ELSEVIER

International Dairy Journal 12 (2002) 427–434

INTERNATIONAL DAIRY JOURNAL

www.elsevier.com/locate/idairyj

# Rheological properties of milk gels made with coagulants of plant origin and chymosin

Cristina L.C. Esteves[a,b], John A. Lucey[a,*], Euclides M.V. Pires[b]

[a] *Department of Food Science, University of Wisconsin-Madison, 1605 Linden Drive, Madison, WI 53706, USA*

[b] *Departamento de Bioquímica, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Apartado 3126, 3000 Coimbra, Portugal*

Received 17 August 2001; accepted 15 November 2001

**Abstract**

The rheological properties of milk gels made using coagulants obtained from the plants *Cynara cardunculus* L. and *Cynara humilis* L. were compared with those of fermentation-produced chymosin, using dynamic low amplitude oscillation as well as large strain (yield) testing. Gelation experiments were performed at 32°C using skim milk powder that had been reconstituted for 2 or 16 h at 32°C. The storage modulus ($G'$), loss tangent ($\tan\delta$) at low frequency (0.002 Hz) and yield stress were higher for chymosin-induced gels than those of plant coagulants, when tested ~6 h after coagulant addition. Plant coagulants were slightly more proteolytic than chymosin, and casein hydrolysis may have resulted in lower gel firmness. Most of the rheological properties were similar for the two plant coagulants, in agreement with their similar enzyme contents. Gelation properties were different in milk reconstituted for 2 or 16 h. This behaviour was probably due to casein hydrolysis by plasmin, as milk reconstituted for 16 h at 32°C had significant levels of degradation of both $\alpha_s$- and $\beta$-caseins. The addition of soybean trypsin inhibitor which inhibits plasmin activity resulted in similar gelation profiles for gels made from milk reconstituted for 16 and 2 h. © 2002 Published by Elsevier Science Ltd.

*Keywords:* Plant coagulants; Rheology; Milk gelation; Chymosin; *Cynara*

## 1. Introduction

Most artisanal varieties of Portuguese cheeses (e.g., Serra da Estrela cheese and Serpa cheese) are produced with coagulants extracted from the style and stigma part of the flowers of *Cynara cardunculus* L. and occasionally with those of *Cynara humilis* L. In this work, the term "coagulant" will be used as a generic designation for milk-clotting enzymes. The coagulants of *Cynara cardunculus* L. and *Cynara humilis* L. are, like chymosin, aspartic proteinases. Both the plant coagulants and chymosin have similar specificities in the cleavage sites as they hydrolyze between hydrophobic amino acids. The *C. cardunculus* L. coagulant contains two enzymes; cardosin A and cardosin B (Esteves, Veríssimo, Faro, & Pires, 1995). Cardosin B is more proteolytic than cardosin A. Both enzymes split the $Phe_{105}$–$Met_{106}$ bond of $\kappa$-casein. Kinetic parameters of cardosin A were similar of those obtained for chymosin, and the values for cardosin B were similar to those of pepsin, when a synthetic peptide was used as a substrate (Veríssimo, Esteves, Faro, & Pires, 1995). In *C. humilis* L. the only aspartic proteinase was a cardosin A-like enzyme (Esteves, 1995). Although *C. cardunculus* L. has been used for cheese production in Portugal for millennia, there are only a few reports on its gelation properties (Vieira de Sá, & Barbosa, 1972; Cordeiro, Jakob, Pais, & Brodelius, 1992; Apodaca, Amigo, & Ramos, 1994); the instruments used in those studies gave empirical results only. Recently, the initial phase of gel formation (up to ~40 min after coagulant addition) was investigated using several mathematical models for gels produced using *C. cardunculus* L., *C. humilis* L. and chymosin (Esteves, Lucey, & Pires, 2001).

Reconstituted skim milk powder (SMP) has been used often in rheological experiments on rennet gels, in order to avoid variations in milk composition. However, the conditions used in the reconstitution of SMP, such as temperature and time, have varied considerably between studies, e.g., 16 h at 30°C (Zoon, van Vliet, & Walstra, 1988), 10 min at 50°C (Lomholt & Qvist, 1997), or 10 h at 25°C (Mellema, 2000). Our preliminary studies on the

*Corresponding author. Tel.: +1-608-265-1195; fax: +1-608-262-6872.

*E-mail address:* jalucey@facstaff.wisc.edu (J.A. Lucey).

0958-6946/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0958-6946(01)00170-4

initial stage of gelation indicated that the rate of increase in the stiffness of the network, as indicated by the storage modulus ($G'$), was influenced by the length of the reconstitution time for SMP (i.e., 2 or 16 h at 32°C). The endogenous milk enzyme plasmin is active at this reconstitution temperature (32°C) (Dulley, 1972). Plasmin is a serine-like proteinase present in milk that can hydrolyze caseins, primarily $\beta$- and $\alpha_s$-caseins (e.g., Snoeren & van Riel, 1979; Visser, 1981; Grufferty & Fox, 1988). Of the caseins, $\kappa$-casein is the most resistant to plasmin hydrolysis (Andrews & Alichanidis, 1983).

The main objective of the present work was to characterize the rheological properties of milk gels made using chymosin and plant coagulants up to the plateau region in $G'$ values (i.e., $\sim$6 h), and their large deformation properties at that stage. The second objective was to study the influence of time used for reconstitution of milk on the gelation behaviour of milk gels made using both chymosin and plant coagulants.

## 2. Materials and methods

### 2.1. Materials

Low heat SMP was supplied by Dairy Farmers of America (Fresno, CA 93727, USA). The plant coagulants from *C. cardunculus* L. and *C. humilis* L. were obtained from the style and stigma part of the flower. The styles and stigmas were removed and left to dry (by air) in the laboratory by leaving them in a dark dry place. The styles and stigma were stored in this form prior to extraction. The strength of the fermentation-produced chymosin used, Maxiren DS (DSM Gist-brocades, 2600 MA Delft, The Netherlands), was 600 International Milk Clotting Units (IMCU $mL^{-1}$) (IDF, 1997). Soybean trypsin inhibitor (STI), type I-S, (Lot. 30K7020) was supplied by Sigma Chemical Co. (St Louis, MO 63178, USA).

### 2.2. Preparation of milk samples

SMP with a 7.05 mg $g^{-1}$ (w/w) of undenatured whey protein nitrogen in non-fat dry milk (Bradley et al., 1992) was reconstituted (9% w/w) in an aqueous solution with $CaCl_2$ (0.1 mg $mL^{-1}$); $NaN_3$ (0.2 mg $mL^{-1}$) was added to prevent bacterial growth. Milk was dispersed at 32°C for 2 or 16 h with gentle agitation using a magnetic stirring unit, and then left at 22°C for at least 1 h before rheological experiments.

### 2.3. Casein hydrolysis during the SMP reconstitution

The degree of casein hydrolysis was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970), with the modifications described by Singh and Creamer (1991). A Mini-Protean 3 Cell unit (Bio-Rad, Hercules, CA 94547, USA) was used. Milk samples were analyzed immediately after milk dissolution, and after incubation of milk at 32°C for 2 and 16 h. Gels were stained with Coomassie Blue G-250 and analyzed by densitometry in a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA). The intact caseins were quantified using the program ImageQuaNT™ Version 4 (Molecular Dynamics, Sunnyvale, CA, USA). In order to inhibit plasmin action on caseins, some milk samples were reconstituted in the presence of STI a serine-type inhibitor (150 μg $mL^{-1}$ of milk). The presence of STI in such concentration was enough to inhibit casein degradation in milk stored for 15 h at 37°C, the optimum temperature for plasmin activity (Dulley, 1972).

### 2.4. Preparation of coagulants

The coagulants were extracted and stored according to Esteves et al. (2001). The quantity of protein used in coagulation assays was 0.025 and 0.013 mg $mL^{-1}$ of milk, for extracts from *C. cardunculus* L. and *C. humilis* L., respectively. Before comparing the gelation properties of *C. cardunculus* L., *C. humilis* L. and chymosin, the same gelation time ($t_g$; $\sim$18 min) was selected for all three coagulants, using milk reconstituted for 16 h as the standard. The amounts of each coagulant necessary to obtain a $t_g$ of $\sim$18 min for milk reconstituted for 16 h were also used for milk reconstituted for 2 h.

### 2.5. Protein quantification

The protein contents of extracts from *C. cardunculus* L. and *C. humilis* L. were determined by the Bradford method (Bradford, 1976) using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA 94547, USA).

### 2.6. Rheological assays

Milk gels are viscoelastic and their small deformation rheological properties can be determined by low amplitude dynamic oscillation (Zoon et al., 1988). A Universal Dynamic Spectrometer, Paar Physica UDS 200 (Physica Messtechnik GmbH, D-70567, Stuttgart, Germany) was used. The measuring geometry (MK 25) consisted of a cone (diam. 7.5 mm and 2° angle) and a plate. Individual gelation assays were performed for a short time (36 min) and measurements were taken every 2 min. Extended gelation assays were followed for $\sim$6 h with measurements taken every 30 min. Before starting the experiments, milk was equilibrated for 15 min at the assay temperature (32°C); 25.5 μL of previously diluted enzyme was then added to 4.25 mL of milk, which was 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, gelation time was considered (arbitrarily) as the time necessary for the gel to reach a $G'$ 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 0.002–1 Hz.

The large deformation properties were studied ~6 h after addition of coagulant. Gels were subjected to a constant shear rate of $\sim 0.01\ s^{-1}$, up to yielding of the gel. The apparent yield stress and shear deformation at yielding were defined as the point when the shear stress started to decrease (Lucey, Teo, Munro, & Singh, 1997).

### 2.7. Statistical analysis

One-way ANOVA was used to analyze the results of casein hydrolysis during milk reconstitution. Two-Way ANOVA was applied to the loss tangent or $\tan\delta$ $(= G''/G')$ results at 0.002 Hz, as well as the apparent stress and shear deformation at yielding. The results of $G'$ as a function of time, as well as $G'$ and $\tan\delta$ as a function of frequency were analyzed using the mixed model procedure. A mixed linear model is a generalization of the standard linear model used in the GLM procedure, the generalization being that the data are permitted to exhibit correlation and nonconstant variability. Least-squares means (LS-means), estimating the marginal means over a balanced population (Searle, Speed, & Milliken, 1980) were compared using the Tukey-Kramer procedure. LS-means are to unbalanced designs what means are to balanced designs. Significance was indicated by $p < 0.05$. Each experiment was repeated at least four times. All statistical analyses were conducted using the SAS program (SAS, 1999).

## 3. Results

### 3.1. Casein hydrolysis during milk reconstitution

When milk was dispersed for 16 h at 32°C, the amount of intact $\alpha_s$-casein was reduced by ~9% ($p < 0.002$) and $\beta$-casein was reduced by ~13% ($p < 0.0009$). For milk reconstituted for 2 h, no casein degradation was detected.

### 3.2. Gelation and small deformation rheological properties

The gelation profiles ($G'$ as a function of time) for chymosin and plant coagulants are shown in Fig. 1a and b. The gelation times for all coagulants, using milk reconstituted for 16 h, were identical (~18 min). The general pattern of gelation curves was similar for chymosin and plant coagulants, although some differences were observed. During the first 2 h of the assay all coagulants had similar $G'$ values, except for the chymosin curve for milk reconstituted for 2 h. In those particular experiments (Fig. 1b), ~1 h after coagulant addition, chymosin had the lowest $G'$ values of all coagulants ($p < 0.04$). Thereafter, it had the fastest rate of increase in $G'$, quickly exceeding the other gelation

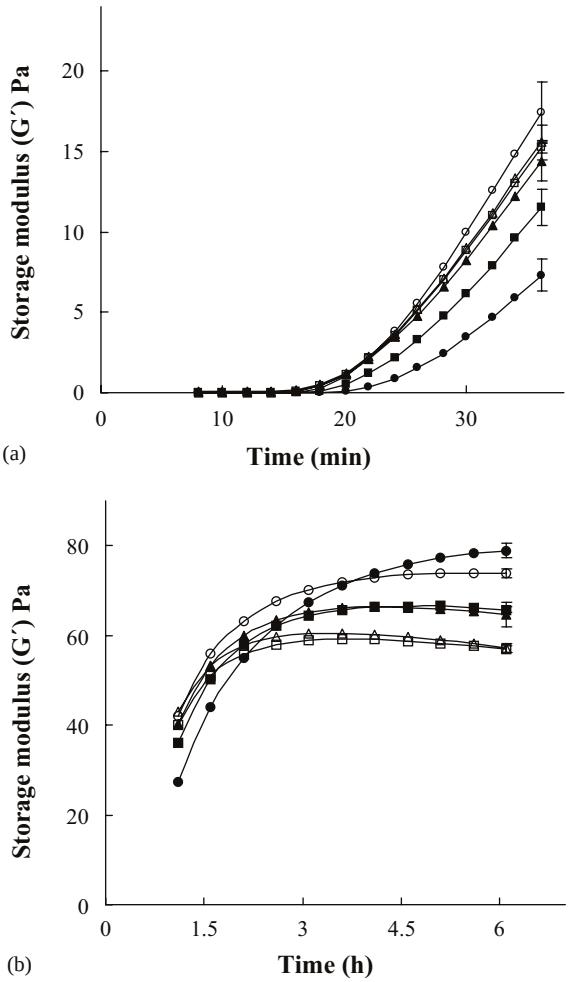


Fig. 1. Storage modulus ($G'$) as a function of time for skim milk gels made at 32°C. Gels were followed for (a) ~40 min and (b) ~6 h after coagulant addition. Gelation was induced by the coagulants from *C. cardunculus* L. for milk reconstituted for 2 (■) and 16 h (□), *C. humilis* L. for milk reconstituted for 2 (▲) and 16 h (△) or chymosin for milk reconstituted for 2 (●) and 16 h (○) at 32°C. Time zero corresponds to addition of coagulant to milk. Values are means ($n \geq 4$) with standard deviations vertical bars for the last data points.

Table 1
Effect of coagulant type on small deformation rheological properties of milk gels determined ~6 h after coagulant addition, for two different milk reconstitution periods

| | Coagulant type | | | | | |
|---|---|---|---|---|---|---|
| | *Cynara cardunculus* L. | | *Cynara humilis* L. | | Chymosin | |
| | Time used for reconstitution of milk at 32°C | | | | | |
| | 2 h | 16 h | 2 h | 16 h | 2 h | 16 h |
| $G'$ (Pa)* | 65.7[b] | 56.9[c] | 64.7[b] | 57.2[c] | 78.9[a] | 73.9[a] |
| Slope of log $G'$ vs. log frequency curve** | 0.236 | 0.237 | 0.228 | 0.242 | 0.245 | 0.257 |
| tan $\delta$ at 0.002 Hz* | 0.481[e,g] | 0.489[e,f] | 0.491[d,f,g] | 0.500[d] | 0.548[h] | 0.528[i] |

*Values are LS-means from replicates ($n \geqslant 4$).
**Values are means from replicates ($n \geqslant 4$).
[a,b,c] LS-means in the row, with different superscripts, are different ($p < 0.007$).
[d–i] LS-means in the row, with different superscripts, are different ($p < 0.0001$).

curves, and by the end of the assay (~6 h) it had the highest $G'$ value (Table 1). $G'$ values for chymosin were higher than those obtained for plant coagulants ~2 and ~4.5 h after addition of coagulant, for milk reconstituted for 16 and 2 h, respectively ($p < 0.008$). No significant differences were obtained between the $G'$ values of *C. cardunculus* L. and *C. humilis* L. gels made from milk reconstituted for 16 h. A similar trend between plant coagulants was found for milk reconstituted for 2 h (Table 1). For plant coagulants, $G'$ tended to plateau ~2.5 and ~3 h after the addition of coagulant to milk reconstituted for 16 and 2 h, respectively. In the case of chymosin, the gelation curves reached a plateau ~2 h later than plant coagulants. At the end of the experiments (~6 h) $G'$ values obtained for plant coagulants were lower than those for chymosin ($p < 0.0005$). For plant coagulants, milk reconstituted for 2 h had higher plateau values for $G'$ than milk reconstituted for 16 h ($p < 0.03$) and no significant differences were obtained with chymosin gels. In experiments where milk was reconstituted for 2 or 16 h in the presence of STI, the resulting curves were similar to those obtained with milk reconstituted for 2 h in the absence of STI (Fig. 2). This trend applied to all coagulants.

The effect of time-scale of applied deformation on rheological properties was determined by a frequency sweep ~6 h after coagulant addition. $G'$ values increased with frequency and the same trends were obtained for all coagulants and milk reconstitution times (Fig. 3). Chymosin showed higher $G'$ values than plant coagulants at frequencies above 0.03 Hz when using milk reconstituted for 2 h ($p < 0.02$). For milk reconstituted for 16 h, $G'$ values for chymosin were higher than the $G'$ values for plant coagulants at frequencies $\geqslant 0.1$ Hz ($p < 0.003$). At lower frequencies no significant differences were observed. Plotting log $G'$ vs. log frequency yielded straight lines ($R^2 \geqslant 0.98$). The slope for all gels varied from ~0.23 to ~0.26. For all coagulants, there was an increase of tan $\delta$ with decreasing frequency (Fig. 4; $p < 0.0001$), in agreement with the results of Zoon et al. (1988). At low frequency (0.002 Hz), regardless of the time to dissolve SMP, tan $\delta$ values for chymosin were higher than those of plant coagulants ($p < 0.0001$). Tan $\delta$ values for *C. humilis* L. gels were significantly higher than those of *C. cardunculus* L. for frequencies lower than 0.1 Hz, when milk was reconstituted for 16 h ($p < 0.05$).

### 3.3. Large deformation rheological properties

The large deformation properties of gels were studied by subjecting the "set" gels (~6 h after coagulant addition) to a constant shear rate (0.01 $s^{-1}$), up to gel yield or fracture. The shear stress at yielding was higher for chymosin than plant coagulants ($p < 0.0001$), for milk reconstituted for 2 or 16 h (Fig. 5; Table 2). No differences were seen between plant coagulants. The shear strain at yield was similar for all coagulants except for chymosin when milk was dissolved for 16 h, when it was higher than the other gels ($p < 0.005$).

## 4. Discussion

All milk gels showed a slow rate of increase in $G'$, taking many hours to plateau (Fig. 1a and b) although $G'$ continued to increase slightly, which suggests that there may be repulsive barriers slowing down further aggregation and particle fusion. In milk reconstituted for 2 h, no casein degradation was detected during the reconstitution period and these gels had a slower rate of increase in $G'$ compared to milk reconstituted for 16 h, where casein degradation was clearly evident (Table 1). Plasmin action on caseins probably changes the physicochemical properties of micelles, namely, molecular weight, three-dimensional structure, hydrophobicity and charge, and this may reduce the repulsive barriers

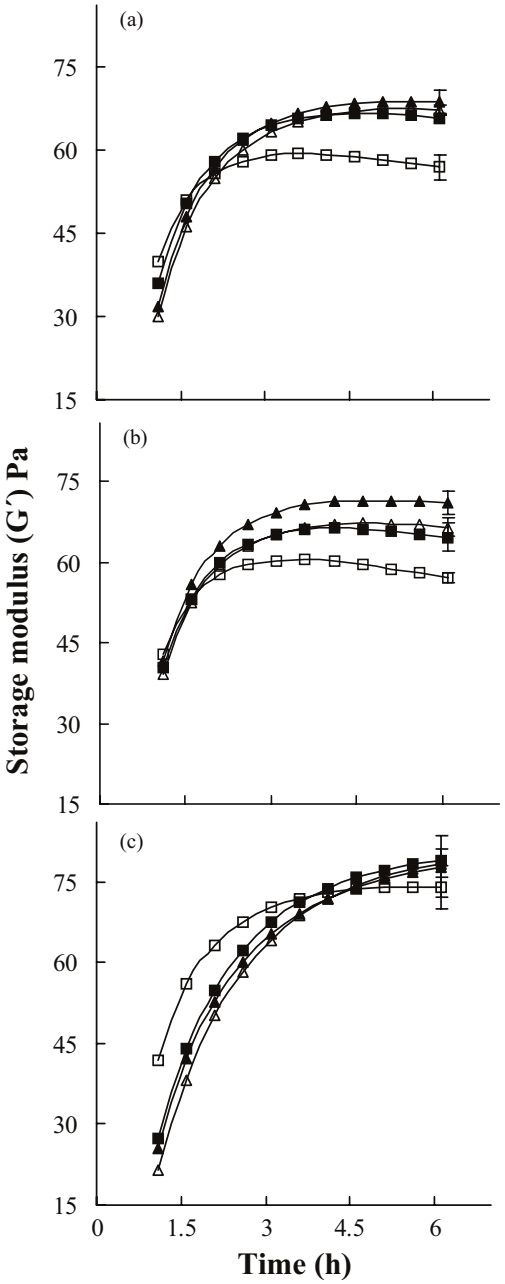


Fig. 2. Storage modulus ($G'$) as a function of time for skim milk gels made at 32°C. Milk was reconstituted in the presence of STI for 2 (▲) and 16 h (△), and in the absence of STI for 2 (■) and 16 h (□). Gelation was induced by the coagulants from (a) *C. cardunculus* L., (b) *C. humilis* L., or (c) by chymosin. Gels were followed for ~6 h after coagulant addition. Time zero corresponds to addition of coagulant to milk. Values are means ($n \geqslant 4$) in the standard deviations (vertical bars) for the last data points.

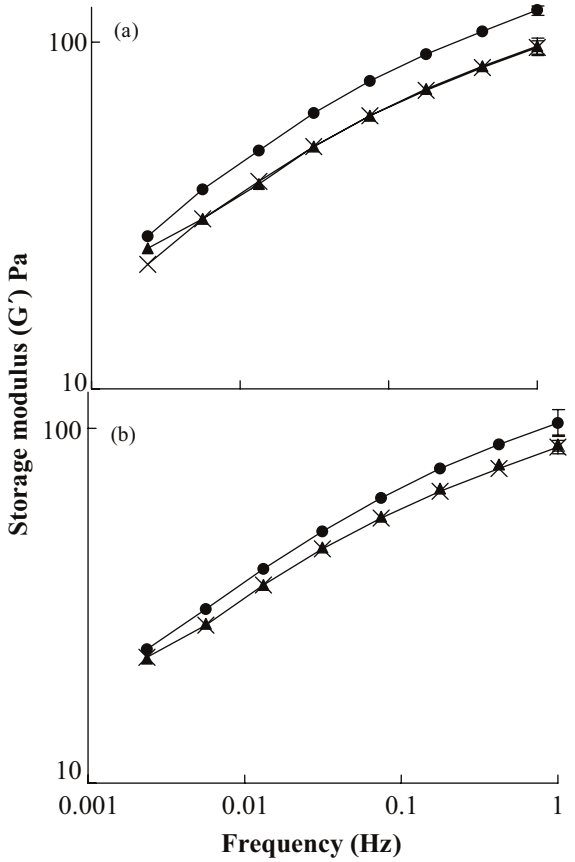


Fig. 3. Storage modulus ($G'$) as a function of frequency for skim milk gelled for ~6 h at 32°C. Milk was reconstituted for (a) 2 and (b) 16 h. Gelation was induced by the coagulants from *C. cardunculus* L. (×), *C. humilis* L. (▲) or by chymosin (●). Values are means ($n \geqslant 4$) with standard deviations (vertical bars) for each data point.

to aggregation and fusion and help accelerate rearrangement processes. As rennet-induced gels are dynamic structures, they evolve with time due to rearrangements in the network structure (van Vliet, 2000). Consequently, the size of aggregates and number of bonds between aggregates increase, and partial fusion of aggregates also occurs (Bremer, van Vliet, & Walstra, 1989). In the initial stages of the gelation process, casein molecules that have been partially hydrolyzed by plasmin, may adopt a more flexible conformation, which would help in the development of gel strength and assist with faster rearrangements of the network structure. An increase in number and strength of bonds between adjacent aggregates should result in a faster rate of increase in $G'$ (Fig. 1a), at least initially. When gel properties were studied over a long time-scale, $G'$ values were lower for milk reconstituted for 16 h than for milk reconstituted for 2 h (Table 1). During gel formation (studied for ~6 h at 32°C) it is possible that caseins will be further hydrolyzed by plasmin. The activity of plasmin itself, or in combination with coagulant, probably leads to the formation of small peptide fragments that do not form part of the gel matrix, as plasmin activity is known to increase protein losses in the whey during cheese-making. In the case of $\beta$-casein, the action of plasmin is complementary to that of coagulants that split peptide bonds in the hydrophobic C-terminal (Fox, Singh, & McSweeney, 1994; Macedo, Faro, & Pires, 1996). A reduction in the

amount of intact casein, which is the gel-forming protein in milk gels, was probably responsible for the lower plateau values for $G'$ in gels made from milk reconstituted for 16 h compared to milk reconstituted for 2 h. Indeed, when the plasmin inhibitor, STI, was added to milk, the pattern of gelation using milk reconstituted for 16 h was similar to that of milk reconstituted for 2 h (Fig. 2).

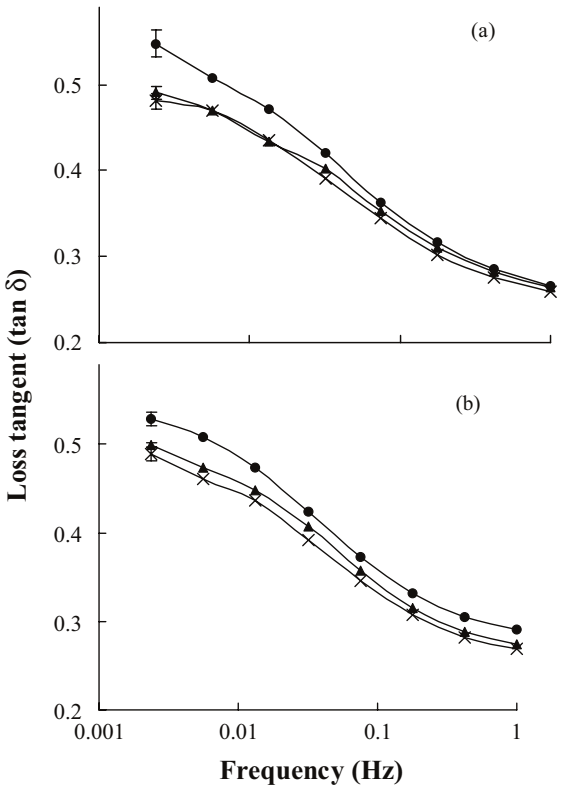


Fig. 4. Loss tangent (tan $\delta$) as a function of frequency for skim milk gelled for ~6 h at 32°C. Milk was reconstituted for (a) 2 and (b) 16 h. Gelation was induced by the coagulants from *C. cardunculus* L. (×), *C. humilis* L. (▲) or by chymosin (●). Values are means ($n \geqslant 4$) with standard deviations (vertical bars) for each data point.

During the initial stages of gelation, chymosin-induced gels made using milk reconstituted for 2 h tended to have a slower rate of increase in stiffness than those made with plant coagulants (Fig. 1a). A similar

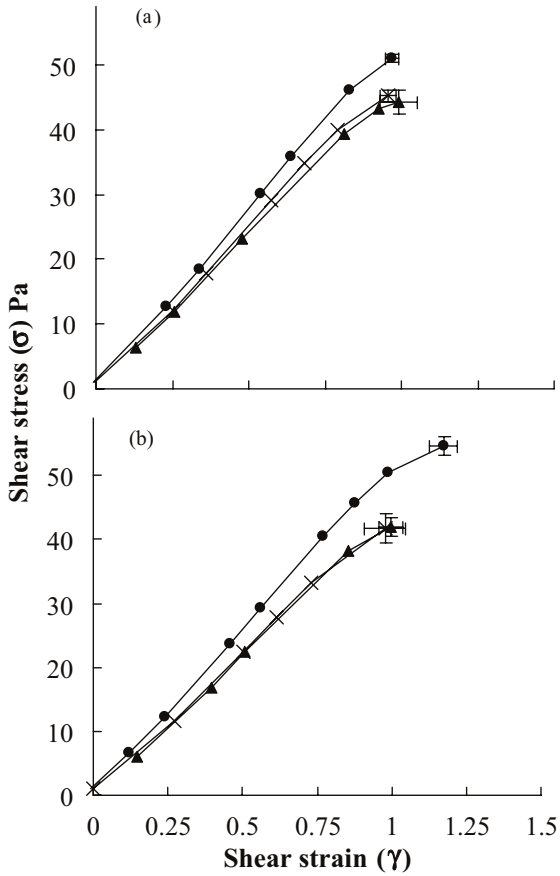


Fig. 5. Shear stress ($\sigma$) as a function of applied deformation ($\gamma$) at a constant shear rate (0.01 $s^{-1}$) for skim milk that was gelled for ~6 h at 32°C. Milk was reconstituted for (a) 2 and (b) 16 h. Gelation was induced by the coagulants from *C. cardunculus* L. (×), *C. humilis* L. (▲) or by chymosin (●). Values are means ($n \geqslant 4$). Vertical and horizontal bars are standard deviations of last data points for strain and shear stress, respectively.

Table 2
Effect of coagulant type on large deformation rheological properties of milk gels determined ~6 h after coagulant addition, for two different milk reconstitution periods

| | Coagulant type | | | | | |
|---|---|---|---|---|---|---|
| | *Cynara cardunculus* L. | | *Cynara humilis* L. | | Chymosin | |
| | Time used for reconstitution of milk at 32°C | | | | | |
| Yield properties | 2 h | 16 h | 2 h | 16 h | 2 h | 16 h |
| Apparent yield stress (Pa)* | 45.2[a] | 41.8[a] | 44.2[a] | 42.0[a] | 50.9[b] | 54.6[b] |
| Apparent yield strain* | 0.96[c] | 0.98[c] | 0.99[c] | 0.99[c] | 0.97[c] | 1.17[d] |

*Values are LS-means from replicates ($n \geqslant 4$); apparent fracture stress and shear deformation at yield were defined as the point when the shear stress started to decrease.

[a,b] LS-means in the row, with different superscripts, are different ($p < 0.0008$).

[c,d] LS-means in the row, with different superscripts, are different ($p < 0.003$).

trend was reported by Esteves et al. (2001) for these coagulants, even though those samples had a shorter coagulation time (~11 min). This behaviour was attributed by these authors as being due to slight differences in specificity and proteolytic activity of plant coagulants on caseins (Esteves et al., 1995; Macedo et al., 1996). The same argument can be used to explain why plateau values for the $G'$ of chymosin gels were significantly higher than both plant coagulants (Fig. 1b). No significant differences were detected in $G'$ values between *C. cardunculus* L. and *C. humilis* L. during the 6 h gelation period. However, at low frequencies, $\tan\delta$ values were higher for *C. humilis* L. gels than for *C. cardunculus* L, when milk was reconstituted for 16 h (Table 1). These two plant coagulants have a common proteinase, cardosin A, but *C. cardunculus* L. also has cardosin B, an enzyme not found in *C. humilis* L. (Esteves, 1995) that is more proteolytic than cardosin A. For all coagulants, there was an increase in $\tan\delta$ with decreasing frequency (Fig. 4); the same trend was reported for milk gels made with calf rennet (Zoon et al., 1988; van Vliet, van Dijk, Zoon, & Walstra, 1991). At low frequency (0.002 Hz) all coagulants exhibited high values of $\tan\delta$ (> 0.4) which indicates an increased susceptibility of the protein-protein bonds to relax at long time-scales and, therefore, indicates a greater susceptibility of coagulant-induced gels to undergo syneresis and rearrangements (van Vliet et al., 1991). A higher degree of rearrangements in gels made with plant coagulants could have caused the lower $G'$ compared with chymosin-induced gels. Apparent yield stress values were significantly higher for chymosin compared to both plant coagulants (Table 2), a result that was in agreement with the trends for $G'$ and suggests that chymosin gels were firmer and stiffer than plant-induced gels. These attributes may influence their cheese-making properties, although the differences were relatively small.

## 5. Conclusions

The trends in rheological properties of coagulant-induced gels produced with chymosin, *C. cardunculus* L. and *C. humilis* L. were generally similar. However, because there were differences in specificity and proteolytic activity of chymosin and *Cynara* sp. coagulants, significant differences in experimental results were detected in some rheological tests. Between plant coagulants, minor differences could be observed in the $\tan\delta$ behaviour, probably due to the additional enzymatic activity in the coagulant of *C. cardunculus* L.

The action of the endogenous enzyme, plasmin, against caseins during the SMP reconstitution procedure, appeared to influence milk gelation properties; this important observation should lead to standardization of milk reconstitution procedures in future rheological studies of rennet-induced gels.

It was also demonstrated that rheological properties are a powerful tool in examining the impact of enzymatic hydrolysis or changes in enzymatic specificity on functional properties of milk proteins.

## Acknowledgements

We wish to thank the Biometry Consulting Group of the College of Agriculture and Life Sciences, UW-Madison, for assistance with statistical analysis.

During this research C.L.C. Esteves was supported by Fundação para a Ciência e a Tecnologia, Portugal.

## References

Andrews, A. T., & Alichanidis, E. (1983). Proteolysis of caseins and the proteose-peptone fraction of bovine milk. *Journal of Dairy Research*, *50*, 275–290.

Apodaca, M., Amigo, L., & Ramos, M. (1994). Study of the milk-clotting and proteolytic activity of calf rennet, fermentation-produced chymosin, vegetable and microbial coagulants. *Milchwissenschaft*, *49*, 13–16.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Bradley, R. L., Arnold, E., Barbano, D. M., Semerad, R. G., Smith, D. E., & Vines, B. K. (1992). Chemical and physical methods, Protein. In R. T. Marshall (Ed.), *Standard methods for the examination of dairy products* (pp. 433–529). Washington, DC: American Public Health Association.

Bremer, L. G. B., van Vliet, T., & Walstra, P. (1989). Theoretical and experimental study of the fractal nature of the structure of casein gels. *Journal of the Chemical Society, Faraday Transactions 1*, *85*, 3359–3372.

Cordeiro, M., Jakob, E., Pais, S., & Brodelius, P. (1992). Milk clotting and proteolytic activities of purified cynarases from *Cynara cardunculus*—a comparison to chymosin. *Milchwissenschaft*, *47*, 683–687.

Dulley, J. R. (1972). Bovine milk protease. *Journal of Dairy Research*, *39*, 1–9.

Esteves, C. L. C. (1995). *Comparative study of biochemical characteristics of the rennets from Cynara cardunculus* L., *Cynara scolymus* L. *and Cynara humilis* L., M.Sc. thesis, University of Coimbra, Portugal.

Esteves, C. L. C., Lucey, J. A., & Pires, E. M. V. (2001). Mathematical modelling of the formation of rennet-induced gels by plant coagulants and chymosin. *Journal of Dairy Research*, *68*, 499–510.

Esteves, C. L., Veríssimo, P. C., Faro, C. J., & Pires, E. V. (1995). Biochemical characterization of the vegetable rennets from the flowers of cardoon: Comparison to calf rennet. *Journal of Dairy Science*, *78*(suppl 1), 145.

Fox, P. F., Singh, T. K., & McSweeney, P. L. H. (1994). Proteolysis in cheese during ripening. In A. T. Andrews, & J. Varley (Eds.), *Biochemistry of milk products* (pp. 1–27). Cambridge: Royal Society of Chemistry.

Grufferty, M., & Fox, P. F. (1988). Milk alkaline proteinase. *Journal of Dairy Research*, *55*, 609–630.

IDF (1997). *Bovine Rennets: Determination of Total Milk-Clotting Activity*. International Dairy Federation, Brussels, Belgium: FIL-IDF Standard no. 157A.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *277*, 680–685.

Lomholt, S. B., & Qvist, K. B. (1997). Relationship between rheological properties and degree of κ-casein proteolysis during renneting of milk. *Journal of Dairy Research*, *64*, 541–549.

Lucey, J. A., Teo, C. T., Munro, P. A., & Singh, H. (1997). Rheological properties at small (dynamic) and large (yield) deformations of acid gels made from heated milk. *Journal of Dairy Research*, *64*, 591–600.

Macedo, I. M. Q., Faro, C. J. F., & Pires, E. M. V. (1996). Caseinolytic specificity of cardosin, an aspartic protease from the cardoon *Cynara cardunculus* L: Action on bovine $\alpha_s$- and $\beta$-casein and comparison with chymosin. *Journal of Agricultural and Food Chemistry*, *44*, 42–47.

Mellema, M. (2000). *Scaling relations between structure and rheology of ageing casein particle gels*. Ph.D. thesis, Wageningen University, the Netherlands.

SAS (1999). *SAS/STAT$^{TM}$ User's Guide* (8.0 eds). Cary, NC: SAS Institute Inc.

Searle, S. R., Speed, F. M., & Milliken, G. A. (1980). Populations marginal means in the linear model: An alternative to least squares means. *The American Statistician*, *34*, 216–221.

Singh, H., & Creamer, L. K. (1991). Changes in the size and composition of protein aggregates on heating reconstituted concentrated skim milk at 120°C. *Journal of Food Science*, *56*, 671–677.

Snoeren, T. H. M., & van Riel, J. A. M. (1979). Milk proteinase, its isolation and action on $\alpha_{s2}$- and $\beta$-casein. *Milchwissenschaft*, *34*, 528–531.

van Vliet, T. (2000). Structure and rheology of gels formed by aggregated protein particles. In K. Nishinari (Ed.), *Hydrocolloids. Part 1* (pp. 367–377). Amsterdam: Elsevier Science.

van Vliet, T., van Dijk, H. J. M., Zoon, P., & Walstra, P. (1991). Relation between syneresis and rheological properties of particle gels. *Colloid and Polymer Science*, *269*, 620–627.

Veríssimo, P. C., Esteves, C. L. C., Faro, C. J. F., & Pires, E. M. V. (1995). The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. *Biotechnology Letters*, *17*, 621–626.

Vieira de Sá, F., & Barbosa, M. (1972). Cheese-making with a vegetable rennet from Cardo (*Cynara cardunculus*). *Journal of Dairy Research*, *39*, 335–343.

Visser, S. (1981). Proteolytic enzymes and their action on milk proteins. A review. *Netherlands Milk and Dairy Journal*, *35*, 65–88.

Zoon, P., van Vliet, T., & Walstra, P. (1988). Rheological properties of rennet-induced skim milk gels. 1. Introduction. *Netherlands Milk and Dairy Journal*, *42*, 249–269.