Action of cardosin A from *Cynara humilis* on ovine and caprine caseinates

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In the Iberian Peninsula, the proteinases present in the flowers of members of the *Cynara* genus, *C. cardunculus*, *C. humilis* and *C. scolymus*, have for many years been successfully used in the manufacture of traditional cheeses from ovine and/or caprine milk on individual farms (Vieira de Sá & Barbosa, 1972; Trujillo *et al.* 1994). In Portugal, *C. cardunculus* is the species most frequently employed. Although commercial thistle was tentatively assumed to be pure in taxonomic terms, accurate analyses have shown that the flowers of *C. cardunculus* are often mixed with flowers of *C. humilis* (Pires *et al.* 1994). The clotting activity of *C. humilis* is due to an aspartic proteinase, currently designated cardosin A and similar to another enzyme obtained from *C. cardunculus*. This enzyme is similar in specificity and activity to chymosin (Pires *et al.* 1994).

The action of cardosin A from *C. cardunculus* upon ovine and caprine caseins has been reported recently (Ramalho-Santos *et al.* 1996; Simões, 1998; Sousa & Malcata, 1998), but as yet there is no information on the proteolytic activity of the proteinase from *C. humilis* upon caseins from milks other than bovine. Caseins from small ruminants' milks are the usual substrates of cardosin during milk coagulation and cheese ripening, and sodium caseinate represents an intermediate system between isolated caseins and the cheese matrix that is free from interference by fat. Thus ovine and caprine caseinates may be useful substrates for investigating the proteolytic activity of cardosin.

The aim of the present study was to compare the action of pure cardosin A, obtained from *C. humilis*, on caprine and ovine caseinates, and to assess the *in vitro* contribution of this enzyme to the overall proteolytic action of thistle rennet.

MATERIALS AND METHODS

Preparation of substrate

Whole caprine and ovine caseins were prepared by isoelectric precipitation from raw milk at pH 4·25 and 4·30 respectively (Sousa & Malcata, 1998).

Preparation of enzyme

Cardosin A was obtained according to the method of Veríssimo *et al.* (1995), with slight modifications. The first step was gel filtration through a Highload 26/60 Sephacryl S-200 column (Pharmacia, S-751 82 Uppsala, Sweden), equilibrated and eluted with 20 mM-Tris-HCl buffer, pH 7·6 at a flow rate of 1·5 ml/min. Purity was

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assayed by SDS-PAGE in a Phastsystem (Pharmacia) using PhastGel homogeneous 20. Protein concentration was determined by the method of Bradford (Robyt & White, 1990) using bovine serum albumin (Merck, D-64293 Darmstadt 1, Germany) as standard.

Characterization of catalytic activity

Whole caprine (or ovine) caseinate was dissolved in 100 mM-sodium phosphate buffer, pH 6·5 at 10 mg/ml. To 10 ml substrate solution, 526 μl of an aqueous solution of cardosin A (180 μg/ml) was added and the mixture was kept at 30 °C with stirring. Samples were withdrawn after 1 min and 1, 3, 6 and 10 h. The reaction was quenched prior to analysis by electrophoresis by addition of an equal volume of double concentrated Tris-HCl buffer containing urea, 2-mercaptoethanol, Bromophenol blue and EDTA (McSweeney *et al.* 1993), and prior to analysis by reversed-phase (RP) HPLC by heating at 95 °C for 30 min. Two parallel experiments were carried out and analyses run in duplicate.

Quantitative characterization by urea-PAGE

Urea-PAGE was carried out using a Protean II xi cell vertical slab unit (Bio-Rad Laboratories, Hercules, CA 94547, USA) according to the Andrews (1983) method (pH 8·9, separation gel 125 g/l, stacking gel 40 g/l) with the modifications proposed by Shalabi & Fox (1987). The gels were stained with Coomassie blue G250 (Bio-Rad) using the method of Blakesley & Boezi (1977). Quantitation of α_s- and β-caseins was by densitometry using a model GS-700 imaging densitometer (Bio-Rad).

Quantitative characterization by reversed-phase HPLC

Samples of hydrolysates (2 ml) were adjusted to pH 4·6 by adding 60 μl acetic acid (333 ml/l); after 10 min, 60 μl 3·33 M-sodium acetate was added. After a further 10 min the samples were centrifuged at 8000 ***g*** for 10 min and the supernatants recovered for further analysis. RP-HPLC was performed following the protocol proposed by Singh *et al.* (1995) with a Beckman system (Beckman Instruments, San Ramon, CA 94583, USA). A Lichrosorb 250 × 4 mm RP-8 (5 μm) column (Merck) was employed with a Lichrocart 4–4 guard column (Merck). Elution was at 30 °C with a flow rate of 1 ml/min using a mixture of two solvents (A and B, 1 ml trifluoroacetic acid/l in water and acetonitrile respectively). The elution pattern was: pure A for 5 min, a linear gradient of 0–500 ml B/l over 55 min, 500 ml B/l for 6 min, a linear gradient to 600 ml B/l over 4 min and 600 ml B/l for 3 min. Samples of 100 μl were injected on to the column and detection was by measuring absorption at 214 nm.

Qualitative characterization by sequencing

Peptide-containing peaks were collected manually from the outlet of the RP-HPLC system and chemically sequenced, up to at least five residues from the N-terminus, via Edman degradation on an automated pulsed liquid-phase protein–peptide sequencer (model 477A, Applied Biosystems, Foster City, CA 94404, USA). The partial sequences thus obtained were checked against the (known) sequences of caseins to find which peptide bonds were cleaved.

RESULTS

The gel filtration step of the aqueous acidic extraction of *C. humilis* flowers removed low molecular mass contaminants, and further separation by ion-exchange

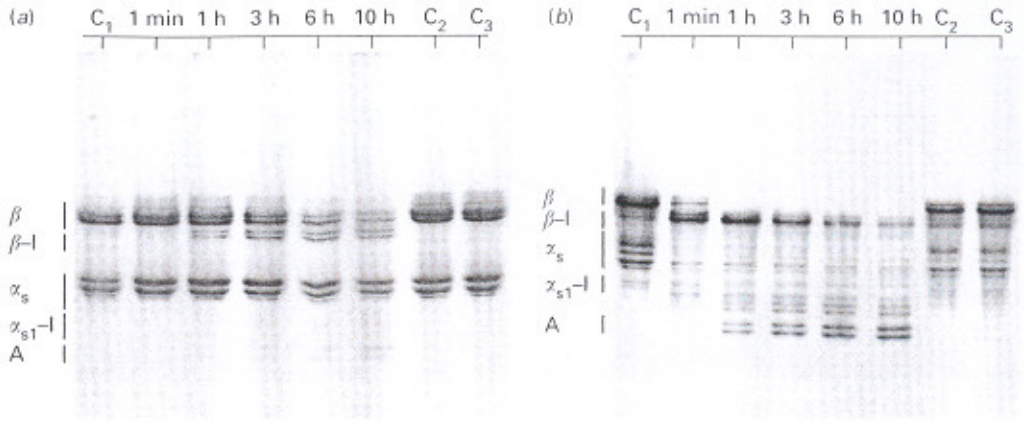

Fig. 1. Urea-PAGE electropherograms of (*a*) ovine and (*b*) caprine sodium caseinate after hydrolysis by cardosin A for the times indicated. C_1, C_2 and C_3 were included as controls: C_1, untreated ovine (or caprine) sodium caseinate; C_2 and C_3, ovine (or caprine) sodium caseinate after incubation in the absence of enzyme for C_2, 1 min and C_3, 10 h respectively. *β*, *β*-Casein; *β*-I, *β*-I-casein; α_s, α_s-casein; α_{s1}-I, α_{s1}-I-casein; A, degradation products of α_{s1}-I-casein.

Table 1. *Hydrolysis of ovine and caprine α_s-casein and β-casein by cardosin A*

(Values are percentage hydrolysed, means ± SD for $n = 2$)

Incubation time, min	α_s-Casein	β-Casein
Ovine		
1	2·7 ± 0·0	3·9 ± 0·4
60	10·5 ± 0·0	4·5 ± 0·1
180	10·9 ± 0·3	18 ± 0·2
360	25·8 ± 0·1	39·6 ± 0·1
600	29·1 ± 0·2	52·1 ± 0·3
Caprine		
1	36·4 ± 0·1	54·7 ± 0·2
60	40·2 ± 0·7	85·0 ± 0·3
180	48·7 ± 1·3	86·0 ± 0·5
360	54·5 ± 0·1	86·6 ± 0·1
600	65·9 ± 0·4	86·6 ± 0·1

chromatography produced two peaks, which were taken to be cardosin A (Veríssimo *et al.* 1995). The purity of these peaks was assayed by SDS-PAGE, which upon molecular mass calibration gave 31 and 15 kDa for the bands corresponding to the two peaks. The fraction corresponding to the second peak eluted was used for the subsequent experiments.

Although both caprine and ovine α_s- and β-caseins were hydrolysed by cardosin A from *C. humilis*, there were several differences in the peptides produced. Hydrolysis of β-casein in both caseinates yielded a pair of bands comparable to that of bovine β-I-casein; they appeared earlier in caprine than in ovine caseinate (Fig. 1). After 10 h digestion, caprine β-casein was degraded more extensively than its ovine counterpart (Table 1). The intense degradation of α_s-casein in caprine caseinate was apparent after 1 min incubation, and was faster than that of its ovine counterpart (Fig. 1, Table 1). Two bands from ovine caseinate and one from caprine caseinate, which may correspond to bovine α_{s1}-I-casein, became visible just after the α_s-casein region (Fig. 1). Two bands (A in Fig. 1) with electrophoretic mobilities

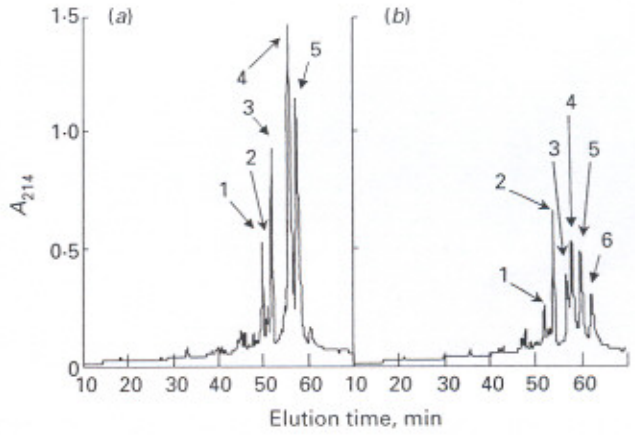

Fig. 2. Reversed-phase HPLC peptide profile of (*a*) ovine and (*b*) caprine sodium caseinate after 10 h hydrolysis by cardosin A. For conditions, see text. Peaks 1–5 (*a*) and 1–6 (*b*) are identified in Table 2.

Table 2. *Specificity of action of cardosin A upon ovine and caprine caseinates*

Peak†	Sequence identified	Source protein	Peptide bond cleaved
Ovine			
1	NH_2-Ser–Gln–Pro–Lys–Val–Leu–	β-Casein	Leu^{165}–Ser^{166}
2	NH_2-Tyr–Tyr–Leu–Pro–Leu–	α_{s1}-Casein	Trp^{164}–Tyr^{165}
3	NH_2-Tyr–Gln–Glu–Pro–Val–	β-Casein	Leu^{190}–Tyr^{191}
4	NH_2-Arg–Glu–Gln–Glu–Glu–	β-Casein	—
5	NH_2-Thr–Asp–Val–Glu–Lys–	β-Casein	Leu^{127}–Thr^{128}
Caprine			
1	NH_2-Tyr–Gln–Lys–Phe–Ala–	α_{s2}-Casein	Tyr^{186}–Tyr^{187}
2	NH_2-Tyr–Gln–Glu–Pro–Val–	β-Casein	Leu^{190}–Tyr^{191}
3	NH_2-Trp–Met–His–Gln–Pro–	β-Casein	Ser^{142}–Trp^{143}
4	NH_2-Arg–Glu–Gln–Glu–Glu–	—	—
5	NH_2-Thr–Asp–Val–Glu–Lys–	β-Casein	Leu^{127}–Thr^{128}
6	NH_2-Gly–Glu–Gln–Glu–Glu–	—	—

† For elution pattern, see Fig. 2.

greater than that of α_{s1}-I-casein were visible in ovine and caprine caseinates hydrolysed for 6 and 1 h respectively, and were probably accounted for by fragments from α_{s1}-I-casein (f(24–?)). Three bands between α_{s1}-I-casein and the A region were visible after 1 h hydrolysis of caprine caseinate (Fig. 1*b*). These may correspond to fragments of β-casein or β-I-casein. In quantitative terms, caprine α_s-casein was hydrolysed to a greater extent than ovine α_s-casein after 10 h incubation (Table 1).

Although caprine caseinate digestion produced more peptides than ovine caseinate digestion, their concentrations (inferred from peak areas and assuming similar response factors) were lower (Fig. 2). This may be a consequence of the different specific action of cardosin upon these two substrates. With ovine sodium caseinate, it was possible to identify four N-terminal sequences corresponding to β-casein and one corresponding to α_{s1}-casein (Table 2). One of the four β-casein peaks (peak 4) was the N-terminal sequence of β-casein. With caprine sodium caseinate, two cleavage sites identified in β-casein were similar to those identified in ovine β-casein, but one additional peptide bond was cleaved (see Table 2). One peptide bond broken in α_{s2}-casein was identified, but no peptide derived from α_{s1}-casein was found

(Table 2). Two peptides could not be associated with either caprine milk casein (Table 2).

DISCUSSION

Our results were similar to those reported by Sousa & Malcata (1998) for the relative rate of hydrolysis of ovine and caprine caseinates, in that caprine caseinate was more extensively hydrolysed than ovine caseinate. Our electrophoretic profiling results for α_{s1}-I casein were consistent with previous studies using *C. cardunculus* (Sousa, 1993; Ramalho-Santos *et al.* 1996; Sousa & Malcata, 1998). No α_{s1}-casein products were detected with caprine caseinate; they were probably present in low concentrations and were not resolved by the techniques employed.

The cardosin A from *C. humilis* behaved as a typical aspartic proteinase, showing a preference for peptide bonds between residues with hydrophobic side chains, as previously reported for bovine caseins by Simões (1998). This enzyme has been previously claimed (Pires *et al.* 1994) to have a specific activity towards bovine caseins similar to that of cardosin A from *C. cardunculus*. Our results indicated that this claim could be extended to ovine caseinate and, to a lesser degree, to caprine caseinate.

A tentative conclusion from this study is that cardosin A from *C. humilis* could replace cardosin A from *C. cardunculus* in the hydrolysis of ovine caseinate.

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