Proteolysis of ovine and caprine caseins in solution by enzymatic extracts from flowers of *Cynara cardunculus*

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Primary proteolysis of ovine and caprine Na-caseinate at 30°C in phosphate buffer at pH 6.5 or 5.5 in the absence of NaCl and at pH 5.2 with 5% (w/v) NaCl by cardosins in aqueous extracts of Cynara cardunculus *flowers was investigated using urea-polyacrylamide gel electrophoresis and reversed-phase high performance liquid chromatography. Caprine caseinate underwent more extensive degradation than ovine caseinate under the same conditions (pH 6.5 and pH 5.5); proteolysis of* β- *and* α_s*-caseins in ovine and, to a lesser extent, in caprine caseinates was reduced in the presence of 5% (w/v) NaCl. Peptide profiles of the pH 4.6-soluble extract had different patterns throughout ripening arising from the different specificity of cardosins toward ovine and caprine Na-caseinates. The major cleavage sites in ovine (caprine) caseinate were Phe105-Met106 (Lys116-Thr117) for* κ*-casein, Leu127-Thr128 and Leu190-Tyr191 (Glu100-Thr101, Leu127-Thr128, Leu136-Pro137 and Leu190-Tyr191) for* β*-casein,* Phe_{23}*-*Val_{24} *(*Phe_{23}*-*Val_{24}*, Trp164-Tyr165 and Tyr173-Thr174) for* α_{s1}*-casein and Phe88-Tyr89 (Ser9-Ser10, Phe88-Tyr89 and Tyr179-Leu180) for* α_{s2}*-casein.* © *1998 Elsevier Science Inc.*

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Introduction

One of the most successful rennets of plant origin, as apparent from long term use in Portugal for the manufacture of farm cheeses from ovine milk, is obtained from the flowers of the thistle, *Cynara cardunculus* L.[1]

An acid proteinase was first isolated from dried thistle flowers by Faro *et al.*[2] and shown to induce milk clotting via cleavage of the sensitive bond Phe105-Met106 in bovine κ-casein.[3,4] The aqueous extract of flowers of *C. cardunculus* was further shown to possess three active proteinases (once termed cynarases or cyprosins, and currently termed cardosins) which have been isolated, purified and partly characterized in terms of activity[5–8] and specificity[4,9,10] toward pure bovine caseins. More recently, two additional aspartic proteinases were isolated from the fresh stigmae of a standard variety of *C. cardunculus* L. Based on the structural and kinetic properties of these enzymes, it was concluded that they result from different genes and are different from the previously reported proteinases of the same plant; hence, they were named cardosin A and cardosin B.[11,12] Each cardosin consists of two subunits with apparent molecular weights of 31 and 15 kDa for cardosin A and 34 and 14 kDa for cardosin B.[12,13] Specificity and kinetic studies were performed using the β-chain of oxidized insulin.[3] Taking advantage of the derived chromophoric peptides, it was reported that cardosin A is similar to chymosin whereas cardosin B resembles pepsin.[13,14]

Although considerably less susceptible than the Phe105-Met106 bond of κ-casein, peptide bonds of α_{s1}-, α_{s2}-, and β-caseins are also hydrolyzed by cardosins (and by chymosin) if subject to appropriate environmental conditions. The susceptibility of bovine Na-caseinate to proteolysis is strongly influenced by the state of aggregation of the substrate[15] which is in turn affected by pH and NaCl. Fundamental information on the action of cardosins on ovine and caprine milks is scarce, and straightforward extrapolation of conclusions obtained with pure bovine caseins to ovine and caprine counterparts is risky. In fact, unlike bovine caseins, ovine caseins exhibit two groups of electrophoretic bands. The group with lower mobility contains β-casein and is divided into two variants, β_1- and

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β_2-casein, which differ in the level of phosphorylation (6 and 5, respectively),[16] although levels ranging continuously from 1–7 have also been reported.[17] The group with higher mobility consists of three bands (α_{s1}-, α_{s2}- and α_{s3}-caseins), designated as a whole as α_s-casein region despite the microheterogeneity arising from variations in the degree of glycosylation and/or phosphorylation coupled with genetic polymorphism.[18–21]

The scope of this work was to experimentally monitor the evolution with time of the breakdown patterns of ovine and caprine caseins by cardosins in crude aqueous extracts of *C. cardunculus* using experimental conditions that parallel milk (pH 6.5) and cheese at early stages of ripening in the absence of salting (pH 5.5) or at early stages of ripening with 5% NaCl (w/v) (pH 5.2) with the goal of furthering knowledge on the nature of action of this plant rennet in cheesemaking and ripening.

Materials and methods

Enzyme

Dried flowers of the wild thistle (*C. cardunculus* L.) were obtained in the Serra da Estrela region (Portugal). The crude extract was prepared by grinding the stigmae of the flowers for 36 s, homogenizing in 0.1 M citrate buffer at pH 5.9, and centrifuging at 12,000 *g* for 5 min. The crude extract (0.1 g of ground flowers ml^{-1} buffer) had a protein concentration of 4.54 mg ml^{-1} as determined by the Coomassie spectrophotometric method using bovine serum albumin as standard.

Milk clotting activity

Rennet clotting activity was measured using low-heat skim milk powder NILAC™ (NIZO, Ede, The Netherlands); the milk substrate was prepared by dissolving 12 g in 100 ml of 10^{-2} M $CaCl_2$ (pH 6.5) at 30°C and 2 ml of this substrate were mixed with 0.2 ml of crude enzyme extract. One rennet unit (RU) was defined as the amount of crude enzyme extract needed to coagulate 10 ml of milk substrate at 30°C in 100 s.

Substrate

Whole ovine and caprine caseins were prepared via isoelectric precipitation of ovine and caprine milks, respectively, by acidification to pH 4.2–4.3 with 6.0 M HCl, heating to 37°C for 30 min and centrifugation at 6,000 *g* for 10 min. The precipitate was recovered by filtration and washed several times with distilled water. The caseins were dispersed in distilled water, the pH adjusted to 7.0 with NaOH and the system allowed to equilibrate for at least 3 h at 4°C before lyophilization.

Hydrolysis of caseins

Ovine and caprine Na-caseinates were dissolved in 100 mM phosphate buffer at pH 6.5 or pH 5.5, or at pH 5.2 in the presence of 5% NaCl, to a final concentration of 10 mg ml^{-1}. The crude enzyme extract (0.270 RU ml^{-1}) was then added at a ratio of 0.526 ml to 10 ml of caseinate (v/v) and the experimental solutions (together with appropriate controls at each pH and salt concentration) were held at 30°C in a thermostated water bath. Aliquots were taken at 1 min, 30 min, 1 h, 3 h, 6 h, 12 h and 24 h. Samples for urea-polyacrylamide gel electrophoresis were mixed with the sample buffer containing mercaptoethanol and urea to inactivate the enzyme and unfold the protein, whereas those for reversed-phase high performance liquid chromatography were heated at 85°C for 30 min to inactivate the enzyme.

Urea polyacrylamide gel electrophoresis (Urea-PAGE)

Samples (0.75 ml) of hydrolysates, obtained and quenched as described above, were prepared for urea-PAGE by adding an equal volume of double-concentrated sample buffer.[22] Urea-PAGE was performed using a Protean II XI vertical slab-gel unit (Bio-Rad Laboratories, Watford, UK) according to the method of Andrews[36] (12.5% T-acrylamide plus bisacrylamide, 4% C-bisacrylamide as a percentage of T) at pH 8.9, with modifications;[23] the gels were stained with Coomasie Blue G-250 (Bio-Rad, Richmond CA) using the method of Blakesley and Boezi.[24] Quantification of intact β_1-, β_2-, α_{s1}-, α_{s2}- and α_{s3}-caseins was by densitometry using a model CD60 densitometer (Desaga, Sarstedt-Gruppe, Germany).

Reversed-phase high performance liquid chromatography (RP-HPLC)

Samples of hydrolysates (2 ml), obtained and quenched as described above, were adjusted to pH 4.6 by addition of 60 μl of 33.3% (w/v) acetic acid, held at room temperature for 10 min and then 60 μl of 3.33 M sodium acetate added.[25] The samples were centrifuged at 6,000 *g* for 10 min and the supernatants recovered for analysis. RP-HPLC was performed by the method of Singh *et al.*[26] using a Beckman system (Beckman Instruments, San Ramon CA). A Lichrosorb RP-8 (5 μm) 250 × 4 mm column (Merck, Darmstadt, Germany) was employed with an Lichrocart 4-4 guard column (Merck). Elution was at 30°C using a mobile phase of two solvents: A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile) at a flow rate of 1.0 ml min^{-1} starting with 100% A for 5 min and then a linear gradient for 55 min up to 50% B, holding at 50% B for 6 min, a linear gradient for 4 min up to 60% B and finally holding at 60% B for 3 min. Absorbance of the eluate was read at 214 nm. Samples (10 mg ml^{-1}) were dissolved in a mixture of solvents A and B (1: 0.01, v/v), filtered through 0.22 μm cellulose acetate filter and an aliquot (75 μl) of the permeate was injected.

Isolation of peptides

Peptides were isolated by RP-HPLC as described above or by electroblotting from urea-PAGE gels using a mini Trans-Blot™ electrophoretic transfer cell (Bio-Rad, Hercules CA). In the latter case, transfer of peptides was at 100 V for 30 min in 10 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer with 10% methanol onto polyvinylidene difluoride membranes (PVDF) with a pore size of 0.22 μm (Bio-Rad). Membranes were rinsed with water and stained in 0.2% Ponceau S in 1% acetic acid for 1 min followed by destaining in water. Bands corresponding to peptides were excised from the membranes and sequenced (5–10 cycles).

Sequence analysis

Peptides were sequenced at the National Food Biotechnology Centre, University College Cork via Edman degradation on an automated pulsed liquid-phase protein-peptide sequencer (Applied Biosystems model 477A, Foster City CA). Liberated amino acids were detected as their phenylthiohydantoin derivatives.

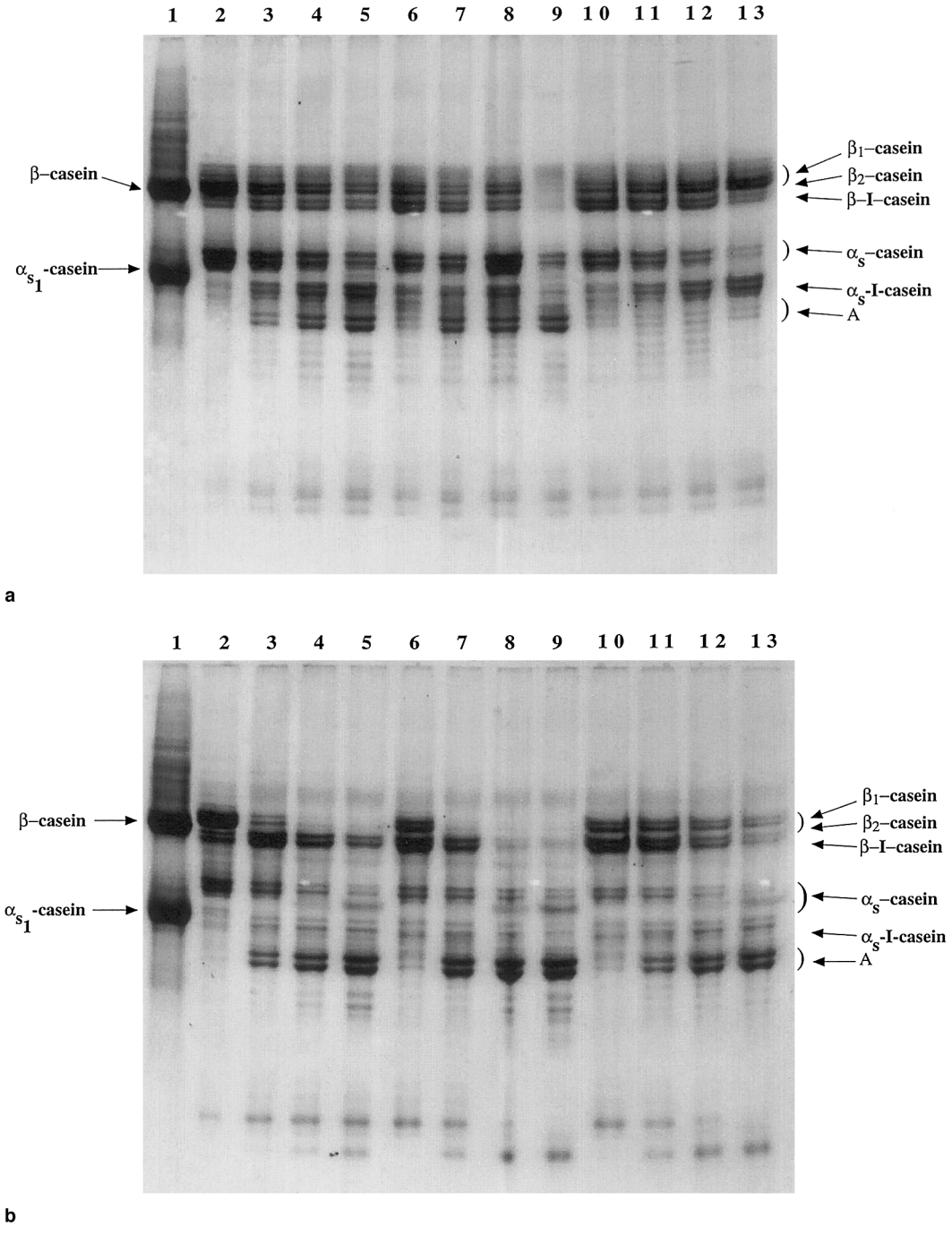
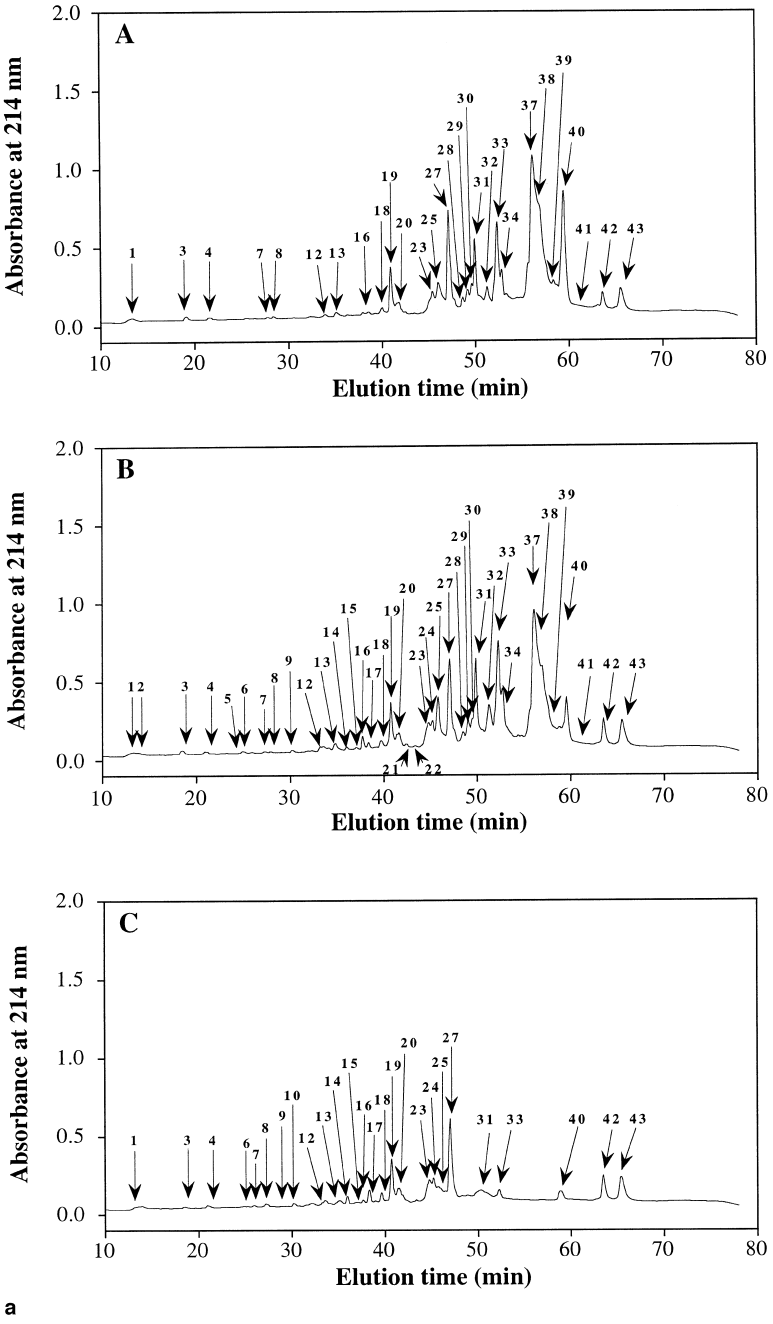


Figure 1 Urea-PAGE electrophoregrams of ovine Na-caseinate (a) and caprine Na-caseinate (b) hydrolyzed by extracts of *C. cardunculus* at 30°C and pH 6.5 (lanes 2–5), pH 5.5 (lanes 6–9), and pH 5.2 with 5% (w/v) NaCl (lanes 10–13) for 1 min, 1 h, 3 h and 6 h, respectively. Bovine Na-caseinate was included as standard in lane 1 of both (a) and (b)

Results and discussion

Experimental data

Urea-PAGE electrophoregrams of ovine and caprine Na-caseinates hydrolyzed by extracts of *C. cardunculus* (0.0142 RU ml^{-1}) at pH 6.5, pH 5.5, and pH 5.2 with 5% NaCl (w/v) throughout the hydrolysis time are shown in *Figures 1a* and *1b.* As expected, the extent of degradation of β- and α_s-caseins in both ovine and caprine Na-caseinates depended on ionic strength and pH. Ovine Na-caseinate at pH 6.5 or 5.5 (*Figure* 1a, lanes 2–8) showed similar rates of hydrolysis of β-caseins (β_1- and β_2-casein) and α_s-caseins for 3 h of hydrolysis; however, β-casein was almost completely degraded in all cases whereas α_s-caseins showed only extensive degradation at pH 5.5 by 6 h of hydrolysis (*Figure 1a*, lane 9). Caprine Na-caseinate exhibited more

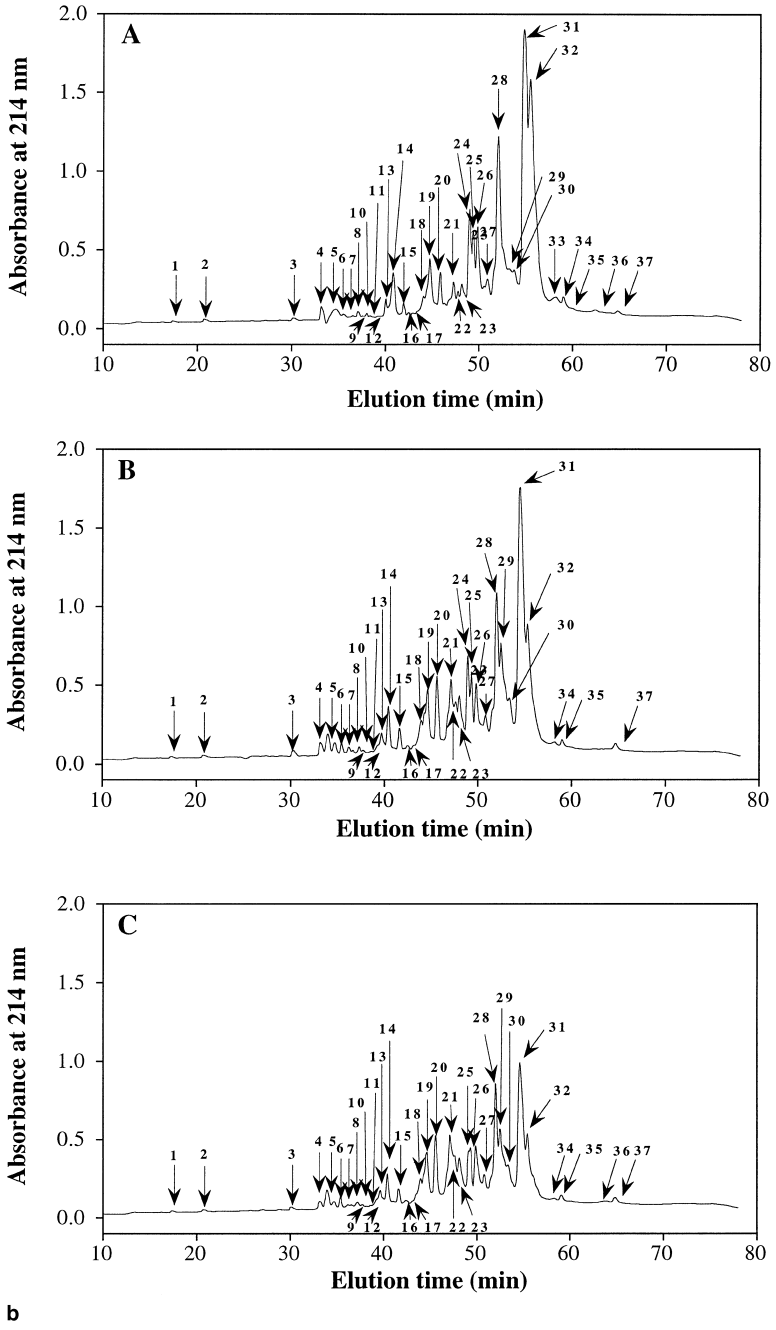


a

Figure 2 RP-HPLC profiles of pH 4.6-soluble peptides from ovine Na-caseinate (a) and caprine Na-caseinate (b) hydrolyzed for 12 h by extracts of *C. cardunculus* at pH 6.5 (A), pH 5.5 (B) and pH 5.2 with 5% (w/v) NaCl (C)

extensive degradation than ovine Na-caseinate under the same conditions (pH 6.5 and pH 5.5); β-casein was completely degraded at pH 6.5 by 3 h whereas complete degradation was observed by 1 h at pH 5.5. Although α_s-caseins were not completely broken down even after 6 h at pH 5.2 in the presence of 5% (w/v) NaCl (*Figures 1a* and *1b*, lanes 10–13), α_s-caseins were hydrolyzed faster than β-caseins in ovine and caprine Na-caseinates.

Under all conditions tested, β-casein in ovine and caprine Na-caseinates was hydrolyzed by cardosins to yield a pair of bands of higher electrophoretic mobility, comparable to that of bovine β-I-casein. This is in agreement with previous reports pertaining to ovine casein hydrolyzed by calf chymosin.[21] Bovine and ovine β-caseins have very similar amino acid sequences. The cleavage sites are probably conserved; the corresponding bonds in ovine β-casein susceptible to chymosin are Leu190-Tyr191 and Ala187-Phe188.[21] In bovine and ovine milk cheeses, β-caseins (β_1- and β_2-casein) were degraded to about the same extent (approximately 33%), but in caprine milk cheeses β-casein was degraded to a greater extent (approximately 44%) by proteinases of *C. cardunculus.*[27] The bonds in bovine



b

Figure 2 Continued

β-casein most susceptible to action by chymosin and proteinases from flowers of *C. cardunculus* are Leu192-Tyr193 and Ala189-Phe190,[9,28] the hydrolysis of which yields β-I-casein.[28] In studies of proteolysis of caprine β-casein by calf rennet, Marcos *et al.*[29] for several varieties of cheeses and Carretero *et al.*[30] for Montsec cheese, the existence of bands accounted for by β-I- and β-II-caseins was reported. Ovine and caprine α_s-caseins were hydrolyzed initially to a set of bands of higher electrophoretic mobility which may correspond to bovine α_{s1}-I-casein. Two bands with greater electrophoretic mobility than the α_{s1}-I-caseins, tentatively denoted as A, were apparent at pH 6.5 and 5.5 after 1 h of hydrolysis in ovine Na-caseinate, and at pH 6.5, 5.5 and 5.2 with 5% (w/v) NaCl after 1 h of hydrolysis in caprine Na-caseinate. Such bands are fragments from α_s-I-caseins (f 24-*) because they apparently contain the same sequence at the N-terminus. Bands with similar electrophoretic mobility and the same corresponding N-terminal sequence were found in solutions of bovine α_{s1}-casein when incubated with extracts from flowers of *C. cardunculus.*[28] Further degradation products with higher electrophoretic mobility and poorly stained (short or medium peptides) could be seen but were not well resolved due to limitations of the analytical techniques employed.

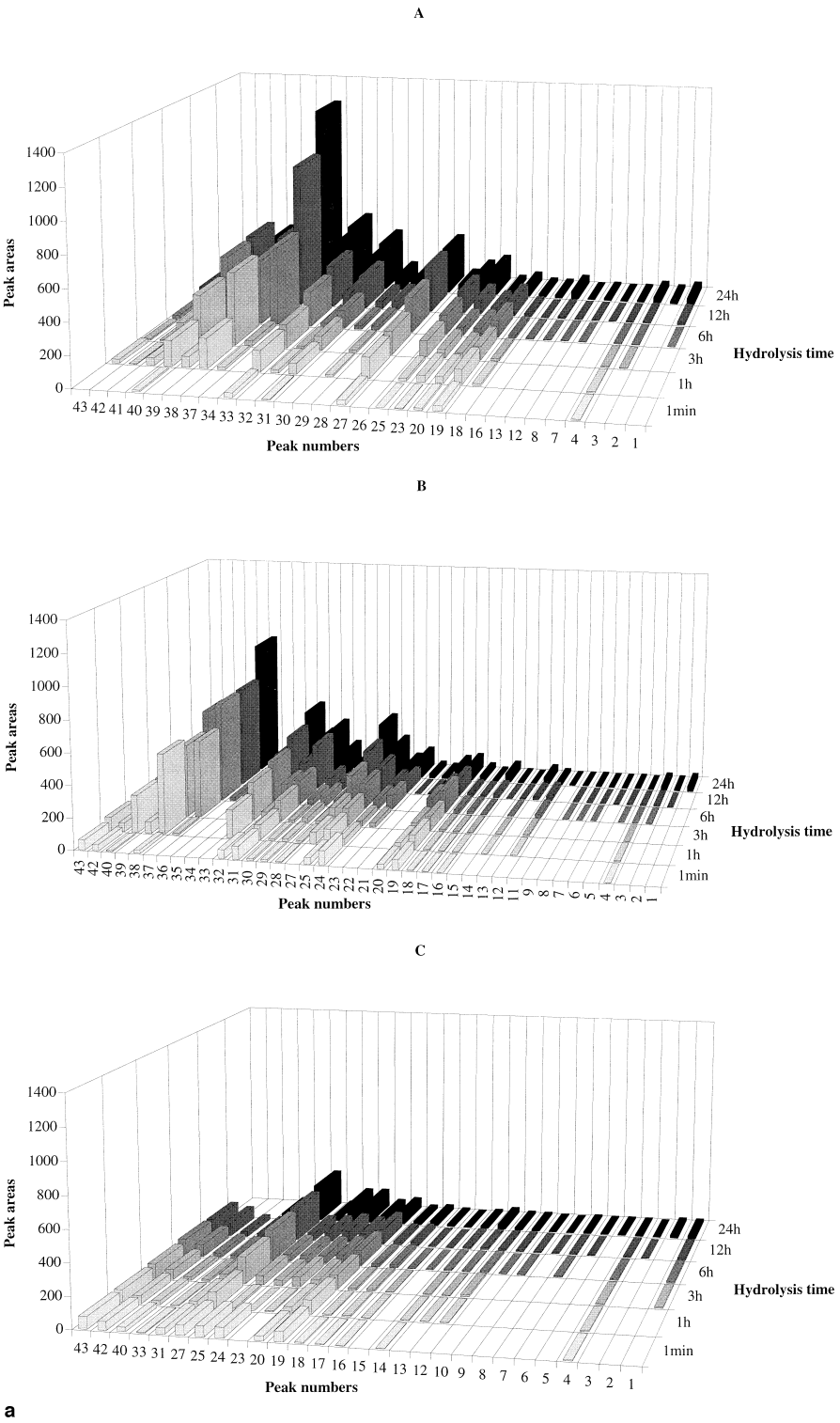


Figure 3 Changes in the area of the RP-HPLC peaks representing the major peptides produced by hydrolysis of ovine Na-caseinate (a) and caprine Na-caseinate (b) catalyzed by extracts of *C. cardunculus* at 30°C, and pH 6.5 (A), pH 5.5 (B) and pH 5.2 with 5% (w/v) NaCl (C) for various times

Proteolysis of β-casein and α_s-caseins in ovine and, to a lesser extent, in caprine Na-caseinates (*Figures 1a* and *1b*) by extracts of *C. cardunculus* was reduced in the presence of 5% (w/v) NaCl at pH 5.2. Sousa[28] reported that proteolysis of bovine β-casein by extracts of *C. cardunculus* at pH 6.5 was increasingly inhibited as NaCl concentration was increased up to 20%. It is also known that proteolysis of bovine β-casein is dependent on pH, ionic strength and

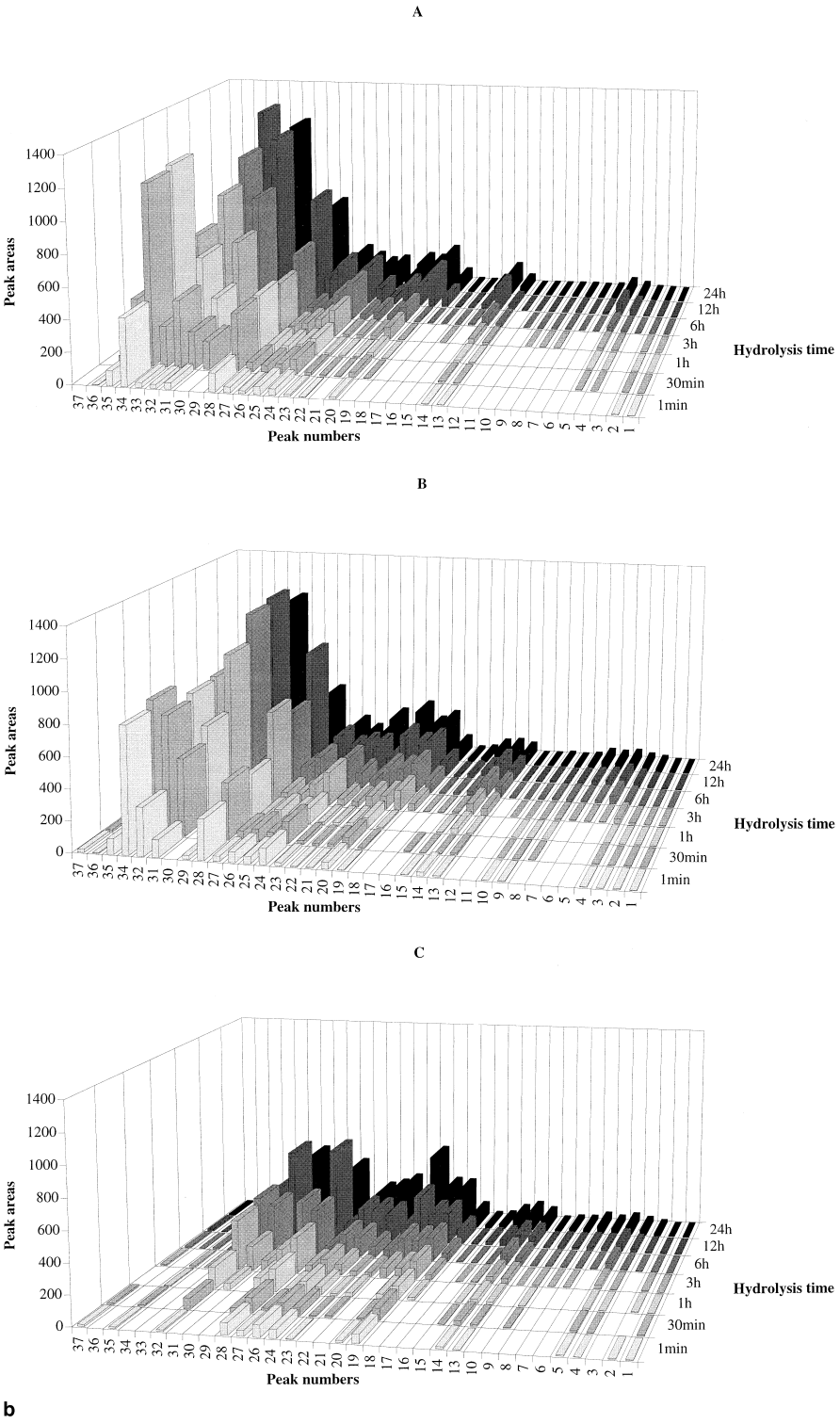


Figure 3 Continued

temperature.[31] Optimum formation of the bovine β-casein peptides β-I, β-II and β-III by calf chymosin occurs at pH 6.4, 4.6 and below 4.6, respectively[32,33]; however, Mulvihill and Fox[33] claimed that inhibition decreased as pH was decreased from 7.0 to 4.6. β-III-casein was not produced in the presence of 2.5% NaCl at pH values below 3.4, but other peptides (e.g., β-IV-casein and β-V-casein) were formed under these conditions (β-IV-casein was also produced in the absence of NaCl at pH 3.4 but only at high enzyme/substrate ratios). Trujillo *et al.*[34] found that caprine β-I-

casein was produced by calf rennet at all pH values and the corresponding electrophoretic band increased in intensity as pH was increased. At low pH values (i.e., not above 4.6), the polypeptide β-II-casein was the main breakdown product detected and β-III-casein was not detected at all at any pH value in the presence of 5% NaCl. Although definitive evidence is lacking, it has been suggested that NaCl exerts an influence on the hydrolysis of β-casein via modification (folding or aggregation) of the substrate rather than of the enzyme.[33] Less marked influence of NaCl on proteolysis of bovine α_s-caseins by extracts of *C. cardunculus* and by chymosin[28] supports this view.

The RP-HPLC peptide profiles in the pH 4.6-soluble fraction of ovine and caprine Na-caseinate hydrolyzed during 12 h at 30°C and pH 6.5, pH 5.5 or pH 5.2 with 5% (w/v) NaCl are shown in *Figures 2a* and *2b*. The peptide profiles in the pH 4.6-soluble fraction of ovine caseinate were virtually similar to those of caprine caseinate after 1 min of hydrolysis (data not shown), but after 12 h of hydrolysis, caprine caseinate (*Figure 2b*) exhibited a higher number (except at pH 5.5) and a higher concentration of peptides than ovine caseinate (*Figure 2a*). This observation may be due to changes in specificity of cardosins on ovine and caprine milk caseins under different ionic strength conditions. Despite the fact that caprine caseinate showed a higher concentration of peptides at pH 6.5 and pH 5.5 than at pH 5.2 with 5% NaCl (*Figure 2b*), the number of peptides produced in caprine caseinate was not too different from that in ovine caseinate. The isolated peptides were sequenced by the Edman procedure from their N-terminus in an attempt to identify them. Ovine κ-casein was cleaved by cardosins at Phe105-Met106 (peptide 19) whereas caprine κ-casein was cleaved by cardosins at Lys116-Thr117 (peptide 14). In bovine κ-casein, the most susceptible bond was Phe105-Met106[4] and it was also shown that such cleavage was able to induce milk clotting as typically happens with acid proteinases. The N-terminal sequences of the peptides designated as 33 [β-(f191-*)], 37 [β-(f1-*)], 38 [β-(f128-*)] and 40 [β-(f128-*)] [β-(f191-*)] (*Figure 2a*) indicated that ovine β-casein was cleaved by cardosins at bonds Leu-190-Tyr191 and Leu127-Thr128, so possible fragments formed are β-(f1/127), β-(f128/192), β-(f128/207) and β-(f191/207); cardosins showed, on the other hand, a β-(f191/207) broader specificity toward caprine β-casein, such as at bonds Glu100-Thr101, Leu127-Thr128, Pro136-Leu137, and Leu192-Tyr193 as indicated by the peptides designated as Leu190-Tyr191 19 [β-(f137-*)], 20 [β-(f128-*)], 28 [β-(f191-*)], 29 [β-(f101-*)], 30 [β-(f128-*)], 31 [β-(f1-*)] and 32 [β-(f128-*)] (*Figure 2b*). Chymosin cleaves bovine β-casein in solution at seven sites which, in decreasing order of rate of attack, are Leu192-Tyr193, Ala189-Phe190, Leu165-Ser166, Gln167-Ser168, Leu163-Ser164, Leu139-Leu140 and Leu127-Thr128 (Visser and Slangen, 1977). Under the experimental conditions used by Charles and Ribadeau-Dumas (1984), only the bonds Ala189-Phe190 and Leu192-Tyr193 of bovine β-casein can be cleaved. Cardosins cleave six bonds over a similar time frame and the relative susceptibility to attack is, in decreasing order, Leu192-Tyr193, Leu191-Leu192, Leu165-Ser166, Phe190-Leu191, Ala189-Phe190 and Leu127-Thr128.[35] Cardosins thus seem to cleave more bonds than chymosin in the bulky, hydrophobic segment Ala189-Phe-Leu-Leu-Tyr193 of bovine β-casein.

The N-terminal sequence of ovine and caprine α_{s1}-casein was identified in peptides designated as 27 (*Figure 2a*) and 19 (*Figure 2b*), respectively, as complementary to the peptide associated with the band identified in urea-PAGE as α_s-I-casein (*Figures 1a* and *1b*); hence, the bond cleaved in α_{s1}-casein was likely Phe23-Val24. Caprine α_{s1}-casein was cleaved by cardosins at the bonds Phe23-Val24, Trp164-Tyr165 and Tyr173-Thr174 as derived from the N-terminal sequences of peptides 24 [α_{s1}-(f165-*)], 28 [α_{s1}-(f24-*)] and 28 [α_{s1}-(f174-*)] (*Figure 2b*). The most susceptible bond to the action of chymosin and cardosins in bovine α_{s1}-casein is Phe23-Phe24.[9,28] Its hydrolysis yields α_{s1}-(f1-23) and (f24-199);[22] the corresponding susceptible bond in ovine α_s-casein with respect to chymosin[21] and to cardosins is Phe23-Val24, so the electrophoretic band designated as α_{s1}-I-casein in ovine and caprine caseins (*Figures 1a* and *1b*) is probably the peptide Val24-Trp199. Macedo *et al.*[35] reported that cardosins can, in addition, cleave the eight bonds Tyr153-Tyr154, Trp164-Tyr165, Tyr165-Tyr166, Tyr166-Val167, Phe145-Tyr146, Leu149-Phe150, Leu156-Asp157 and Ala163-Trp164 whereas chymosin can only cleave the Phe23-Phe24 bond of bovine α_{s1}-casein. McSweeney *et al.*[22] identified at pH 6.5 and 5% NaCl the following seven cleavage sites of bovine α_{s1}-casein by chymosin: Phe28-Pro29, Leu40-Ser41, Leu149-Phe150, Phe153-Tyr154, Leu156-Asp157, Tyr159-Pro160 and Trp164-Tyr165. These peptide bonds were also cleaved at pH 5.2 in the presence of 5% NaCl. In addition, Leu11-Pro12, Phe32-Gly33, Leu101-Lys102, Leu142-Ala143 and Phe179-Ser180 could also be cleaved. Five of these bonds were also cleaved by cardosins at pH 6.5,[22] i.e., Phe23-Phe24, Leu149-Phe150, Phe153-Tyr154, Leu156-Asp157 and Trp164-Tyr165, but cardosins could not cleave the two bonds containing a prolyl residue at the C-terminal side.

Ovine α_{s2}-casein was cleaved by cardosins at Phe88-Tyr89 as derived from the N-terminal sequence of the peptide denoted as 31 [α_{s2}-(f89-*)] (*Figure 2a*), and caprine α_{s2}-casein was cleaved by cardosins at Ser9-Ser10, Phe88-Tyr89 and Tyr179-Leu180, thus leading to peptides 19 [α_{s2}-(f180-*)], 20 [α_{s2}-(f89-*)], 25 [α_{s2}-(f89-*)] and 28 [α_{s2}-(f10-*)] (*Figure 2b*); in bovine α_{s2}-casein, cardosins catalyzed the hydrolysis of two peptide bonds between rather hydrophobic amino acids (such as Phe88-Tyr89 and Tyr95-Leu96), thus releasing the heptapeptide Tyr89-Tyr95.[35] Grappin *et al.* claimed that bovine α_{s2}-casein was resistant to catalytic action by chymosin, but McSweeney *et al.* argued that chymosin could act upon both Phe88-Tyr89 and Tyr95-Leu96.

Changes in the relative peak area of the major peptides produced by cardosins after 1 min, 1 h, 3 h, 6 h, 12 h and 24 h are represented in *Figures 3a* and *3b* for ovine and caprine caseinates. The peptide profiles of the ovine caseinate hydrolysates were similar to one another, although those peptide peaks denoted as 26 and 41 were present only at pH 6.5, and those peptide peaks denoted as 11, 21, 22, 35 and 36 were present only at pH 5.5. At pH 6.5 after 1 min of hydrolysis (*Figure 3a-A*), the primary peptides apparent were those denoted as 4, 19, 20, 23, 25, 27, 31, 33 and 40. The peptides denoted as 23, 27 [α_{s1}-(f1-*)], 31 [α_{s2}-

(f89-*)] and 33 [β-(f191-*)] showed the highest specific formation rates, such as 8.02, 4.77, 1.31 × 10 and 1.30 × 10 UA (units of absorbance) min^{-1} RU^{-1}, respectively. At the same pH but after 1 h of hydrolysis (*Figure 3a-A*), the secondary peptides denoted as 32, 37 [β-(f1-*)] and 38 [β-(f128-*)] were those that exibited the highest formation rates, such as 1.04 × 10, 4.70 × 10 and 1.05 × 10 UA min^{-1} RU^{-1}, respectively. They did not reach a plateau during the whole period of study (24 h). At pH 5.5 after 1 min of hydrolysis (*Figure 3a-B*), the primary peptides apparent were those denoted as 4, 16–20, 24, 25, 28–32, 38, 40, 42 and 43. Peptides denoted as 16, 18, 20, 24, 25, 29, 30 and 32 displayed the highest formation rates, such as 3.21, 1.42, 2.63, 1.57, 3.79, 2.49, 7.96 and 6.45 UA min^{-1} RU^{-1}. The secondary peptides denoted as 27, 33 and 37 (*Figure 3a-B*) showed the highest formation rates by 1 h, such as 8.58, 7.30 and 2.19 × 10 UA min^{-1} RU^{-1}, respectively. The peptides produced at pH 5.2 with 5% (w/v) NaCl after 1 min of hydrolysis (*Figure 3a-C*) were, with the exception of peptide denoted as 10, also produced at either pH 6.5 or pH 5.5. The major primary and secondary peptides were those denoted as 24 and 23, respectively, with formation rates of 4.75 and 3.38 UA min^{-1} RU^{-1}, respectively. The peptide profiles of the caprine hydrolysates at pHs 6.5, 5.5 and 5.2 with 5% (w/v) NaCl (*Figure 3b, A-C*) had a number of peptides in common. Hydrolysates from caprine caseinate produced at pH 6.5 after 1 min (*Figure 3b-A*) included as primary peptides those denoted as 1, 2, 13, 14, 20, 22–28 and 31–36. The highest formation rates were observed for those peptides denoted as 20 [β-(f128-*) + α_{s2}-(f89-*)], 24 [α_{s1}-(f165-*)], 26, 28 [α_{s1}-(f24-*) + α_{s1}-(f174-*) + β-(f191-*) + α_{s2}-(f10-*)], 31 [β-(f1-*)] and 32 [β-(f128-*)], such as 7.87, 4.85, 9.99, 2.20 × 10, 5.37 × 10 and 4.13 × 10 UA min^{-1} RU^{-1}, respectively. At pH 5.5 after 1 min of hydrolysis (*Figure 3b-A*), the primary peptides were those denoted as 1–4, 9, 10, 13–15, 19–29, 31, 32 and 34–37 whereas the ones that showed the highest formation rates were 19–23, 28, 29 and 31, such as 1.34 × 10, 9.18, 1.64 × 10, 7.86, 1.26 × 10, 8.37, 1.14 × 10 and 4.85 × 10 UA min^{-1} RU^{-1}, respectively. The secondary peptides produced from caprine caseinate at pH 6.5 that showed the highest formation rates were 19 [α_{s1}-(f1-*) + α_{s2}-(f180-*) + β-(f137-*)], 21 and 29 [β-(f101-*)], such as 1.18 × 10, 8.95 and 1.97 × 10 UA min^{-1} RU^{-1}, respectively, but at pH 5.5 no secondary peptide was found that exhibited a significant rate of formation. All peptides produced at pH 5.2 and 5% (w/v) NaCl (*Figure 3b-C*) were also produced either at pH 6.5 or pH 5.5. The major primary and secondary peptides were denoted as 28 and 21 with corresponding formation rates of 1.48 × 10 and 2.44 × 10 UA min^{-1} RU^{-1}, respectively.

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