Effect of Insoluble Calcium Concentration on Rennet Coagulation Properties of Milk

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ABSTRACT

Rennet-induced gels were made from milk acidified to various pH values or milk at pH 6.0 that had added EDTA. The objective was to examine the effect of removing insoluble Ca (INS Ca) from casein micelles (CM) on rennet gelation properties. For the pH trial, diluted lactic acid was added to reconstituted skim milk to decrease the pH to 6.4, 6.0, 5.8, 5.6, and 5.4. For the EDTA trial, EDTA was slowly added (0, 2, 4, and 6 mM) to reconstituted skim milk, and the final pH values were subsequently adjusted to pH 6.0. Dynamic low amplitude oscillatory rheology was used to monitor gel development. The Ca content of CM and rennet wheys made from these milks was measured using inductively coupled plasma spectroscopy. The INS Ca content of milk was altered by the acidification pH values or level of EDTA added. In all samples, the storage modulus (G') exhibited a maximum (GM), with a decrease in G' during longer aging times. Gels made at pH 6.4 had higher GM compared with gels made at pH 6.7 probably due to the reduction in electrostatic repulsion, whereas the INS Ca content only slightly decreased. The highest GM value of gels was observed at pH 6.4 and the GM value decreased with decreasing pH from 6.4 to 5.4. This was due to an excessive loss of INS Ca from CM. There was a decrease in GM with the increase in the concentration of added EDTA, which was probably due to the loss of colloidal calcium phosphate, which weakens the integrity of CM. Loss tangent (LT) values at GM increased with a reduction in milk pH and the addition of EDTA to milk. Rennet gels at the point of the GM were subjected to constant low shearing to fracture the gels. With a reduction in INS Ca content, the yield stress decreased, whereas LT values increased indicating a weaker, more flexible casein network. Microstructure of rennet-induced gels near the GM point and 2 to 10 h after this point was studied using fluores-

cence microscopy. At GM, gels made from milk acidified to pH 6.4 exhibited more branched, interconnected networks, whereas strands and clusters became larger with a reduction in milk pH to 5.4. Gels made from milk with EDTA added had more finely dispersed protein clusters compared with gels made from milk with no EDTA added. These microscopic observations supported the effect of loss of INS Ca on GM and LT. There was a decrease in apparent interconnectivity between strands in gel microstructure during aging, which agreed with the decrease in G' after GM. It can be concluded that low levels of solubilization of INS Ca and the decrease in milk pH resulted in an increase in GM. With greater losses of INS Ca there was excessive reduction in cross-linking within CM, which resulted in weaker, more flexible rennet gels. This complex behavior cannot be explained by adhesive hard sphere models for CM or rennet gels made from these CM.

Key words: casein micelle, insoluble calcium, rheology, rennet coagulation

INTRODUCTION

Gelation of caseins is the first key step in cheese manufacture. The addition of chymosin to the milk initiates the destabilization of casein micelles (CM) via hydrolysis of κ -CN, which is the first phase of rennet coagulation (Dalglish, 1992). Once a sufficient degree of destabilized paracasein micelles are produced, aggregation begins, which is the second phase (Hyslop, 2003), and this leads to the formation of a 3-dimensional space-filling gel, which is the third phase (Horne and Banks, 2004). In contrast to the first and second stages of rennet coagulation, there have been fewer studies on the third stage of curd development. Rennet-induced milk gels are viscoelastic, and their dynamic rheological properties can be characterized using both the viscous and elastic components. In general, these 2 shear moduli sigmoidally increase with time during rennet curd formation.

The native CM has been treated as an adhesive hard sphere particle (de Kruif, 1998). In the hard sphere model, stability and instability properties of native CM

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are ascribed to the hairy layer ("brush") of κ -CN on their surface. This model has been used to quantitatively model the viscosity of renneted skim milk as a function of time where it goes through a minimum and then an increase in viscosity before a gel is formed. However, the hard sphere model is thermodynamic in origin and it has no inherent time scale. It is not able to predict behavior after the gel point; for example, dynamics of network formation. Its limitations were well documented in acidified milk gel formation; that is, it cannot take into account the importance of the internal structure of CM on gel properties (Horne, 2003).

In the dual-binding model for the structure and formation of CM (Horne, 1998), individual CN molecules are considered as block copolymers. Micellar assembly is viewed as a polymerization process occurring as a result of hydrophobic interactions or by bridging via insoluble calcium (INS Ca) phosphate (Horne, 1998). The balance between electrostatic repulsion and attractive hydrophobic interaction controls the degree of incorporation of individual CN molecules in the assembly of CM. Thus, the dual-binding model for CM views INS Ca as a key bridging material. It is well known that INS Ca, or colloidal calcium phosphate as it is often called, is solubilized as milk pH decreases (Dalglish and Law, 1989). It can therefore be predicted that the loss of INS Ca from within CM should modify the internal structural integrity and alter the mechanical properties of gels made from these modified CM. This aspect is quite relevant for textural properties of cheese made from milk renneted at lower pH; that is, cheese made from direct acidified milk (Choi et al., 2004).

Rheological properties of rennet-induced gels have been extensively studied usually at the normal milk pH (Horne, 1995; Esteves et al., 2001; Udabage et al., 2001; Srinivasan and Lucey, 2002), and also at lower pH values (van Hooydonk et al., 1986; Zoon et al., 1989; Roefs et al., 1990; Mellema et al., 2000, 2002; Vetier et al., 2000). Nonetheless, the effect of INS Ca in rennet gelation properties has not been extensively studied and the effect of altering renneting pH and loss of INS Ca from CM on rennet gelation properties have not been interpreted in the context of the new dual-binding model for CM.

It is our hypothesis that modifications of internal micellar interactions could affect the gelation properties of rennet-induced gels. The objective of this study was to examine the effect of removing INS Ca from CM on the rennet gelation properties of skim milk. Two approaches were used. In the first, the INS Ca content of CM was altered by acidification of milks with diluted lactic acid. However, because pH and INS Ca content vary in these samples, we did another treatment where

pH was constant (~6.0) and EDTA was used to chelate Ca.

MATERIALS AND METHODS

Materials

Low-heat skim milk powder was supplied by Dairy Farmers of America (Fresno, CA). Soybean trypsin inhibitor, type I-S (T 9003), and acridine orange were purchased from Sigma Chemical Co. (St. Louis, MO). Commercial double-strength fermentation-produced chymosin (Chymostar) was supplied by Rhodia Inc. (Madison, WI). Lactic acid (88% wt/wt) was supplied by Chr. Hansen (Milwaukee, WI); EDTA and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were purchased from Fisher Scientific (Fairlawn, NJ).

Preparation of Milk Samples

Low-heat skim milk powder was reconstituted in demineralized water to 8.7% (wt/wt) solids, and stirred at room temperature for 30 min; NaN_3 (0.2 mg/mL) and soybean trypsin inhibitor (0.15 mg/mL) were added to prevent bacterial growth and inhibit plasmin activity, respectively. The milk solutions were equilibrated by stirring at 32°C for 2 h using a magnetic stirring unit, and immediately cooled to ~4°C. For the experiments in which skim milk solutions were acidified (i.e., the pH trial), predetermined amounts of diluted lactic acid (1:4, acid:water) were slowly added to milk samples to decrease the pH of milk to 6.4, 6.0, 5.8, 5.6, and 5.4; about half the estimated total amount of lactic acid that was required was initially added and the remaining acid was slowly added until the desired pH was reached. For the addition of EDTA to cold (~4°C) milk (i.e., EDTA trial), the EDTA was slowly added to obtain final concentrations of 0, 2, 4, and 6 mM EDTA, and the milk was stirred for 1 h. Predetermined amounts of diluted lactic acid were slowly added to milks that had added EDTA to have all samples at pH 6.0. All milk samples were then warmed to 32°C, and their pH values were rechecked and adjusted to be within ± 0.05 of the desired pH values. Milk samples were stored at 4°C for about 6 h to allow for equilibration. To help reduce the gelation time, 50 $\mu\text{g/mL}$ of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was added at the time that NaN_3 was added for the pH trial, and 120 $\mu\text{g/mL}$ of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was added 30 min before the addition of rennet for the EDTA trial.

Chemical Analyses

Total solids, fat, CN, and total protein were determined as described by Marshall (1992). Soluble Ca in milk samples was defined as Ca content in rennet whey $\times$ correction factor (0.998) for the volume of CN precipi-

Table 1. Chemical composition of reconstituted skim milks acidified to different pH values

	pH of acidification						SEM ¹	LSD ²
	6.7	6.4	6.0	5.8	5.6	5.4		
Total solids, %	8.76 ^{ab}	8.71 ^{ab}	8.78 ^{ab}	8.82 ^a	8.69 ^b	8.69 ^b	0.04	0.12
Fat, %	0.09 ^a	0.0 ^a	0.08 ^a	0.07 ^a	0.07 ^a	0.07 ^a	0.01	0.05
Total protein, %	3.08 ^a	3.09 ^a	3.09 ^a	3.10 ^a	3.08 ^a	3.08 ^a	0.02	0.08
Casein, %	2.41 ^a	2.44 ^a	2.43 ^a	2.45 ^a	2.43 ^a	2.43 ^a	0.02	0.08
Total Ca, mg/100 mL	99.8 ^{ab}	97.9 ^c	100.2 ^a	99.4 ^{ab}	99.0 ^{bc}	98.3 ^c	0.3	1.04
Insoluble Ca, mg/100 mL	70.4 ^a	66.0 ^b	45.9 ^c	34.9 ^d	21.9 ^e	11.9 ^f	1.0	3.25
Soluble Ca, mg/100 mL	29.4 ^a	31.9 ^a	54.2 ^b	64.4 ^c	77.0 ^d	86.4 ^e	1.0	3.34

^{a-f}Means with different superscript letters within the same row are significantly different ($P < 0.05$).

¹SEM = standard error of the mean.

²LSD = least significant difference.

tate; insoluble Ca = total Ca in milk – soluble Ca in milk. For Ca analysis, ashed samples were solubilized with 25% HNO₃ and diluted to 1% HNO₃ with double-deionized water. Concentrations of Ca were quantified by inductively coupled argon plasma emission spectroscopy (model Varian Vista-Pro AX, Varian Australia Pty Ltd., Clayton, Victoria, Australia). Wavelength used for Ca analysis was 317.9 nm.

Rheological Properties

A universal dynamic spectrometer (Paar Physica UDS 200, Physica Messtechnik GmbH, Stuttgart, Germany) was used to determine the rheological properties of rennet gels. During deformation, the nondestructive rheological properties can be determined by low amplitude dynamic oscillation with the measurement of the storage modulus (**G'**) and loss tangent (**LT**), which is the ratio of viscous to elastic properties. The cup and bob measuring geometry consisting of 2 coaxial cylinders (one of external diameter 25.0 mm, the other of internal diameter 27.5 mm) was used. Treated milk samples that had been stored at 4°C for ~6 h were warmed to 40°C for 1 h, cooled to 32°C, and held for 15

min at 32°C. Then, 43.1 µL of diluted rennet (1:120 for pH trial; 1:75 for EDTA trial) was added to 20 mL of milk. After addition of rennet to milk, the mixture was stirred for 2 min and 12.75 mL of milk sample was transferred to the measuring geometry of the rheometer. Vegetable oil was placed on the surface to prevent evaporation. Samples were oscillated at a frequency of 0.1 Hz, strain applied was 0.05%, and measurements were taken every min until gels attained their maximum **G'** (**GM**). The large deformation properties of rennet gels made in situ were also determined. When gels attained their GM, a constant shear rate of 0.01 s⁻¹ was applied to the gel. Yield stress was defined as the point when the shear stress started to decrease. The constant shear rate technique for determining an apparent yield stress and shear deformation at yielding has been described previously (Lucey et al., 1997).

Fluorescence Microscopy

Acridine orange (0.2% wt/vol), dissolved in water, was used as fluorescent protein dye. Three hundred microliters of acridine orange was added to 50 mL of milk sample and mixed for 15 min. Diluted rennet was added

Table 2. Chemical composition of reconstituted skim milk with different EDTA concentrations added to milk at pH 6.0

	Normal milk (pH 6.7)	EDTA concentration (mM)				SEM ¹	LSD ²
		0	2	4	6		
Total solids, %	8.62 ^c	8.67 ^{bc}	8.76 ^{abc}	8.82 ^{ab}	8.84 ^a	0.04	0.15
Fat, %	0.08 ^a	0.08 ^a	0.07 ^a	0.08 ^a	0.09 ^a	0.01	0.04
Total protein, %	3.06 ^c	3.04 ^c	3.08 ^{bc}	3.11 ^{ab}	3.14 ^a	0.01	0.04
Casein, %	2.39 ^a	2.40 ^a	2.40 ^a	2.41 ^a	2.42 ^a	0.01	0.04
Total Ca, mg/100 mL	98.3 ^a	97.2 ^a	98.6 ^a	95.3 ^a	96.4 ^a	1.4	4.69
Insoluble Ca, mg/100 mL	74.1 ^a	48.3 ^b	43.6 ^b	30.5 ^c	20.6 ^d	2.4	7.72
Soluble Ca, mg/100 mL	24.1 ^d	48.8 ^c	55.0 ^c	64.8 ^b	75.8 ^a	2.5	8.07

^{a-d}Means with different superscript letters within the same row are significantly different ($P < 0.05$).

¹SEM = standard error of the mean.

²LSD = least significant difference.

with stirring for 2 min. A few drops of the mixture were transferred to a concave slide and then incubated at 32°C for the predetermined time. The microstructure of rennet-induced gels was observed using a fluorescence microscope (Axioskop 40, Carl Zeiss Light Microscopy, Gottingen, Germany) equipped with motorized stage (z-drive) (Axioskop z mot plus, Carl Zeiss Inc., New York, NY). A series of images from different positions in the gel matrix were acquired with the aid of z-stack module (AxioVision 3.1, Carl Zeiss Vision GmbH, Munchen-Hallbergmoos, Germany). To eliminate out-of-focus light in fluorescence microscopy, the nearest-neighbor algorithm as a 3-dimensional deconvolution was applied (Verveer et al., 1999; Schaefer et al., 2001). This algorithm is based on a simple 2-dimensional subtraction of the out-of-focus information, which is applied to all image planes in the z-stack (Carl Zeiss Vision, 2002).

Statistical Analysis

An ANOVA was carried out using the SAS program (SAS Institute, 2001) to see if there were effects of acidification pH values or addition of EDTA on milk and mineral composition, and on rheological properties. The differences in least squares means were determined using LSD. Significance was established at $P < 0.05$.

RESULTS

Milk Composition

Because milk composition, including fat and CN contents, is well known to affect coagulation properties of milk (Choi and Ng-Kwai-Hang, 2003), reconstituted skim milks were used in this study; the composition of the milk is shown in Tables 1 and 2. As expected, no significant ($P > 0.70$) differences in fat and CN contents were found in the various samples used for the pH and EDTA trials. The slightly lower CN content in the EDTA trials compared with the pH trials suggests that the total Ca content of milk in the EDTA trial would be expected to be slightly lower than that in pH trial, which was confirmed in Table 2. The N content of EDTA is about 7.52%, and its contribution to the total protein content in EDTA trial was estimated to be about 0.035, 0.071, and 0.107 g of protein per 100 mL of milk for 2, 4, and 6 mM EDTA, respectively. The contribution of EDTA to total protein (Table 2) was not taken into consideration in the results shown in Table 2. The CN content in the EDTA trial was determined by assuming that N in EDTA behaved as non-CN nitrogen. Dilution effects on the total solids by the addition of lactic acid in the pH trial (Table 1) and contribution by the addition of

Table 3. Mean squares and probability for rheological properties of rennet-induced gels

Source	df	GM ¹	LT ¹	σ_{yield} ¹
pH values	5	2,442.1**	0.12**	1,480**
Error	12	3.2	0.0003	1.7
EDTA concentrations	4	1,333**	0.025**	1,148**
Error	10	18.85	0.0001	33.48

¹GM = maximum storage modulus; LT = loss tangent; σ_{yield} = yield stress.

** $P < 0.0001$.

EDTA to total solids (Table 2) were not taken into account.

Solubilization of Insoluble Ca from Casein Micelles

Tables 1 and 2 show the degree of solubilization of Ca from CM into the serum phase as a function of milk pH or concentration of added EDTA. It should be noted that the total Ca contribution from Ca chloride was estimated to be 1.36 mg/100 g of milk in the pH trial and 3.27 mg/100 g of milk in the EDTA trial. Total Ca content of milk was similar in both trials. As expected, acidification of milk samples and the addition of EDTA had statistically significant ($P < 0.01$) effects on the INS Ca content. For the pH trial, the INS Ca content of milk at pH 6.7 was 70.4 mg/100 mL and the INS Ca content decreased to 11.9 mg/100 mL in milk at pH 5.4. The INS Ca content of milk at pH 6.0 with no added EDTA was 48.3 mg/100 mL and the INS Ca content decreased to 20.6 mg/100 mL with the addition of 6 mM EDTA.

Small Deformation Rheological Properties of Rennet-Induced Gels

The effects of different pH values of milk (Table 3) or the various concentrations of EDTA added to milk (Table 3) on the GM values of rennet gels were found to be highly significant ($P < 0.001$). The G' profiles of milk samples acidified to different pH values and milks with different concentrations of EDTA are shown in Figures 1a and 2a, respectively. In all samples, it was observed that the G' profile exhibited a maximum and thereafter, decreased (data not shown), presumably because of a reaction such as microsyneresis (Roefs et al., 1990). This decrease in G' after the GM occurred sooner in low pH samples. The GM values at pH 6.7, 6.4, 6.0, 5.8, 5.6, and 5.4 were 69, 75, 57, 41, 16, and 6 Pa, respectively (Figure 1a). The GM values of gels made from normal milk at pH 6.7 were slightly lower than those of gels at pH 6.4 ($P < 0.01$). This result is in agreement with that of Zoon et al. (1989) and Mellema et al. (2000). The GM value of rennet gels was highest

Figure 1. a) Storage modulus (G') and b) loss tangent (LT) as a function of time for rennet-induced milk gels made from milk acidified to pH 6.7 (■), 6.4 (□), 6.0 (▼), 5.8 (▽), 5.6 (●), and pH 5.4 (○). Results are means of triplicates with error bars for standard deviation.

at pH 6.4 and decreased with decreasing pH from 6.4 to 5.4 ($P < 0.01$), which agrees with results of Zoon et al. (1989) and Vetier et al. (2000). The gelation time (Figure 1a), defined as the point when gel had a G' of ≥ 0.1 Pa, of milk samples at pH 6.7, 6.4, 6.0, 5.8, 5.6, and 5.4 were 243, 148, 87, 70, 49, and 34 min, respectively (Figure 1a), and increased with an increase in milk pH. This is in agreement with other reports (Zoon et al., 1989). The GM values of gels made from normal milk

at pH 6.7 and milks at pH 6.0 with 0, 2, 4, and 6 mM EDTA were 76, 56, 42, 48, and 18 Pa, respectively (Figure 2a). For both trials, the gels made from normal milk at pH 6.7 had slightly different GM values (76 and 69 Pa in the EDTA and pH trials, respectively) presumably due to the slightly different CaCl_2 concentrations and rennet concentrations used in the 2 trials. The GM value for normal milk at pH 6.7 was higher compared with all the EDTA-treated milks ($P < 0.01$). This was

Figure 2. a) Storage modulus (G') and b) loss tangent (LT) as a function of time for rennet-induced milk gels made with different EDTA levels and subsequently adjusted to pH 6.0; normal milk at pH 6.7 (■), and milk at pH 6.0 with 0 (▼), 2 (▽), 4 (●), and 6 mM (○) added EDTA. Results are means of triplicates with error bars for standard deviation.

probably due to the loss of CCP crosslinks by EDTA. Milk samples with 4 mM EDTA added had slightly higher (but not significant, $P > 0.12$) GM values than milks with 2 mM EDTA. The lowest GM values among the milk samples with added EDTA were obtained in the milks with 6 mM EDTA. The gelation time (Figure 2a) for milk samples at pH 6.0 with 0, 2, 4, and 6 mM EDTA added was 15, 21, 170, and 615 min, respectively.

The gelation time increased with an increase in the concentration of added EDTA. This was probably due to lower Ca^{2+} activity even after CaCl_2 was added, because Ca^{2+} is critical for the aggregation of rennet-altered CM (Lucey and Fox, 1993).

The acidification of milk to different pH values or the concentration of EDTA added to milk had a highly significant effect ($P < 0.001$) on the LT values of rennet-

induced gels at GM (Table 3). The LT profiles as a function of time for the pH trial are shown in Figure 1b. The LT values decreased at gelation and thereafter remained constant or slightly increased. The LT values after gelation increased with decreasing pH. The LT values of rennet-induced gels at GM for milks acidified to pH 6.7, 6.4, 6.0, 5.8, 5.6, and 5.4 were 0.35, 0.35, 0.41, 0.46, 0.59, and 0.89, respectively (Figure 1b). These LT values were significantly ($P < 0.01$) different from one another except the LT values for gels at pH 6.7 and 6.4 ($P > 0.74$). The LT values after gelation in gels made from normal milk at pH 6.7 and in gels made from milk at pH 6.0 with 0, 2, 4, and 6 mM EDTA were 0.35, 0.41, 0.45, 0.53, and 0.58, respectively (Figure 2b). These LT values were significantly ($P < 0.01$) different.

Large Deformation Rheological Properties of Rennet-Induced Gels

Altering milk pH values or adding various concentrations of EDTA to milk had a highly significant effect on yield stress ($P < 0.01$) of rennet-induced gels as can be seen from the ANOVA results (Table 3). Shear stress profiles as a function of strain for rennet gels with different pH values are shown in Figure 3a. Yield stress values in rennet gels made with pH 6.7, 6.4, 6.0, 5.8, 5.6, and 5.4 were 45, 54, 49, 35, 7, and 2 Pa, respectively. With a reduction in pH from 6.4 to 5.4, there was large decrease in yield stress from 54 to 2 Pa ($P < 0.01$). Yield stress of rennet gels made from milk at pH 6.7 were lower than those at pH 6.4 and 6.0, and higher than those at pH 5.8, 5.6, and 5.4. The shear stress profiles for rennet gels made from milk at pH 6.0 with EDTA added are shown in Figure 3b. The yield stress of gels made from normal milk (pH 6.7) was 54 Pa, and the corresponding values in gels made at pH 6.0 with 0, 2, 4, and 6 mM EDTA were 56, 40, 25, and 10 Pa, respectively. There was no significant ($P > 0.62$) difference in yield stress of gels made from milks at pH 6.7 or pH 6.0.

Microstructure

The micrographs of rennet-induced gels made with different milk pH values and levels of added EDTA are presented in Figures 4 and 5, respectively. Gels were examined at their GM and also several hours after the GM to understand possible structural changes during aging (i.e., microsyneresis). Microstructures observed at GM for pH trial are shown in Figures 4a, b, c, g, h, and i, and microstructures observed after GM (~2 to 6 h) in Figures 4d, e, f, j, k, and l. Larger pores were seen as the pH decreased from 5.8 (Figure 4g) to 5.4 (Figure 4i). The micrographs for gels examined at the GM for

the EDTA trial are shown in Figures 5a, b, c, g, and h and examined after GM (~3 to 10 h) are shown in Figure 5d, e, f, i, and j. In gels made from milk with EDTA added, there were more finely dispersed protein clusters and thinner strands (Figure 5c, g, and h) compared with gels made from milk with no EDTA added (Figure 5a and b). Micrographs obtained several hours after GM shows that considerable macroscopic changes had occurred compared with gels observed at GM. For both trials the apparent pore size of gels at GM became larger with aging.

DISCUSSION

The rennet gelation behavior of milk gels is influenced by many variables including pH and the INS Ca content. In this study we investigated the influence of reducing the INS Ca content at constant pH and when pH was allowed to vary. The effect of the loss of INS Ca on the properties of CM can be viewed in the context of the dual-binding model for CM (Horne, 1998). Attractive hydrophobic interactions, Ca phosphate crosslinks, and electrostatic interactions are the main forces that help maintain the internal stability of CM. Horne (1998) proposed that electrostatic repulsion and hydrophobic interactions can be viewed as being in balance; that is, if electrostatic repulsion decreases, then the effects of hydrophobic interactions are felt as an increase in attraction between CN residues. Modification of the attractive or repulsive interactions modulates CN interactions and micelle integrity (Horne, 1998). Therefore, the loss of INS Ca from within CM should reduce the number of Ca phosphate crosslinks, and should modify the localized balance between hydrophobic interaction and electrostatic repulsion. It is also possible that the loss of INS Ca exposes charged phosphoserine groups that would increase the local electrostatic repulsion between CN (Lucey et al., 2003). It was our hypothesis that modifications of internal CM interactions could affect the gelation properties of rennet-induced gels.

Significant amounts of INS Ca content were dissolved from CM with a reduction in pH (Table 1), and by the addition of EDTA to milk (Table 2), which is in agreement with many previous studies (Dalglish and Law, 1989). The highest GM of rennet-induced gels made at different pH values occurred at pH 6.4, not pH 6.7 (Figure 1a). Although there was only a small amount loss of INS Ca during acidification of milk from 6.7 to pH 6.4 (Table 1), there was a substantial reduction in electrostatic repulsion due to the reduction in zeta potential (Walstra and Jenness, 1984). In this pH range it appears that the reduction in electrostatic repulsion more than compensated for the small loss of INS Ca,

Figure 3. Shear stress as a function of applied deformation (strain) at a constant shear rate ($\sim 0.01 \text{ s}^{-1}$) for a) rennet-induced milk gels made from milk acidified to pH 6.7 (■), 6.4 (□), 6.0 (▼), 5.8 (▽), 5.6 (●), and pH 5.4 (○); b) rennet gels made with different EDTA levels and subsequently adjusted to pH 6.0; normal milk at pH 6.7 (■), and milk at pH 6.0 with 0 (▼), 2 (▽), 4 (●), and 6 mM (○) added EDTA. Results are means of triplicates with error bars for standard deviation.

resulting in milk at pH 6.4 having the highest GM value (Figure 1a). Therefore, this can be viewed as having the net interaction balance shifted in favor of enhanced attractive hydrophobic interactions. The GM values of gels decreased as milk pH values were reduced from 6.4 to 5.4. This was probably due to extensive loss of INS Ca (Table 1), which reduced the number of attrac-

tive INS Ca crosslinks. This trend was confirmed in rennet gels made from milk that had EDTA added and in which the milk pH values were all adjusted to pH 6.0. This addition of EDTA caused the loss of attractive INS Ca bridges, which weakened the gels. As a result of the decreased attractive interaction and possibly increased electrostatic repulsion (although this might be

Figure 4. Microstructure of rennet-induced gels made from milk at different acidification pH values examined at the time of the maximum value for storage modulus (G'), milk at pH 6.7 (a), 6.4 (b), 6.0 (c), 5.8 (g), 5.6 (h), and 5.4 (i), and gels examined between 2 and 6 h after this maximum in G' , milk at pH 6.7 (d), 6.4 (e), 6.0 (f), 5.8 (j), 5.6 (k), and 5.4 (l). The protein matrix is white and pores are dark. Scale bar = 50 μm .

Figure 5. Microstructure of rennet-induced gels made with different EDTA concentrations examined at the time of maximum value for the storage modulus (G'), milk at pH 6.7 (a), and milk at pH 6.0 with 0 (b), 2 (c), 4 (g), and 6 mM (h) added EDTA, and gels examined between 3 and 10 h after this maximum in G' , milk at pH 6.7 (d), and milk at pH 6.0 with 0 (e), 2 (f), 4 (i), and 6 mM (j) added EDTA. The protein matrix is white and pores are dark. Scale bar = 50 μm .

less important because these samples had a constant pH value), the GM values decreased with an increase in EDTA concentration from 0 to 2 mM (Figure 2a). The GM values for gels made from milk with 4 mM added EDTA were similar to those with 2 mM EDTA. This could be due to the very long aging time it took to reach GM; milks with 4 mM EDTA needed 1,635 min to reach GM, whereas the milk with 2 mM EDTA took only 335 min. The extremely long aging time given to the 4 mM EDTA sample might have allowed greater bond formation, rearrangement, or micelle fusion. The loss of internal INS Ca bonds as indicated by higher LT (Figure 2b) should loosen the integrity of CM and should also promote more internal rearrangements. In contrast, gels made from milk samples with 6 mM EDTA added only had a low GM value, presumably because of excessive loss of INS Ca crosslinks from the CM with this level of EDTA (Table 2).

The LT parameter may indicate relaxation behavior of bonds within the time scale of the measurement (Zoon et al., 1989; van Vliet et al., 1991). Higher LT values indicate an increased susceptibility for rearrangements. The LT values at GM increased with a reduction in milk pH (Figure 1b) and with an increase in the concentration of EDTA added to milk (Figure 2b). This suggests that weaker, more flexible CN networks were formed in gels made from milk in which the CM had lower INS Ca content. The microstructure of gels made at pH 6.4 (Figure 4b) and 6.0 (Figure 4c) had smaller pores compared with gels made at pH 5.8 (Figure 4g), 5.6 (Figure 4h), and 5.4 (Figure 4i). The trends from the micrographs agreed with the lower GM, higher LT, and lower yield stress values obtained in low pH milk samples. Yield stress decreased with a reduction in INS Ca of CM (Figure 3), which may be attributed to occurrence of large pores and weaker interactions between CN particles (Lucey et al., 1997). There was a decrease in apparent interconnectivity between strands and clusters in the gel microstructure during aging (Figures 4 and 5), probably due to rearrangements and microsyneresis (probably aided by ongoing proteolysis). This observation agreed with the decrease in G' during aging (results not shown).

The formation of rennet-induced milk gels has been considered as an adhesive hard sphere particle gel (de Kruif, 1998). The hard sphere model ignores the internal structure and bonding within the CM (de Kruif, 1998). This adhesive hard sphere models predict that pH controls stickiness of the hairs ("brush") of CM; that is, the lower the pH, the stickier or less repulsive the CM. The results of the present study suggest that the internal integrity plays a critical role in the rheological and microstructural properties of rennet gels. In this study, it was found that the dual-binding model

for CM (Horne, 1998) was useful in explaining the effect of altering pH and INS Ca content on the properties of rennet gels.

CONCLUSIONS

This study demonstrated that the INS Ca content of CM has a major effect on the rheological properties of rennet-induced gels. This indicates that internal structural features are important for the properties of these gels and that hard sphere particle gel models do not predict this behavior, which is a limitation of these "surface" models. The loss of INS Ca reduced CN cross-linking and may have increased repulsion between the newly exposed phosphoserine residues, resulting in weaker gels. The loss of INS Ca also increased the LT values of gels, indicating an increase in the mobility of bonds in the network. In this study altering the internal micellar CN interactions altered the properties of rennet-induced gels, and presumably the properties of cheeses made from these gels.

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