Effects of the Concentration of Insoluble Calcium Phosphate Associated with Casein Micelles on the Functionality of Directly Acidified Cheese

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ABSTRACT

Directly acidified cheeses with different insoluble Ca (INS Ca) contents were made to test the hypothesis that the removal of INS Ca from casein micelles (CM) would directly contribute to the softening and flow behavior of cheese at high temperature. Skim milk was directly acidified with dilute lactic acid to pH values of 6.0, 5.8, 5.6, or 5.4 to remove INS Ca (pH trial). Lowering milk pH also reduced protein charge repulsion, which could influence melt. In a second treatment, EDTA (0, 2, 4, or 6 mM) was added to skim milk that was subsequently acidified to pH 6.0 (EDTA trial). Both types of milks were then made into directly acidified cheese. Cheese properties were determined at approximately 10 h after pressing to reduce possible confounding effects of proteolysis. The INS Ca content was determined by the acid-base titration method. Dynamic low-amplitude oscillatory rheology was used to measure the viscoelastic properties of cheese during heating from 5 to 80°C. The composition of all cheeses was as similar as possible, with cheese-making procedures being modified to obtain similar moisture contents (~55%). Insoluble Ca contents of cheeses significantly decreased with a reduction in pH or with the addition of EDTA to skim milk. The pH values of cheeses in the pH trial varied, but all cheeses in the EDTA trial had similar pH values (~5.73). In the pH trial, the reduction in cheese pH and consequent decrease in INS Ca content resulted in a reduction in the G' values of cheeses at 20°C. In contrast, the G' values at 20°C in cheeses from the EDTA trial increased with EDTA addition up to 4 mM EDTA. The G' values at 70°C of cheeses from the pH trial decreased with a decrease in cheese pH, and a similar decrease was observed in the G' values of cheese from the EDTA trial with an increase in EDTA concentration even though these cheeses had a similar pH value. In both trials, loss tangent (LT) values increased with tem-

peratures >30°C and reached a maximum at approximately 70°C. In the pH trial, LT values at 70°C increased from 1.50 to 4.24 with a decrease in cheese pH from 5.78 to 5.21. The LT values increased from 1.43 to 3.23 with an increase in the concentration of added EDTA from 0 to 6 mM. In the EDTA trial, the decrease in G' and increase in LT values at 70°C were due to the reduction in INS Ca content, because the pH values of these cheeses were the same. It can be concluded that the loss of INS Ca increases the melting in cheeses that have the same pH and gross chemical composition, and removal of INS Ca can even make cheese at high pH (~5.73) exhibit reasonable melt characteristics.

Key words: cheese functionality, texture, insoluble calcium, rheology

INTRODUCTION

In the United States a large amount of cheese is used as an ingredient such as that for pizza topping and in cheese sticks (Lucey et al., 2003). In these applications, specific functional characteristics such as different degrees of melting or stretching are required. Numerous studies have been reported to try to identify factors responsible for controlling the melting and stretching of heated cheese. These factors include the total calcium content of cheese (Metzger et al., 2001; Boutrou et al., 2002; Joshi et al., 2004; McMahon et al., 2005), pH value (Lawrence et al., 1987; Pastorino et al., 2003; Sheehan and Guinee, 2004), type of acid used for directly acidified cheese (Keller et al., 1974), stretching temperature of Mozzarella (Mulvaney et al., 1997), and draining pH (Lee et al., 2005). Recently, some basic mechanisms have been proposed to help explain how these various factors influence the melting and textural behavior of cheese (Lucey et al., 2003).

It is well known that total Ca content in cheese has a significant effect on the melting of cheese (Keller et al., 1974; Lucey and Fox, 1993). Kimura et al. (1992) used electrodialysis to demineralize skim milk and reduce the total Ca content of skim milk by 40 to 70%. The pH of string cheese made from demineralized milk

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was between 6.50 and 5.95 and cheese with a pH of 6.1 and 55% demineralization had the best stringiness. Joshi et al. (2003, 2004) reported that lowering the total calcium content in cheese by the combined use of acetic acid and glucono- δ -lactone helped increase melting. O'Mahony et al. (2006) modified the total Ca and insoluble (INS) Ca contents of Cheddar cheese by immersion of slices of cheese in a synthetic aqueous phase buffer. By altering the Ca concentration of the buffer, the Ca content of the cheese could be modified. O'Mahony et al. (2006) reported that cheese with low total and low INS Ca contents had greater meltability compared with the same cheese with greater total and INS Ca contents. There has been growing evidence that the Ca associated with casein micelles (CM); that is, INS Ca (instead of total Ca) is more important for controlling the melting behavior of cheese (Lucey and Fox, 1993; Lucey et al., 2003, 2005; Lee et al., 2005). The limited number of studies on the direct role of INS Ca in cheese texture is probably due to the difficulties in quantifying the INS Ca content of cheese. However, several methods have been developed to quantify the INS Ca content of cheese and changes in this concentration during ripening (Hassan et al., 2004).

Most studies on the effect of cheese pH and INS Ca on the melting behavior of cheese did not separate the effect of INS Ca content from that of cheese pH (protein charge). This complication occurs because a reduction in pH is always accompanied by the inevitable loss of INS Ca. It is well known that lowering the pH of cheesemilk (e.g., by preacidification) results in a reduction in the Ca content of cheese, a less dense protein matrix, and improved melt (e.g., McMahon et al., 2005). Based on the recent model for cheese texture (Lucey et al., 2003), electrostatic repulsion, crosslinking by INS Ca, and attractive hydrophobic interactions are the main interactions that control cheese melting and the behavior of cheese at elevated temperatures. Therefore, changes in pH and INS Ca content would be expected to modulate these interactions.

The objective of this study was to examine the effects of the loss of INS Ca from the CM on the melting of cheese that occurs during heating. We wanted to determine if the loss of INS Ca from cheese without a change in pH could induce an increase in meltability. We are not aware of any studies that have quantified the INS Ca content of directly acidified cheese and investigated the quantitative relationships between the actual INS Ca in cheese and cheese melting and rheological behavior. In a previous study (Choi et al., 2007) we investigated the gelation properties of rennet-induced gels made with reconstituted skim milk. These milk samples were very similar to those used to make the direct-acid cheeses in this study. One objective was to try to identify

if some gelation characteristics were related to the meltability of direct-acid cheese. Because many other factors such as cheese composition and proteolysis are known to affect cheese functionality, we tried to minimize these effects by making cheeses with similar moisture contents and measuring rheological properties of the cheeses within 10 h postmanufacture (to reduce the extent of proteolysis). It has recently been reported (O'Mahony et al., 2005) that most of the early texture changes during cheese ripening are due to partial solubilization of colloidal calcium phosphate associated with the *para*-CN matrix of the curd (i.e., INS Ca).

MATERIALS AND METHODS

Cheese Manufacturing

Directly acidified cheeses (4 full replicates) were manufactured from pasteurized skim milk (73°C for 15 s) obtained from the University of Wisconsin-Madison Dairy Plant. Four 21-L mini-cheese vats equipped with a computer-controlled cooking program and an automatic variable speed agitator were used to make the cheeses in this study. Twenty kilograms of skim milk was transferred into each vat. Skim milk was then cooled and maintained at 4°C until the desired pH was achieved. In the pH trial, the pH of skim milk was decreased to 6.0, 5.8, 5.6, or 5.4 by the addition of diluted (1:4, acid:water) lactic acid (Chr. Hansen, Milwaukee, WI). To slowly acidify milks, about half of the amount of diluted lactic acid needed to obtain the desired pH value was added at one time (the total amount required was predetermined before cheese making). This process was repeated with full agitation every 10 min until the target pH was attained. In the EDTA trial, EDTA ($\text{Na}_2\text{C}_{10}\text{H}_{14}\text{O}_8\text{N}_2 \cdot 2\text{H}_2\text{O}$, Fisher Scientific, Suwanee, GA) was slowly added with stirring for 1 h at 4°C to obtain final concentrations of 0, 2 (0.07%), 4 (0.15%), or 6 (0.22%) mM EDTA. The pH values at which 0, 2, 4, and 6 mM EDTA were added to milk at 4°C were 6.76, 6.68, 6.54, and 6.44, respectively. It was impossible to have coagulation at levels of EDTA greater than 6 mM with a reasonable concentration of rennet. Predetermined amounts of diluted lactic acid were slowly added to the EDTA-treated milk samples to obtain a final pH of 6.0. All treated milk samples were kept at 4°C for 1 h with stirring and their pH was checked and readjusted if necessary. Milks were then warmed to 32°C and stirred for another 1 h.

For chemical analysis, milk samples were taken and NaN_3 (0.2 mg/mL, Fisher Scientific) was added to inhibit bacterial growth. For cheese making, double-strength chymosin (Chymostar, Rhodia, Madison, WI) was added to the milk. It should be noted that in the pH trial the amount of rennet used in all the milk

samples was kept constant, but for the EDTA trial the rennet concentration was varied to obtain similar gelation times. The coagulum was cut with 0.63-cm knives, allowed to heal for 5 min, and then gently (manually) stirred for 5 min before cooking. Cooking time and temperature were slightly varied to achieve similar moisture content (~55%) in all cheeses. Whey was drained and curd was allowed to mat. Curd stretching properties were subjectively tested 3 times by taking approximately 40 g of curd from the vat and soaking the curd in hot water ($74 \pm 1^\circ\text{C}$) for 1 min. An experienced cheesemaker stretched the hot curd by hand, and physical and visual observations of stretch properties were recorded. After stretching, the curd was put back in hot water for 1 min and the same curd tested again for stretching. This was repeated a third time. Curd was salted (15 g/kg of curd weight) and the salted curd was packed into hoops that were lined with cheesecloth and pressed (approximately 100 to 200 kPa) at ambient temperature for ~10 h. The viscoelastic properties of cheese were measured about 10 h after pressing to reduce the effect of ripening (proteolysis) anticipated to occur on longer storage.

Chemical Analysis

Milk samples were analyzed for total solids, fat, protein, and CN (Marshall, 1992). Total Ca in milk, rennet whey, and cheese was analyzed by inductively-coupled argon plasma emission spectroscopy (ICP; Choi et al., 2007). Total Ca in rennet whey, which was corrected for the volume of precipitate, was considered as the soluble Ca in milk. The total solids, fat, and protein of cheese were also determined (Marshall, 1992). Sodium in cheese was analyzed by ICP using a wavelength of 330.2 nm and was converted to salt (NaCl). Cheese was analyzed for INS Ca content by the acid-base titration method (Hassan et al., 2004), and for cheese pH by the quinhydrone method (Marshall, 1992). In the titration method, the milk and cheese buffering curves were integrated between the pH limits of 4.1 and the point at which 2 curves intersected. All analyses were done at least in triplicate.

Dynamic Low-Amplitude Oscillatory Rheology

The rheological properties of cheese were determined as described by Lucey et al. (2005). A dynamic shear test was performed using a controlled-stress rheometer (Universal Dynamic Spectrometer, Paar Physica UDS 200, Physica Messtechnik GmbH, Stuttgart, Germany) with a serrated plate measuring system (MP31, 50-mm-diameter). Rheological properties of cheese were evaluated with an applied strain of 0.2% and a fre-

quency of 0.1 Hz. Temperature sweeps were performed from 5 to 80°C at $1^\circ\text{C}/\text{min}$. Storage modulus (G') and loss tangent (LT) were the parameters determined from dynamic low-amplitude oscillatory rheology (DLAOR) tests (Lucey et al., 2005). Cheese samples were sliced into disks of approximately 2.2 mm thickness and 50 mm diameter. Slices were stored in a plastic bag at 6°C for at least 3 h before testing. Samples were glued to the bottom heating (Peltier) plate of the rheometer using cyanoacrylate glue. Use of the glue and the serrated plate prevented slippage of the sample (Tunick et al., 1990). The exposed surface at the edges of the sample was covered with a thin layer of vegetable oil to prevent it from drying out. During loading, the normal force readings were kept at approximately 1.0 N to ensure good contact between the serrated plate and the cheese sample without excessive deformation of samples; data acquisition was begun only after a relatively constant normal force reading of ~0.8 N was obtained (i.e., a low and relatively stable normal force after relaxation of the stress applied during loading).

Statistical Analysis

An ANOVA was carried out using SAS software (SAS Institute, 2001) to investigate the influence of acidification pH values or EDTA addition on milk and cheese composition. Because each cheese might have slightly different concentrations of moisture, fat, protein, salt, and pH, these parameters were included in the statistical model as a covariate when the effect of acidification pH values of milk or addition of EDTA to milk on rheological properties of cheese was analyzed. Differences in least squares means were determined using Fisher's protected LSD. Significance was indicated at $P < 0.05$. Possible association between the rheological properties of direct acid cheese and the gelation characteristic of rennet-induced gels that we recently reported (Choi et al., 2007) was investigated using Pearson correlation coefficients.

RESULTS

Milk and Cheese Composition

The gross chemical composition of milk used to make the directly acidified cheeses is summarized in Tables 1 (pH trial) and 2 (EDTA trial). Total solids, fat, CN, and total Ca contents of milk were similar ($P > 0.05$) across pH treatments or different EDTA concentrations. In contrast, the INS Ca content of milk was significantly ($P < 0.05$) reduced by preacidification of milk to lower pH values (Table 1) or by the use of increasing concentrations of EDTA (Table 2). Both treatments were intended to remove INS Ca from CM, and these

Table 1. Chemical composition of milks acidified to different pH values, and the resulting direct acid cheeses made from these milks

Composition	pH of acidification				SEM	LSD
	6.0	5.8	5.6	5.4		
Milk						
Total solids, %	9.01	9.06	9.05	9.08	0.05	0.15
Fat, %	0.17	0.16	0.16	0.16	0.01	0.05
Casein, %	2.37	2.39	2.37	2.38	0.03	0.12
Total Ca, mg/100 g of milk	99.7	98.7	98.2	98.2	0.9	2.77
Insoluble Ca, mg/100 g of milk	51.2 ^a	39.0 ^b	27.0 ^c	17.7 ^d	1.4	4.43
Cheese						
Moisture, %	55.6	54.0	55.1	55.6	0.6	1.98
Fat, %	2.1	2.2	1.7	1.6	0.1	0.59
Protein, %	34.1	35.8	34.8	35.3	0.5	1.80
Salt/NaCl, %	0.95	1.04	0.94	1.03	0.06	0.21
pH of cheese	5.78 ^a	5.59 ^b	5.37 ^c	5.21 ^d	0.01	0.05
Total Ca, mg/100 g of cheese	823 ^a	686 ^b	469 ^c	337 ^d	16	52.2
Insoluble Ca, mg/100 g of cheese	498 ^a	401 ^b	241 ^c	138 ^d	17	54
Insoluble Ca, % of total Ca	60 ^a	58 ^{ab}	51 ^b	41 ^c	2	7

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

results agreed with the previous report by Choi et al. (2007), and these trends were expected. An average total Ca content (99 mg/100 g of milk) of milk in the pH trial was slightly lower than that (103 mg/100 g of milk) in the EDTA trial. These trials were done on different days and at different times of the year; therefore the slight differences could be due to some seasonal, lactational, nutritional, or genetic factors that affect the salt concentration in milk (Tsioulpas et al., 2007).

The composition of the cheese resulting from 2 types of milks is shown in Tables 1 and 2 for the pH and EDTA trials, respectively. The moisture content of cheese was similar in the pH trial. Although cheese-making procedures (i.e., cooking temperature and time) were modified to try to have similar moisture content in all

cheeses, cheeses made with milk containing 4 mM EDTA had a significantly lower moisture content compared with 0 or 6 mM EDTA cheeses. Fat, protein, and salt contents of cheese were similar ($P > 0.05$) in both trials. It is known that differences in cheese composition would affect rheological properties of cheese, and we included cheese compositional effects (e.g., moisture content) in our statistical analysis to investigate the effect of INS Ca content in cheese on rheological properties of cheese. The cheeses made with milks directly acidified to 6.0, 5.8, 5.6, and 5.4 had cheese pH values of 5.78, 5.59, 5.37, and 5.21, respectively (Table 1). The pH values (5.73 ± 0.08) in the EDTA treatment cheeses were similar ($P > 0.05$) due to adjustment of final milk pH to 6.0 after the addition of EDTA. It should be noted

Table 2. Chemical composition of milks with different EDTA concentrations added to milk at pH 6.0, and the resulting direct acid cheeses from these milks

Composition	EDTA concentration (mM)				SEM	LSD
	0	2	4	6		
Milk						
Total solids, %	9.01	8.98	9.10	9.20	0.08	0.25
Fat, %	0.16	0.13	0.15	0.15	0.02	0.07
Casein, %	2.36	2.32	2.36	2.37	0.01	0.06
Total Ca, mg/100 g of milk	103.5	102.7	103.5	103.0	0.9	2.97
Insoluble Ca, mg/100 g of milk	58.0 ^a	52.0 ^b	46.2 ^c	40.2 ^d	1.7	5.40
Cheese						
Moisture, %	55.2 ^a	54.3 ^{ab}	53.0 ^b	55.8 ^a	0.5	1.74
Fat, %	2.6	2.5	1.7	1.8	0.5	1.84
Protein, %	34.4	34.5	36.3	34.2	0.5	1.81
Salt/NaCl, %	0.89	0.93	0.97	1.00	0.07	0.23
pH	5.76	5.69	5.72	5.73	0.04	0.14
Total Ca, mg/100 g of cheese	827 ^a	799 ^b	760 ^c	640 ^d	6	19.4
Insoluble Ca, mg/100 g of cheese	511 ^a	469 ^b	421 ^c	375 ^d	7	24.4
Insoluble Ca, % of total Ca	61 ^a	58 ^b	55 ^b	58 ^{ab}	1	3.2

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

Figure 1. Acid-base buffering curves for cheese titrated from initial cheese pH to pH 3.0 with 0.5 *N* HCl and then back-titrated to pH 9.0 with 0.5 *N* NaOH; cheese made from milk acidified to pH 6.0 (a), 5.8 (b), 5.6 (c), or pH 5.4 (d) and cheese made from milk that had 0 (e), 2 (f), 4 (g), or 6 mM (h) EDTA added before cheese making. Vertical dashed line indicates buffering peak at pH 4.8.

that the pH values of all the cheeses were lower than those of milks used for the coagulation step; this was presumably due to some fermentation of lactose by non-starter bacteria.

The acid-base buffering curves that were used to quantify the INS Ca content of cheeses for the pH and EDTA trials are shown in Figure 1. The buffering peak in the vicinity of pH ~4.8 decreased with a decrease in milk pH by preacidification (Figure 1, panels a to d), which indicated a reduction in the INS Ca content of cheese (Lucey and Fox, 1993). A similar trend was observed in cheese with increasing amounts of EDTA added to milk (Figure 1, panels e to h). Total Ca and INS Ca contents in the pH trial (Table 1) were 823 and 498 mg/100 g of cheese made with milk acidified to pH 6.0; this decreased to 337 and 138 mg/100 g of cheese made from milk acidified to pH 5.4. Similarly, a reduction in total Ca and INS Ca contents was observed as a result of adding EDTA to milk (Table 2); that is, total Ca and INS Ca contents were 827 and 511 mg/100 g of cheese made with 0 mM EDTA added, and this decreased to 640 and 375 mg/100 g of cheese made with 6 mM EDTA added. A change in total Ca content of cheese in both trials paralleled the change in INS Ca content in milk.

Rheological Properties of Cheeses

The viscoelastic properties of the prepared cheeses are shown in Figures 2 and 3. The G' values for cheese made from milk acidified to different pH values (Figure 2a) and from EDTA-treated milks (Figure 2b) decreased with an increase in heating temperature. A decrease in G' indicates a softening of these cheeses. Changes in LT during heating are shown in Figure 3a and 3b for the pH and EDTA trials, respectively. The LT values began to increase at about 40°C and exhibited a maximum (LT_{max}) at approximately 70°C, which indicates the point of highest bond mobility (shortest relaxation time; Lucey et al., 2005).

Two representative temperatures, 20 and 70°C, were chosen to compare low and high temperature behavior of cheese because the viscoelastic properties of cheese depend on temperature (Lucey et al., 2003). Statistical results are shown in Table 3. In the pH trial, the acidification pH values of milk before cheese making had a significant ($P < 0.01$) effect on the rheological properties of cheese such as G' and LT at 20 and 70°C, LT_{max} , and temperature at LT_{max} . The same rheological properties except the LT values at 20°C ($P > 0.13$) in the EDTA trial were affected by the concentration of EDTA added to milk ($P < 0.05$).

Figure 2. Storage modulus (G') as a function of temperature for cheese (a) made from milk acidified to pH 6.0 ($\blacktriangledown$), 5.8 (∇), 5.6 ($\bullet$), or pH 5.4 ($\circ$) and cheese (b) made from milk that had 0 ($\blacktriangledown$), 2 (∇), 4 ($\bullet$), or 6 mM ($\circ$) EDTA added before cheese making. Cheese was heated from 5 to 80 $^{\circ}\text{C}$ at 1 $^{\circ}\text{C}/\text{min}$. Error bars indicate standard deviation ($n = 4$). Insert is the low temperature region magnified from 5 to 40 $^{\circ}\text{C}$.

The G' values at 20 $^{\circ}\text{C}$ decreased (Table 4) as the cheese pH values and INS Ca content in cheeses were reduced as a result of acidification of milk pH (Table 1). In contrast, when INS Ca was chelated by EDTA at milk pH 6.0, cheeses showed the opposite trend at the 20 $^{\circ}\text{C}$ measurement temperature; that is, a reduction in the INS Ca content, but a similar cheese pH (Table 2) increased the G' values from 19.8 kPa in cheese made with 0 mM EDTA to 26.0 kPa in cheese made with 4 mM EDTA added (Table 4). Cheese made from milk with 6 mM EDTA had the lowest G' values of all the EDTA-treated samples at the 20 $^{\circ}\text{C}$ measurement temperature (Table 4). The G' values at 70 $^{\circ}\text{C}$ of all cheeses in the pH trial decreased (Table 4) from 0.47 to 0.02 kPa with a decrease in cheese pH from 5.78 to 5.21

Figure 3. Loss tangent (LT) as a function of temperature for cheese (a) made from milk acidified to pH 6.0 ($\blacktriangledown$), 5.8 (∇), 5.6 ($\bullet$), or pH 5.4 ($\circ$) and cheese (b) made from milk that had 0 ($\blacktriangledown$), 2 (∇), 4 ($\bullet$), or 6 mM ($\circ$) EDTA added before cheese making. Cheese was heated from 5 to 80 $^{\circ}\text{C}$ at 1 $^{\circ}\text{C}/\text{min}$. Vertical and horizontal error bars are standard deviations for loss tangent and for the temperature of the loss tangent, respectively ($n = 4$).

(Table 1). The G' values at 70 $^{\circ}\text{C}$ in EDTA-treated cheeses decreased (Table 4) from 0.42 to 0.12 kPa with an increase in EDTA from 0 to 6 mM. Although the pH values of the EDTA-treated cheeses were similar (Table 2), the INS Ca content decreased from 511 to 375 mg/100 g of cheese with an increase in EDTA concentration from 0 to 6 mM. The G' value at 70 $^{\circ}\text{C}$ of cheese made from milk acidified to pH 5.4 was very low (0.02 kPa) compared with any of the G' values at 70 $^{\circ}\text{C}$ in the EDTA-treated cheeses (>0.12 kPa; Table 4). This may be due to excessive loss of crosslinks as indicated by the very low INS Ca content in this pH 5.4 cheese (Table 1).

The LT values at 20 $^{\circ}\text{C}$ increased ($P < 0.05$) from 0.25 to 0.35 with a reduction in cheese pH values in cheese

Table 3. Mean squares and probabilities for effects on small deformation rheological properties of cheeses made from milk acidified to different pH values (pH trial) and with different concentrations of EDTA added to milk (EDTA trial)

Source of variation	df	Storage modulus (Pa)		Loss tangent (LT)		LT maximum (LT _{max})	Temperature at LT _{max}
		20°C	70°C	20°C	70°C		
pH trial							
pH	3	48,969,548***	138,737**	0.005***	4.66***	6.11***	29.58***
Moisture	1	67,205	880	0.00002	0.013	0.0001	0.111
Fat	1	402,225	6,704	0.0007*	0.21	0.24	1.825
Protein	1	154,883	3,616	0.0001	0.07	0.0009	0.76
Salt	1	821,467	1,483	0.000001	0.04	0.028	12.22
Error	8	981,070	92,334	0.001	0.65	0.0735	2.25
EDTA trial							
EDTA	3	56,305,138***	15,928*	0.0001	1.958***	1.9560***	20.42**
Moisture	1	805,546	269	0.00004	0.07*	0.0607*	3.33
Fat	1	3,745,815	180	0.00005	0.1*	0.0853*	1.38
Protein	1	345,431	0.11	0.00003	0.007	0.0030	0.28
Salt	1	4,057,625	13.57	0.00004	0.005	0.0021	28.78**
pH	1	15,792,982*	21,539*	0.0001	0.104*	0.1059*	1.35
Error	7	2,301,832	3,113	0.00003	0.0124	0.0098	1.548

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

made from milk acidified to pH 6.0 to 5.4, but were not significantly different ($P > 0.13$) with a change in the EDTA concentrations (Table 4). In cheese made from milk acidified from pH 6.0 to 5.4, the LT values at 70°C increased from 1.50 to 4.24, respectively (Table 4). The LT values at 70°C increased from 1.43 in cheese made with 0 mM EDTA to 3.23 in cheese made with 6 mM EDTA. This increase in LT values was due to a decrease in the INS Ca content (and total Ca content). A greater LT_{max} value indicates a greater propensity of cheese to melt and flow when heated (O'Mahony et al., 2006). The LT_{max} values for cheese made from milk preacidified to pH 6.0, 5.8, 5.6, and 5.4 were 1.52, 2.73, 4.34, and 4.93, respectively (Table 4). The LT_{max} values of cheese made from milk with 0, 2, 4, and 6 mM EDTA added were 1.45, 1.85, 2.43, and 3.24, respectively. Temperatures at LT_{max} were 67, 72, 66, and 62°C for cheeses made from milk acidified to pH values 6.0, 5.8, 5.6, and 5.4,

respectively. The LT_{max} temperatures were 71, 72, and 70°C for cheeses made with 2, 4, and 6 mM EDTA added, respectively, and occurred at higher temperatures than in the control (0 mM EDTA). Cheeses made from milk acidified to 5.4 gave the lowest temperature of LT_{max} (Table 4), which may be due to excessive loss of attractive INS Ca crosslinks (Table 1). Lucey et al. (2005) showed that temperatures at which LT_{max} occurred decreased with cheese ripening. In our trials, fresh cheeses (~10 h after pressing) were subjected to rheological measurement, and proteolysis effects were probably negligible.

Physical and Visual Attributes of Hot Curd

Physical and visual attributes of hot curd during first hand stretching are shown in Table 5. When stretchability of curd at 74°C was evaluated for cheeses made

Table 4. Small deformation rheological properties of cheeses made with different acidification pH values of milk and with various concentrations of EDTA added to cheese milk (mean ± SD)

Treatment	Storage modulus (Pa)		Loss tangent (LT)		LT maximum (LT _{max})	Temperature at LT _{max} (°C)
	20°C	70°C	20°C	70°C		
pH trial						
pH 6.0	20,250 ^a ± 1,464	471 ^a ± 101	0.25 ^c ± 0.02	1.50 ^c ± 0.18	1.52 ^a ± 0.17	67 ^b ± 2
pH 5.8	18,845 ^a ± 865	311 ^a ± 168	0.26 ^c ± 0.01	2.70 ^b ± 0.33	2.73 ^b ± 0.34	72 ^a ± 1
pH 5.6	12,985 ^b ± 417	47 ^b ± 10	0.29 ^b ± 0.01	4.16 ^a ± 0.14	4.34 ^c ± 0.16	66 ^b ± 1
pH 5.4	9,573 ^c ± 1,054	20 ^b ± 8	0.35 ^a ± 0.02	4.24 ^a ± 0.45	4.93 ^d ± 0.43	62 ^c ± 1
EDTA trial						
0 mM	19,752 ^b ± 1,837	420 ^a ± 88	0.23 ± 0.01	1.43 ^a ± 0.13	1.45 ^a ± 0.11	66 ^a ± 3
2 mM	20,459 ^b ± 2,359	371 ^{ab} ± 130	0.24 ± 0.01	1.85 ^b ± 0.15	1.85 ^b ± 0.17	71 ^b ± 1
4 mM	25,952 ^a ± 1,781	308 ^b ± 100	0.25 ± 0.01	2.41 ^c ± 0.17	2.43 ^c ± 0.16	72 ^b ± 1
6 mM	17,025 ^c ± 1,896	118 ^c ± 46	0.25 ± 0.01	3.23 ^d ± 0.22	3.24 ^d ± 0.22	70 ^b ± 2

^{a-d}Means with different letters within the same column in each trial are significantly different ($P < 0.05$).

Table 5. Physical and visual properties of hot curd observed during first hand-stretching while curd was held in water at 74±1°C for 1 min

Treatment	Physical and visual properties	
	Tension properties ¹	Visual properties
pH values of acidification		
6.0	Very tough, no stretching, no flow, breakage at the start of stretching	White shiny surface (translucent), rough, fibrous, and dried surface, clear feathering ²
5.8	Soft, stretching better than curd made from milk at pH 6.0, but still breakable in the middle of stretching	Less white surface than cheese made with milk at pH 6.0, still feathering but less obvious
5.6	Good stretchability without curd breakage, less sticky than pH 5.4 curd	Yellowish transparent color and easy to stretch, low stickiness, no feathering
5.4	Flowing under its own weight, much softer and soupy	Very homogeneous surface, yellowish transparent, difficult to deal with curd as it was very sticky
EDTA concentrations added to milk at pH 6.0 (mM)		
0	The same as direct acid cheese made with milk at pH 6.0	The same as direct acid cheese made with milk at pH 6.0
2	Limited stretching but softer than cheese with 0 mM EDTA, breakage in the middle of the stretching	Still fibrous, whitish surface, feathering
4	Stretchable similar to curd made with milk at pH 5.8, no breakage	A little yellowish surface, no feathering
6	Good stretchability like curd from milk acidified to pH 5.6, no breakage	Yellow homogeneous surface, sticky but less sticky than curd made with milk directly acidified to pH 5.4

¹Hand feeling when hot curd was stretched by both hands.

²A fibrous rod-like shape at the end of broken curd observed at the stretching of hot curd.

from milk acidified from pH 6.0 to 5.4, tough curd at pH 6.0 became stretchable and soupy at pH 5.4 due to a reduction in INS Ca crosslinks and a decrease in cheese pH values. An increase in stretchability with increasing EDTA levels, which was observed in cheese at similar pH values, occurred with a loss of INS Ca crosslinks. The hand-tension properties determined during stretching generally agreed with the trends in the G' and LT values. In DLAOR tests, very small strains (0.2%) within the linear viscoelastic region were applied but the stretching properties were large deformation tests. There were changes in the color of curd (from white to yellow) as the INS Ca was lost and this was probably due to a change in the state of casein aggregation with the loss of INS Ca.

Correlations Between Rennet Coagulation and Cheese Properties

The properties of rennet-induced gels made from milks acidified to various pH values or with EDTA added were previously studied by our group (Choi et al., 2007). Correlation coefficients between rheological properties of rennet-induced gels (using data from the study of Choi et al., 2007) and those of cheese from the pH and EDTA trials are shown in Table 6. There were no significant correlations ($P > 0.05$, data not shown) between total Ca content in milk and cheese properties

such as G' and LT values at 70°C, LT_{max} , total Ca and INS Ca content, or cheese pH. The INS Ca content in milk was significantly correlated with cheese properties; for example, G' ($r = 0.84$, $P < 0.001$) and LT values at 70°C ($r = -0.92$, $P < 0.001$), LT_{max} ($r = -0.92$, $P < 0.001$), total Ca ($r = 0.88$, $P < 0.001$), and INS Ca content ($r = 0.89$, $P < 0.001$), as well as cheese pH ($r = 0.68$, $P < 0.001$). The maximum G' value (GM) in rennet gels was positively correlated with cheese properties including the G' value at 70°C ($r = 0.86$, $P < 0.001$), total Ca ($r = 0.92$, $P < 0.001$) and INS Ca contents ($r = 0.90$, $P < 0.001$), and cheese pH ($r = 0.75$, $P < 0.001$). The GM

Table 6. Pearson correlation coefficients between rheological properties¹ of rennet-induced gels and direct acid cheeses

Variable	Rennet-induced gels			
	Insoluble Ca	GM	LT	σ_{yield}
Direct acid cheeses				
G'_{70}	0.84***	0.86***	-0.76***	0.87***
LT_{70}	-0.92***	-0.92***	0.82***	-0.94***
LT_{max}	-0.92***	-0.93***	0.87***	-0.93***
Total Ca	0.88***	0.92***	-0.91***	0.87***
Insoluble Ca	0.89***	0.90***	-0.92***	0.90***
Cheese pH	0.68***	0.75***	-0.80***	0.70***

¹ GM = maximum storage modulus; LT = loss tangent at GM ; σ_{yield} = yield stress; G'_{70} = storage modulus value at 70°C; LT_{70} = loss tangent value at 70°C; LT_{max} = maximum loss tangent.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

in rennet gels was significantly negatively correlated with cheese rheological properties, including LT value at 70°C ($r = -0.92$, $P < 0.001$) and $LT_{\max}$ ($r = -0.93$, $P < 0.001$). The correlations between yield stress of gels (defined as the point when the shear stress started to decrease) and cheese properties were similar to GM. The LT value at GM in rennet-induced gels was negatively correlated with cheese properties including G' value at 70°C ($r = -0.76$, $P < 0.001$), total Ca ($r = -0.91$, $P < 0.001$) and INS Ca ($r = -0.92$, $P < 0.001$), and cheese pH ($r = -0.80$, $P < 0.001$), but positively correlated with the LT value of cheese at 70°C ($r = 0.82$, $P < 0.001$) and $LT_{\max}$ ($r = 0.87$, $P < 0.001$).

DISCUSSION

In this study cheeses with variable amounts of INS Ca contents were made to test our hypothesis that the loss of INS Ca from CM alters the rheological properties of cheese even if the pH values of the cheeses are similar. We believe that the INS Ca that is bound to CN molecules in cheese, and not the total Ca in cheese, is involved in stress-carrying bonds between CN particles and directly contributes to the viscoelastic properties of cheese (Lucey et al., 2003). From the recent cheese texture model (Lucey et al., 2003), CN interactions determine cheese texture, and when melting occurs electrostatic repulsion is greater than the attractive interactions. The interactions between CN particles can be altered to favor either electrostatic repulsion or attractive interactions. Acidification of milk reduces electrostatic repulsion on the caseins and reduces INS Ca crosslinks. In this study EDTA was added to milk whose pH was subsequently adjusted to pH 6.0 to have similar electrostatic repulsion but different degree of attractive interactions (amount of INS Ca crosslinks). This experimental approach helped resolve the usual complication that cheese pH and INS Ca content vary concomitantly.

The G' values at 70°C for cheese made from milk acidified to pH 5.4 were significantly lower than in cheese made from milk preacidified to pH 6.0 (Table 4). This was due to considerable loss of INS Ca crosslinks. In relation to the cheese texture model, low pH reduced attractive interactions, and cheese became meltable. Lee et al. (2005) showed that in cheese below pH 5, electrostatic attractions with the approach of the CN isoelectric point would dominate even if there was a reduction in other attractive interactions such as INS Ca crosslinks. The G' values at 70°C in cheese decreased with an increase in EDTA concentration (Table 4). Because cheese in the EDTA trial had similar pH values (and the slight differences were also adjusted in statistical analysis), the observed decrease in G' values was only caused by reduction of INS Ca (i.e., INS Ca cross-

links). In the context of the model of Lucey et al. (2003) the loss of INS Ca resulted in the loss of attractive interactions (crosslinks), and this helped to allow the melting of cheese to occur at high pH values. It is likely that the loss of INS Ca increased bond mobility (i.e., increased relaxation) by reducing crosslinks between the caseins.

This loosening of interactions between and within CN particles (Horne, 1998) may help explain the observed behavior of G' values at 20°C. At 20°C, the hydrophobic interactions within CN are weak, which allows CN particles to swell. It is possible that swelling resulted in greater contact area between neighboring particles, which could increase the number of bonds or their strength in the gel system. O'Mahony et al. (2006) reported that in cheese at pH ~5.25, there was increased swelling and hydration of the *para*-CN matrix of the cheese with a reduction in INS Ca. In the EDTA trial, the G' values at 20°C exhibited a significant increase with the addition of up to 4 mM EDTA. The removal of INS Ca by the addition of EDTA in the range of 0 to 4 mM facilitates greater rearrangement and molecular mobility of CM, which may have helped increase swelling of CN particles and contact area. The INS Ca crosslinks transmit stress under deformation, and excessive loss of INS Ca probably reduced the G' value at 20°C of cheese with 6 mM EDTA. An initial increase in G' value with some loss of INS Ca and then a decrease were reported in yogurt-type gels made with added trisodium citrate (Ozcan-Yilsay et al., 2007). In contrast, the G' values at 20°C in the pH trial exhibited the expected trend; that is, a decrease in G' values at 20°C in cheeses with a decrease in the acidification pH values of milk. This decrease in the G' values at 20°C with a decrease in milk pH was probably due to the extensive loss of INS Ca crosslinks with a decrease in pH.

The direct-acid cheeses used in this study can be considered somewhat similar to low moisture rennet-induced gels (i.e., they have similar pH values and very little proteolysis). In our previous study (Choi et al., 2007) it was found that reducing the INS Ca content of CM increased CN bond mobility and the flexibility of the rennet gel networks. Our hypothesis was that increased bond mobility in the rennet-induced gels should also increase the bond mobility in direct-acid cheeses made from these gels. The hypothesis is that INS Ca rather than total Ca plays a crucial role in bonding within CM. Direct support for this was provided by the observation that total Ca was not, but INS Ca in milk was, highly correlated with rheological properties of direct acid cheese (Table 6). For example, there was a significant negative correlation ($r = -0.92$, $P < 0.001$) between INS Ca content in milk and the LT value at 70°C in cheese. Loss of INS Ca in milk reduced

CN cross-linking, which resulted in weaker, more flexible gels as indicated by low GM and yield stress, and high LT at GM in rennet gels. When these gels were converted into cheese without any further large change in pH, it appeared that the properties of these rennet gels were significantly correlated with cheese properties such as the G' and LT values at 70°C, LT_{max} , total Ca and INS Ca content, and cheese pH. Rennet gels with high LT values exhibit greater flexibility and bond mobility, and cheese made from these gels had good meltability.

CONCLUSIONS

This study demonstrated that INS Ca content in cheese plays a key role in determining the melting and flow behavior of heated cheese. The reduction in INS Ca obtained by decreasing cheese pH resulted in an increase in the LT values at high temperature. Cheeses treated with EDTA but still having a high pH value also exhibited an increase in LT values because of the reduction in INS Ca content. A cheese texture model that views the texture properties of cheese as being the result of the net strength of the attractive and repulsive CN interactions was useful in explaining the effects of INS Ca on cheese melting. It was also observed that increasing the flexibility of bonds in rennet-induced gels was significantly related to the ability of cheese to melt at high temperature.

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