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On the activity and specificity of cardosin B, a plant proteinase, on ovine caseins

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Abstract

The proteolytic activity of cardosin B, an aspartic proteinase from the thistle, *Cynara cardunculus*, on ovine α_s-caseins and β-caseins (independently or present together in sodium caseinate) was followed by urea polyacrylamide gel electrophoresis and reversed phase high performance liquid chromatography. This enzyme degraded both types of caseins, but not to the same degree. In sodium-caseinate, by 10 h at 30°C, α_s-caseins were more susceptible to proteolysis by cardosin B than β-casein whereas, in isolated form, the reverse was observed. Sequencing of the peptides produced by hydrolysis of Na-caseinate showed that the major cleavage sites in α_{s1}-casein were Leu156-Asp157 and Trp164-Tyr165 whereas, in β-casein, they were Leu127-Thr128, Leu165-Ser166 and Leu90-Tyr191. The bonds Trpl64-Tyr165 and Leu165-Ser166 were the most susceptible to cardosin B when this enzyme acted upon isolated α_{s1}- and β-casein, respectively.

Keywords: Rennet substitute; Proteolysis; Electrophoresis; RP-HPLC

1. Introduction

All enzymes employed commercially in milk coagulation are aspartic proteinases, with acidic pH optima, and possess high levels of homology between their primary structures and of similarity between their catalytic mechanisms. Calf rennet, originally obtained from the abomasa of recent-born ruminant calves, contains chymosin (and pepsin to a lesser extent) and has been the coagulant most extensively used in cheesemaking. Shortage of supply of that traditional rennet has urged the search for new coagulating preparations for eventual use as rennet substitutes. Plant proteinases are interesting because they are natural products which can be easily extracted by aqueous infusion.

Cynara cardunculus L., a species of thistle related to the globe artichoke, can be found in dry, stony, uncultivated areas all over Portugal (Roseiro, 1991); since Roman times, aqueous extracts of its flowers have been used as a milk coagulant in the manufacture of various types of Portuguese traditional ewe's milk cheeses, especially Serra da Estrela cheese (Vieira de Sá & Barbosa, 1972; Campos, Guerra, Aguilar, Ventura & Camacho, 1990). Flowers of *C. cardunculus* were claimed to contain one aspartic proteinase with two glycosylated subunits with molecular weights 31 and 16 kDa; that enzyme was found to induce milk coagulation through cleavage of the Phe105–Met106 bond in bovine κ-casein (Faro, 1991). However, more recent studies have shown that, in fact, there are two distinct aspartic proteinases present in the thistle, viz. cardosins A and B (Veríssimo et al., 1996). These two enzymes appear chiefly in the female part of the flowers of *C. cardunculus*, i.e. in the upper area (where the ratio of the concentration of cardosin A to cardosin B is higher); in the lower part of the flowers, only cardosin B is present (Castanheira, 1998). Cardosin A consists of two subunits with apparent molecular weights 31 and 15 kDa, whereas cardosin B consists of two subunits with apparent molecular weights 34 and 14 kDa; it has been claimed that these enzymes are similar, in terms of specificity and activity, to chymosin and pepsin, respectively (Pires et al., 1994; Veríssimo, Esteves, Faro & Pires, 1995). Sousa (1993) and Macedo, Faro & Pires (1993) reported that the primary cleavage site on bovine α_{s1}-casein is Phe23–Phe24; other peptide bonds can also be cleaved in that casein, viz. Phe145–Tyr146, Leu149–Phe150, Tyr153–Tyr154,

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Leu156–Asp157, A1a163–Trp164. Trp164–Tyr165, Tyr165–Tyr166 and Tyr166–Val167 (Macedo et al., 1993). In bovine α_{s2}-casein, cardosins catalyse the hydrolysis of the peptide bonds Phe88–Tyr89 and Tyr95–Leu96 (Macedo et al., 1996). Sousa and Malcata (1998) monitored the primary proteolysis of ovine caseinate by both cardosins in crude aqueous extracts of *C. cardunculus*; the major cleavage sites in ovine caseinate were found to be Phe105–Met106 for κ-casein, Leu127–Thr128 and Leu190–Tyr191 for β-casein, Phe23–Val24 for α_{s1}-casein, and Phe88–Tyr89 for α_{s2}-casein. Studies, pertaining to the independent action of cardosins A and B toward bovine caseins, made it clear that in α_{s1}-casein the two enzymes cleave, primarily, the bonds Phe23–Phe24, Phe153–Phe154 and Trp164–Tyr165; cardosin A also cleaves the bond Tyr165–Tyr166, whereas cardosin B cleaves, in addition, Phe150–Arg151 (Ramalho-Santos, Veríssimo, Faro & Pires, 1996). The peptide bond Leu192–Tyr193 of β-casein is the most susceptible to attack by both cardosins; the peptide bonds Leu127–Thr128 and Leu165–Ser166 can also be cleaved, but their lability is different depending on the cardosin that is used (Simões, 1998). It was also reported that both enzymes cleave the Phe105–Met106 bond in bovine κ-casein. Even though the independent action of cardosins A and B on bovine caseins has been reported, no data pertaining to the independent action of those two aspartic proteinases on ovine caseins have, to our knowledge, been made available to date.

Since proteolysis is the most important biochemical event during cheese ripening, in which the enzymes contributed by the rennet play a relevant role (especially in the first stages thereof), it is of utmost importance to evaluate the degradation patterns of caseins in model systems that mimic actual cheesemaking because of their effects on yield, texture and flavour of the final cheese. Hence, this communication reports studies on the action of cardosin B upon ovine Na-caseinate, and also upon α_s- and β-caseins isolated therefrom.

2. Materials and methods

2.1. Preparation of enzyme

The method described by Veríssimo et al. (1995), with slight modifications, was followed to purify cardosin B; stigmae of dried flowers of *C. cardunculus* were separated and homogenized in a mortar and pestle at the ratio of 1 g of flowers per 12 ml of aqueous 0.1 mol/l citric acid (pH 3.0). The homogenate was centrifuged at 6000 *g* for 20 min, and a 2-ml aliquot of the supernatant was applied to a Highload 26/60 Sephacryl S-200 column (Pharmacia, Uppsala, Sweden), after proper conditioning, and eluted with 20 mmol/l Tris-HU buffer (pH 7.6) at a flow rate of 1.5 ml/min. The fraction of the eluate corresponding to the proteinases of interest (eluted at 135 ml) was applied to a Mono Q HR 5/5 column (Pharmacia), from which the protein was eluted with the same buffer at a flow rate of 0.75 ml/min under a linear gradient of 0–0.5 mol/l NaCl within 30 min; the fraction corresponding to the peak eluted at 17.25 ml was collected as cardosin B. Purity was double-checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis in a Phastsystem (Pharmacia) using PhastGel homogeneous 20.

2.2. Quantification of protein

Protein concentration was determined by the method of Bradford (Robyt & White, 1990). Bovine serum albumin (Merck, Darmstadt, Germany) was used as reference in the preparation of the calibration curve.

2.3. Preparation of sodium caseinate

Whole ovine caseins were obtained by isoelectric precipitation from raw milk by acidification to pH 4.3 with 6 mol/l HCl, with stirring. The mixture of caseins and whey was warmed to 45°C and held at that temperature for 45 min. The caseins were recovered by filtration through a clean cloth and washed several times with deionized water. The caseins were then resuspended in deionized water (to the initial volume) and pH was adjusted to 7.0 with 1 mol/l NaOH. The suspension was allowed to equilibrate at 4°C for at least 2 h, and then lyophilized and stored until use.

Ovine α_s- and β-caseins were isolated by the procedure of Mercier, Maubois, Poznanski, and Ribadeau-Dumas (1968), with modifications; these caseins were prepared through fractionation of Na-caseinate (1.5 g per 15 ml 0.01 mol/l Tris-HUCl buffer) by chromatography in a column (75×2.5 cm) containing DEAE-cellulose using 0.01 mol/l Tris-HUCl buffer (pH 7.0), with 4.5 mol/l urea, 0.01 mol/l imidazol and 0.1% (w/v) β-mercaptoethanol as eluent, via a linear gradient 0–0.4 mol/l NaCl within 25 h at the flow rate of 80 ml/h; experiments were carried out at room temperature. The α_s- and β-casein fractions obtained were independently pooled, dialyzed against deionized water, lyophilized and stored until use.

2.4. Enzymatic hydrolysis

Whole Na-caseinate, as well as α_s- and β-caseins were dissolved in 100 mmol/l phosphate buffer (pH 6.5) to a final concentration of 1% (w/v) and allowed to stabilize at 30°C; 0.05% (w/v) sodium azide was then added to inhibit adventitious microflora. Hydrolysis, at 30°C, was started with addition of 526 μl of cardosin B extract (180 μg/ml) per 10 ml of substrate solution. Samples of hydrolysates were taken after 1 min and 1, 3, 6 and 10 h; the reaction was

quenched prior to analysis by electrophoresis via addition of double-concentrated buffer at 50% (v/v) (McSweeney, Olson, Fox, Healy & Hojrup, 1993), and prior to analysis by RP-HPLC via heating at 95°C for 30 min.

2.5. Protein and peptide profiling by electrophoresis

Urea polyacrylamide gel electrophoresis (urea-PAGE) (at 12.5% for the separation gel and 4% for the stacking gel, both at pH 8.9) was performed on samples of casein hydrolysates using the method of Andrews (1983) with modifications (Shalabi & Fox, 1987). Electrophoresis was carried out in a Protean II xi cell vertical slab unit (Bio-Rad Laboratories, Watford, UK) using a model 1000/500 power supply (from Bio-Rad Laboratories). Gels were stained with Coomassie Blue G250 (Bio-Rad Laboratories) following the method of Blakesley and Boezi (1977); α_s- and β-caseins were quantified by densitometry using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratoires, Hercules CA, USA).

2.6. Peptide profiling by chromatography

Samples of hydrolysates (2 ml) were adjusted to pH 4.6 by addition of 60 μl of 33.3% (v/v) acetic acid and held at that temperature for 10 min; then, 60 μl of 3.33 mol/l sodium acetate was added. The samples were held for a further 10 min and centrifuged at 8000 *g* for 10 min, and the supernatants were recovered for further analysis.

The method of Singh, Fox & Healy (1995) was used to analyse the casein hydrolysates by reversed-phase high performance liquid chromatography (RP-HPLC). Chromatographic analysis was performed in a Beckman system (Beckman Instruments, San Ramon CA, USA) consisting of an autosampler (model 502), a solvent delivery system with two pumps (programable solvent module 126), a diode array detector (module 168) and a personal computer with the Gold™ software v.6.01 for data acquisition and analysis. A Lichrosorb 250×4 mm RP-8 (5 μm) column (Merck) with a Lichrocart 4-4 guard column (Merck) was used at 30°C. The protein was eluted at a flow rate of 1 ml/min, using a mixture of two solvents: solvent A, i.e. 0.1% (v/v) trifluoroacetic acid (TFA) in water, and solvent B, i.e. 0.1% (v/v) trifluoroacetic acid (TFA) in acetonitrile. The gradient pattern followed was: 100% (v/v) A over 5 min, a linear gradient to 50% (v/v) B over 55 min, 50% (v/v) B over 6 min, a linear gradient to 60% (v/v) B over 4 min and 60% (v/v) B over 3 min. Aliquots of 100 μl were injected onto the column and detection was by absorbance at 214 nm.

2.7. Peptide sequencing

Peptide-containing peaks were collected manually from the outlet of the RP-HPLC system and sequenced up to 5–10 N-terminal residues by the Edman iterative degradation procedure; the partial sequences thus obtained were checked against the (known) sequences of caseins to determine the peptide bonds cleaved by the proteinases.

3. Results and discussion

The electrophoretic patterns of hydrolysates of ovine caseinate, isolated β-casein and isolated α_s-casein are shown in Figs. 1–3, respectively. The percentage of α_s- and β-caseins hydrolysed, both in ovine caseinate and in isolated form, are shown in Fig. 4. Two main groups of ovine milk caseins were identified, a realization that is consistent with previous observations (Richardson & Creamer, 1976); the group with higher mobility represents the α_s-casein region, whereas that with lower mobility is the β-casein region (see Figs. 1–3).

In caseinate hydrolysates, degradation of either α_s- or β-caseins was observed as early as by 1 min (see lane 2 of Fig. 1); it was apparent that α_s-caseins were hydrolysed faster than β-caseins (see Fig. 4), reaching percent degradations of 76 and 52%, respectively, by 1 min. It is also clear from inspection of Fig. 1 that, by this time, only a small amount of α_s-casein remains unhydrolysed. Two bands appear ahead of β-casein (see bands A and B in lane 2 of Fig. 1), as well as three bands with higher electrophoretic mobilities than α_s-caseins (see bands C, D and E in lane 2 of Fig. 1). Bands A, B, C, and D tended to disappear as incubation progressed, while band E and other bands with mobilities between those of α_s-caseins

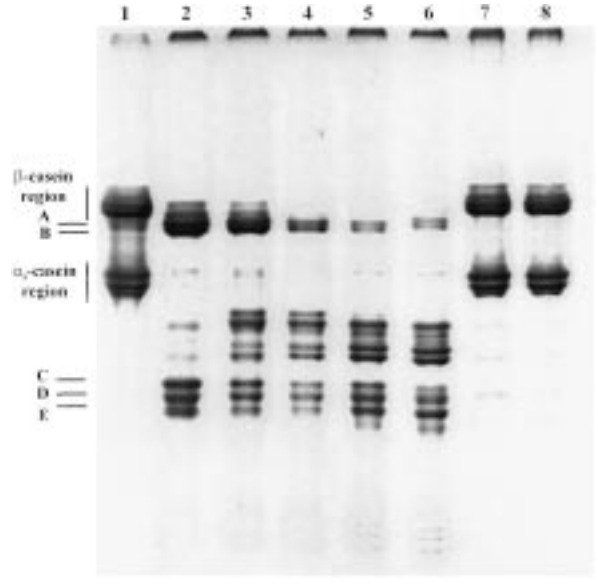

Fig. 1. Urea-PAGE electrophoregram, of ovine Na-caseinate after hydrolysis by cardosin B for 1 min and 1, 3, 6 and 10 h (lanes 2–6). Lane 1 contains native ovine Na-caseinate, and lanes 7 and 8 contain ovine Na-caseinate after incubation for 1 min and 10 h, respectively, in the absence of enzyme, all included as controls.

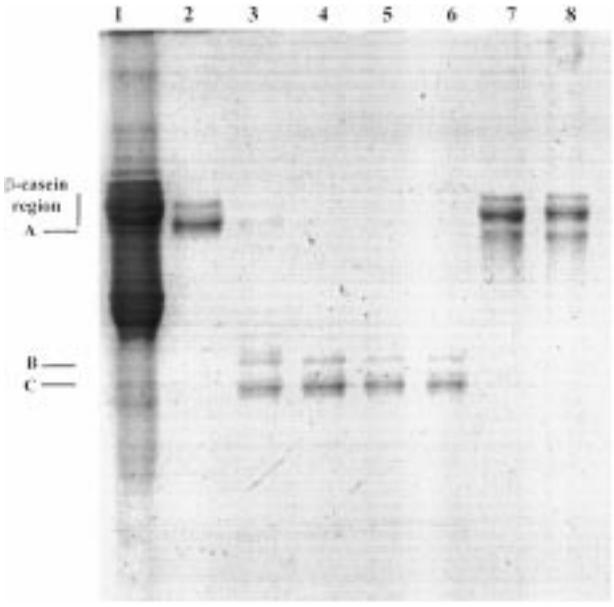

Fig. 2. Urea-PAGE electrophoregram of isolated β-casein after hydrolysis by cardosin B for 1 min and 1, 3, 6 and 10 h (lanes 2–6). Lane 1 contains native ovine Na-caseinate, and lanes 7 and 8 contain ovine β-casein after incubation for 1 min and 10 h, respectively, in the absence of enzyme, all included as controls.

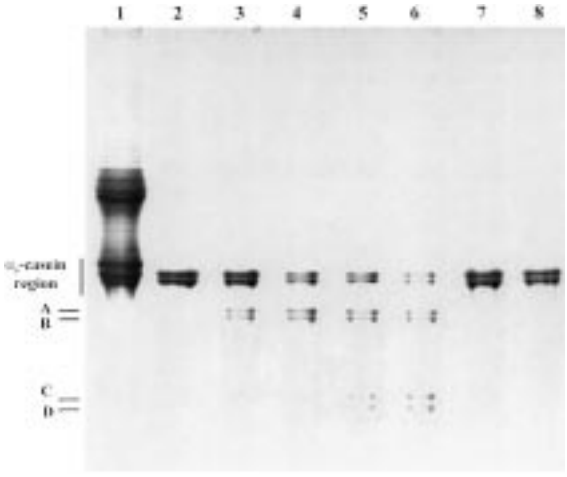

Fig. 3. Urea-PAGE electrophoregram of isolated α_s-casein after hydrolysis by cardosin B for 1 min and 1, 3, 6 and 10 h (lanes 2–6). Lane 1 contains native ovine Na-caseinate, and lanes 7 and 8 contain ovine α_s-casein after incubation for 1 min and 10 h, respectively, in the absence of enzyme, all included as controls.

and of band C became more intense (see lanes 3–6 of Fig. 1). α_s-Caseins were hydrolysed more rapidly than β-caseins, up to 100 and 87%, respectively, by 10 h. It should be noted that a residual amount of proteinaceous material still remains by 10 h; however, it is probably not accounted for by α_s-casein, but is, rather, a polypeptide resulting from degradation of α_s-casein or of β-casein. This observation is comparable to what happens in cheese, since it has also been claimed that β-caseins were less susceptible to proleolysis than α_s-caseins in the manufacture of raw ewe's milk cheese that use extracts of *C. cardunculus* as coagulant (Sousa & Malcata, 1997).

With respect to cleavage of isolated β-caseins by cardosin B, one band is apparent ahead of the β-casein region, by 1 min of hydrolysis, which eventually vanishes as incubation time elapses (see band A in lanes 2–6 of Fig. 2). This band is comparable to that of bovine β-I casein (Sousa, 1993), which results from cleavage of both Leu192–Tyr193 and A1a189–Phe190 (Macedo et al., 1993; Sousa, 1993). In ovine β-casein, the corresponding breakdown sites are Leu190–Tyr191 and A1a187–Phe188 (Whyte, 1995). Studies encompassing the action of cardosin B on separated bovine β-caseins showed that the Leu192–Tyr193 bond was also cleaved, as well as Leu127–Thr128 and Leu165–Ser166 (Simões, 1998). After 1 h of hydrolysis, two bands displaying higher electrophoretic mobilities than that of band A could be observed, which became more intense with time (see bands B and C in lanes 3–6 of Fig. 2); by 10 h of incubation, β-caseins were fully degraded. The state of aggregation and globular conformation of caseins seems to affect proteolysis (Dalgleish, 1987); in fact, the aforementioned results show that β-caseins are, in separated (and possibly denaturated) form, more quickly and extensively degraded than in caseinate (and possibly structured) form (see Fig. 4). The higher resistance to hydrolysis in the latter case may be attributed to the complex set of interactions within caseins in Na-caseinate (which encompass native α_s-caseins, β-caseins and κ-caseins), and also to the high amount of fat in ovine milk, which changes accessibility of the enzyme to the labile peptide bonds.

When in isolated form, α_s-caseins were extensively hydrolysed and reached a degradation level of 81% by 10 h of incubation. Two bands, displaying higher mobilities than α_s-caseins, were apparent after 1 h of incubation and their density increased as hydrolysis time elapsed (see bands A and B in lanes 3–6 of Fig. 3). Two other bands, C and D; appeared by 6 h of reaction and remained still thereafter. Despite reports by Dalgleish (1987), α_s-caseins were hydrolysed to a lesser extent in isolated form than in caseinate form by cardosin B. These results might be rationalized in view of the spatial distribution of caseins within the caseinate structure: the putative bulky location of α_s-caseins (in a more hydrophobic environment) may favour hydrolysis when compared with isolated (and hence fully exposed) α_s-caseins. Further work is, however, needed in order to fully clarify this point. It should be emphasized that differences observed between degradation patterns of α_s- and β-caseins (present together in Na-caseinate or separated from one another) are not at all due to a dilution factor, since the concentration of substrate protein was in all cases normalized by its initial concentration.

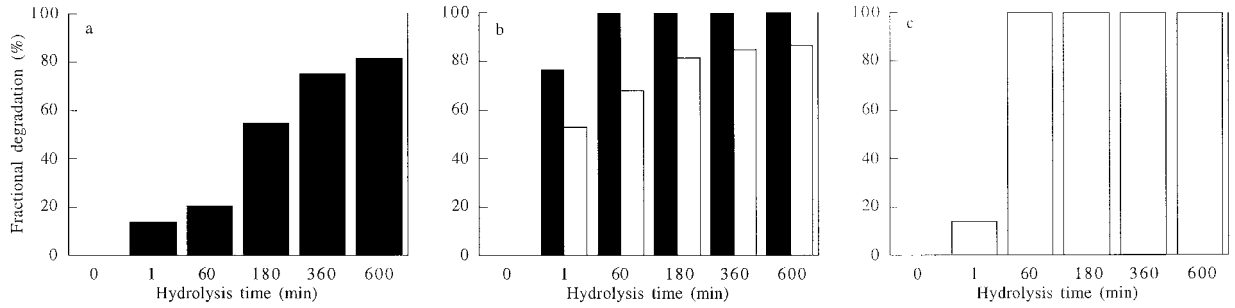

Fig. 4. Percentage of hydrolysis of ovine caseins (α_s-casein: ■; β-casein: □) in isolated form (α_s-casein: a, β-casein: c), and in the presence of each other in Na-caseinate form (b), effected by cardosin B, versus incubation time.

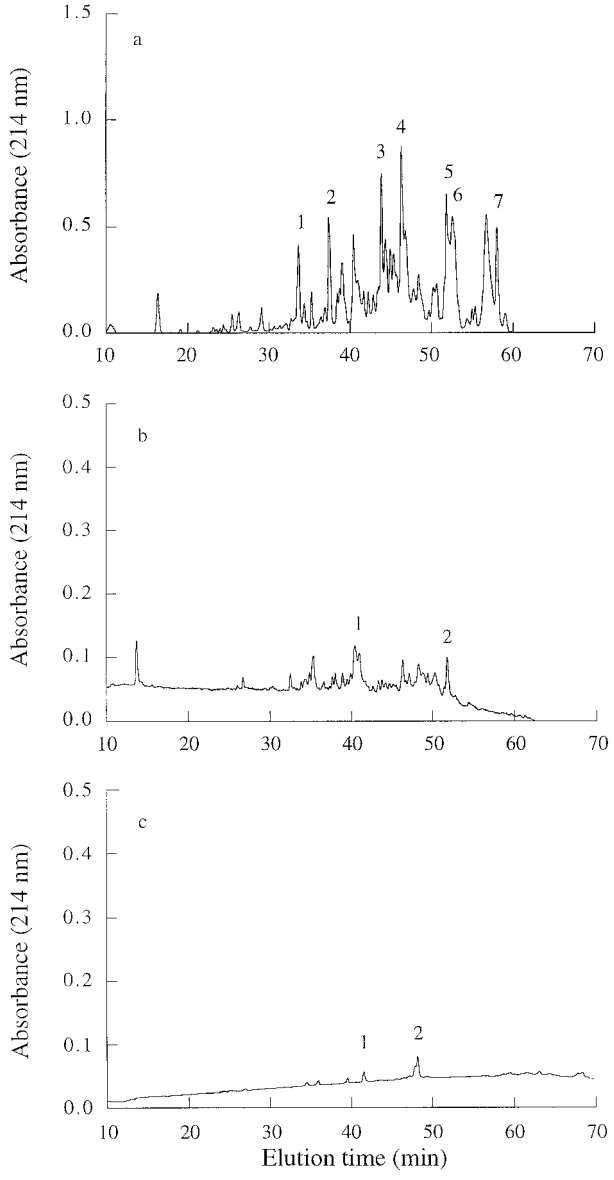

Fig. 5. RP-HPLC peptide profile of ovine sodium caseinate (a), isolated β-casein (b) and isolated α_s-casein (c) by 10 h of hydrolysis carried out by cardosin B.

The RP-HPLC peptide profiles of hydrolysates of ovine caseinate, isolated ovine β-casein and isolated ovine α_s-casein, obtained via action of cardosin B for 10 h, are shown in Fig. 5. Seven major peaks were visible in the peptide profile of ovine caseinate hydrolysate. Sequencing of those peptides indicated that cardosin B can cleave peptide bonds in both α_s- and β-caseins. The N-terminal sequences of the peptides, designated as 1 [α_1-(f157-*)], 3 and 5 [α_{s1}-(f165-*)] in Fig. 5a, indicated that α_{s1}-casein was cleaved by cardosin B at bonds Leu156–Asp157 and Trp164–Tyr165. The N-terminal sequence of ovine α_{s1}-casein was identified in peptide 4. β-Casein was cleaved by cardosin B at Leu165–Ser166, Leu190–Tyr191 and Leu127–Thr128, as derived from the N-terminal sequence of the peptides denoted as 2 [β-(f166-*), 6 [β-(f191-*)] and 7 [β-(f128-*)], respectively (Fig. 5a). The peptide bonds Leu190–Tyr191 and Leu127–Thr128 were also claimed (Sousa & Malcata, 1998) to be broken in ovine sodium caseinate by cardosins A and B mixed with one another.

With respect to isolated β-casein, the N-terminal sequence of peptide 2 [β-f166-*)] (Fig. 5b) indicated that ovine β-casein was broken by cardosin B at peptide bond Leu165–Ser166; Leu165–Ser166 was also cleaved by cardosin B in bovine β-casein, as well as Leu127–Thr128 and Leu192–Tyr193 (Simões, 1998).

With respect to isolated α_s-caseins, two major peaks became apparent by 10 h of hydrolysis (Fig. 5c). α_{s1}-Casein was cleaved by cardosin B at the peptide bond Trp164–Tyr165, as apparent from sequencing of the peptide designated as 2 [α_{s1}-(f165-*)]. The corresponding bond cleaved in bovine α_{s1}-casein is Trp164–Tyr165; the bonds Phe23–Phe24, Trp153–Tyr154 and Phe150–Arg151 can, in addition, be cleaved (Ramalho-Santos et al., 1996).

4. Conclusions

Significant differences exist with respect to the action of cardosin B from *C. cardunculus* upon ovine α_s- and

β-caseins. When α_s- and β-caseins are present together in sodium-caseinate, α_s-caseins are much more susceptible to proteolysis than β-caseins, whereas the reverse is observed when the caseins are tested independently. In sodium caseinate, α_{s1}-casein is cleaved by cardosin B at bonds Leu156–Asp157 and Trp164–Tyr165, whereas β-casein is cleaved at peptide bonds Leu127–Thr128, Leu165–Ser166 and Leu190–Tyr191. The bonds Leu165–Ser166 and Trpl64-Tyr165 are cleaved by cardosin B in isolated β-casein and isolated α_{s1}-casein, respectively.

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