Effect of insoluble calcium concentration on endogenous syneresis rate in rennet-coagulated bovine milk

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ABSTRACT

The rennet coagulation of milk has been extensively studied. Mathematical modeling of the gelation process has been performed, mainly for the purpose of predicting the gel point. Rheological profiles of rennet gels during aging (long reaction times) have indicated that the gel stiffness (modulus) attains a maximum and thereafter decreases. We wanted to model this type of behavior and used the Carlson model, which includes terms for the proteolysis of κ -casein hairs (creating active sites) and the crosslinking of these activated sites. To account for the observed decrease in the gel modulus with time, we modified the Carlson model by adding an exponential decay term, which we ascribe to endogenous syneresis. We believe that this decay (i.e., syneresis rate) would likely be influenced by the mobility of bonds within casein micelles (in gels as indicated by the rheological loss tangent parameter). To modify the internal structural bonding of casein micelles, reconstituted skim milk was acidified to pH values 6.4, 6.0, 5.8, 5.6, and 5.4, or EDTA was added to milk at concentrations of 0, 2, 4, and 6 mM, and the final pH values of EDTA-treated samples were subsequently adjusted to pH 6.0. These treatments were then used to prepare rennet gel samples that were monitored by dynamic low amplitude oscillatory rheometry. When the modified Carlson model was fitted to the actual experimental storage modulus values of each sample, it fitted the data reasonably well (especially the pH trial data). As the pH values of milk decreased, the modulus values at infinite reaction time (G'_{∞}) increased; however, G'_{∞} decreased with an increase in the EDTA concentration. In the pH trial, the rate constants for the proteolysis of κ -casein hairs and the crosslinking of these activated sites exhibited a maximum at pH 5.6 and 6.0, respectively. The rate constant for endogenous syneresis increased at pH values

<6.0. The rate constant for endogenous syneresis was significantly positively correlated ($r \geq 0.96$) with the loss tangent values of gels (indicating greater mobility), probably due to the loss of insoluble calcium phosphate crosslinking within micelles, which was significantly negatively correlated ($r \geq 0.81$) with the rate constant for endogenous syneresis. In the EDTA trial, with an increase in the EDTA concentration no maximum was observed in the rate constants related to proteolysis of κ -casein hairs or crosslinking of these activated sites. The rate constant for endogenous syneresis decreased at higher EDTA levels. The different rheological/modeling behavior in the EDTA trials was likely due to the very significant inhibition of rennet gelation induced by the use of EDTA, which also resulted in extremely long reaction times. Our modified Carlson model fit our experimental pH trial data very well, which indicates that the rennet gel system has the potential to synerese from the start; indeed this ability is an innate property of the casein micelle. Endogenous syneresis was enhanced by the loss of insoluble calcium phosphate crosslinking within casein micelles as this increased bond mobility within rennet gels.

Key words: casein, gelation, rennet, syneresis

INTRODUCTION

Syneresis (shrinkage) of rennet gels is of utmost importance in controlling moisture levels, cheese yield, and product quality in cheese manufacture, yet the process(es) are still imperfectly understood (Dejmek and Walstra, 2004). A distinction has to be made between endogenous syneresis (i.e., syneresis due to contraction of the gel under absence of external forces) and syneresis due to external pressure (e.g., pressing). In this study, we restrict ourselves to endogenous syneresis. Syneresis is regarded as the final process in the transformation of milk into cheese through the expulsion and separation of whey from the coagulated grains. The major portion of syneresis occurs after the cutting of the curd as a result of mechanical work (e.g., stirring) on the shrinking curd grains but spontaneous

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endogenous syneresis must also be ongoing before the cutting time (Dejmek and Walstra, 2004). One of the questions we seek to answer is how early in the aggregation/coagulation is syneresis an active process.

Physical descriptions of the syneresis process tend to be mechanical in nature and speak of relaxation in pre-stressed bonds (van Vliet et al., 1991). The shrinkage and compression of curd grains after cutting fits well with this picture. Another description sees the shrinking curd-grain as a self-squeezing sponge (Tijskens and De Baerdemaeker, 2004). But what is the origin of such squeezing forces, and how can they be manipulated to control the rate of syneresis? Again, based on studies of syneresis of rennet (pH 6.6) and acid skim milk gels (pH 4.6), prepared at different temperatures, van Vliet et al. (1991) concluded that syneresis increased with increasing rheological loss tangent (**LT**), a measure of relaxation ability of the gel, but was not dependent on the elastic shear modulus of these gels. Mellema et al. (2002) interpreted such behavior mainly in terms of rearrangements involving strands and particles within the gel, thereby implicitly limiting the syneresis process to later stages of gel aggregation and aging.

More recently, Bittante et al. (2013) reported extended duration studies of maturing rennet gels, monitored using the Formagraph. Accepting the limitations of this instrument as a "rheometer," their gels show a maximum in "elasticity" as they mature, and the authors interpret the further decline as being the result of syneresis, following an earlier interpretation by Zoon et al. (1988). By fitting their cure curves to a modified Douillard model, they obtain estimates for the rate of syneresis in rennet gels made from a large number of milks collected in a major trial studying genetic aspects of milk coagulation properties. They did not, therefore, make any attempt to interpret this behavior in terms of a mechanistic understanding of endogenous syneresis, nor did they extend their study to varying reaction parameters, such as pH or temperature, known to influence the rate of syneresis (Dejmek and Walstra, 2004).

In this paper, we seek to cover both those omissions, that is, to measure syneresis rates as a function of milk pH and colloidal calcium phosphate content, and to interpret these results in terms of a proposed mechanism for syneresis. We also seek to explain the relationship between gel relaxation behavior (through **LT**) and syneresis rate observed by van Vliet et al. (1991) in their limited study.

Our starting point is the classical mechanism of rennet coagulation where the addition of rennet to milk destabilizes casein micelles (**CM**) and initiates gel development. The process is considered to take place in 3 partly overlapping stages: hydrolysis of κ -CN, aggregation of activated **CM**, and gelation. After aggregation,

2 reactions can occur during gelation. First, more particles (i.e., renneted **CM**) are continuously incorporated into percolation strands as the reaction time elapses. This contributes to the mechanical development of a 3-dimensional particle gel network (Horne and Banks, 2004). Second, some proteolytic reactions caused by rennet and other indigenous enzymes (Srinivasan and Lucey, 2002), and macro/microsyneresis (Roefs et al., 1990; Dejmek and Walstra, 2004) via inter/intra-rearrangements of the particles, can take place. During long reaction times syneresis and possibly proteolysis reactions could lead to a weakening of the rennet gel network. This mechanism/framework, largely based on studies carried out at natural pH, does not consider possible effects involving changes in micellar integrity and internal **CM** structure at pH values encountered in normal cheese-making conditions. Decreasing pH leads to the solubilization of some or all of the colloidal calcium phosphate (**CCP**), thereby considerably weakening the micellar structure. Choi et al. (2008) addressed this issue, showing that depleting the **CCP** of the **CM** directly affected the functionality of cheese produced from those milks. In demonstrating this, they added to the growing evidence that the **Ca** associated with **CM** [that is, insoluble (**INS**) **Ca** (instead of total **Ca**)] is more important for controlling the melting behavior of cheese (Lucey and Fox, 1993). It is our contention that this **INS Ca** in pH-adjusted cheeses should be interpreted as the remnant **CCP** cross-links from **CM** whose integrity is now more reliant on the much weaker and more ephemeral hydrophobic linkages. Such micellar structures will be more prone to inter/intra-micellar rearrangement reactions due to greater bond mobility and relaxation, and thereby would be expected to also influence gel syneresis rates.

In prolonged studies of curd formation, Choi et al. (2007) observed a maximum in elastic shear modulus, G' (**GM**), followed by a decay. As did Bittante et al. (2013), we have included a multiplicative exponential decay term in our mathematical model. This includes the full Carlson equation (Carlson et al., 1987), rather than the Douillard equation (Douillard, 1973) to which the Carlson expression is reduced when a coagulation time is substituted for its first term. As a multiplicative term, the added exponential decay is operative from the earliest stages of the reaction, indeed from time zero. It accommodates the relaxation and rearrangement processes occurring as manifested in endogenous syneresis.

Syneresis rates were measured as a function of the **INS Ca** content of **CM**. The **INS Ca** concentration of **CM** was modified by 2 approaches (Choi et al., 2007). In pH trial, milk samples were acidified to pH values from 6.7 to 5.4. Because using different pH values of

milk also modifies other types of reactions, we also used a second approach. In this EDTA trial, various concentrations of EDTA, ranging from 0 to 6 mM, were added to milk and their pH values were all adjusted to pH 6.0 to have similar protein charge. Thus, in the EDTA trial, we had gels with similar pH values, but with different INS Ca contents.

To interpret the changes in endogenous syneresis rate, we make use of the gel model developed by Horne (1995) for particle gel networks. This mechanical model successfully related the ultimate gel strength of the curd network to the number of micelles and their size, assuming particle integrity was maintained throughout. Relaxing this condition (particle integrity) has allowed us to picture syneresis behavior, as described in the Discussion.

MATERIALS AND METHODS

Preparation of Milk Samples and Measurement of Rheological Properties

Preparation of milk samples and measurement of the rheological properties were reported previously (Choi et al., 2007). Briefly, low heat skim milk powder (Dairy Farmers of America, Fresno, CA) reconstituted in demineralized water to 8.7% (wt/wt) solids was stirred at room temperature for 30 min; NaN_3 (0.2 mg/mL, Fisher Scientific, Fairlawn, NJ) and soybean trypsin inhibitor (0.15 mg/mL, Sigma Chemical Co., St. Louis, MO) were added. The milk solutions were equilibrated by stirring at 32°C for 2 h and immediately cooled to ~4°C. For the experiments where skim milk solutions were acidified (i.e., 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. For the addition of EDTA to cold (~4°C) milk (i.e., EDTA trial), 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 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, for the pH trial 50 $\mu\text{g/mL}$ of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Fisher Scientific) was added at the same time that NaN_3 was added, and for the EDTA trial 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.

A Universal Dynamic Spectrometer (Paar Physica UDS 200, Physica Messtechnik GmbH, Stuttgart,

Germany) was used to measure the G' and LT values of rennet gels. The cup and bob measuring geometry consisting of 2 coaxial cylinders (diameters 25.0 and 27.5 mm) was used. Treated milk samples 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 (Chymostar, Rhodia Inc., Madison, WI, diluted 1:120 for pH trial; 1:75 for EDTA trial) was added to 20 mL of milk. The mixture was stirred for 2 min and 12.75 mL of milk sample was transferred to the cup. Vegetable oil was placed on the surface to prevent evaporation. Sample was oscillated at a frequency of 0.1 Hz, and strain applied was 0.5%. Monitoring of gel development followed the reaction for one half-life beyond their maximum G' value. Gels made with 2 and 4 mM EDTA exhibited so slow a decay that gel properties were monitored for 3 half-lives. One half-life of decay was defined as the time interval for the maximum G' value to decay to half its height.

Calcium and Phosphorus Analyses

The Ca and P contents of milks were analyzed as described previously by Choi et al. (2007). Briefly, ashed samples were solubilized with 1 mL of 25% HNO_3 and diluted to 1% HNO_3 with double-deionized water. The concentration of Ca and P was quantified using an Inductively Coupled Argon Plasma Emission Spectrometer (model Varian Vista-Pro AX, Varian Australia Pty Ltd., Clayton, Victoria, Australia). Wavelengths used for Ca and P were 317.9 and 214.9 nm, respectively. Soluble Ca or P in milk samples was defined as Ca or P content in rennet whey $\times$ correction factor (0.998) for the volume of CN precipitate (IDF, 1964): INS Ca or $\text{INS P} = \text{total Ca or total P in milk} - \text{soluble Ca or soluble P in milk}$.

Carlson and Modified Carlson Models

The G' values measured as a function of reaction time were substituted for the shear modulus in the modified Carlson model (Carlson et al., 1987) in Equation [2]. The original Carlson model is shown in Equation [1]:

$$G' = G'_\infty \left[1 - \left(\frac{k_l}{k_l - k_f} \right) e^{-k_f t^*} + \left(\frac{k_f}{k_l - k_f} \right) e^{-k_l t^*} \right], \quad [1]$$

where G'_∞ is G' at $t = \infty$, $t^* = t - t_g$, t_g is gelation time (i.e., reaction time at which G' value is ≥ 0.1 Pa), k_f is the rate constant for the creation of active sites, and k_l is the rate constant for the destruction of active sites. The modified Carlson model is shown in Equation [2]:

$$G' = G'_{\infty} \left[1 - \left(\frac{k_l}{k_l - k_f} \right) e^{-k_f t^*} + \left(\frac{k_f}{k_l - k_f} \right) e^{-k_l t^*} \right] * [e^{-k_s t^*}], \quad [2]$$

where k_s is the rate constant for macro/microsyneresis.

Data Fitting and Statistical Analyses

Our initial strategy was to solve 4 simultaneous equations to obtain initial values for iteration; first, second, and third graphical derivatives of the gelation profiles gave 3 equations and the modified Carlson model provided the fourth equation. We tried to solve these equations for unknown variables (G'_{∞} , k_f , k_l , and k_s) using pencil and paper (manually) or with Mathematica software (Wolfram Research Inc., Champaign, IL), but these approaches proved unsatisfactory. Mathematica software gave another mathematical equations instead of numerical solutions. Alternatively, estimates of some of these parameters were available from literature (Carlson et al., 1987; O'Callaghan and Guinee, 1995), and a wider range of values was selected to include these reported values. These values were used as starting values for the iterative fitting procedure, PROC NLIN procedure from SAS (version 8.2, SAS Institute Inc., Cary, NC). This procedure fits nonlinear regression models by least squares. The Gauss-Newton method (Gallant, 1987) was chosen as the fitting algorithm. One-way ANOVA and Fisher's protected least significance difference were applied to the parameter values obtained for the modified Carlson model. Two-way ANOVA for the effect of reaction time at 2 time points (i.e., GM and the end of experiment) and treatments on LT values was carried out. Pearson correlation coefficients were obtained among INS Ca contents, LT values at 2 time points, and k_s . Significance was indicated by $P < 0.05$. All statistical analyses were conducted using SAS.

RESULTS

Changes of Insoluble Ca and P Contents in Casein Micelles

A detailed explanation was previously given on how milk constituents varied as a result of acidification pH values and the addition of EDTA to milk (Choi et al., 2007), but some important results will be briefly emphasized with regard to changes in the Ca and P contents in CM. Total Ca and P contents in milk for both trials remained constant because no loss of these 2 minerals occurred before or after the treatments. The total Ca content of milk was about 15 mg/100 mL higher than total P content (Figure 1), which is in agreement with

published literature (Law and Leaver, 1998). However, the INS Ca and INS P contents in CM were altered as a result of our manipulations. For the pH trial (Figure 1a) the INS Ca and INS P contents in CM at pH 6.7 were 70.4 and 44.9 mg/100 mL, respectively, which decreased to 11.9 and 18.5 mg/100 mL in milk at pH 5.4, respectively. The INS Ca and INS P contents of milk at pH 6.0 without added EDTA was 48.3 and 33.2 mg/100 mL, respectively, and these concentrations decreased to 20.6 and 15.3 mg/100 mL, respectively, with the addition of 6 mM EDTA (Figure 1b).

Rheological Properties of Rennet-Induced Gels

Representative G' values as a function of reaction time for rennet-induced gels made from milk acidified to various pH values, and from milk with various concentrations of added EDTA are shown in Figures 2a and 3a, respectively. All kinetic profiles (Figure 2a and 3a) for gel formation exhibited 2 features, a GM and a

Figure 1. Total and insoluble Ca and P contents in reconstituted skim milks (a) acidified to different pH values, and (b) with different EDTA concentrations added to milk at pH 6.0. Total Ca (■); insoluble Ca (□); total P (▼); insoluble P (▽). The Ca data were taken from Choi et al. (2007). Values are means of triplicates with error bars for standard error of means.

decrease in G' values after GM with prolonged reaction times. The significance of the GM was interpreted as being due to the disruption of the internal structure of CM (Choi et al., 2007). The decrease in G' values we consider as due to reactions, such as, macro/microsyneresis (Zoon et al., 1988; van Vliet et al., 1989, 1991; Roefs et al., 1990), which occurred sooner in lower pH samples (Figure 2a), and later when higher concentrations of EDTA were added to milk (Figure 3a). The LT values in rennet gels at GM, and at the end of gelation, increased with a decrease in pH values of acidification or with an increase in EDTA concentrations (Table 1). These results are similar to those reported by Roefs et al. (1990). However, no statistical difference ($P < 0.05$) was present between the LT values at GM or at the end of experiment, except for the LT values obtained from rennet gels made from milk with pH value of 6.4.

Fitting of Modified Carlson Model to Gelation Profiles

All experimental gelation profiles were fitted with the modified Carlson model (Equation [2]). The fitted G' values corresponding to the experimental values for the pH and EDTA trials are included in Figures 2a and 3a, respectively. All 6 fitted curves were close to the respective experimental gelation profiles for the milk samples with pH values of 6.7, 6.4, 6.0, 5.8, 5.6, and 5.4 (Figure 2a). The 4 predicted gelation curves for samples from milk with no EDTA added at pH values of 6.7 and 6.0, and with 2 and 4 mM EDTA added looked similar to their experimentally measured profiles (Figure 3a), but some deviation of the fitted curve from the experimental one occurred at reaction time of 1,800 min in the gelation profile from milk made with 4 mM EDTA. The cause appeared to originate in the G' behavior at very long reaction times in this particular profile, its value tending to a constant, stable value rather than a decrease to zero. We have no doubt that inclusion of a constant in the modified Carlson model would give a much better fit to the data of this sample (i.e., instead of the $[e^{-k_s t^*}]$ term, a term such as $[e^{-k_s t^*} + b]$ would have produced a better fit).

Residuals for G' values (observed G' values – fitted G' values) are presented in Figures 2b and 3b for the pH and EDTA trials, respectively. Residuals were not randomly distributed for both treatments; rather, they exhibited some systematic repeated patterns. Esteves et al. (2001) also reported similar patterns for the residuals obtained from the fitting of the Carlson model (equation 1) to the G' values in rennet-induced gels made from milk at pH ~6.7. However, the range for the residuals in our treatment was much greater than those

reported by Esteves et al. (2001). This was probably due to different experimental conditions including different pH values for the CM and reaction times; reaction times measured by Esteves et al. (2001) were only 40 min compared with our prolonged reaction times (up to 54 h). For the comparison to be made among different pH values or different EDTA concentrations, reduced residuals normalized to maximum residual G' were obtained (Figure 2c and 3c). The reduced residuals in both trials had similar trends to residuals reported by Esteves et al. (2001).

Model Parameters

Parameter values were obtained by the fitting of actual G' values in each gelation profile to the modified Carlson model as a function of reaction time. These parameters are shown in Figure 4. With a decrease in the pH values of milk before the addition of rennet, the G' values at theoretically infinite reaction time (G'_∞ , which is experimentally unattainable, though readily derived by curve-fitting) increased from 242 to 640 Pa for pH 6.7 and 5.4, respectively (Figure 4a). As pH values were reduced from 6.7 to 5.6, the rate constant, k_f , for the creation of bonding sites on CM by rennet-mediated hydrolysis of κ -CN, went through a minimum (0.0016 min^{-1}) at pH 6.0 (Figure 4b), but k_i , the rate constant at which the active sites form bonds with other active sites of CM, the aggregation rate constant, passed through a maximum (0.0257 min^{-1}) at pH 6.0 (Figure 4c). The rate constant for macro/microsyneresis, k_s , also showed the same trend as the k_f at pH 6.0, the k_s had the lowest value of 0.0015 min^{-1} (Figure 4d). We note also that k_s shows a similar trend to G'_∞ (cf. Figures 4a and 4d). At pH 6.7, k_f values were similar to k_i . Our values for k_f and k_i were lower than those reported by Carlson et al. (1987), but close to those reported by O'Callaghan and Guinee (1995); k_f is dependent on enzyme concentration.

The parameters obtained from the EDTA trial are shown in Figures 4e to 4h. With an increase in concentration of EDTA from 0 to 6 mM, G'_∞ (Figure 4e), k_f (Figure 4f), and k_i (Figure 4g) decreased from 1,130, 0.0064, and 0.009 to 22 Pa, 0.0025 min^{-1} , and 0.0025 min^{-1} , respectively. Maximum k_s , the rate constant for syneresis, was observed in gelation profiles for rennet gels made from milk with 2 mM EDTA added, and the maximum k_s decreased to 0.0001 min^{-1} when 6 mM EDTA was added (Figure 4h). The rennet-induced gels made from milk at pH 6.7, which served as a control in both trials, had different values for the 4 parameters. This is probably due to slightly different concentrations of CaCl_2 added to shorten the gelation time, as well as

Figure 2. Typical development of actual experimental (a, closed symbol) storage modulus (G') and fitted (a, open symbol) G' values to modified Carlson model as a function of reaction time for rennet-induced milk gels made from milk acidified to pH 6.7 (black hexagon, open hexagon), 6.4 ($\blacklozenge$, $\lozenge$), 6.0 ($\blacktriangledown$, $\triangledown$), 5.8 ($\blacktriangle$, $\triangle$), 5.6 ($\blacksquare$, $\square$), and 5.4 ($\bullet$, $\circ$). Residuals (b) were difference between experimental and fitted G' , and reduced residuals (c) were residuals that were normalized to maximum residual G' . Results shown are for a single gelation curve at different pH values.

the different rennet concentrations used to coagulate milk in both treatments.

Possible associations among INS Ca contents, LT values at GM, LT values at the end of gelation, and k_s are shown in Table 2. For the pH trial, significant negative correlations were found between INS Ca contents and LT values of rennet gels measured at GM and at the end of gelation with r -values of -0.85 ($P < 0.01$) and -0.76 ($P < 0.01$), respectively. The k_s was negatively correlated with INS Ca contents, and was positively

correlated with both LT values. However, for EDTA trial, k_s was negatively correlated with both LT values, and not significantly correlated with INS Ca contents ($r = 0.32$, $P > 0.05$).

DISCUSSION

The gelation profiles (Figures 2a and 3a) presented 2 important characteristics: the presence of GM and a decrease in G' values after GM with very long re-

Table 1. Loss tangent values at the maximum storage modulus (GM) and at the end of rennet gelation (END) for the pH and EDTA trials

		pH value of acidification						
pH trial		6.7	6.4	6.0	5.8	5.6	5.4	SEM
LT	GM	0.35 ^a	0.36 ^a	0.40 ^a	0.45 ^a	0.57 ^a	0.88 ^a	0.01
	END	0.39 ^a	0.41 ^b	0.40 ^a	0.42 ^a	0.54 ^a	0.88 ^a	0.01
		EDTA concentration (mM) at pH 6.0						
EDTA trial		pH 6.7 at 0 mM EDTA	0	2	4	6		
LT	GM	0.33 ^a	0.38 ^a	0.41 ^a	0.51 ^a	0.58 ^a		0.01
	END	0.36 ^a	0.38 ^a	0.40 ^a	0.53 ^a	0.60 ^a		0.01

^{a,b}Means with different superscript letters within the same column are significantly different ($P < 0.05$).

Figure 3. Typical development of actual experimental (a. closed symbol) storage modulus (G'), fitted (a. open symbol) G' values to modified Carlson model as a function of reaction time for rennet-induced gels made with different EDTA levels and subsequently adjusted to pH 6.0; normal milk at pH 6.7 (black hexagon, open hexagon), and milk at pH 6.0 with 0 ($\bullet$, $\circ$), 2 ($\blacksquare$, $\square$), 4 ($\blacktriangle$, $\triangle$), and 6 mM ($\blacktriangledown$, $\triangledown$) added EDTA. (b) and (c) are residuals and reduced residuals, respectively. Results shown are for a single gelation curve at different EDTA concentration.

Figure 4. Values (means $\pm$ SEM) of the parameters for modified Carlson model applied to a storage modulus as a function of reaction time from gelation time to the end of gelation obtained from the pH and EDTA trials.

action times. Because a trypsin inhibitor was added to all milk samples, it was very unlikely that plasmin (a trypsin-like enzyme)-mediated proteolysis initiated inter/intra-rearrangements of CN particles and thereby caused a reduction in G' . Instead, it is very likely that the loss of internal bonds within CM increased the propensity for rearrangements of CN molecules within and

between aggregated CM in these gels. We believe that these intra/inter-rearrangements result in a weakening of these gels leading to endogenous syneresis, which was supported by the microstructural changes previously observed in these gels during aging (Choi et al., 2007). In this study, our aim was to investigate/quantify rate constants for the production of the rennet-induced gels

Table 2. Correlation coefficients among rate constants for macro/microsyneresis (k_s), loss tangent (LT) values at maximum storage modulus (GM) and at the end of gelation (END), and insoluble (INS) Ca contents in rennet-induced gels for the pH and EDTA trials

Item	pH trial			EDTA trial		
	INS Ca	LT at GM	LT at END	INS Ca	LT at GM	LT at END
LT at GM	−0.85**			−0.91**		
LT at END	−0.76**	0.98**		−0.86**	0.98**	
k_s	−0.81**	0.98**	0.96**	0.32	−0.60*	−0.69**

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

made from CM with modified internal bonding, implementing a modified Carlson model for this task, and to relate this to ongoing endogenous syneresis behavior.

Our modified Carlson model (Equation [2]) reproduced the observed behavior very well (Figures 2a and 3a). The modified equation consists essentially of 3 terms, G'_∞ , the bracketed term describing the kinetics of the creation of activated micelles and their removal by incorporation into aggregates and coagulum, and finally the exponential decay in elastic modulus, which we ascribed to endogenous syneresis.

If k_s , which is the rate constant within the exponential decay term, was zero, then we would have observed sigmoidal curves which tended to G'_∞ . However, we did not observe sigmoidal curves (Figure 2a), which indicates that this decay term was not zero. The decay term also operates from the earliest moments of the reaction (because our modified Carlson model fit the experimental data very well) indicated that the system had the potential to synerese from the start also; indeed this ability is an innate property of the CM.

This argues against rearrangements of strands or micelles in the gel network as being the sole factor responsible for the kinetic growth of G' values (Mellema et al., 2002). Mellema et al. (2002) claimed that in rennet gels all of the growth in gel strength could be ascribed to rearrangements, because they were using the fractal model developed by Bremer et al. (1989). This fractal model requires that at the gelation time all of the particles are incorporated into the gel network, otherwise they cannot make the assumption that the volume fraction occupied by the cluster is the same as the volume fraction of CM in the system. Mellema et al. (2002) found no free CM in their specific experiments, but micellar aggregates (dimers, trimers, and polymers) might have been present in the sol, but trapped in the network, and they would not contribute to the gel strength until they were incorporated into the network. Indeed they might have been partially connected to the network but as dangling ends, which were not yet connected enough to form complete load-bearing strands and hence were not significantly contributing to the G' values. The Carlson model successfully describes the aggregation and growth of the network, the incorporation of micellar aggregates, and the closing of these dangling loops. Rearrangements, both internal and intramicellar, may also contribute, but only after a network has been formed to then rearrange.

Internal bonding of CM was modified by depleting the levels of INS Ca and INS P (Figure 1). The particle size of CM hardly changes within the pH range of 6.7 to 5.4 (Roefs et al., 1985; Marchin et al., 2007), and the CM porosity increases as increasing amounts of CN become solubilized with decreasing pH values

(Vreeman et al., 1989). These suggest that the hard sphere feature of CM in our samples was not preserved (i.e., the CM were soft or at least had different internal bonding from native CM). When rennet-induced gels were made from these milk samples, the LT values (both at GM and at the end of experiment) increased (Table 1) with a decrease in pH (especially at pH <6.0; Table 1) as internal bonding diminished with a decrease in CCP. This indicates that protein-protein bonds became more mobile with shorter relaxation times, which would be expected to facilitate macro/microsyneresis (Roefs et al., 1990). In Horne's dual-bonding model for the structure of CM (Horne, 1998), CCP is an important material that supports the integrity of CM. This loss of CCP would reduce the number of bonds within CM or casein-based gel networks, which affects macro/microsyneresis. The LT values at the end of gelation were the same as those at GM except for one gelation profile (obtained from milk samples with pH value of 6.4), which may indicate that the overall nature of the bonds does not change significantly in gels beyond GM over the remainder of gel development.

In the model described by Horne (1995), G'_∞ is the shear modulus achieved at infinite (unattainable) reaction time when all particles are incorporated into the gel network. van Vliet and Walstra (1985) wrote G' as

$$G' = CN \frac{d^2F}{dx^2},$$

where N is the number of stress-bearing strands per unit area in a cross-section perpendicular to x , the direction of external force or stress; C is a characteristic length determining the geometry of the network; and dF is the change in Gibbs free energy when the elements are moved apart over a distance dx , and is therefore related to the bond strength in systems where enthalpic effects dominate over entropic, as argued by van Vliet and Walstra (1985). Bond strength will be influenced by the remaining (electrostatic) repulsion on CN following κ -CN proteolysis. Horne (1995) assumed this bond strength to be constant, but reducing pH decreases micellar charge and consequently electrostatic repulsion, deepening the potential energy well that is seen as bond strength, and consequently increasing the calculated G'_∞ in the pH trial (Figure 4a).

In both trials, CaCl_2 was added to accelerate the gelation, more being added in the EDTA trial. The effect of addition of Ca^{2+} was explained by Horne and Lucey (2014) as reducing the remnant electrostatic charge on CNs through binding of Ca^{2+} . This reduced electrostatic charge would explain the higher G'_∞ obtained at pH 6.0 without EDTA compared with the sample made at pH 6.7 (Figure 4e). The poor fit in the plot

obtained with 4 mM added EDTA and the lack of a defined maximum with 6 mM added EDTA, suggest that disruption of internal bonds has been too severe in these systems. The downturn in the values of G'_{∞} when EDTA is included might also be explained by the excessive softening of the CM through the removal of internal bonds (as demonstrated by the loss of INS Ca from CM and increased LT values of gels).

As far as rate constants (Figure 4) are concerned, the following 2 points should be kept in mind. First, as parameters were highly correlated, some caution should be exercised in the interpretation of these parameters. Second, our fitting method was not the same as that used in the original Carlson model (Carlson et al., 1987). The model parameters reported by Carlson et al. (1987) were estimated from a Formagraph instrument and outputs were converted into some measure of elasticity for inclusion in their model. Our parameters were directly measured using small strain rheology. We obtained 4 rate parameters simultaneously by iteration until the sum of square error term was minimal, but Carlson et al. (1987) were able to use a different approach. Because they did several experiments on the same milk under the same conditions of pH and temperature, this allowed them to obtain values of G'_{∞} and k_i at an excess concentration of rennet. Having obtained, and fixed, G'_{∞} and k_i , they then obtained k_f as a function of rennet concentration. Because we changed the reaction pH or CCP contents, we anticipated that this would influence all 4 fitting parameters and hence our chosen option was to fit all 4 parameters simultaneously in the same calculation. The k_f is a rate of the first of 2 reactions where rennet creates reactive crosslinking sites on the surface of CM, and k_i is a rate of the second reaction where reactive sites form crosslinks, leading to gel network.

We observed the maximum k_f value at pH 5.6 (Figure 4b). van Hooydonk et al., 1986 reported that rennet has optimum activity in milk at pH $\sim$ 6.0. The pH optimum for the hydrolysis of κ -CN by chymosin has been reported as around 5.2 (Humme, 1972). Another possible complicating factor during acidification of milk is that chymosin adsorption onto CM increases with a decrease in pH (Larsson et al., 1997). Carlson et al. (1987) reported relatively similar k_f values at pH 6.6 and 6.3 in their renneting experiments.

It is generally considered that the rate of aggregation (in our model the k_i value) of renneted CM increases with a decrease in pH (van Hooydonk et al., 1986); a reduction also occurs in the extent of hydrolysis of the macropeptide required to initiate gel formation with a reduction in pH (van Hooydonk et al., 1986). Certainly, below pH 6.0, or in the presence of added EDTA, reducing CCP content of the CM has an unfavorable

influence on the rate of aggregation and gel formation (Figure 4). Acidification of milk in the presence of Ca-chelating agents reduces the CCP concentration, resulting in the inhibition of rennet coagulation, which does not occur below pH 6.2 [i.e., when approximately 30% of CCP has been solubilized (Shalabi and Fox, 1982)]. Severe disruption of the CM and solubilization of the caseins inhibits gel formation. In our experiments, we added Ca^{2+} to the EDTA samples, which allowed coagulation to still occur, but the k_i value did not change much in the different treatments (Figure 4g). In the case of EDTA addition, the sequestration of calcium may increase micellar charge, leading to an increased repulsion and lower aggregation rates. Acidification of milk in the absence of EDTA (i.e., our pH trial) decreased the CCP content of CM, but in this case the Ca^{2+} concentration increased, which helped offset the deleterious effect of the reduced CCP content on rennet coagulation (Shalabi and Fox, 1982). With a decrease in pH, electrostatic repulsion diminishes, and the k_i value (rate of aggregation) increased with a maximum observed at pH 6.0 (Figure 4c). However, at pH values <6.0 , the k_i value decreased again, possibly due to excessive losses of CCP (disruption of the CM), which negatively affect rennet coagulation.

More definitive conclusions can be drawn from our calculated values of k_s , the rate constant within the exponential decay term, which was added as a third multiplicative term to the modified Carlson model (Equation [2]) to describe the effects of endogenous syneresis. As to why endogenous syneresis could bring about a decrease in elastic modulus, we offer the possible depiction of gel formation/aging shown in Figure 5. This shows, in a series of snapshots, the progress of the coagulation reaction, from individual micelles in Figure 5a, through small aggregates in Figure 5b, to percolating clusters in Figure 5c, and then in Figure 5d, the change in cluster morphology as groups of linked micelles within the cluster coalesce into larger particles with fewer stress-bearing links between them (facilitated by high bond mobility). The last step reduces the measurable (actual) elastic modulus and the coalescence displaces trapped (interstitial) water from the aggregates (syneresis). Note that this depiction is limited to this particular snapshot (Figure 5d) purely for illustrative purposes. That our measurements fit the modified Carlson model so well indicates that syneresis is ongoing from the earliest stages of aggregation, and would also be occurring, say, in the time-frame of snapshot Figure 5b when we only have small aggregates but not the percolating clusters that would define gelation.

In our pH trial, k_s increased as the pH was reduced from pH 6.0 to 5.4. As we reduced the pH and decreased internal bonding through the removal of CCP,

Figure 5. Schematic representation of macro/microsyneresis occurring in our gel system. (a) Rennet-converted micelle (18 monomers in this diagram) begins to move around with dimers, trimers, or quadramers being about to be formed as reaction progresses. (b) Some time afterward, gelation occurs as monomers, dimers, trimers, or quadramers are incorporated into percolation strands, increasing G' . (c) Most of renneted micelles are participating in the gel network, resulting in maximum in G' . (d) However, after GM, bonds within and between micelles in small aggregates (2 dimers, 2 trimers, and 2 quadramers in diagram d) are redistributed, which can be envisaged as rearranging into blob- or spherical-like aggregates. These would look to all intents and purposes as larger micelles going into aggregation phase, and fewer of them would be present. So gels would be weaker as predicted by the number of stress-carrying bonds that could be created between them. Also it would be a first-order effect because it is within the micelle or aggregate once formed. The diagram in (d) ties in with the micrographs (Choi et al., 2007).

this made it easier for the aggregating micellar clusters to rearrange into larger particles and promoted syneresis. The larger particles also mean larger pore sizes, a further factor enhancing syneresis. Larger aggregates and pore sizes were found in micrographs taken 2 to 6 h after GM, as previously reported by Choi et al. (2007).

In the EDTA trial, the highest k_s values were observed in gels made with 2 mM added EDTA, but very low values for k_s were obtained with 4 and 6 mM EDTA. The changes in k_s throughout this series were consistent with the sizes of aggregates seen previously in micrographs taken between 3 to 10 h after GM (Choi et al., 2007). The very low values of k_s in the samples made with 4 or 6 mM EDTA compared with gels made with 2 mM EDTA, may be related to the extremely long gelation times used in these profiles (170 and 615 min for samples with 4 and 6 mM EDTA, respectively). Much of the coalescence/internal rearrangements that are enhanced by removal of INS Ca may have already

occurred in small aggregates before gelation being detectable, shifting the processes of syneresis and gelation into different time-frames from the picture (Figure 5) envisaged.

Just as indicated by van Vliet et al. (1991), we found highly significant correlations between the rate constants for syneresis and the LT values of our gels (Table 2). The poor correlation for the EDTA trial we have already explained above. The LT values were also highly correlated with the levels of INS Ca. Decreasing the levels of internal bonding in CM therefore promoted endogenous syneresis.

CONCLUSIONS

Using a modified Carlson model, we investigated the effect of the depletion of internal micellar bonds, such as those cross-linked to calcium phosphate, on extended duration cure curves measured during rennet gel forma-

tion. These cure curves show a maximum in gel elasticity followed by a mono-exponential decay. The decay in gel strength we ascribe to endogenous syneresis, an innate property of the CM. The rate of syneresis can be enhanced by decreasing internal micellar bonding brought about by reducing milk pH or by addition of EDTA at a fixed milk pH value.

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