Effect of pH on the Gelation Properties of Skim Milk Gels Made From Plant Coagulants and Chymosin

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ABSTRACT

The effect of three milk pH values, 6.0, 6.3 and 6.7, on gelation properties was monitored by small amplitude oscillatory rheology as well as a large deformation (yield) test for gels induced by the plant coagulants, *Cynara cardunculus* L. and *Cynara humilis* L., and chymosin. Gel microstructure was studied using confocal scanning laser microscopy. For each coagulant, a decrease in pH of milk resulted in a decrease in gelation time (t_g), and an increase in the rate of increase in storage modulus (G'). At pH 6.0 and 6.3, plant coagulant-induced gels reached a maximum value in G' ($G'_{\max}$) followed by a decrease in G' values during the rest of the experiment, reflecting higher proteolytic activity of plant coagulants towards caseins as determined by SDS-PAGE. Gels produced at pH 6.0 and 6.3, exhibited a minimum in loss tangent ($\tan \delta$) followed by slight increase in $\tan \delta$ during gel aging, that may have been associated with faster rearrangements of the gel network structure. In gels aged for ~6 h, the values for G' , $\tan \delta$ at low frequency (0.006 Hz) and yield stress were higher for chymosin than for plant-induced gels. For gels with the same pH value, no major differences were observed in microstructure between coagulants. Gels made at low pH values (6.3 and 6.0) appeared to have a denser or more interconnected structure than gels made at pH 6.7. Our results suggest that, at a low pH, the type of coagulant used in gelation is likely to have a considerably impact on gel/cheese structure.

(**Key words:** gelation pH, plant coagulants, rheology, milk coagulation)

Abbreviation key: CCP = colloidal calcium phosphate, CSLM = confocal scanning laser microscopy, dG'/dt = rate of increase of storage modulus, dG''/dt =

rate of increase of loss modulus, G' = storage modulus, G'' = loss modulus, $G'_{\max}$ = maximum value of storage modulus, $\tan \delta$ = loss tangent, t_g = gelation time.

INTRODUCTION

The coagulant of *Cynara cardunculus* L. is commonly used in Portugal for cheesemaking (e.g., Serra da Estrela cheese). Because the cheeses produced with *C. cardunculus* L. have unique texture and flavor characteristics they have been designated with Protected Designation of Origin (EEC, 1996). A related plant species, *Cynara humilis* L., is also used for cheesemaking.

Milk pH is an important environmental factor in the gelation phase of cheese making. During the production of most cheeses in Western countries, it is common practice to add lactic acid bacteria to milk, generically known as “starters,” to develop acidity and promote coagulation. The pH of milk affects both the enzymatic and aggregation reactions. By lowering pH, there is a decrease in the colloidal stability of milk. The pH of milk directly influences enzymatic activity of coagulant. Both chymosin and plant coagulants have maximum proteolytic activity on casein at pH ~6.0 (Van Hooydonk et al., 1986b; Faro, 1991).

Plant coagulants share many features with chymosin; they are aspartic proteinases, they hydrolyze the Phe₁₀₅-Met₁₀₆ bond of κ -casein and have a similar catalytic coefficient (K_{cat}/K_m) towards κ -casein (Macedo et al., 1993). However, plant coagulants are slightly more proteolytic on caseins and have a broader specificity than chymosin. The coagulant obtained from *C. cardunculus* L. has two aspartic proteinases named cardosin A and cardosin B (Pires, 1998). These enzymes have been isolated and characterized at the biochemical and molecular levels (Pires, 1998; Faro et al., 1999; Egas et al., 2000; Vieira et al., 2001). Only a cardosin A-like proteinase was detected in the coagulant of *C. humilis* L. (Esteves, 1995). Cardosin B is more proteolytic on casein than cardosin A (Esteves et al., 1995; Pires, 1998).

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In our previous studies on plant coagulants, we investigated the suitability of various mathematical models for the milk gelation process (Esteves et al., 2001), and performed a detailed comparison of the rheological properties of plant- with chymosin-induced gels at the natural pH of milk (~6.5 to 6.7) (Esteves et al., 2002). The pH of milk is an important parameter in gelation and cheese quality. The pH of milk also influences the activity of coagulants. In the present study, we studied the effect of pH on the gelation characteristics of skim milk gels produced by the plant coagulants *C. cardunculus* L. and *C. humilis* L., and chymosin.

MATERIALS AND METHODS

Materials

Low-heat nonfat dry milk (**NDM**) as supplied by Dairy Farmers of America (Fresno, CA). The plant coagulants from *C. cardunculus* L. and *C. humilis* L. were obtained as described by Esteves et al. (2001). The fermentation-produced chymosin used was Maxiren DS (DSM Gist-Brocades, 2600 MA Delft, The Netherlands), and its strength was 600 International Milk Clotting Units (IMCU/ml) (IDF, 1997). Soybean trypsin inhibitor, type I-S, (Lot. 30K7020) was supplied by Sigma Chemical Co. (St. Louis, MO).

Preparation of Milk Samples

Nonfat dry milk with 7.05 mg/g (wt/wt) of undenatured whey protein nitrogen in NDM was used (Bradley et al., 1992). NDM was reconstituted (9%, wt/wt) in an aqueous solution with CaCl_2 (0.1 mg/ml); NaN_3 (0.2 mg/ml) was added to prevent bacterial growth along with soybean trypsin inhibitor (0.15 mg/ml), which inhibits plasmin activity. Milk was dispersed at 32°C for 2 h with gentle agitation and then left at 22°C for at least 1 h. The pH of the reconstituted milk was ~6.7. In experiments with milk at pH 6.3 and 6.0, pH was slowly adjusted with lactic acid (9% vol/vol) with the dilution of milk not exceeding 2.7% (vol/vol). After the desired pH was reached milk was left for 15 min.

Rheological Assays

A Universal Dynamic Spectrometer, Paar Physica UDS 200 (Physica Messtechnik GmbH, D-70567, Stuttgart, Germany) was used in the rheological assays. The measuring geometry (MK 25) consisted of a cone (diameter 75 mm and 2° angle) and a plate.

Before comparing the gelation properties of *C. cardunculus* L., *C. humilis* L., and chymosin, the same gelation time (t_g ; ~19 min) was assigned to all three coagulants in milk gelation experiments conducted at

the natural pH of milk (pH ~ 6.7). The amounts of each coagulant necessary to obtain a t_g of ~19 min with milk at pH 6.7, was also used in the experiments at pH 6.3 and 6.0. In additional experiments at pH 6.0, smaller amounts of coagulants were used for *C. cardunculus* L., *C. humilis* L., and chymosin samples, which were diluted 8.8, 8.5 and 4.7-fold, respectively, compared to their concentration used at pH 6.7. All gelation assays were performed at 32°C and were followed for ~6 h after the addition of coagulant to milk. In the assays at pH 6.3 and 6.0, after a delay of 2 min, measurements were taken in the following sequence, every 30 s during first 2 min, every 1 min during next 2 min, every 2 min for 30 min, and then every 30 min for 5.5 h. Gels produced at pH 6.7 were tested every 2 min during the first 30 min of assay, and then every 30 min for the rest of the experiment. Before starting the assays, milk was equilibrated for 15 min at 32°C, 25.5 μl of previously diluted enzyme was added to 4.25 ml of milk, which was then mixed thoroughly, and the mixture immediately transferred to the plate of the rheometer. The exposed edge of the cone and plate geometry was covered with vegetable oil to prevent dehydration of the sample.

In the gelation experiments, samples were oscillated at a frequency of 0.1 Hz and the strain applied was 0.03, which is within the linear viscoelastic region for rennet gels (Zoon et al., 1988). In the present work, t_g was (arbitrarily) considered the time necessary for the gel to reach a storage modulus (G') value of 0.5 Pa. The effect of the time-scale of deformation on the rheological properties was determined by a frequency sweep ~6 h after the addition of coagulant; frequency was varied from 0.006 to 1 Hz. Loss tangent ($\tan \delta$), is the ratio between the viscous modulus (G'') and the elastic modulus, i.e., $\tan \delta = G''/G'$.

The large deformation properties were studied ~6 h after addition of coagulant. Gels were subjected to a shear rate of 0.01 s^{-1} , up to yielding of the gel. The yield stress and shear deformation at yielding were defined as the point when the shear stress started to decrease.

Confocal Scanning Laser Microscopy (CSLM)

The use of CSLM for evaluating the microstructure of milk gels has been reported by Srinivasan and Lucey (2002). The fluorescent protein dye, Acridine orange (~0.2%, wt/vol), was dissolved in demineralized water, and several drops were added to milk. Milk samples were stirred for ~2 min to disperse the dye. Milk was warmed to 32°C and rennet added. After stirring for 2 min, a few drops of the mixture were transferred to special object slides with a cavity, and a coverslip was placed over the sample. The slide was then placed in a petri dish and held in a temperature-controlled incubator.

Table 1. Effect of pH on gelation time for gels induced by the coagulants *Cynara cardunculus* L., *Cynara humilis* L. and chymosin.¹

Type of coagulant	Gelation time (s) ³		
	6.0 pH	6.3 pH	6.7 pH ²
<i>Cynara cardunculus</i> L.	192 (6.5) ^{aA}	309 (15.3) ^{bA}	1136 (39.1) ^{cA}
<i>Cynara humilis</i> L.	193 (7.1) ^{aA}	323 (10.5) ^{bA}	1112 (37.2) ^{cA}
Chymosin	224 (16.8) ^{aA}	335 (20.0) ^{bA}	1123 (44.2) ^{cA}

^{a,b}Means with different superscripts within each coagulant, and ^{A,B}means within each pH value are different ($P < 0.05$).

¹Values are means from replicates ($n = 3$) with SD in brackets.

²Milk at natural pH.

³Time point when gels had a storage modulus of 0.5 Pa.

tor (model 650F, Fisher Scientific, Hanover, IL) at 32°C for approximately 6 h. The gels were then examined on a Bio-Rad MRC 1024 CSLM (Hemel, Hempstead, UK) attached to an inverted Nikon Eclipse TE 300 microscope, which had a 60× oil immersion objective with a numerical aperture of 1.4. The CSLM has an air-cooled Ar/Kr laser that was used with an excitation wavelength of 488 nm. Many fields were viewed and typical micrographs were reported.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The extent of degradation of caseins during the gelation phase (i.e., 6 h after addition of coagulants to milk) was determined by SDS-PAGE on a mini-Protein 3 electrophoresis unit (Biorad Laboratories, Richmond, CA) using the method of Laemmli (1970) described by the manufacturer. The separating gel was composed of 16% acrylamide (2.67% C) made up in Tris/HCl buffer, pH 8.8, and the stacking gel was composed of 4% acrylamide (2.76% C) in Tris/HCl buffer, pH 6.8. Both gel buffers contained 0.1% SDS. Gel samples were dispersed by vortexing for 2 min in sample buffer containing an additional 2% SDS and 0.05% mercaptoethanol, and the solutions heated at 95°C for 5 min. The gels were run at 200 V for about 45 min and then stained with 0.025% Coomassie Blue G-250 dissolved in 7% acetic acid and 40% methanol solution. Gels were destained by washing with several changes of 7% acetic acid and 40% methanol solution. Two different samples volumes were used for SDS-PAGE: a) milk gels were diluted 1:2 with sample buffer and 5 μ l loaded; and b) milk gels were diluted 1:12.3 with sample buffer and 10 μ l loaded.

Statistical Analysis

All statistical analyses were conducted using the SAS program (SAS, 1999). The mixed model procedure, Proc Mixed, was used for the analysis of results. The t_g re-

sults were log-transformed because they did not follow a normal distribution. Means were compared using the Tukey-Kramer procedure. Significance was indicated by $P < 0.05$. Each experiment was repeated three times.

RESULTS

Gelation Time

Lowering milk pH greatly decreased t_g for all coagulants ($P < 0.001$; Table 1). The decrease in t_g was more pronounced when the pH of milk was reduced from pH 6.7 to 6.3 compared with reducing the pH of milk from 6.3 to 6.0. At both pH 6.3 and 6.0, t_g values were very short. No significant differences in t_g were obtained between the three coagulants at any of the pH values used in this study.

G' as a function of time. Changing pH had a considerable impact on the shape of the gelation profiles of all coagulants (Figure 1). Lowering pH accelerated the gel formation process. In gels made at pH 6.0 and 6.3, the G' profiles were similar when the values were increasing rapidly, but shortly after gelation (i.e., <1 h after coagulant addition) the gelation curves of plant coagulants and chymosin started to exhibit considerable differences (Figure 1). Plant coagulant-induced gels exhibited a clear maximum in G' values ($G'_{\max}$) that was followed by a steady decrease in G' values until the end of the experiment (Figure 1b, c). The final G' values of chymosin gels (~6 h after addition of coagulant) were greater than those obtained for plant coagulant-induced gels (Figure 1). At pH 6.7, the relative gelation behavior of chymosin- and plant-induced gels was different compared with gels made at pH 6.0 and 6.3. The gelation profiles of chymosin and plant coagulants at the natural pH of milk were described elsewhere (Esteves et al., 2001, 2002).

There were differences between coagulants in the $G'_{\max}$ values, which in some (but not all) cases occurred well before the end of the experiment. At each pH value investigated, chymosin had larger $G'_{\max}$ values than

Figure 1. Storage modulus, G' , as a function of time, t , for skim milk gels obtained at pH values 6.7 (a), 6.3 (b), and 6.0 (c) using the coagulants *Cynara cardunculus* L. (x), *Cynara humilis* L. (▲), and chymosin (●). G' vs t at pH 6.0 (panel 1d), of plant coagulants and chymosin diluted ~8.7- and 4.7-fold, respectively. Time zero corresponds to addition of coagulant to milk. Values are means ($n = 3$) and vertical bars are the standard deviations for the last data points. At pH 6.0, at the lower concentration of coagulant, syneresis was observed in plant-induced gels ~4 h after the addition of coagulant to milk. The results for that part of the gelation profile were not plotted in panel 1d.

plant coagulants (Table 2). The pattern for $G'_{\max}$ was similar for both plant coagulants. The G' values at the end of the experiments (~6 h after coagulant addition) were also different for plant coagulants compared with chymosin (Figure 1; Table 3). At the end of gelation, regardless of milk pH, gels produced with chymosin had higher G' values than those produced with plant coagulants ($P < 0.004$). The highest G' values at the end of gelation were obtained for chymosin-induced gels at pH 6.3 ($P < 0.004$; Table 3). At the end of the experiments, the lowest G' values in gels produced with plant coagulants were observed in milk at pH 6.0 ($P < 0.002$). Regardless of milk pH, both plant coagulants had similar G' values at the end of gelation.

For all coagulants, t_g value at pH 6.0 was considerably shorter than t_g at pH 6.7 (Table 1). Additional experiments were performed to compare the gelation profiles at pH 6.0 and 6.7 when they had a similar t_g .

In those experiments, plant coagulants and chymosin were diluted 8.8 to 8.5 and 4.7 times, respectively. In gels produced at pH 6.0 with plant coagulants at a low enzyme concentration, syneresis was evident ~4 h after coagulant addition to milk, and these results were not considered for data analysis. Regardless of chymosin concentration used to gel milk at pH 6.0, there was no significant difference in $G'_{\max}$ values (Table 2); a similar result was obtained at the end of gelation. That was not the case for plant-induced gels, where the lower coagulant concentration produced the highest G' values, at least at the pH values investigated.

Tan δ Values

Figure 2 shows the results of the changes in tan δ with time during the gelation process. In contrast to pH 6.7, in gels formed at pH 6.3 and 6.0, there was

Table 2. Effect of pH on the maximum value for storage modulus, $G'_{\max}$, for gels induced by the coagulants *Cynara cardunculus* L., *Cynara humilis* L. and chymosin.¹

Type of coagulant	$G'_{\max}$ (Pa) ⁴			
	6.0 pH	6.3 pH	6.7 pH ²	6.0 _{low enzyme conc.} pH ³
<i>Cynara cardunculus</i> L.	74.1 (1.2) ^{abA}	77.3 (3.2) ^{aA}	69.6 (2.3) ^{bA}	86.6 (2.3) ^{cA}
<i>Cynara humilis</i> L.	78.8 (3.1) ^{aA}	82.3 (2.4) ^{aA}	71.2 (2.0) ^{bA}	88.7 (1.2) ^{cA}
Chymosin	100.4 (4.1) ^{aB}	100.4 (3.5) ^{aB}	83.3 (1.0) ^{bB}	101.9 (2.8) ^{aB}

^{a,b,c}Means with different superscripts within each coagulant and ^{A,B}means within each pH value are different ($P < 0.05$).

¹Values are means from replicates ($n = 3$) with SD in brackets.

²Milk at the natural pH.

³Plant coagulants and chymosin diluted ~8.7 and 4.7 times, respectively. Gelation time (~19 min) was similar to gels made at pH 6.7.

⁴Maximum value of storage modulus reached during milk gelation.

increase in $\tan \delta$ values during gel aging (Figure 2b, c). As expected, in the beginning of gelation, regardless of pH value, there was a sudden decrease in $\tan \delta$ values. At pH 6.0 and 6.3, the initial decrease in $\tan \delta$ values was followed by a slight increase during the rest of the experiment. Although the absolute difference in the $\tan \delta$ values at this minimum and at the end (~6 h) of the gelation phase was small, the increase in $\tan \delta$ values was significant ($P < 0.001$). This was also observed when a lower concentration of coagulant was used to coagulate milk at pH 6.0 (Figure 2d). All $\tan \delta$ curves obtained for *C. humilis* L. tended to have an intermediate behavior between chymosin and *C. cardunculus* L.

Frequency Sweeps

These tests were performed ~6 h after the coagulant was added to milk. The $\tan \delta$ values at low frequency (0.006 Hz) were significantly influenced by both pH value of milk and type of coagulant used ($P < 0.0001$). At low frequency, regardless of pH of milk, $\tan \delta$ values for chymosin were higher than those of plant coagulants and no differences were obtained between plant coagu-

lants (Table 3). For all coagulants, the $\tan \delta$ values at pH 6.0 were higher than at 6.7, although the difference between values was small.

Large Deformation Rheology

Table 3 shows the yield stress and strain values obtained in gels ~6 h after coagulant addition to milk. Regardless of pH of milk, yield stress values for chymosin were higher than those obtained for plant coagulants, and no differences were obtained between plant coagulants. Plant coagulants had the highest yield stress values at pH 6.3. In the case of chymosin, stress values at pH 6.0 and 6.3 were higher than at pH 6.7. At each of the pH values tested, shear strain was not significantly different between coagulants. However, there was a significant effect of pH value used ($P < 0.0001$). For all coagulants the yield strain values for milk at the natural pH (6.7) were significantly lower than at the other pH values.

Gel Microstructure

The effect of pH on the microstructure of the coagulant-induced gels is shown in Figure 3. For gels with

Table 3. Effect of pH of milk on rheological properties obtained in milk gels ~6 h after coagulant addition to milk. Gels were produced by *Cynara cardunculus* L., *Cynara humilis* L. and chymosin.

	<i>Cynara cardunculus</i> L.			<i>Cynara humilis</i> L.			Chymosin		
	6.0 pH	6.3 pH	6.7 pH ¹	6.0 pH	6.3 pH	6.7 pH ¹	6.0 pH	6.3 pH	6.7 pH ¹
G' (Pa) ²	51.8 ^b	66.0 ^c _{fg}	69.2 ^{eg} _h	54.9 ^b	71.5 ^{cde}	70.0 ^d _{fg}	90.7 ^a	101.9 ⁱ	83.3 ^b
$\tan \delta$ ³	0.490 ^{be-i}	0.480 ^f _{nop}	0.474 ⁱ _{mpq}	0.503 ^{b-d}	0.488 ^{ce} _{j-m}	0.474 ^h _{loq}	0.541 ^a	0.532 ^a	0.502 ^d _{gkn}
Yield									
Stress (Pa) ⁴	45.1 ^b _{fgh}	61.2 ⁱ	45.0 ^{eh} _j	47.3 ^{b-e}	63.0 ⁱ	43.6 ^d _{gj}	70.7 ^a	76.5 ^a	50.8 ^{cf}
Yield strain ⁴	1.239 ^a _{hij}	1.397 ^d _{gjl}	1.012 ^m	1.360 ^{ae} _{-g}	1.490 ^c _{fikl}	0.981 ^m	1.547 ^{a-d}	1.470 ^b _{ehkl}	1.067 ^m

^{a-q}Means with different superscripts within each rheological parameter are different ($P < 0.05$).

¹Milk at the natural pH.

² G' was measured at frequency 0.1 Hz.

³ $\tan \delta$ was measured at frequency 0.006 Hz.

⁴Apparent yield stress and strain were obtained at a shear rate of 0.01 s^{-1} .

Figure 2. Loss tangent, $\tan \delta$, as a function of time, t , for skim milk gels obtained at pH values 6.7 (a), 6.3 (b), 6.0 (c) using the coagulants *Cynara cardunculus* L. (x), *Cynara humilis* L. (▲), and chymosin (●). G' vs t , at pH 6.0 (panel 2d), of plant coagulants and chymosin diluted ~8.7- and 4.7-fold, respectively. Time zero corresponds to addition of coagulant to milk. Values are means ($n = 3$) and vertical bars are the standard deviations for the last data points. At pH 6.0, at the lower concentration of coagulant, syneresis was observed in plant-induced gels ~4 h after the addition of coagulant to milk. The results for that part of the gelation profile were not plotted in panel 2d.

the same pH value, no major differences were observed in microstructure between coagulants. Gels made at low pH values, i.e., 6.3 and 6.0 (Figure 3d to i), appeared to have a denser or more interconnected structure than gels made at pH 6.7 (Figure 3a to c).

SDS-PAGE

The degradation of caseins by coagulants during the 6 h gelation phase at 32°C is shown in Figure 4. Several unidentified peptides (U1-3) can be observed in the gels made with plant coagulants (lanes 1 to 6), and another unidentified band, U4 was observed in all gels made with various coagulants (Figure 4a). The κ -casein band had virtually disappeared in all the gels made with

coagulants, presumably reflecting the hydrolysis of κ -casein necessary to initiate gelation. In SDS-PAGE gels with a larger sample volume (Figure 4b) an additional band (U5) was present in untreated milk and in gels made with chymosin (lanes 7 to 9), and this band was presumably γ -casein. This γ -casein band was absent in gels made with plant coagulants (lanes 1 to 6) as it was presumably further degraded. Small peptides (U6) could be observed in the gels made with plant coagulants (Figure 4b).

DISCUSSION

The pH of milk had a large impact on the lag time for the initiation of gelation and on the resulting shape

Figure 3. Confocal scanning laser micrographs of skim milk gels made at 32°C at pH 6.7 (a to c), 6.3 (d to f) and 6.0 (g to i) induced by *Cynara cardunculus* L. (a, d, g), *Cynara humilis* L. (b, e, h) and chymosin (c, f, i). Micrographs were taken 6 h after the addition of coagulant to milk. Protein matrix is white and dark areas are pores. Scale bar = 10 μm .

of the gelation curves. For all coagulants, lowering pH resulted in shorter t_g . Other authors also observed that pattern of results when calf rennet or chymosin was used as coagulant (Ernstrom and Wong, 1974; Kowalchuk and Olson, 1977). By lowering the pH of milk, there is a decrease in the electrostatic repulsion between the casein micelles due to a decrease in the charge density on κ -casein, which is the casein that provides (much of the) colloidal stability to milk (Wade et al., 1996; Van Hooydonk et al., 1986a; De Kruif, 1999).

By lowering the pH of milk, the initial G' values increased faster, i.e., dG'/dt values were larger (Figure 1); Zoon et al. (1989) also found similar results. The G'_{max} and yield strain values tended to be larger at

low pH, which indicates that there were differences between gels produced at different pH values. Decreasing pH from 6.7 up to 6.0 results in some solubilization of CCP (Van Hooydonk et al., 1986a; Le Gräet and Brulé, 1993) and protonation of negatively charged casein groups, phosphoseryl and carboxyl (Horne, 1998). The released Ca^{2+} may interact with carboxyl groups (Byler and Farrell, 1989; Dalgleish and Law, 1989). The overall impact of lowering pH from 6.7 up to 6.0 is probably a decrease in the net negative charge on casein micelles, which favors hydrophobic attractions. Consequently, both bond formation between casein particles and particle fusion are likely to proceed faster, resulting in larger G' values, which was observed in our study.

Figure 4. SDS-PAGE electrophoretograms of milk gels made at 32°C induced by *Cynara cardunculus* L. (lanes 1 to 3), *Cynara humilis* L. (lanes 4 to 6), and chymosin (lanes 7 to 9) at pH 6.7 (lanes 1, 4, 7), 6.3 (2, 5, 8) and 6.0 (3, 6, 9), untreated milk is in lane M. (a) normal protein loading and (b) overloaded protein (see text for details). Various unidentified peptides resulting from casein hydrolysis are indicated by letters U.

At pH 6.0 and 6.3, the gelation curves of plant coagulant-induced gels exhibited a $G'_{\max}$ that was followed by a steady decrease in G' values (Figure 1). In the case of chymosin, only a slight decrease in G' values was observed at pH 6.0. A decrease in G' values during gelation may be an indication of extensive rearrangements of the casein network (Mellema, 2000). Some loss of colloidal calcium phosphate (CCP) within micelles at low pH values removes some "cross-linking" material, which may make micelles more prone to re-

arrangements, especially if this is also encouraged by additional proteolysis by the coagulant. Loss of CCP makes caseins more accessible to proteolysis (Fox, 1970). Both chymosin and plant coagulants have their maximum activity on caseins at pH ~6.0 (Van Hooydonk et al., 1986b; Faro, 1991). At the end of the experiments (~6 h) G' values at pH 6.0 were lower than at pH 6.3 (Table 3).

Regardless of milk pH, the G' values at maximum value and at the end of gelation were higher for chy-

mosin than plant coagulants. The same pattern of results was found for yield stress values, what indicates that chymosin gels were firmer than those obtained with plant coagulants. That pattern of results was probably due to the higher nonspecific proteolysis of the plant coagulants (Macedo et al., 1996), which we observed in this study (Figure 4). Plant coagulants hydrolyzed more of the caseins at lower pH than at pH 6–7. Generally, the α_s - and β -casein bands are less intense (i.e., more hydrolyzed) in the plant coagulant gels compared with chymosin samples. The similar general proteolytic activity of the two plant coagulants towards caseins agrees with previous results (Esteves et al., 1995; Pires, 1998).

In the case of plant coagulants, the amount of coagulant added to milk influenced the G' values of the gel. For both plant coagulants, gels produced at pH 6.0 with diluted coagulant (~8.7-fold) had the largest G' values of all plant-induced gels investigated in the present study. Because much less coagulant was used in these experiments, it was likely that there was less proteolysis of caseins, and so there may have been fewer rearrangements of the gel network during most of the experiment.

Tan δ values obtained at low frequency (0.006 Hz) at the end of gelation (~6 h after coagulant addition to milk) were higher for chymosin than plant coagulants, which is a further indication of differences (structural) between chymosin and plant coagulants. The tan δ vs. t profiles varied with pH. At pH 6.0 and 6.3, all coagulants produced tan δ vs. t curves that initially exhibited a sudden decrease at the point of gelation and after a minimum in tan δ values was attained, the tan δ values slightly increased (Figure 2). To the best of our knowledge, this is the first time that a slight increase in tan δ values has been reported during the gelation of coagulant-induced gels. A relatively large increase in tan δ values during gelation has been observed in acid-induced gelation of heated milk (Lucey et al., 1997) and in milk gel systems that have the concomitant action of both coagulant and acid (Lucey et al., 2000). In the present work, milk was not preheated, and there was no acid production during gelation. In both of these two previous examples the increase in tan δ was postulated to be caused by the loss of CCP from micelles that were already part of the gel network. However, in our study, gels produced from milk that was acidified to pH 6.0 and left for 16 h before coagulation still showed an increase in tan δ values suggesting that slow solubilization of CCP was not responsible for this effect (results not shown).

It was noted that the increase in tan δ was only observed under conditions where the initial dG'/dt was fast. In this scenario, rearrangements in the gel net-

work structure are likely to occur faster, which may be a possible cause of the slight increase in tan δ values. In additional experiments with milk at the natural pH (6.7) but with conditions that promote faster gelation and rearrangements, such as high gelation temperature and/or high enzyme concentration, a slight increase in tan δ was also observed (data not shown). The tan δ vs. t curves of *C. humilis* L. tended to have an intermediate behavior between those of chymosin and *C. cardunculus* L.. This may be an indication that gels produced with *C. humilis* L. were more similar to chymosin-induced gels than those of *C. cardunculus* L., although no significant differences were found between G' vs. t curves of plant coagulants.

CONCLUSIONS

The milk pH had a great influence on the gelation properties of both plant coagulants and chymosin. For all coagulants, by lowering pH from the natural pH of milk, t_g became shorter and the rate of increase in G' , or rate of gel firming, increased. When rapid gelation occurred, at pH 6.0 and 6.3, there was a slight increase in tan δ values during gel aging. This was likely to be due to rearrangements in the gel network structure. For the tan δ parameter, *C. humilis* L. tended to have an intermediate behavior between *C. cardunculus* L. and chymosin.

At a low pH, a decrease in G' values was observed during gel aging, especially for the plant gels probably due to the additional proteolysis that occurs in these gels. It is recommended that a lower concentration of plant coagulants should be used in the gelation of milk at low pH, to avoid the possible negative impact of extensive casein proteolysis on the texture and flavor of cheese.

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