Comparative studies on the gelling properties of cardosins extracted from *Cynara cardunculus* and chymosin on cow's skim milk

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Abstract

A comparative study was developed on the clotting activities and gelling properties of cardosins A and B, extracted from dried flowers of *Cynara cardunculus*, and chymosin on cow's skim milk, at various pH values. The determination of the total milk-clotting activity was performed following an international standard, whereas a rheometer was employed to measure the viscoelastic properties of the gels subsequently formed: the evolution of the complex modulus (G^*) and the phase angle (δ) was monitored with time. The G^* values of the milk gels were higher for cardosins than for chymosin at pH 6.6, but the reverse held at pH 6.4 and 6.2. The δ values were identical for all three enzymes tested. Chymosin exhibited the highest specific milk clotting activity, followed by cardosin B. The clotting activity of chymosin seems to be more influenced than that of cardosins by the pH of milk.

Introduction

Milk-clotting enzymes are the primary active agents in cheesemaking; coagulation of milk is a crucial step, which involves the enzyme-mediated cleavage of κ-casein at the peptide bond Phe105-Met106 that renders the casein micelles unstable, and eventually causes aggregation that yields a clot and a gel afterwards (de Koning, 1967; Delfour, Jollès, Alais, & Jollès, 1965; Jollès, Alais, & Jollès, 1968). In addition to such a specific proteolytic activity, milk-clotting enzymes usually possess a broader proteolytic activity towards α_s- and β-caseins, which eventually aids in ripening. Chymosin (EC 3.4.23.4), extracted from the abomasa of sucking-calves (calf rennet), was the first and still is the most used milk-clotting enzyme in traditional cheesemaking worldwide. In Portugal, however, aqueous extracts of the flowers of *Cynara cardunculus*, a plant similar to the globe artichoke, have been used for ages as milk coagulant in the manufacture of traditional ewe's and/or goat's milk cheeses. The milk-clotting activity of said extract is accounted for by two aspartic proteinases, named cardosins A and B, which in terms of activity and specificity resemble chymosin and pepsin, respectively (Veríssimo, Esteves, Faro, & Pires, 1995).

The primary proteolysis brought about by the coagulant affects not only the flavour and texture of the ripened cheese, but also the curd firming rate, with consequences upon the cheese yield (Lawrence, 1991). However, the magnitude of this effect is greatly influenced by several factors, including the type and concentration of enzyme, the previous thermal treatment of the milk, the pH, and a number of individual components adventitious or added to milk (Kowalchyk & Olson, 1977). Therefore, the assessment of the clotting power of rennet enzymes has always been of great importance from scientific, technological and commercial viewpoints.

Several methodologies have been used since the end of the 19th century to characterize the milk-clotting activity; most of them depend on visual observation of clot formation in a reference milk substrate (Soxhlet, 1877; Berridge, 1952). However, older methods lack an accurate definition of the total milk-clotting activity (Andrén, 1998), which has been adequately overcome by the IDF Standard 157A: 1997; internationally

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recognized reference rennet powders, with an overall milk-clotting activity of 1000 IMCU g^{-1} (IMCU = International Milk-Clotting Units), are currently in use. This IDF standard hence allows a reliable comparison of the total milk-clotting activity between rennets and coagulants.

Although determination of the ability of enzymes to effect milk coagulation is important, determination of the physical evolution of the gel is not of less importance; hence, studies on the structure and mechanical properties of milk gels have been carried out using viscometers (Scott-Blair & Oosthuizen, 1961; Kopelman & Cogan, 1976; Korolczuk & Maubois, 1987) and penetrometers (Steinholt, 1973). The Formagraph, originally designed to perform assays with the flour of cereals, was also adapted to record coagulation properties of cheese-milk (McMahon & Brown, 1982), although its measurements are somewhat empirical. Nowadays, a dynamic shear rheometer (e.g. Bohlin) is preferred, via a dynamic test that monitors the milk coagulation process throughout time. This technique allows continuous measurement of the viscoelastic properties of the milk gel, and it does not interfere with the gelation process itself; the signal is indeed related to physical properties, and measurements can be taken during the whole coagulation process (Bohlin, Hegg, & Ljusberg-Wahren, 1984).

Despite the studies by Esteves, Lucey, and Pires (2001, 2002) encompassing the rheological properties of milk gels manufactured with coagulants from *C. cardunculus* and *Cynara humilis*, no studies have to date focused on the rheological properties imparted on the milk gels by the isolated enzymes, cardosins A and B, from *C. cardunculus*. Such a knowledge is of great importance to better understand the contribution of each cardosin to the general milk-clotting activity and gelling properties of that plant coagulant.

The aim of this research effort was thus to make a comparative evaluation of the milk-clotting activity and the gelling properties exhibited independently by cardosin A and cardosin B from *C. cardunculus*, as well as by chymosin taken as control, on cow's skim milk at various pH values, after proper addition of $CaCl_2$. The aforementioned IDF Standard was used to determine the total milk-clotting activity, whereas a dynamic shear rheometer (Bohlin VOR) was employed to measure the viscoelastic properties of the gels subsequently formed.

Materials and methods

Substrates

The milk used in the experiments was reconstituted low-heat, low-fat spray dried milk powder (RSM) (from Maryland and Virginia Milk Producers Cooperative Association, USA), prepared according to the procedure described in IDF Standard 157A: 1997 (International Dairy Federation, 1997). The milk (prepared fresh each day) was used for 3 daily measurements, and the experiments were run during 3 days in a row. The milk was stored at 4°C, and the amount needed for each analysis was preheated to 30°C for 15 min right before the experiment started.

In addition to the original level of Ca in the milk (reconstituted in deionised water) which produced a milk pH of 6.6, two other different pH values, 6.4 and 6.2, were obtained by using a $CaCl_2$ solution that was 0.05% and 0.11% (w/v), respectively, in the preparation of the milk substrate. In order to prevent bacterial growth, 0.03% (w/v) azide was added to both milk substrate and buffer.

Enzymes

The plant clotting enzymes were obtained according to the following protocol. The stigmae and stylets of dried flowers of *C. cardunculus* were duly separated, and homogenized using a mortar and pestle, at the ratio of 1 g of flowers per 10 mL of aqueous 0.1 M citric acid (pH 3.0). The homogenate was centrifuged at 5000*g* for 20 min; a 10 mL-sample of the supernatant was then applied to a Sephacryl S-100 column ($2.5 \times 45\,cm^2$) (Pharmacia, Uppsala, Sweden), after proper equilibration, and resolved using 20 mM Tris-HCl buffer (pH 7.6) as eluent at a flow rate of 1.5 mL min^{-1} in an FPLC system (Pharmacia). The peaks were detected by absorbance at 280 nm. The fraction collected at the outlet stream, which corresponded to the proteases of interest (i.e. the second peak eluted), was applied to a HiTrap™ Q HP column (Pharmacia); elution proceeded with two buffers: buffer A (i.e. 20 mM Tris-HCl, pH 7.6) and buffer B (i.e. 20 mM NaCl in 20 mM Tris-HCl, pH 7.6), at a flow rate of 6 mL min^{-1}, under a linear gradient from 0% to 30% B within 11 min. The fractions corresponding to the first two peaks (i.e. cardosin A) were pooled together, and the fraction corresponding to the third peak (i.e. cardosin B) was collected independently. All fractions were further dialysed overnight at 4°C against plain water, and lyophilised prior to use.

The (chromatographically pure) chymosin used was prepared as described by Andrén, Björck, and Claesson (1980); its total milk-clotting activity was 174,000 IMCU g^{-1}.

Stock solutions of all enzymes under study were prepared from the corresponding lyophilisates in a 0.1 M phosphate buffer (pH 5.7), with the concentrations of 10 g L^{-1} for either cardosin A or cardosin B, and 1 g L^{-1} for chymosin, and stored at 4°C; samples of all stock solutions were diluted just before use down to the concentration required, so as to achieve approximately the same gelling time.

Quantitative characterization of enzyme proteins

The protein concentration of each enzyme solution was determined by the phenol reagent method for biological fluids, using the micro-protein determination (Sigma Diagnostics®, St. Louis, MO, USA).

Qualitative characterization of enzyme proteins

Pre-determined amounts of each enzyme solution were placed in separate Eppendorf vials, and 100 μL of sodium dodecyl sulphate (SDS) was added to each one. The vials were heated at 90°C for 10 min in a heating block, and then cooled down to room temperature. The separation of the proteins was carried out on an automated electrophoresis system Phastsystem (Pharmacia) using a PhastGel gradient 8–25 (Pharmacia): each gel had a stacking gel zone (4.5% T, 3% C) and a continuous 8–25% gradient gel zone with 2% cross-linking; staining was with Coomassie brilliant blue.

Milk-clotting activity assay

The total milk-clotting activity of each enzyme was measured according to IDF Standard 157A: 1997; the results were expressed in IMCU g^{-1}.

Milk-clot rheological assay

Each enzyme solution (500 μL), diluted as appropriate to give a gelling time of ca. 15 min, was added to a 10 mL sample of RSM, previously heated to 30°C, and mixed by end-over-end rotation. The mixture was then poured into the measuring cup (C25) of a Bohlin VOR Rheometer (Metric Analys AB, Stockholm, Sweden) which was stoppered with a special cover. The oscillation frequency used was 0.5 Hz, the amplitude was 5% and the torsion bar was 1 g cm; the temperature of measurement was 30°C, and data acquisition proceeded for 4 h. The values of the complex modulus (G^*) and the phase angle (δ) were then determined.

Experimental design

All three enzymes were studied in triplicate on 3 consecutive days and in random order, so as to cover each pH value.

Results and discussion

Purification of cardosins

The acidic extract of the flowers of *C. cardunculus* was subject to gel filtration; a typical chromatogram is shown in Fig. 1a. The second peak possessed milk-clotting activity, and was further purified via ion exchange chromatography under a linear gradient; a typical chromatogram is shown in Fig. 1b; the chromatographic peaks associated with the fractions containing cardosins A and B are duly marked (after a posteriori confirmation by SDS-PAGE).

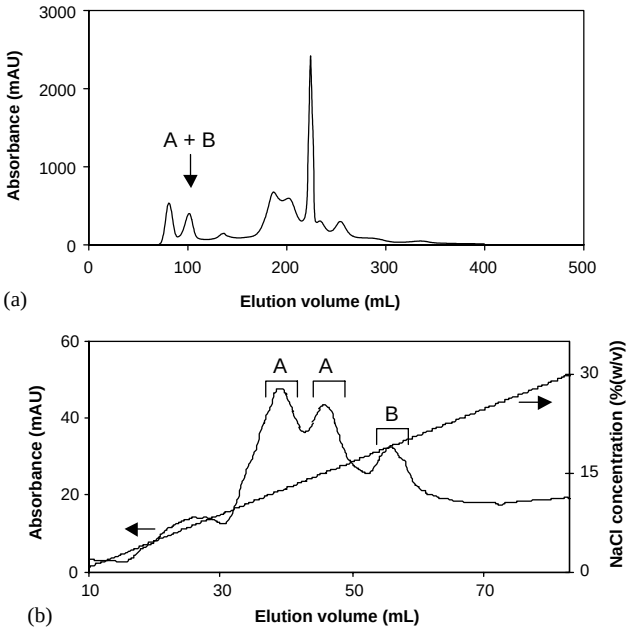

Fig. 1. Typical chromatograms obtained by (a) gel filtration and (b) ion exchange, during enzyme purification, with indication of cardosins A and B.

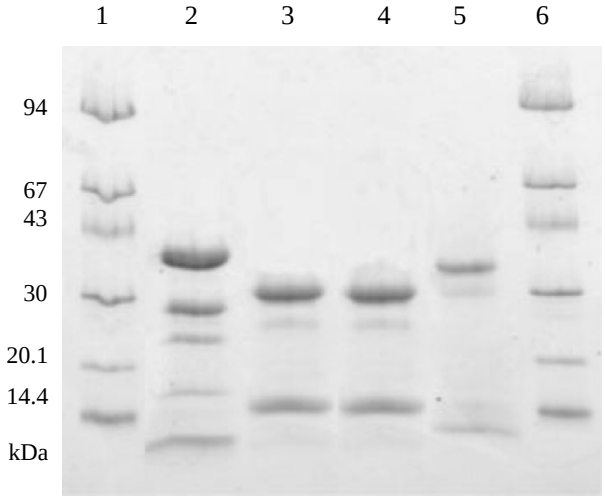

Fig. 2. Typical SDS-PAGE electrophoretogram after enzyme purification, with identification of chymosin (lane 2), cardosin A (lanes 3 and 4) and cardosin B (lane 5). Molecular weight markers were also included for reference (lanes 1 and 6).

The electrophoretogram (Fig. 2) yields the profile of the 3 peaks resolved by ion exchange chromatography: peaks 1 and 2 correspond to fractions containing

polypeptides with molecular weights of ca. 30 and 15 kDa, which correspond to the subunits of cardosin A (lanes 3 and 4 in Fig. 2), whereas peak 3 contains polypeptides with molecular weights of ca. 31 and 14 kDa, which resemble the subunits of cardosin B (lane 5 in Fig. 2). Similar results were reported by Veríssimo et al. (1995) for proteases from *C. cardunculus*, even though their purification procedure was not exactly identical to ours. Although the lane produced by chymosin is accounted for by 5 bands, only the upper band possesses proteolytic activity, so our preparation can be deemed as representative of the pure enzyme.

Our measurements of the milk-clotting activity of the pure enzymes indicated that cardosins A and B exhibit strengths of 290 and 680 IMCU g^{-1}, respectively. The protein contents of the cardosin powders are 0.25 and 0.09 mg g^{-1} for cardosins A and B, respectively; this gives total milk-clotting activities of 1160 and 7556 IMCU g^{-1} of protein, respectively. The cardosin A powder was purer (i.e. contained more protein), whereas the cardosin B powder had a higher specific activity; the specific activity of chymosin was, however, much higher, viz. 174,000 IMCU g^{-1} powder, and even higher values have been reported elsewhere (Harboe & Budtz, 1999). The protein value found for chymosin (1.5 mg protein per mg of powder) is somewhat strange; however, remember that a phenol reagent method for biological fluids was used, and apparently chymosin contains a higher average of phenol-binding amino acids than the proteins in the reference sample. In any case, all three enzymes were analysed by the same method at the same time, so the results are fully comparable.

Gelling properties of coagulant enzymes

All enzymes, i.e. cardosin A and cardosin B from *C. cardunculus*, as well as chymosin were tested for their milk gelling properties at various pH values obtained by $CaCl_2$ addition. The evolution of the complex modulus (G^*) with time is shown in Fig. 3. Since comparison of the effect of coagulant specificity on curd firming rate requires identical gelling times for full validity (Marshall, Hatfield, & Green, 1982; McMahon & Brown, 1982, 1985), enzyme stock solutions were diluted as appropriate prior to each experiment so as to yield a gelling time of ca. 15 min. The results obtained for G^* were then further correlated so as to correspond to a gelling time of exactly 15.3 min, i.e. the results were multiplied by the quota of the measured gelling time for 15.3 min so as to be able to compare the curd firming rate of the enzymes originating from exactly the same gelling point.

After the gelling point at pH 6.2, there was a sigmoidal increase in G^* with time (Fig. 3a); Bohlin et al. (1984) pointed out that this is the typical behaviour of the secondary phase of milk coagulation. The G^*

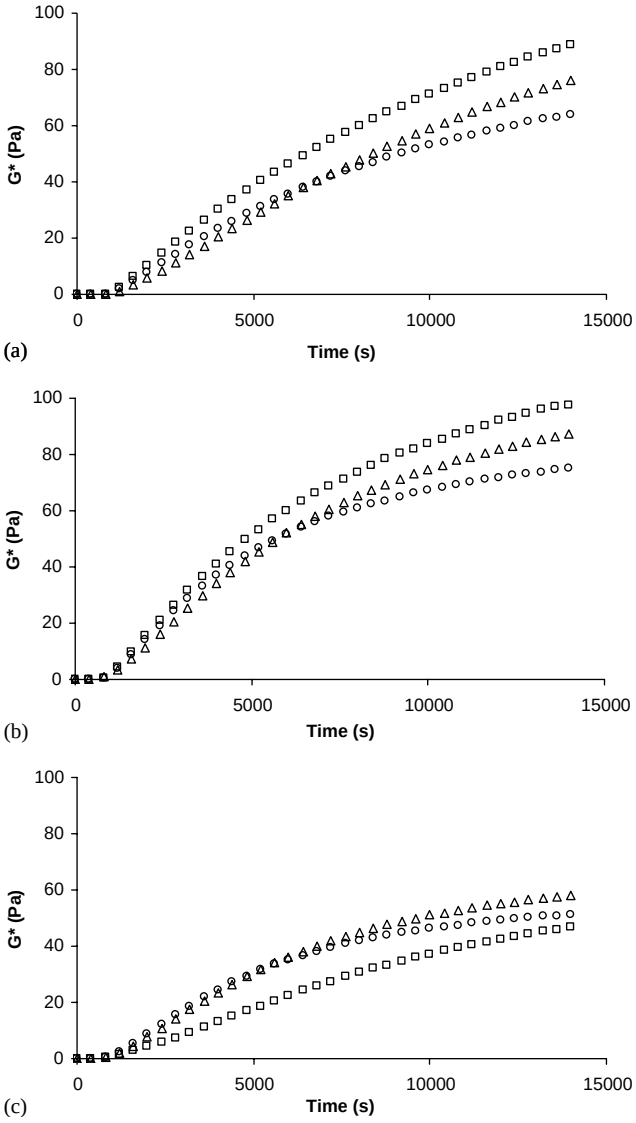

Fig. 3. Complex modulus (G^*) during coagulation by cardosin A (○), cardosin B (△) and chymosin (□), at pH 6.2 (a), pH 6.4 (b) and pH 6.6 (c). Standard error of the means: 1.01, 6.78 and 3.14 Pa (cardosin A), 1.55, 8.96 and 2.72 Pa (cardosin B) and 2.55, 6.68 and 1.16 Pa (chymosin) for pH 6.2, 6.4 and 6.6, respectively.

values for chymosin were higher than those for cardosin B, which were in turn higher than those for cardosin A. The results found at pH 6.4 were very similar to those at pH 6.2, but the actual G^* values corresponding to each enzyme were higher (Fig. 3b); at both pH values, the first G^* values for cardosin B were lower than those for cardosin A. Approximately 6000 s after addition of coagulant, the G^* curve for cardosin B intersects that for cardosin A. At pH 6.6, the experimental results obtained were different from those produced at pH 6.2 or 6.4 (Fig. 3c). Although the G^* values increased over time, a plateau was not reached in the case of chymosin; there is, however, a reduced tendency to observe a sigmoidal shape of the curve G^* vs. t at higher pH values

(and lower $CaCl_2$ concentrations) (Bohlin et al., 1984). The G^* values for cardosin B were above those for cardosin A, which were in turn higher than those for chymosin.

The increase of G^* while pH is decreased owing to addition of $CaCl_2$ is tentatively explained by the reduction of electrostatic repulsion between micelles, as well as the increase in Ca^{2+} activity owing to solubilization of calcium phosphate which may lead to faster structural rearrangements, and subsequent micelle aggregation (Zoon, van Vliet, & Walstra, 1989; López, Lomholt, & Qvist, 1998).

With cardosin A, the highest value of G^* (i.e. 75 Pa) was obtained at pH 6.4, whereas the lowest (i.e. 50 Pa) was at pH 6.6. The G^* values were very similar at pH 6.2 and 6.6, during the first 6000 s. In what concerns cardosin B, the maximum value of G^* (i.e. 88 Pa) was also obtained at pH 6.4, whereas the lowest (i.e. 58 Pa) was at pH 6.6. For the first 7000 s, the G^* values at pH 6.6 were higher than those at pH 6.2. In the case of chymosin, as happened with both cardosins, the highest G^* value (i.e. 99 Pa) was reached at pH 6.4, whereas the lowest (i.e. 47 Pa) was at pH 6.6; however, the difference between the results at pH 6.2 and 6.4, on one hand, and the results at pH 6.6, on the other, was more pronounced than for the plant enzymes, thus suggesting that chymosin is much more sensitive to pH than cardosins.

The curd firming rates (measured between 2000 and 3000 s), brought about by the various coagulants at the three pH values tested, are depicted in Table 1. At pH 6.2 and 6.4, the curd firming rate for chymosin was higher that those for cardosins A and B, which had similar values. At pH 6.6, the values for cardosins A and B were again similar to each other, but higher than the chymosin counterpart. Esteves et al. (2001, 2002), who studied the rheological properties of reconstituted skim milk coagulated with crude aqueous extract of *C. cardunculus* and chymosin at pH 6.6–6.7 (i.e. 0.1 g $CaCl_2$ added per liter of reconstituted milk), reported similar results: the slope of the G^* vs. t plot for the plant coagulant laid above that for chymosin.

With regard to the phase angle, δ (data not shown), a sudden decrease is observed when the secondary phase of coagulation starts, i.e. at the transition from solution to gel form; afterwards, and during the whole build-up of the gel strength, the phase angle remains essentially constant. In agreement with results reported by Zoon et al. (1989) and López et al. (1998), it was observed that the δ values for all enzymes at the different situations studied were essentially identical (ca. 20°C); this means that the phase angle (or tan δ, for that matter) is independent of pH. The phase angle is supposedly a measure of the type of linkage on which the network is based (Bohlin et al., 1984), so its constancy during the secondary phase of coagulation was anticipated.

The amount of each enzyme needed for a gelling time of 15.3 min at various pH values is presented in Table 2. Chymosin was found to have the highest specific activity at all 3 pH values tested, followed by cardosin B. The specific activity differed by a factor of ca. 10 between chymosin and cardosin B, which in turn was found to have a specific activity ca. 10-fold higher than that of cardosin A. Hence, the specific activity of chymosin was found to be ca. 100-fold higher than that for cardosin A. The milk-clotting activity of the cardosins was less influenced by pH than that of chymosin, which in turn was shown (Andrén & Reedtz, 1990) to be less sensitive to pH than bovine pepsin. In this study, the influence of pH was most pronounced between the pH values 6.4 and 6.6: ca. 11-, 6- and 3-fold higher amount of chymosin, cardosin B and cardosin A, respectively, was needed at pH 6.6 to produce the same milk-clotting activity than at pH 6.4. McMahon and Brown (1985) found, on the other hand, no significant differences in curd firmness among commercial coagulants (namely chymosin, calf rennet, *Mucor miehei* proteinase, *Mucor pusillus* proteinase, adult bovine rennet, calf rennet-porcine pepsin and bovine pepsin), 3 h after enzyme addition to milk at pH 6.45.

Cardosins A and B are present in the pistils of the flowers of *C. cardunculus*, and the milk-clotting activity of said plant extracts has been known for centuries, and appropriately taken advantage of in the manufacture of traditional Portuguese cheeses at the farm level from ewe's and goat's milk (Faro et al., 1999; Silva & Malcata, 1999, 2000a, b), which are considered as exquisite foods. It is known that a great variability exists in the final quality of said cheeses. Although some studies have been done on the gelling properties of the

Table 1
Curd firming rates, dG^*/dt ($\times 10^2$), by the various coagulants measured between 2000 and 3000 s

pH	Firming rate, dG^*/dt (Pa/min)		
	Cardosin A	Cardosin B	Chymosin
6.2	0.80 ± 0.05	0.71 ± 0.05	1.03 ± 0.10
6.4	1.25 ± 0.39	1.16 ± 0.49	1.38 ± 0.38
6.6	0.85 ± 0.20	0.81 ± 0.19	0.42 ± 0.07

Table 2
Amount of each enzyme ($\times 10^7$) needed at the various pH values to produce a gelling time of 15.3 min for 10 mL of RSM

pH	Protein (mg)		
	Cardosin A	Cardosin B	Chymosin
6.2	2179	339	20
6.4	2615	373	26
6.6	8715	2196	294

proteases present in the aforementioned plant coagulants, no data were to date available concerning the properties of each cardosin, and its independent contribution to the overall milk-clotting activity and gelling properties. This work has shown that cardosins exhibit a very different rheological behaviour between themselves, and also when compared with chymosin. This knowledge is important in order to rationally reduce said variability in cheesemaking using extracts from *C. cardunculus*.

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References

Andrén, A. (1998). Milk-clotting activity of various rennets and coagulants: Background and information regarding IDF standards. *Bulletin of the International Dairy Federation*, *332*, 9–14.

Andrén, A., Björck, L., & Claesson, O. (1980). Quantification of chymosin (rennin) and pepsin in bovine abomasa by rocket immunoelectrophoresis. *Swedish Journal of Agricultural Research*, *10*, 123–130.

Andrén, A., & von Reedtz, C. (1990). The effects of chromatographically pure bovin chymosin and pepsin A on cheese curd firmness. *Journal of Dairy Research*, *57*, 109–117.

Berridge, N. J. (1952). Some observations on the determination of the activity of rennet. *Analyst*, *77*, 57–62.

Bohlin, L., Hegg, P.-O., & Ljusberg-Wahren, H. (1984). Viscoelastic properties of coagulating milk. *Journal of Dairy Science*, *67*(4), 729–734.

de Koning, P. J. (1967). *Studies of rennin and genetic variants of caseins.* Ph.D. thesis, University of Amsterdam, Amsterdam.

Delfour, A., Jollès, J., Alais, C., & Jollès, P. (1965). Caseinoglycopeptides: Characterisation of a methionine residue and of the *N*-terminal sequence. *Biochemistry and Biophysic Research Communications*, *19*, 425–455.

Esteves, C. L., 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*(3), 499–510.

Esteves, C. L., Lucey, J. A., & Pires, E. M. V. (2002). Rheological properties of milk gels made with coagulants of plant origin and chymosin. *International Dairy Journal*, *12*, 427–434.

Faro, C., Ramalho-Santos, M., Vieira, M., Mendes, A., Simões, I., Andrade, R., Veríssimo, P., Lin, X.-L., Tang, J., & Pires, E. (1999). Cloning and characterisation of cDNA encoding cardosin A, and RGD-containing plant aspartic protinase. *Journal of Biological Chemistry*, *274*(40), 28724–28729.

Harboe, M., & Budtz, P. (1999). The production, action and application of rennet and coagulants. In B. A. Law (Ed.), *Technology of cheesemaking* (pp. 33–65). Sheffield, UK: Sheffield Academic Press.

IDF (1997). *Bovine rennets. Determination of total milk-clotting activity.* International IDF Standard 157A. International Dairy Federation, Brussels, Belgium.

Jollès, J., Alais, C., & Jollès, P. (1968). The tryptic peptide with the rennin-sensitive linkage of cow's κ-casein. *Biochimica et Biophysica Acta*, *168*, 591–593.

Kopelman, I. J., & Cogan, U. (1976). Determination of clotting power of milk clotting enzymes. *Journal of Dairy Science*, *59*(2), 196.

Korolczuk, J., & Maubois, J. L. (1987). Computerized viscometric method for studying rennet coagulation of milk. *Journal of Textural Studies*, *18*(2), 157–172.

Kowalchyk, A. W., & Olson, N. F. (1977). Effects of pH and temperature on the secondary phase of milk-clotting by rennet. *Journal of Dairy Science*, *60*(8), 1256–1259.

Lawrence, R. C. (1991). Processing conditions. *International Dairy Federation Issue No. 9301, Factors affecting the yield of cheese* (pp. 64–78). Brussels, Belgium: International Dairy Federation.

López, M. B., Lomholt, S. B., & Qvist, K. B. (1998). Rheological properties and cutting time of rennet gels. Effect of pH and enzyme concentration. *International Dairy Journal*, *8*(4), 289–293.

Marshall, R. J., Hatfiels, D. S., & Green, M. L. (1982). Assessment of two instruments for continuous measurement of the curd-firming of rennet milk. *Journal of Dairy Research*, *49*(1), 127–135.

McMahon, D. J., & Brown, R. J. (1982). Evaluation of formagraph for comparing rennet solutions. *Journal of Dairy Science*, *65*(8), 1639–1642.

McMahon, D. J., & Brown, R. J. (1985). Effects of enzyme type on milk coagulation. *Journal of Dairy Science*, *68*(3), 628–632.

Scott-Blair, G. W., & Oosthuizen, J. C. (1961). A viscometric study of the breakdown of casein in milk by rennin and rennet. *Journal of Dairy Research*, *28*, 165.

Silva, S. V., & Malcata, F. X. (1999). On the activity and specificity of cardosin B, a plant proteinase, on ovine caseins. *Food Chemistry*, *67*(4), 373–378.

Silva, S. V., & Malcata, F. X. (2000a). Action of cardosin A from *Cynara humilis* on ovine and caprine caseinates. *Journal of Dairy Research*, *67*(3), 449–454.

Silva, S. V., & Malcata, F. X. (2000b). Comparative catalytic activity of two plant proteinases upon caprine caseins in solution. *Food Chemistry*, *71*(2), 207–214.

Soxhlet, F. (1877). Die Darstellung haltbarer Labflüssigkeiten. *Milch-Zeitung*, *6*, 497–501.

Steinholt, K. (1973). The use of an Instron Universal Testing Instrument in studying the rigidity of milk during coagulation by rennin. *Milchwissenschaft*, *28*(2), 94.

Veríssimo, P., Esteves, C., Faro, C., & Pires, E. (1995). The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. *Biotechnology Letters*, *17*(6), 621–626.

Zoon, P., van Vliet, T., & Walstra, P. (1989). Rheological properties of rennet-induced skim milk gels. 4. The effect of pH and NaCl. *Netherlands Milk and Dairy Journal*, *43*(1), 17–34.