protein concentrate powders (MPC; protein concentrations of 56, 70, and 90%)

Item	SM	MPC56	MPC70	MPC90
Protein (%)	36	56	70	90
Lactose (%)	49.1	18.3	10.2	0.45
Ca ²⁺ (mg/g)	12	19	21	24
Ca ²⁺ :protein (mg/g)	34.3	33.9	30	26.7
Mg ²⁺ (mg/g)	1	0.98	0.91	0.85
K ⁺ (mg/g)	18	11	6.8	2
Na ⁺ (mg/g)	3.9	2.2	1.5	0.45
S (mg/g)	2.8	4	4.5	5.6
P (mg/g)	9.4	13	14	15
Ca:P (mg/mg)	1.27	1.46	1.5	1.6

Determination of Calcium Content in Soluble and Insoluble Fractions. Before addition of CaCl₂ to the renneting experiments, aliquots (10 mL) of the dispersions were separated by centrifugation at 84,000 $\times g$ for 30 min at 25°C, as previously described. Total calcium in the supernatants was determined and expressed as soluble calcium. The amount of colloidal calcium, defined as the amount of calcium present in the insoluble fraction, was calculated as the difference between the total calcium in the sample and the amount of soluble calcium.

RESULTS AND DISCUSSION

Characterization of MPC Powders

The relative amounts of total calcium and the relative ratios of calcium to phosphorus in the MPC powders were SM < MPC56 < MPC70 < MPC90 (Table 1). However, it is important to note that higher protein MPC (for example, MPC90) had less total calcium per protein. These results imply that the calcium present in insoluble calcium phosphate complexes increased with the protein concentration in the powders.

Results on the average particle size of MPC dispersions are summarized in Table 2. Figure 2 shows the particle size distribution of MPC90 dispersions after different treatment combinations. Mixing the samples at room temperature for up to 3 h did not significantly reduce the particle size. Most particles were greater than 30 μ m in diameter (Figure 2A). These large particles will eventually sediment under quiescent conditions. After shearing at room temperature (Figure 2A), most CN aggregates were still >20 μ m. Heating at 60°C for 30 min with or without shear applied was enough to produce monomodal dispersions of MPC56 and MPC70 (Table 1). For MPC90, both heating at 40°C and heating at 60°C for 30 min and shear were necessary to obtain average particle sizes that were not significantly different from those of well-dispersed MPC56 and MPC70.

Table 2. Average particle size [$d_{(4,3)}$] for 3 commercial milk protein concentrates (MPC; protein concentrations of 56, 70, and 90%) after 1 h of hydration, as a function of the temperature applied (20, 40, or 60°C for 30 min) and shear (Y) or no shear applied (N)

Item	20°C		40°C		60°C	
	N	Y	N	Y	N	Y
MPC56	29.56 ± 3.6 ^a	1.97 ± 0.09 ^b	1.22 ± 0.63 ^b	0.15 ± 0.01 ^b	0.13 ± 0.01 ^b	0.14 ± 0.01 ^b
MPC70	56.23 ± 5.44 ^c	10.73 ± 0.26 ^d	0.44 ± 0.05 ^b	0.14 ± 0.01 ^b	0.14 ± 0.01 ^b	0.14 ± 0.01 ^b
MPC90	111.6 ± 2.46 ^e	41.71 ± 0.32 ^f	53.42 ± 5.22 ^c	1.36 ± 0.03 ^b	44.39 ± 4.05 ^f	0.92 ± 0.01 ^b

^{a-f}Different superscript letters indicate differences at $P < 0.05$.

However, even under those heating and shear conditions, traces of large CN particles (10 to 70 μm) could be measured in MPC90 dispersions (Figure 2B).

These results agreed with several earlier reports on the challenges of reconstituting MPC powders. Barbosa-Canovas et al. (2005) suggested that shear aids in the dispersion of the powders by facilitating the fusion of small nonporous particles, which will otherwise stay insoluble as gel-like, semihydrated particles. McKenna (2000) demonstrated that MPC85 powders stored at 20°C for 6 mo contained large particles of fused CN

micelles that did not dissociate when dissolved in water at 45°C for 30 min. Contrary to our results, Castro-Morel and Harper (2002) found no correlation between solubility and protein content of 37 different MPC powders from 10 different countries. However, in our study only 3 MPC were used, because our objective was not to survey the available MPC on the market, but to determine the factors affecting the renneting behavior of MPC. A heating treatment of 60°C for 30 min, followed by 5 min of shear was applied to all powders to ensure a particle size distribution of the suspensions

Figure 2. Particle size distribution (volume % vs. diameter) of a commercial milk protein concentrate (MPC90) dispersed under selected treatment combinations. Powders dispersed at 20°C (A) for 1 h; 3 h; 1 h and 5 min of shear (1 h + s); 1 h and 5 min of shear and overnight storage at 4°C (1 h + s + o); or (B) dispersed for 1 h at 20°C and then heated at 40°C for 30 min (1 h + 40°C); 1 h at 20°C and then heated at 60°C for 30 min (1 h + 60°C); 3 h at 20°C and then heated at 60°C for 30 min (3 h + 60°C); 1 h at 20°C, heated at 60°C for 30 min, and then sheared for 5 min (1 h + 60°C + s). Distributions shown are the average of 2 replicate experiments.

that was not significantly different among the different MPC samples.

Solubility of MPC is also affected by storage temperature (Anema et al., 2006) or by processing conditions, such as preheating temperature (Castro-Morel and Harper, 2003), the presence of calcium (Carr, 2002) and serum components at drying (Gaiani et al., 2005). Gaiani et al. (2005) reported that adding milk ultrafiltrate to native phosphocaseinates before spray-drying favored rapid rehydration, probably because of the hygroscopic nature of the components present in the ultrafiltrate. Therefore, it may be possible that the low content of serum material in the high-protein MPC powders (Table 1) may be one of the causes for their inferior dispersability when compared with MPC56 or reconstituted SM. Davenel et al. (1997) proposed that reducing the number of calcium phosphate bridges between the CN micelles, which would normally be produced during the drying of milk products, would improve the solubility of the MPC powders.

Another important aspect of the characterization of MPC was the extent of whey protein denaturation. For this reason, the amount of residual native protein was quantified for MPC samples, relative to SM, which were dispersed by mixing at 20°C for 1 h and were sheared for 5 min. The percentages of native whey protein in supernatants were $99.35 \pm 0.15\%$, $66.18 \pm 0.03\%$, and $61.76 \pm 0.01\%$ for MPC56, MPC70, and MPC90, respectively. Similar amounts of native whey proteins have been reported by Castro-Morel and Harper (2002) for various commercial MPC and by Vasbinder et al. (2003) for milk heated between 70 and 85°C. The results indicate that MPC70 and MPC90 showed a higher degree of whey protein denaturation compared with MPC56, which contained as much native whey protein as a low-heat SM.

The process of dispersing MPC chosen in this research (dissolving at 20°C for 1 h, heating at 60°C for 30 min, and shearing for 5 min) caused a decrease in the relative percentage of residual native protein to $86.76 \pm 0.23\%$, $61.76 \pm 0.26\%$, and $55.88 \pm 0.09\%$ for MPC56, MPC70, and MPC90, respectively.

The differences in the protein aggregates present in the supernatant after centrifugation were also studied by using preparative size-exclusion chromatography. The elution patterns for the centrifuged supernatants (Figure 3) were similar to those described in previous literature (Anema and Klostermeyer, 1997; Anema and Li, 2000, 2003; Donato and Dalgleish, 2006). The first peak (eluting at 48 min) was mostly lipid material, with very little protein (Donato and Dalgleish, 2006). The large peak eluting at approximately 90 min corresponded to the residual whey proteins present in the supernatant. This peak was lower for MPC90 and

MPC70 than for SM or MPC56, confirming the results of residual native whey protein discussed above. The last peak, occurring at 110 min in SM, was also reported as nonprotein material (Guyomar'ch et al., 2003). Sodium dodecyl sulfate-PAGE analysis of the wide protein peak eluting between 60 and 90 min demonstrated that it was composed of protein aggregates containing small quantities of CN, larger amounts of β -LG, and traces of α -LA. These observations suggest that MPC90 and MPC70 contained more of these aggregates than SM and MPC56. These heat-induced protein aggregates have been reported previously for heated milk, and their presence affected acid-induced gelation of milk (Guyomar'ch et al., 2003; Donato and Dalgleish, 2006). The aggregates in MPC90 were distributed over a wide size range and eluted later than the aggregates of the other supernatant samples. This could be caused by heating at different pH values. In fact, it has been reported that increasing the pH of milk before heating increases the polydispersity of the heat-induced aggregate peak, resulting in a shift in the elution profile of these aggregates to longer times (Donato and Dalgleish, 2006).

Renetting Behavior of MPC Dispersions

Figure 4 illustrates the difference in rheological behavior and kinetics of CMP release for the MPC samples dispersed in water. For these experiments, a small amount of rennet was used to obtain accurate measurements of the CMP release. In all cases, a plateau of CMP was obtained after approximately 60 min from the addition of rennet. The maximum amounts of CMP released were significantly lower for MPC with higher protein concentration than for MPC56, which showed a behavior similar to that of reconstituted SM (with a plateau at approximately 100% CMP; data for SM not shown). The lower amounts of CMP released from MPC70 and MPC90 were most likely caused by the higher level of heat-induced protein denaturation, which is known to impair both primary (κ -CN hydrolysis) and secondary (aggregation) phases of rennet-induced CN coagulation (Van Hooydonk et al., 1987; Calvo et al., 1995).

Figure 5 illustrates the differences among the intensities of protein bands in SDS-PAGE of MPC supernatants treated under nonreducing (lanes 1 to 4) and reducing conditions (lanes 7 to 10). The electrophoretic analysis demonstrated that all the κ -CN present in the supernatants after centrifugation were involved in disulfide bonds with β -LG, and α -LA seemed to be less affected than β -LG. Similarly, Havea (2006) recovered disulfide-linked aggregates of mainly κ -CN and β -LG from the soluble and insoluble fractions of MPC85 dis-

Figure 3. Elution profiles of centrifuged supernatant of reconstituted skim milk powder (SM) or commercial milk protein concentrates (MPC56, MPC70, MPC90; 5% protein) dissolved in water for 1 h and then heated at 60°C for 30 min and sheared for 5 min.

persions. Because the κ -CN present in soluble complexes with whey proteins may not be a good substrate for rennet (Singh et al., 1988), this may support our findings and explain the lower plateau value of the percentage of CMP release in MPC90 and MPC70 compared with MPC56 (Figure 4).

The renneting behavior of the MPC dispersions (all at 5% protein) also showed differences among the different MPC. The rate of increase of the elastic modulus, G' , was slower for MPC56 than for MPC90 and MPC70. The gelling time for MPC56 was 29.2 ± 4.0 min and was significantly longer than that of MPC90 suspensions (14.4 ± 1.1 min). The MPC70 dispersions showed intermediate times (18.8 ± 1.0 min). These results are

in disagreement with previous reports: Kuo and Harper (2003a,b) showed, in fact, that the gelation time, determined visually, did not depend on the type of MPC. The G' value determined at 3 h also demonstrated that MPC90 dispersions had a lower stiffness modulus than MPC56 and MPC70.

When comparing the rheological data to the CMP release data, it was clear that MPC samples showed gelation at different levels of CMP release. Although SM showed gelation after 90% of κ -CN hydrolysis (data not shown), in MPC samples, the gel point occurred when 58% (MPC90), 74% (MPC70) and 87% (MPC56) of the CMP (compared with SM) was released (Figure 4). It is known that in unheated milk, the secondary

Figure 4. Storage modulus, G' (right axis, solid symbols), and CN macropeptide (CMP) release (left axis, open symbols) as a function of time for rennet gels made from commercial milk protein concentrates MPC90 (▲△), MPC70 (■□), and MPC56 (●○) reconstituted in water (5% protein). Values are the average of 3 replicate experiments. The CMP values are shown as percentages of CMP ($\text{CMP}_t/\text{CMP}_\infty$, where CMP_∞ is the amount of CMP released at plateau from skim milk powder reconstituted at 5% protein). Dotted lines denote gelling points.

Figure 5. Sodium dodecyl sulfate-PAGE of centrifugal supernatants under reducing (lanes 1 to 6) and nonreducing (lanes 7 to 10) conditions for dispersions of commercial milk protein concentrates MPC56 (lanes 1 and 7), MPC70 (lanes 2 and 8), and MPC90 (lanes 3 and 9), and skim milk powder (lanes 4 and 10). Lanes 5 and 6 are sodium caseinate and whey protein isolate standards.

phase of rennet aggregation occurs only when most of the κ -CN is hydrolyzed from the CN micelle (Sandra et al., 2007). The data shown in Figure 4 for MPC56 seem to agree with this mechanism. On the other hand, in MPC70 and MPC90, less CMP needed to be released before the CN micelles started to aggregate. This was most likely caused by the higher amount of denatured whey proteins and the presence of whey protein-CN aggregates in the serum phase (see Table 1 and Figure 3). In the MPC70 and MPC90 dispersions, the micelles had less κ -CN on their surface (more bare patches) and would therefore show aggregation with a lower extent of CMP release. In support of this hypothesis, Singh and Fox (1986) reported that κ -CN-depleted micelles obtained from heated milk (90°C and pH 7.3) were sensitive to rennet coagulation when redispersed in synthetic milk ultrafiltrate, but rennetability was impaired when the same micelles were kept in their own serum adjusted to pH 6.7. This difference in behavior can be easily explained, because the reassociation of κ -CN and whey protein complexes with the micelles is pH dependent (Anema and Li, 2000, 2003).

The lower availability in soluble calcium and the increased colloidal calcium relative to total calcium (Tables 1 and 3) are obvious reasons for the difference in the aggregation of the CN micelles (McMahon et al., 1993; de la Fuente, 1998). Table 3 illustrates the amounts of calcium and phosphorus present in the soluble phase compared with the total dispersion. All samples showed similar insoluble calcium-to-phosphorus ratios. This would suggest that differences in coagulation behavior depended not on the colloidal calcium (insoluble fraction) but on the ratio between soluble and insoluble calcium.

To test the effects of the presence of soluble calcium in the ionic equilibrium and in the gelling behavior of the CN micelles of the various MPC dispersions, the MPC dispersions were extensively dialyzed against skim milk (5% total protein). Statistical analysis (ANOVA) confirmed that dialysis did not affect the ratio of insoluble calcium and phosphorus, but increased the content of soluble calcium and phosphorus (data not shown). Dialysis of the MPC dispersions restored the equilibrium of free ions and caused dissociation of the CN from the micelles. Sodium dodecyl sulfate-PAGE (Figure 6) indicated that dialysis increased the amount of soluble CN. This was also supported by size-exclusion chromatography data on supernatants of dialyzed MPC dispersions (data not shown).

The gelling point of the MPC dispersions dialyzed against SM (Figure 7B) shifted to longer times than those of nondialyzed samples (Figure 7A). The gelation time (determined when $\tan \delta = 1$ in rheological experiments) of MPC after dialysis were 52.7 ± 0.9 , 81 ± 13 , and 126 ± 14 min for MPC56, MPC70, and MPC90, respectively. Note that for these experiments, a higher amount of rennet was added than in the experiments shown in Figure 4. The impact of dialysis was radically dependent on the protein concentration of the original MPC powders. After dialysis, MPC56 showed a significantly longer gelation time than for the same dispersions in water, but the final G' value was not significantly different, demonstrating that if the milk protein

Table 3. Amounts of calcium and phosphate present in milk protein concentrate (MPC; protein concentrations of 56, 70, and 90%) dispersions (5% total protein) and in the supernatant after centrifugation (soluble calcium and phosphate)

Item	SM ¹	MPC56	MPC70	MPC90
Total calcium (mg/g)	1.7 $\pm$ 0.0	1.55 $\pm$ 0.7	1.4 $\pm$ 0.0	1.25 $\pm$ 0.7
Soluble calcium (mg/g)	0.52 $\pm$ 0.1	0.35 $\pm$ 0.3	0.29 $\pm$ 0.5	0.22 $\pm$ 0.1
Total phosphorus (mg/g)	1.4 $\pm$ 0.01	1.1 $\pm$ 0.14	0.95 $\pm$ 0.1	0.77 $\pm$ 0.0
Soluble phosphorus (mg/g)	0.65 $\pm$ 0.07	0.34 $\pm$ 0.07	0.25 $\pm$ 0.02	0.13 $\pm$ 0.01
Total calcium:phosphorus ratio (wt/wt)	1.21	1.40	1.47	1.62
Insoluble calcium:phosphorus ratio (wt/wt)	1.57	1.57	1.57	1.62
Soluble:insoluble calcium (wt/wt)	0.44	0.29	0.26	0.21

¹SM = skim milk powder.

Figure 6. Effect of the reequilibration by dialysis of milk protein concentrate (MPC) dispersions against reconstituted skim milk (5% protein). Sodium dodecyl sulfate-PAGE of centrifugal supernatants of MPC56 (lanes 1 and 7), MPC70 (lanes 2 and 8), and MPC90 (lanes 3 and 9) before (lanes 1 to 3) and after (lanes 7 to 9) dialysis conditions. Lane 4: centrifugal supernatant of reconstituted skim milk powder; lanes 5 and 6: sodium caseinate and whey protein isolate standards, respectively.

concentration process is mild, the changes in the CN micelle functionality are also limited. On the other hand, MPC70 and MPC90 were affected by dialysis, and the increased amount of soluble calcium in these systems hindered the aggregation of the CN, with significantly longer gelling times and very low gel stiffness moduli. In the case of MPC90, after dialysis the suspensions did not show a visible gelation. This behavior has never been reported, and although the calcium phosphate present in the insoluble fraction (which should be related to the colloidal fraction) was similar among the MPC samples (Table 3), the increase in the soluble salt fraction for the MPC70 and MPC90 dispersions strongly affected the colloidal interactions among the CN.

These results with dispersions of MPC dissolved in water were compared with those dissolved in SM (Table 4). Table 5 illustrates the rheological properties of rennet gels from SM added with MPC (6% protein). In this

Figure 7. Storage modulus, G' , as a function of time for rennet gels made from milk protein concentrate (MPC) or skim milk powder in water (5% protein solutions) that had been given heat treatment 60°C and shear (A) and dialyzed (B). Values are taken from representative master curves of 3 replicates.

Table 4. Amounts of calcium and phosphate present in reconstituted skim milk (SM) with added milk protein concentrate (MPC; protein concentrations of 56, 70, and 90%; 6% total protein) as total and soluble (present in the supernatant after centrifugation)

Item	SM	MPC56	MPC70	MPC90
Total calcium (mg/g)	1.9 ± 0.2	1.95 ± 0.07	1.85 ± 0.07	1.75 ± 0.08
Soluble calcium (mg/g)	0.53 ± 0.01	0.47 ± 0.01	0.45 ± 0.03	0.39 ± 0.02
Total phosphorus (mg/g)	1.6 ± 0.14	1.5 ± 0.01	1.4 ± 0.01	1.3 ± 0.01
Soluble phosphorus (mg/g)	0.71 ± 0.03	0.57 ± 0.01	0.52 ± 0.02	0.59 ± 0.2
Total calcium:phosphorus ratio (wt/wt)	1.18	1.3	1.32	1.34
Insoluble calcium:phosphorus ratio (wt/wt)	1.54	1.58	1.58	1.95

case also, the gelation time depended on the type of MPC. Significantly shorter gelation times were recorded for MPC70 and MPC90 dissolved in SM. On the other hand, the gelation time of MPC56 added to milk was not different from that of the SM control sample. The effect of dialysis was greatly reduced compared with MPC dispersions dissolved in water, but it was still shown clearly that after dialysis, MPC70 and MPC90 showed significantly longer gelling times. From the data on gelling time and the G' modulus (Table 5), it is possible to hypothesize that in these samples, the gelation was dominated by the CN micelles from milk, and after dialysis against milk, the samples showed a behavior closer to that of the original milk.

CONCLUSIONS

The rheological behavior of the rennet gels of CN from MPC was consistent with the current view that the micelle is a hydrophobic colloid in which the energy of interaction among the molecules is the sum of electrostatic repulsion and hydrophobic attraction (Horne, 1998; Dalgleish et al., 2004). However, the present results demonstrate that the level of total calcium and phosphate present in milk and the ratio of insoluble to soluble ions are crucial to the aggregative stability of

the protein. The gel strength of MPC dispersions rich in insoluble calcium (nondialyzed MPC samples) was higher than that in the dialyzed samples, suggesting stronger interactions within the micelle after gelation. This was somewhat reversed when the soluble calcium was increased by dialysis against reconstituted milk.

Our study showed a clear relationship between the calcium activity, the insoluble calcium:phosphate ratio, and micelle integrity on the rheological behavior of MPC. These results support only in part the view of Udabage et al. (2001), who proposed that the effects of colloidal calcium phosphate within the micelle dominate the gel formation of renneted milks. More than the colloidal calcium phosphate, the ratio between the ionic and colloidal calcium is critical to the gel behavior. After dialysis, the gelling points and the rate of increase of G' values of the MPC samples were close to the values for SM. These findings lead to the conclusion that the mineral equilibrium in MPC solutions plays a pivotal role in the aggregation of rennet-induced MPC gels.

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Table 5. Gel points and modulus (G') of rennet-induced gels for reconstituted milk at 6% protein as a function of type of milk protein concentrate (MPC; protein concentrations of 56, 70, and 90%) added¹

Item	Gel point (min)	G' value at 3 h (Pa)
SM ²	73.8 ± 4.8 ^a	103 ± 23 ^{ac}
MPC56 + SM	63.3 ± 1.5 ^b	210 ± 15 ^b
MPC56 + SM, dialyzed	78.2 ± 4.2 ^a	133 ± 31 ^a
MPC70 + SM	52.42 ± 6.7 ^d	208 ± 19 ^b
MPC70 + SM, dialyzed	88.9 ± 1.8 ^c	100 ± 4 ^c
MPC90 + SM	43.1 ± 4.6 ^d	194 ± 16 ^b
MPC90 + SM, dialyzed	77.6 ± 2.9 ^a	106 ± 10 ^{ac}

^{a-d}Within the same column, different superscript letters indicated significant differences at $P < 0.05$.

¹The rheological parameters are indicated for the same samples after dialysis.

²SM = skim milk powder.

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