

Partial calcium depletion during membrane filtration affects gelation of reconstituted milk protein concentrates

H. Eshpari,^{*1} R. Jimenez-Flores,[†] P. S. Tong,[†] and M. Corredig^{*2}

^{*}Department of Food Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

[†]Dairy Products Technology Center, California Polytechnic State University, San Luis Obispo 93407

ABSTRACT

Milk protein concentrate powders (MPC) with improved rehydration properties are often manufactured using processing steps, such as acidification and high-pressure processing, and with addition of other ingredients, such as sodium chloride, during their production. These steps are known to increase the amount of serum caseins or modify the mineral equilibrium, hence improving solubility of the retentates. The processing functionality of the micelles may be affected. The aim of this study was to investigate the effects of partial acidification by adding glucono- δ -lactone (GDL) to skim milk during membrane filtration on the structural changes of the casein micelles by observing their chymosin-induced coagulation behavior, as such coagulation is affected by both the supramolecular structure of the caseins and calcium equilibrium. Milk protein concentrates were prepared by preacidification with GDL to pH 6 using ultrafiltration (UF) and diafiltration (DF) followed by spray-drying. Reconstituted UF and DF samples (3.2% protein) treated with GDL showed significantly increased amounts of soluble calcium and nonsedimentable caseins compared with their respective controls, as measured by ion chromatography and sodium dodecyl sulfate-PAGE electrophoresis, respectively. The primary phase of chymosin-induced gelation was not significantly different between treatments as measured by the amount of caseino-macropptide released. The rheological properties of the reconstituted MPC powders were determined immediately after addition of chymosin, both before and after dialysis against skim milk, to ensure similar serum composition for all samples. Reconstituted samples before dialysis showed no gelation (defined as $\tan \delta = 1$), and after re-equilibration only control UF and DF samples showed gelation. The

gelation properties of reconstituted MPC powders were negatively affected by the presence of soluble casein, and positively affected by the amount of both soluble and insoluble calcium present after reconstitution. This work, testing the chymosin-induced gelation behavior of various reconstituted MPC samples, clearly demonstrated that a decrease in pH to 6.0 during membrane filtration affects the integrity of the casein micelles supramolecular structure with important consequences to their processing functionality.

Key words: milk protein concentrate, casein, calcium depletion, chymosin-induced gelation

INTRODUCTION

Milk protein concentrate (MPC) powders are manufactured from skim milk through membrane filtration and spray drying. The major components of these shelf-stable food ingredients are caseins, whey proteins, lactose, fat, and minerals, in varying proportions depending on the extent of concentration and the type of membrane separation employed.

For optimal utilization of MPC it is important to clearly understand their functionality after reconstitution. The details of the chymosin-induced gelation properties of reconstituted MPC powders have been previously reported (Ferrer et al., 2008; O'Mahony et al., 2009; Martin et al., 2010; Hunter et al., 2011), as MPC can be employed in the cheesemaking processes to increase cheese yield and productivity. The chymosin-induced gelation of casein micelles consists of 2 phases. In the initial phase, the enzyme chymosin specifically cleaves the Phe105-Met106 bond of κ -CN, which is present on the casein micelle surface. This reaction causes a reduction of the steric and electrostatic repulsion forces between micelles (Dalglish, 1984; Sandra and Dalglish, 2007). At a lower pH, a release of soluble calcium occurs, chymosin increases its activity (van Hooydonk et al., 1986), and a partial collapse of the κ -CN layer takes place due to a decrease in electrostatic repulsion (De Kruif and Zhulina, 1996). Once most of the κ -CN has been cleaved, the micelles are able to approach

one another and aggregate in the presence of calcium (Fox et al., 2000; Sandra et al., 2011). The equilibrium between soluble and colloidal calcium in the micelles is critical to the formation of chymosin-induced gels (Choi et al., 2007). Ferrer et al. (2008) reported that mineral equilibrium in MPC solutions plays a crucial role in the aggregation of chymosin-induced MPC gels. Furthermore, Kuo and Harper (2003) reported that chymosin-induced gels made with MPC56 were stiffer than those prepared with MPC85 tested at equivalent protein levels. It was recently demonstrated (Sandra and Corredig, 2013) that the presence of nonmicellar casein in the soluble phase may have a negative effect on the chymosin-induced gelation properties of MPC powders. Partial calcium depletion by the addition of sodium chloride (Sikand et al., 2013) chelating agents, cation exchange chromatography, or acidification can affect functional properties of MPC (Bhaskar et al., 2001). However, the effect of mineral distribution and the state of casein micelles on the technological properties of MPC is still not fully understood and the molecular mechanisms behind such effects have not been fully elucidated. Solubilization of colloidal calcium phosphate from casein micelles during membrane filtration (e.g., through acidification) may affect the structural organization of these protein particles. The aim of our study was to investigate the effects of addition of glucono- δ -lactone (**GDL**) to skim milk during membrane filtration on the structural changes of the casein micelles by studying their chymosin-induced gelation behavior after reconstitution, as chymosin-induced gelation is affected by both the calcium equilibrium and their supramolecular structure.

MATERIALS AND METHODS

Materials

Pasteurized skim milk was obtained from Producer's Dairy Foods Inc. (Fresno, CA). Analytical grade reagents were from Sigma-Aldrich Chemical Ltd. (St. Louis, MO). Glucono- δ -lactone was purchased from Roquette America, Inc. (Geneva, IL). Ultrapure water (Milli-Q Ultrapure Water Purification Systems, Billerica, MA) was used to prepare all the solutions.

Preparation of MPC65 and MPC80

The MPC powders were manufactured in duplicate, either by UF (65% protein; **MPC65**) or by UF followed by diafiltration (80% protein; **MPC80**) using pasteurized (72°C for 16 s) skim milk. The GDL (3.25 g/L) was added to cold milk (4°C) under continuous stirring to reach pH 6.0 in a time period of ~5 h be-

fore membrane filtration. Ultrafiltration began at $5.8 \pm 1.5^\circ\text{C}$ (mean \pm SD). During UF, the temperature was allowed to increase in such a way that, by the end of the UF process, the temperature was $20 \pm 1.6^\circ\text{C}$. Controls were prepared at the native milk pH (~pH 6.6). It is important to note that this research studied the effect of pH adjustment (to 6.0) only before starting the UF, and no pH control occurred throughout the UF or diafiltration (**DF**) steps. The MPC65 and MPC80 powders were manufactured in the pilot plant of Dairy Products Technology Center at California Polytechnic State University (San Luis Obispo) with a cross-flow membrane pilot-plant unit (R12 model, Niro Inc., Hudson, WI) equipped with dual 10-kDa cut-off, spiral-wound, polyethersulfone membranes (Snyder Filtration, Vacaville, CA). The liquid MPC was spray dried with a pilot Niro Filtermat Spray Dryer (Niro Inc.) to approximately 3.5% moisture, and the obtained MPC powders were immediately collected and upon cooling to ambient temperature ($23 \pm 2^\circ\text{C}$) sealed in airtight bags for further analysis. Total protein present in the powders was determined by Kjeldahl.

Powder Rehydration

The MPC powders were reconstituted in Ultrapure water (Milli-Q Ultrapure Water Purification Systems) to a final protein concentration of 3.2% (wt/wt) using a household kitchen blender for 6 min at high speed. Water was heated to 66°C using a heat-stir plate before reconstitution. Each reconstituted sample was then divided into 2 equal portions; one portion was stored in a tightly sealed container at 4°C overnight. The other portion of the rehydrated samples (prepared as described above) was dialyzed against skim milk at 4°C, also overnight, to allow enough time for full rehydration and obtain samples with similar rehydration history before and after dialysis. Dialysis was carried out using an 8,000-Da cut-off membrane (Spectra/Por, Spectrum Laboratories Inc., Rancho Dominguez, CA) to restore, as much as possible, the original milk serum composition and be able to compare the gelation behavior of the casein micelles between treatments. The control samples were named **UFC** and **DFC** before and **UFC-D** and **DFC-D** after dialysis and the GDL-treated samples were named **UFG** and **DFG** before and **UFG-D** and **DFG-D** after dialysis for UF and DF samples, respectively.

Characterization of Rehydrated Samples

The protein was measured by the Dumas method (Leco FP-528; Leco Corp., St. Joseph, MI) and the pH was measured using an Accumet pH meter model 925

(Thermo Fisher Scientific, Waltham, MA). All analyses were carried out in duplicate with separate batches of reconstituted MPC samples.

The particle size distribution of the samples was determined using integrated light scattering (Mastersizer S, Malvern, Southborough, MA) immediately after reconstitution or after overnight storage at 4°C. The refractive index used was 1.39 and 1.33 for casein and water, respectively (Ferrer et al., 2008).

SDS-PAGE of the Soluble Fraction

The protein composition of the soluble fraction of the MPC samples reconstituted at 3.2% equal protein (as described herein) and a pasteurized skim milk sample (to evaluate the effect of MPC manufacturing process) was analyzed using SDS-PAGE both before and after dialysis. The centrifugal supernatant obtained from centrifugation at $65,000 \times g$ for 30 min at 25°C was mixed (1:1 ratio) with electrophoresis buffer containing 1 M Tris HCl buffer pH 6.8, 75% glycerol, 10% SDS, 2-mercaptoethanol, and 1% bromophenol blue. The solution was heated and mixed at 95°C for 5 min using a thermomixer (model 5436; Eppendorf, Hauppauge, NY). Samples were cooled to room temperature and 10 μ L of each sample was then loaded onto the gels. The SDS-PAGE was carried out in a vertical slab gel of 1.5 mm thickness with 15% acrylamide resolving gel and 4% stacking gel in a Bio-Rad mini-protean electrophoresis system (Bio-Rad Laboratories, Hercules, CA) at a constant voltage of 175 V. Gels were then treated with Coomassie blue R-250 for 45 min (Bio-Rad) and destained with 45% MilliQ water, 45% methanol, and 10% glacial acetic acid for 1 h, and then with the same solution diluted 1:1 in water overnight. The gels were scanned using a Bio-Rad Gel Doc EZ Imager equipped with Image Lab 3.0 software (Bio-Rad) and the protein bands were quantified by laser scanning densitometry. The areas of the scanned bands of α_s - and β -CN were proportional to the protein concentration in the centrifugal supernatants of each sample.

Calcium Concentration

The concentration of calcium in different fractions (total, soluble, and permeable) of reconstituted powders (equal to 3.2% protein) as well as a skim milk sample (for comparison) was measured in duplicate for each sample, using ion chromatography, by an Advanced Compact IC (Metrohm AG, Zurich, Switzerland), using a silica gel column (Metrosep C2 150/40 packed with 7 mm silica gel; Metrohm AG) at 30°C following the method described by Rahimi-Yazdi et al. (2010). The calcium measured in the bulk powder solu-

tions was referred to as total, representing the total amount of calcium in the reconstituted MPC samples. The amount of calcium in the centrifugal supernatant of reconstituted powders ($65,000 \times g$ for 1 h at 25°C) was referred to as soluble (nonsedimentable) calcium, containing both protein-bound and protein-free calcium fractions of the sample. In a separate experiment, the reconstituted powders were filtered using a Prep/Scale™-TEF 1ft2 cartridge ultrafiltration unit (10-kDa cut-off Regenerated cellulose, Millipore, Billerica, MA) to separate the protein-free permeate; the calcium measured was referred to as permeable calcium or non-protein bound soluble calcium. It is recognized that the amount of ionic (protein-free) calcium plays a major role in chymosin-induced gelation, surface, and rheological properties of milk proteins (De la Fuente 1998); hence, it is important to measure the concentration of both soluble and permeable calcium.

Nonsedimentable Caseins

The amount of caseins recovered in the supernatant of the reconstituted powders after ultracentrifugation at $65,000 \times g$ for 1 h at 25°C (Beckman L8-80M, nvT-90 rotor, Beckman Inc., Palo Alto, CA) was analyzed using SDS-PAGE. Aliquots of the MPC powders (re-suspended at 3.2% wt/wt equal protein concentration) were ultracentrifuged and the caseins present in the centrifugal supernatants were analyzed using SDS-PAGE.

Gelation Experiments

The MPC samples reconstituted (either in water or in water and then dialyzed against skim milk) were equilibrated at 30°C, and chymosin ($790 \pm 5\%$ international milk clotting units (IMCU)/mL strength Chymax Ultra; Chr. Hansen, Milwaukee, WI) was then added at a final concentration of 0.034 IMCU/mL. The samples were stirred for 30 s after chymosin addition.

Caseinomacropeptide Release

The release of caseinomacropeptide (CMP) by chymosin was monitored at controlled temperature of 30°C, according to an established method (López-Fandino et al., 1993). Upon addition of chymosin to the MPC samples at the concentration mentioned above, each sample was immediately distributed into 2-mL aliquots in different test tubes. The reaction of chymosin was stopped at certain time points (up to 2 h) by addition of 4 mL of 3% perchloric acid in each tube and vortexing subsequently. The tubes were then refrigerated overnight before collecting the supernatant. The

collected supernatants were centrifuged at $4,500 \times g$ for 15 min at 22°C (model 5415D centrifuge; Eppendorf), filtered through 0.45- μm Millex-GV filter units (Thermo Fisher Scientific), and analyzed by reverse phase-HPLC using a SpectraSystem LC with degasser, pump, autosampler, and UV detector set to 210 nm (Thermo Fisher Scientific). The sample (100 μL) was injected into a column (Pharmacia Biotech mRPC C2/C18 ST 4.6/100; GE Biotech, Piscataway, NJ) with a Vydac C-4 guard column (MandelScientific, Guelph, ON, Canada) kept at 40°C. The elution was carried out with 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 90% acetonitrile using a nonlinear gradient as previously described (López-Fandino et al., 1993). The total peak area of the chromatograms was integrated using ChromQuest software 5.0 version 3.2.1 (Thermo Fisher Scientific). The maximum peak area after gelation point for each sample was considered as 100% of the CMP released that could be detected.

To determine the calcium concentration at the onset of gelation, chymosin was added to the reconstituted MPC samples as described above. At the time the CMP release reached a plateau (~ 70 min after addition of chymosin), a solution of pepstatin-A inhibitor in dimethyl sulfoxide (1 mg/mL) was added to each sample at 50 $\mu\text{L}/\text{mL}$ concentration and the mixture was stirred well using a vortex. Each mixture was then centrifuged at the conditions described above to prepare samples for soluble and permeable calcium measurements.

Gel Formation

A controlled stress rheometer (AR 1000; TA Instruments, New Castle, DE) was employed to conduct the rheological measurements, using a conical concentric cylinder geometry (5,920 μm fixed gap, 15 mm cylinder radius, 14 mm rotor outer radius, and 42 mm cylinder immersed height). The temperature was maintained at 30°C using an external water bath (Isotemp 3016; Thermo Fisher Scientific). Measurements were conducted as previously described by Sandra et al. (2011) at 1 Hz, initial oscillation stress of 1.8 mPa, and strain of 0.01. The gel point was defined as $\tan \delta = 1$. Data analysis was carried out using the rheology Advantage analysis software, version 5.0.38 (TA Instruments).

Statistical Analysis

The sample treatments and analyses in the present study were carried out in duplicate. Values were means of replicate measurements and the differences between the means of the treatments were compared using one-way ANOVA at a significance level of $P < 0.05$. The statistical analysis was performed using the GLM com-

mand in Minitab (v.16.1, Minitab Inc., State College, PA). Difference between the treatments were tested using Tukey's honestly significant difference intervals with $\alpha = 0.05$.

RESULTS AND DISCUSSION

Characterization of the Reconstituted MPC

Integrated light-scattering measurements of the reconstituted powders revealed no significant differences in the particle size distribution between treatments. All dispersions, both before and after dialysis against skim milk, showed a multimodal distribution of sizes, with a large peak appearing between 0.01 and 1 μm of diameter and a small population of particles between 10 and 100 μm (data not shown). These results indicated that the rehydration was sufficient to obtain in the reconstituted milk a distribution of particle size comparable to that of the original milk.

The pH values, amounts of soluble and total calcium, and protein content of the MPC dispersions reconstituted in water before and after dialysis against milk, as well as pasteurized skim milk, are shown in Table 1. After reconstitution in water, the MPC samples showed the highest pH values for the control samples (6.97 and 7.10 for UFC and DFC, respectively) and the lowest values for the acidified sample (6.50 and 6.68 for UFG and DFG, respectively). The DF samples had a higher pH than the UF samples. After dialysis against skim milk (pH = 6.66) all the samples recovered the original pH. As pH affects chymosin activity (Fox et al., 2000), it is an important parameter to control.

The values of the total and the soluble calcium (as defined in Materials and Methods) after reconstitution and dialysis against milk are also shown in Table 1. Before dialysis, the value of total calcium for all MPC samples was significantly lower than that of skim milk (SM; 1,140 mg/kg), with the DFG (553 mg/kg) showing the lowest value of all the treatments, although only significantly lower than the UFC (756 mg/kg). Dialysis against SM restored the amount of total calcium in the control samples to the same level as in SM. The amount of total calcium in the acidified samples also increased after dialysis, although remained at a significantly lower level compared with that of SM and MPC control.

The differences in calcium content were mostly derived from the differences in soluble calcium. The concentration of soluble calcium for all MPC samples was significantly lower than that of SM (409 mg/kg). The acidified milk MPC (UFG and DFG) showed higher values of soluble calcium compared with the corresponding controls (UFC and DFC). Dialysis against milk restored the amount of soluble calcium in all MPC

Table 1. Values for pH, protein, total and soluble calcium concentrations, and the ratio of insoluble calcium per protein (means \pm SD) in skim milk (SM) and milk protein concentrate powder reconstituted in water (3.2% wt/wt), equal protein (wt/wt), and the same samples after dialysis against skim milk¹

Sample	pH	Total protein (% wt/wt)	Total calcium (mg/kg)	Soluble calcium (mg/kg)	Insoluble calcium-to-protein ratio (wt/wt)
SM	6.66 \pm 0.00 ^b	3.21 \pm 0.03 ^a	1139 \pm 43 ^a	408 \pm 1 ^a	0.023 \pm 0.001 ^a
UFC	6.97 \pm 0.00 ^a	3.20 \pm 0.08 ^a	756 \pm 20 ^b	136 \pm 30 ^c	0.019 \pm 0.001 ^{a,b}
UFG	6.50 \pm 0.00 ^c	3.16 \pm 0.02 ^a	639 \pm 46 ^{b,c}	243 \pm 11 ^b	0.013 \pm 0.002 ^b
DFC	7.10 \pm 0.02 ^a	3.20 \pm 0.01 ^a	672 \pm 9 ^{b,c}	115 \pm 8 ^c	0.017 \pm 0.002 ^{a,b}
DFG	6.68 \pm 0.04 ^b	3.18 \pm 0.03 ^a	553 \pm 26 ^c	183 \pm 22 ^{b,c}	0.012 \pm 0.001 ^b
SM	6.66 \pm 0.00 ^a	3.21 \pm 0.03 ^a	1140 \pm 43 ^a	409 \pm 1 ^a	0.023 \pm 0.001 ^a
UFC-D	6.65 \pm 0.01 ^a	3.21 \pm 0.01 ^a	1144 \pm 65 ^a	400 \pm 1 ^a	0.023 \pm 0.001 ^a
UFG-D	6.64 \pm 0.00 ^a	3.20 \pm 0.02 ^a	895 \pm 40 ^b	418 \pm 5 ^a	0.016 \pm 0.001 ^b
DFC-D	6.65 \pm 0.01 ^a	3.20 \pm 0.03 ^a	1261 \pm 97 ^a	371 \pm 20 ^a	0.022 \pm 0.001 ^a
DFG-D	6.65 \pm 0.00 ^a	3.19 \pm 0.01 ^a	922 \pm 30 ^b	405 \pm 15 ^a	0.015 \pm 0.002 ^b

^{a-c}Within a column, superscript letters indicate significant differences for $P < 0.05$.

¹UFC = UF control; UFG: UF acidified with glucono- δ -lactone (GDL); DFC = diafiltration (DF) control; DFG = DF acidified with GDL. After dialysis: UFC-D, UFG-D, DFC-D, and DFG-D.

samples to levels similar to SM. This will become important in the determination of the gelation behavior, as soluble calcium plays a major role in the chymosin-induced interactions of casein micelles (Dalglish, 1983; Sandra et al., 2011).

Although most of the difference in calcium content in the retentates could be attributed to the soluble calcium, it was also possible to conclude that lower levels of colloidal calcium were present in the acidified retentates (UFG and DFG), as shown in Table 1. Before dialysis, the ratio of insoluble calcium per protein in the acidified UF and DF samples (UFG and DFG) was significantly lower than the same ratio in SM. In spite of the recovery of soluble calcium after dialysis, the ratio of insoluble calcium per protein in the acidified UF and DF samples was not recovered and remained at a significantly lower level compared with SM and the dialyzed control samples (UFC-D and DFC-D). The significantly lower ratio of insoluble calcium to protein observed in the acidified MPC compared with the control MPC, both before and after dialysis against milk, suggested that the acidification of milk during membrane filtration caused a lasting change in the soluble and insoluble calcium equilibrium. This change should affect the processing functionality of the casein micelles (Fox et al., 2000; Choi et al., 2007).

Protein Composition of the Centrifugal Supernatants

In addition to whey proteins, caseins were present in the centrifugal supernatants of the reconstituted MPC. The band intensities were measured by scanning densitometry, and the areas, weighted by the total area of whey proteins, are shown in Figure 1. All area ratios were compared with SM samples. As all retentates were reconstituted at the same protein concentration, and

the band intensity of whey proteins was not significantly different between samples. All MPC samples, both before and after dialysis, exhibited significantly higher concentrations of soluble caseins (α_s and β) compared with SM. Reconstituted MPC samples made with acidified milk (UFG and DFG) showed significantly higher concentrations of α_s - and β -CN compared with control MPC samples (UFC and DFC). This would indicate that acidification at pH 6 not only caused calcium release (see Table 1), but extensive solubilization of micellar casein. Whereas β -CN is known to be prone to solubilization, as for example with temperature changes (O'Mahony et al., 2009), this is not the case for α_s -CN, which are associated with the calcium phosphate nano-clusters in the core of the casein micelles (Dalglish and Corredig, 2012).

In the case of β -CN, only the UFC retentates showed a similar area ratio compared with skim milk. Dialysis against milk, whereby soluble calcium increased in the serum phase (Table 1), caused an increase in soluble β -CN even for UFC (see UFC-D compared with UFC). Control DFC showed a higher level of β -CN or whey protein compared with UFC, and again dialysis against milk showed a further increase in the solubilization.

The solubilization of β -CN was higher for the acidified retentates, with UFG significantly higher than UFC, with dialysis against milk showing even higher solubilization. Diafiltration of the acidified retentates showed the highest extent of β -CN solubilization, with DFG showing significantly higher β -CN or whey protein area ratios; in this case, there seemed to be a plateau in the β -CN release. Earlier work on β -CN release with cooling also demonstrated that β -CN release reaches a plateau (Rahimi-Yazdi et al., 2014).

In the case of α_s -CN, all retentate samples showed a higher level compare with skim milk (Figure 1, black

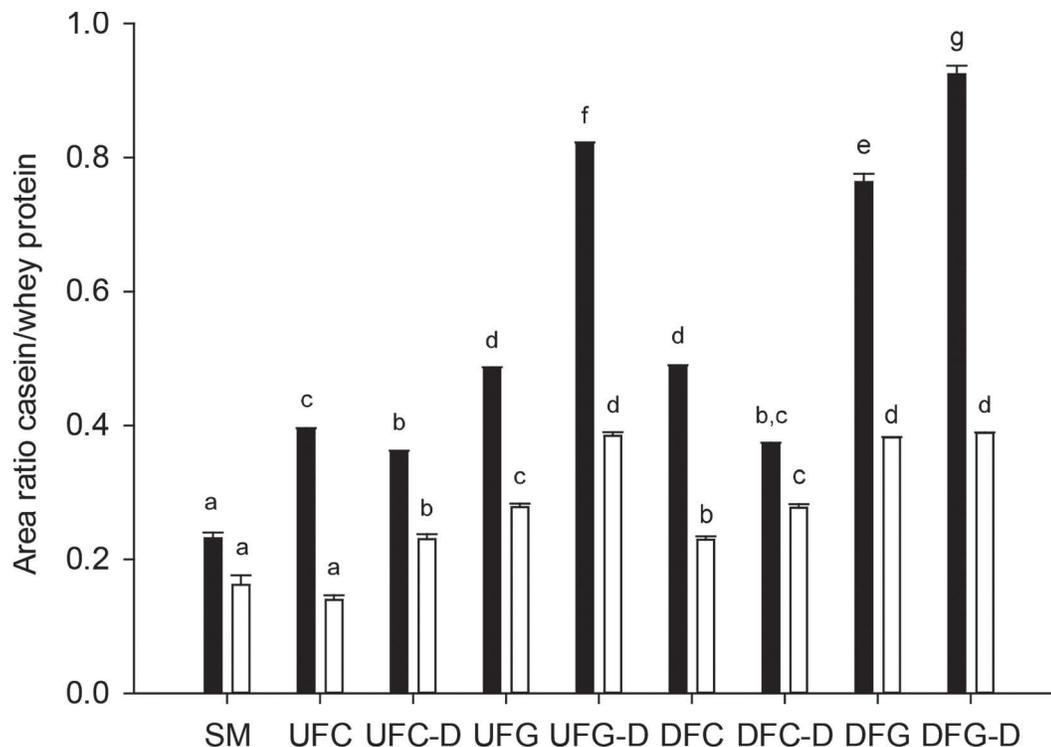


Figure 1. Ratio of α_s -CN (black bars) and β -CN (white bars) to whey protein (as measured by SDS electrophoresis) in the centrifugal supernatant of skim milk (SM) and milk protein concentrate samples reconstituted in water (3.2% protein, wt/wt). UFC = UF control; UFG = UF acidified with glucono- δ -lactone (GDL); DFC = diafiltration (DF) control; DFG = DF acidified with GDL. After dialysis UFC-D, UFG-D, DFC-D, and DFG-D. Letters (a-g) indicate significant differences within casein type as determined by Tukey's honestly significant procedure using $\alpha = 0.05$. Error bars represent standard deviation.

bars). A difference was evident in the behavior of control retentates compared with acidified retentates. Both UFC and DFC showed a higher level of α_s -CN compared with SM, and DFC was higher than UFC, indicating an increase in unsedimentable casein in the retentates, with a higher extent of solubilization with DF. In this case, unlike for β -CN, there was a decrease of α_s -CN after dialysis against SM (in UFC-D and DFC-D samples compared with corresponding UFC and DFC). It was concluded that the additional soluble calcium caused reaggregation of the α_s -CN in these retentates.

Acidified retentates showed a higher level of α_s -CN-to-whey area ratio than the control samples, consistent with a higher level of dissociation in these retentates. The UFG supernatants showed values similar to those of DFC supernatants, and DFG was higher than DFC. Unlike control retentates, in this case dialysis of acidified retentates against SM caused an increase in soluble α_s -CN. These differences would suggest a difference in the behavior of the retentates during further processing, when for example, MCP are added to milk for fortification and standardization of dairy products. It is indeed expected that the concentration of soluble caseins in the samples will be an important factor af-

fecting the chymosin-induced gelation properties of the casein micelles in the reconstituted MPC.

Chymosin-Induced Gelation

The kinetics of CMP release for the various reconstituted MPC were compared with a pasteurized SM sample. The amount of CMP released was calculated relative to the maximum amount of CMP released by the sample once it reached a plateau. It is known that micellar aggregation typically occurs at a CMP release above 85% for milk samples (Dalglish, 1983; Sandra and Dalglish, 2007). Figure 2 illustrates the percent CMP released as a function of time before (Figure 2A) and after (Figure 2B) re-equilibration of the serum phase by dialysis.

After reconstitution (Figure 2A), some initial differences were noted in the rate of CMP release across the various MPC samples, although all samples reached the critical value of 85% CMP release at ~ 70 min after addition of chymosin. Both UF and DF controls showed a lower extent of CMP release, whereas UF and DF acidified showed a significantly higher rate of release. The differences observed in the kinetics of hydrolysis

during the initial stages after addition of the enzyme can be attributed to the differences in pH and soluble calcium present in the reconstituted MPC (Table 1). Although considering that DFG and SM had statistically equivalent pH values (Table 1) yet showed differences in their CMP release, it can be concluded that the differences in the soluble calcium play a major role in this regard. After re-equilibration of the serum phase to concentrations of calcium and pH similar to skim milk (Figure 2B), all treatments showed comparable kinetics of CMP release.

Chymosin-induced aggregation was followed using small amplitude oscillatory stress, and no apparent gel formation occurred in reconstituted MPC samples, albeit all samples showed that 85% CMP release was reached with or without dialysis against milk (see Fig-

ure 2). The only samples, other than SM, that formed a measurable gel were UFC-D and DFC-D. Figure 3 compares the development of the rheological parameters of these reconstituted samples to those of SM. The lack of aggregation of the reconstituted MPC samples can be attributed to the low concentration of soluble calcium present in the samples, as previously demonstrated (Dalglish, 1983; Martin et al., 2010; Sandra et al., 2011). A minimum concentration of 2 mM calcium chloride added to the serum phase is required for the reconstituted MPC to form a gel (Martin et al., 2010). However, the inhibition of gel formation in the acidified MPC samples after reconstitution and dialysis against milk (UFG-D and DFG-D) may not be explained by the low concentration of soluble calcium or differences in pH, as these 2 samples showed a statistically equivalent level of soluble calcium and pH value compared with those of skim milk (see Table 1). The lack of gelation is due to the presence of high concentrations of caseins in the soluble phase, as those contribute to the inhibition of the secondary stage of casein aggregation (Gaygadzhev et al., 2012).

Table 2 summarizes the statistical differences for samples represented in Figure 3. It is important to note that all samples contained similar protein concentration, as well as soluble calcium and pH. The gelation time (defined as the point $\tan \delta = 1$) was much shorter in skim milk compared with UFC-D and DFC-D. The values of $\tan \delta$ at the plateau were not significantly different across the 3 treatments, indicating similarities in the type of linkages in the gel, although with different stiffness (modulus) values, as previously shown (Sandra et al., 2011). A delayed onset of G' (storage modulus) development and $\tan \delta$ plateau was observed for the reconstituted MPC samples compared with SM. Clearly, DF samples showed a further delay compared with UF controls.

Previous reports have discussed the effect of calcium concentration (in both soluble and insoluble phases) and the amount of sedimentable (micellar) casein in chymosin-induced gelation of milk (Udabage et al. 2001; Philippe et al., 2003). It has also been demonstrated (Gaygadzhev et al., 2012; Sandra and Corredig, 2013) that chymosin-induced gelation of casein micelles is delayed by the presence of soluble caseins. Soluble calcium is key to the aggregation stage of chymosin-induced gelation; hence, it needs to be closely analyzed to demonstrate the effect of soluble caseins on casein gelation. In Figure 4 and Table 3, the concentration of soluble calcium was measured as both soluble as unsedimentable by centrifugation and permeable (i.e., not associated with protein). All gels prepared with reconstituted retentates were analyzed for soluble and permeable calcium after 70 min from chymosin addi-

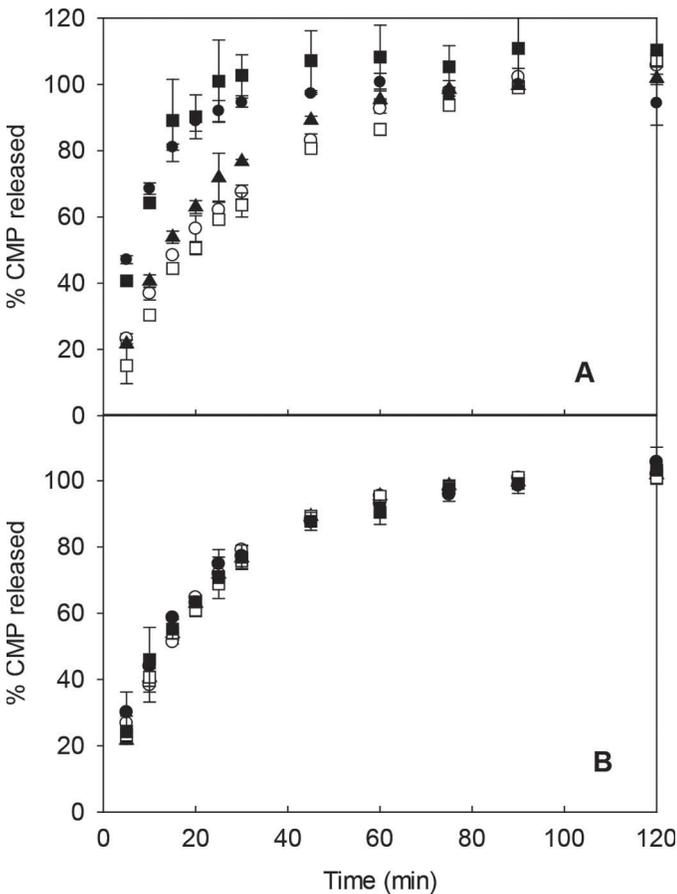


Figure 2. Amount of caseinomaclopeptide (CMP) released as a function of time skim milk (SM; ▲) and milk protein concentrate samples reconstituted (3.2% protein, wt/wt) (A) and after dialysis against skim milk (B). UFC = UF control (○); UFG = UF acidified with glucono- δ -lactone (GDL; ●); DFC = diafiltration (DF) control (□); DFG = DF acidified with GDL (■). Values are the average of at least 2 independent experiments and error bars represent standard deviation. The plateau value is calculated as the maximum area of CMP released.

Table 2. Summary of rheological parameters (means \pm SD) measured during gelation for skim milk (SM) and milk protein concentrates reconstituted in water at 3.2% (wt/wt), equal protein, and dialyzed against milk¹

Item	SM	UFC-D	DFC-D
Gelation time (min)	21 \pm 0.7 ^a	38 \pm 1.5 ^b	49 \pm 4 ^c
G' (storage modulus) 70 min after chymosin addition (Pa)	52 \pm 14 ^a	5.8 \pm 1.6 ^b	2.2 \pm 0.3 ^c
Tan δ value at plateau	0.25 \pm 0.01 ^a	0.26 \pm 0.01 ^a	0.26 \pm 0.02 ^a

^{a-c}Within a row, different superscript letters indicate statistical differences for $P < 0.05$.

¹UFC-D = UF control; DFC-D = DF control. All other treatments did not show gelation. Gelation time was defined as tan $\delta = 1$. Values are the average of 2 independent experiments with standard deviation.

tion and compared with those prepared with skim milk. We have clearly shown that, although the amount of soluble calcium was similar in all reconstituted retentates, the amount of permeable calcium changed in the reconstituted samples before dialysis against milk. After dialysis, both soluble and permeable calcium were

similar among the treatments in spite of the fact that only UFC-D and DFC-D showed the formation of a gel; DFC-D showed a modulus $G' > 1$ Pa. Skim milk had a significantly higher soluble and permeable calcium compared with the reconstituted retentates at 70 min after addition of chymosin. It is possible to assume that some of the calcium may become associated with the chymosin-modified caseins, as the initial soluble calcium in UF milk was comparable to that of SM. The results clearly demonstrated that the inhibition of aggregation can be attributed to the higher level of soluble caseins.

CONCLUSIONS

Partial acidification (to pH 6.0) of milk with GDL before membrane filtration modified the serum composition and compromised the integrity of the supramolecular structure of the casein micelles. The changes in the serum composition depend on the history before reconstitution, but also on the environmental conditions used during reconstitution. This has important implications in dairy processing, by demonstrating that the integrity of the casein micelles will depend on the environment in which retentate powders were reconstituted. The gelation properties of reconstituted MPC powders were negatively affected by the presence of soluble casein, and positively affected by the amount of both soluble and insoluble calcium present after reconstitution. Under these conditions, although there seemed to be sufficient CMP release to cause instability to the micelles, rearrangements must occur that modify the colloidal properties of the casein micelles and κ -CN is no longer critical to the stabilization of the polyelectrolyte layer. It has been previously proposed that in the presence of a sufficient amount of soluble caseins and calcium, after hydrolysis of κ -CN, the soluble caseins associate with the reactive surface of the casein micelles, inhibiting aggregation of these protein particles (Gaydadjev et al., 2012; Sandra and Corredig, 2013). This study provided further evidence of the shielding effect of soluble caseins on the active

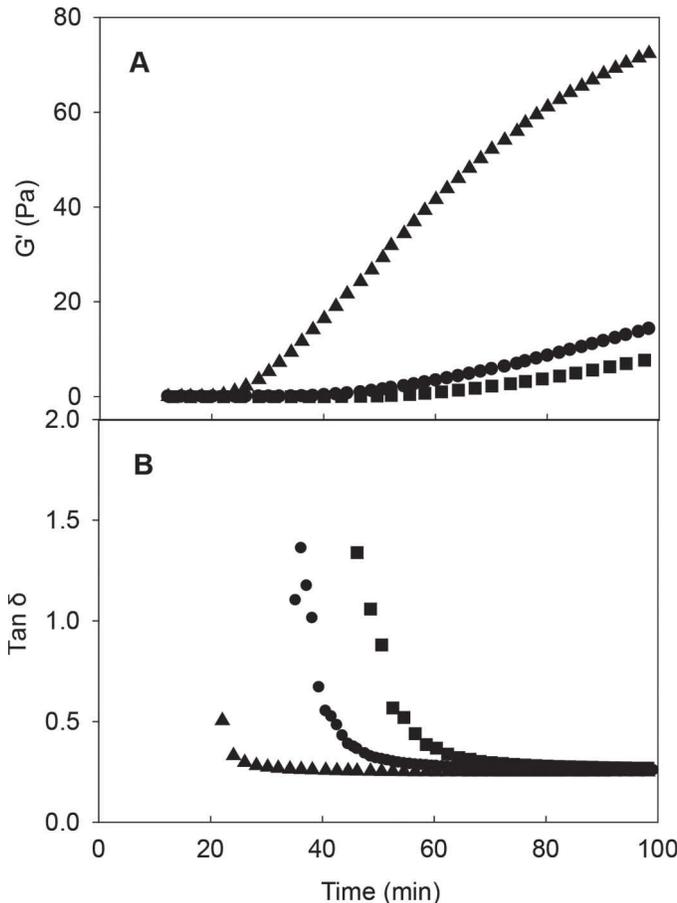


Figure 3. Development of G' (storage modulus; A) and Tan δ (B) measured by small amplitude oscillatory stress as a function of time after addition of chymosin for skim milk (SM; \blacktriangle) and milk protein concentrate samples reconstituted (3.2% protein, wt/wt). UF control after dialysis = \bullet ; diafiltration control after dialysis = \blacksquare . Runs are representative of 2 different replicates.

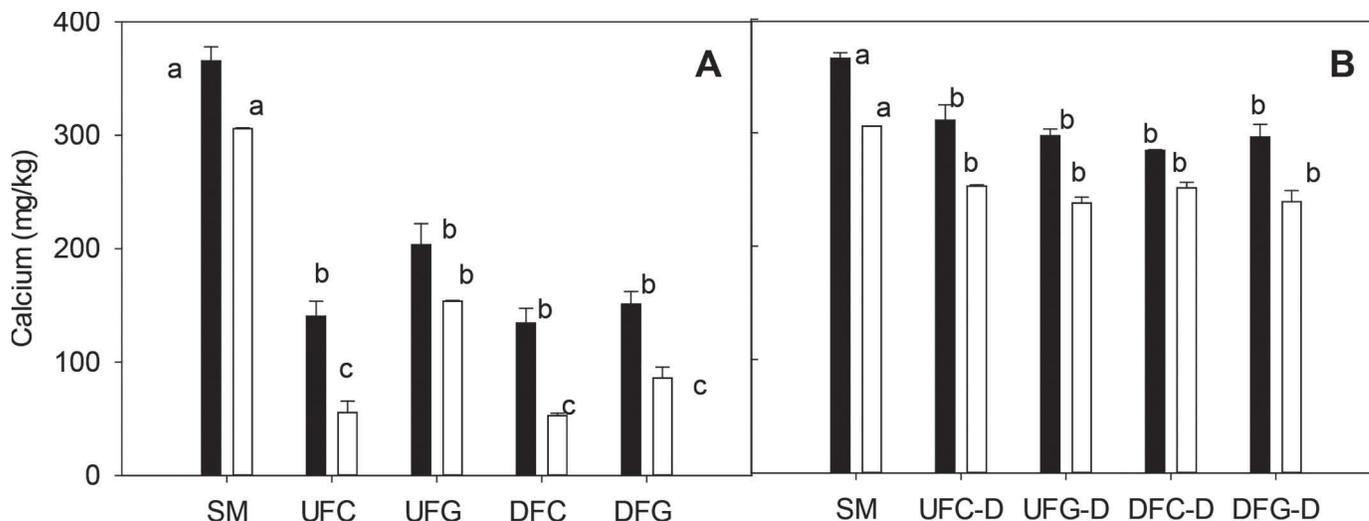


Figure 4. Soluble (black bar) and permeable (white) calcium concentration measured 70 min after chymosin addition for skim milk (SM) and milk protein concentrates reconstituted (3.2% protein, wt/wt) (A) and the same samples after dialysis against skim milk (B). UFC = UF control; UFG: UF acidified with glucono- δ -lactone (GDL); DFC = diafiltration (DF) control; DFG = DF acidified with GDL. Different letters (a–c) indicate significant differences at $P < 0.05$. Error bars represent standard deviation of 2 independent experiments.

sites of casein micelles, responsible for the disruption of the secondary stage of chymosin-induced gelation in the acidified MPC.

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Table 3. Values for soluble and permeable calcium concentrations (means \pm SD) measured 70 min after chymosin addition for skim milk (SM) and milk protein concentrate reconstituted in water (3.2% wt/wt), equal protein, and the same samples after dialysis against SM¹

Sample	Soluble calcium (mg/kg)	Permeable calcium (mg/kg)
SM	365.52 \pm 12.58 ^a	305.80 \pm 0.84 ^a
UFC	140.26 \pm 13.41 ^b	55.44 \pm 10.11 ^c
UFG	203.51 \pm 18.60 ^b	153.72 \pm 0.73 ^b
DFC	134.30 \pm 13.06 ^b	52.71 \pm 2.06 ^c
DFG	151.20 \pm 11.14 ^b	85.89 \pm 9.70 ^c
SM	365.52 \pm 12.58 ^a	305.80 \pm 0.84 ^a
UFC-D	310.99 \pm 13.64 ^b	252.99 \pm 1.12 ^b
UFG-D	297.46 \pm 5.80 ^b	238.02 \pm 5.20 ^b
DFC-D	284.29 \pm 0.84 ^b	251.33 \pm 5.03 ^b
DFG-D	296.18 \pm 11.40 ^b	239.29 \pm 9.70 ^b

^{a–c}Within a column and either before or after dialysis, superscript letters indicate significant differences at $P < 0.05$.

¹UFC = UF control; UFG = UF acidified with glucono- δ -lactone (GDL); DFC = diafiltration (DF) control; DFG = DF acidified with GDL. After dialysis: UFC-D, UFG-D, DFC-D, and DFG-D.

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