

Proteolysis of ovine caseins by cardosin A, an aspartic acid proteinase from *Cynara cardunculus* L.

Sofia V. Silva, F. Xavier Malcata*

Escola Superior de Biotecnologia, Universidade Católica Portuguesa,
Rua Dr. António Bernardino de Almeida, 4200 Porto, Portugal

(Received 6 October 1997; accepted 3 April 1998)

Abstract — The breakdown of α_s -caseins and β -caseins (in the form of α_s -caseins, the form of β -caseins, and the form of a mixture of α_s - and β -caseins in Na-caseinate) by cardosin A, one of the major two proteinases present in the flowers of *Cynara cardunculus* L., was experimentally studied via urea polyacrylamide gel electrophoresis. In Na-caseinate, α_s - and β -caseins were degraded up to 46 and 76 %, respectively, by 10 h of hydrolysis. In separated form, α_s -caseins reached a level of degradation up to 67 % while β -caseins were quickly and extensively degraded up to 76 %. In general, β -caseins seemed to be more susceptible to proteolysis than α_s -caseins. © Inra/Elsevier, Paris.

milk protein / enzyme / plant rennet / electrophoresis

Résumé — Protéolyse des caséines de brebis par la cardosine A, une protéase acide aspartique de *Cynara cardunculus* L. La dégradation des caséines α_s et des caséines β (sous la forme de caséines α_s , de caséines β , et d'un mélange de caséines α_s et β d'un caséinate du sodium) par la cardosine A, une des protéinases majeures présentes dans les fleurs de *Cynara cardunculus* L., a été étudiée par électrophorèse en gel de polyacrylamide à l'urée. Dans le caséinate de sodium, les caséines α_s et β ont été respectivement dégradées jusqu'à 46 et 76 %, après 10 h d'hydrolyse ; sous forme séparée, les caséines α_s ont atteint des niveaux de dégradation de 67 % ; pourtant, les caséines β ont été dégradées plus rapidement et extensivement jusqu'à 76 %. En général, les caséines β doivent être plus sensibles à la protéolyse que les caséines α_s . © Inra/Elsevier, Paris.

protéine laitière / enzyme / présure végétale / électrophorèse

* Correspondence and reprints.

1. INTRODUCTION

Milk clotting by proteolytic enzymes is a crucial operation in most forms of cheesemaking. Calf rennet was the first (and still considered the best) mixture of milk clotting enzymes, mainly chymosin and pepsin. Chymosin accounts for more than 90% of the milk clotting activity in rennets extracted from the abomasa of very young animals, but as the animal ages the ratio of chymosin to pepsin decreases [8]. The worldwide supply of animal rennet is currently insufficient to satisfy the growing demand by the cheese industry and consequently the search for alternative sources of milk clotting enzymes has gained renewed interest. Although rennets from genetically engineered microorganisms have been produced in higher and higher numbers, several wild plants can also produce proteolytic enzymes which have, nevertheless, not attracted extensive attention. The exception to this rule is a clotting aqueous extract from the flowers of the wild thistle (*Cynara cardunculus L.*), which has been successfully used for centuries in cheesemaking in Portugal [15] and bordering regions of Spain. The clotting activity of this extract was first attributed to the presence of three proteolytic enzymes synthesized by the stigmata of those flowers [3], and such (tentatively termed) cynarases 1, 2 and 3 were purified and partially characterized [6]. Two new aspartic acid proteinases, (tentatively named) cardosins A and B, were later isolated and are believed to be genetically different from the aforementioned cynarases [17]; in terms of activity and specificity, cardosins A and B are remarkably similar to chymosin and pepsin, respectively [16].

In addition to this clotting activity, the enzymes present in rennet and entrapped in the curd can also bring about protein breakdown during cheese ripening [4, 5], and are thus responsible for the release of various peptides with biochemical, rheological and sensory roles in cheese [4]. It has been firmly established that the acid proteinases

present in rennet are highly specific in their action towards the Phe105-Met106 bond of κ -casein, although they can also attack caseins other than κ -casein at much lower rates [4]; on the other hand, despite the fact that the action of cardosins A and B on bovine α_{s1} -casein was studied [10], fundamental work encompassing the action of these proteinases on ovine caseins remains scarce. Hence, the aim of this work was to study the action of cardosin A on ovine Na-caseinate, and on families of ovine caseins isolated therefrom, to eventually better understand what happens during ripening of traditional Portuguese cheeses which use this enzyme in their manufacture protocol.

2. MATERIALS AND METHODS

2.1. Substrate preparation

Ovine sodium-caseinate was prepared from raw milk by acidification to pH 4.3 with direct addition of 6 mol·L⁻¹ HCl under stirring, warming up to 45 °C and holding at that temperature for 45 min. The precipitate was then recovered by filtration through a clean cloth, washed several times with deionized water and resuspended in deionized water, and its pH adjusted to 7.0 with 1 mol·L⁻¹ NaOH. The solution was allowed to equilibrate at 4 °C for at least 2 h and then lyophilized.

Ovine α_s -caseins and β -caseins were prepared from lyophilized Na-caseinate via semipreparative separation by chromatography with DEAE-cellulose using 0.01 mol·L⁻¹ Tris-HCl buffer (pH 7.0) containing 4.5 mol·L⁻¹ urea, 0.01 mol·L⁻¹ imidazol and 0.1% (w/v) β -mercaptoethanol as eluent via a linear gradient of 0–0.4 mol·L⁻¹ of sodium chloride within 25 h at the flow rate of 80 mL·h⁻¹. The various α_s -casein and β -casein fractions were independently pooled together, dialyzed against deionized water and lyophilized.

2.2. Enzyme preparation

Dried flowers of the thistle (*C. cardunculus L.*) were homogenized at the ratio of 1 g of ground flowers to 12 mL of citric acid (pH 3.6) and centrifuged at 6 000 g for 20 min. The super-

natant was collected and a 2-mL aliquot was applied to a Highload 26/60 Sephacryl S-200 column (Pharmacia, Uppsala, Sweden) after proper equilibration, and eluted with 20 mmol·L⁻¹ Tris-HCl buffer (pH 7.6) at a flow rate of 1.5 mL·min⁻¹. The various fractions corresponding to the peak of absorbance eluted at 135 mL were pooled together and applied to a Mono Q HR 5/5 column (Pharmacia), from which they were eluted with the same buffer at the flow rate of 0.75 mL·min⁻¹ under a linear gradient of 0–0.5 mol·L⁻¹ sodium chloride within 30 min, and the fraction corresponding to the peaks of absorbance at the eluted volume of 12.75–16.5 mL was duly collected as cardosin A; assay for purity was done via polyacrylamide gel electrophoresis with sodium dodecyl sulfate in a Phastsystem (Pharmacia) using the PhastGel gradient 8–25.

2.3. Enzymatic hydrolysis

Whole Na-caseinate, separated α_s -caseins and separated β -caseins (as appropriate) were dissolved in 100 mmol·L⁻¹ phosphate buffer (pH 6.5) so as to yield 1% (w/v) final concentration, and sodium azide (0.05% w/v) was added to prevent bacterial growth. The preparation of cardosin A (180 μ g·mL⁻¹) was then added at the ratio of 0.526 mL per mL of substrate solution and the mixture was incubated at 30 °C. Samples were taken after 1 min, 1 h, 3 h, 6 h and 10 h of hydrolysis and the reaction was quenched prior to analysis via addition of double concentrated buffer at 50% (v/v) [7] in the case of samples for electrophoresis.

2.4. Protein and peptide profiling by electrophoresis

Urea polyacrylamide gel electrophoresis (urea-PAGE) was performed on samples of casein hydrolyzates following the method of Andrews [1] with slight modifications [12]. Electrophoresis was carried out in a Protean II xi cell vertical slab unit (Bio-Rad Laboratories, Watford, UK); the power supply, model 1000/500 (Bio-Rad Laboratories), was set at 280 mV for the stacking gel and then increased to 300 mV for the separating gel. Gels were stained with Coomassie Blue G250 (Bio-Rad Laboratoires) using the method of Blakesley and Boezi [2]. Quantification of α_s - and β -caseins was done by densitometry using a model GS-700 imaging densitometer (Bio-Rad Laboratoires, Hercules, CA, USA).

3. RESULTS AND DISCUSSION

The electrophoretogram of ovine Na-caseinate after hydrolysis is shown in *figure 1*, whereas those of separated α_s -caseins and separated β -caseins after hydrolysis are shown in *figures 2* and *3*, respectively. The ratio of concentrations of intact α_s - and β -caseins to their initial concentrations, both in ovine caseinate and in separated forms, are shown in *figure 4*.

Sodium caseinate hydrolyzates contained two major groups of electrophoretic bands (see *figure 1*). Richardson and Creamer [11] pointed out that the group with the highest electrophoretic mobility is accounted for by α_s -caseins, which may be further subdivided into three variants (α_{s1} -, α_{s2} - and α_{s3} -casein), whereas the group with the lowest mobility is accounted for by β -caseins, which may be further subdivided into two variants (β_1 - and β_2 -casein), as represented as α_s -casein and β -casein regions, respectively, in *figure 1*. All caseins underwent degradation after as little as 1 min of incubation, and β -caseins were hydrolyzed faster than α_s -caseins (see *figure 4*); the percent degradation of the β -casein family as a whole was 26.6%, whereas that of the α_s -casein family was 17.6%. After this time, breakdown of whole ovine Na-caseinate by cardosin A led to two bands with higher electrophoretic mobility than the β -casein region, which disappeared quickly (see bands A and B of lane 4 in *figure 1*). One band exists that is located right after the α_s -casein region but which vanishes by 10 h of incubation (see band C of lane 6 in *figure 1*), whereas two bands with higher mobility became more intense as hydrolysis time elapsed (see bands D and E of lanes 2–6 in *figure 1*). Hydrolysis of β -caseins occurred to a much greater extent (up to 75.9%) than hydrolysis of α_s -caseins (up to 46.5%) (see *figure 4*), and it also took place more quickly.

With respect to cleavage of separated α_s -caseins by cardosin A, a set of bands

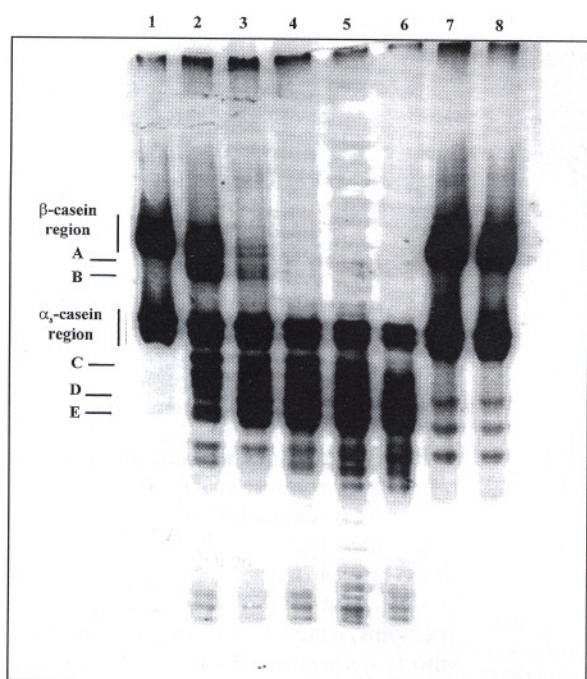
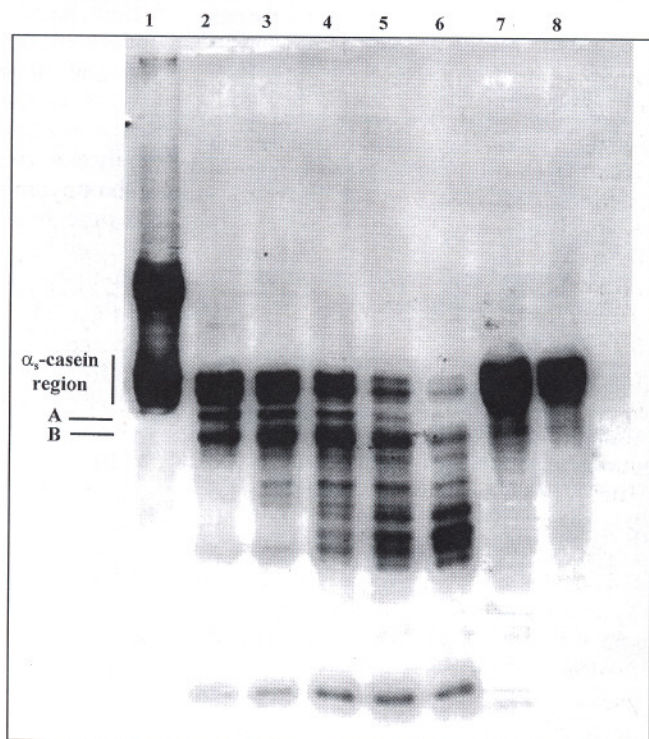


Figure 1. Urea-PAGE electrophoregram of Na-caseinate after incubation for 1 min, 1 h, 3 h, 6 h and 10 h (lanes 2–6, respectively) with cardosin A; lane 1 contains plain ovine Na-caseinate, and lanes 7 and 8 contain plain ovine Na-caseinate, after incubation for 1 min and 10 h, all included as controls.

Figure 1. Diagramme électrophorétique urée-EGPA de caséinate de sodium après incubation pendant 1 min, 1 h, 3 h, 6 h et 10 h (lignes 2–6, respectivement) avec cardosine A ; la ligne 1 contient du caséinate de sodium de brebis pur, et les lignes 7 et 8 contiennent du caséinate de sodium après incubation pendant 1 min et 10 h ; utilisés comme contrôles.

Figure 2. Urea-PAGE electrophoregram of separated α_s -caseins after incubation for 1 min, 1 h, 3 h, 6 h and 10 h (lanes 2–6, respectively) with cardosin A; lane 1 contains plain ovine Na-caseinate, and lanes 7 and 8 contain plain ovine α_s -caseins, after incubation for 1 min and 10 h, all included as controls.

Figure 2. Diagramme électrophorétique urée-EGPA de caséines α_s après incubation pendant 1 min, 1 h, 3 h, 6 h et 10 h (lignes 2–6, respectivement) avec cardosine A ; la ligne 1 contient du caséinate de sodium de brebis pur, et les lignes 7 et 8 contiennent des caséines α_s après incubation pendant 1 min et 10 h ; utilisés comme contrôles.



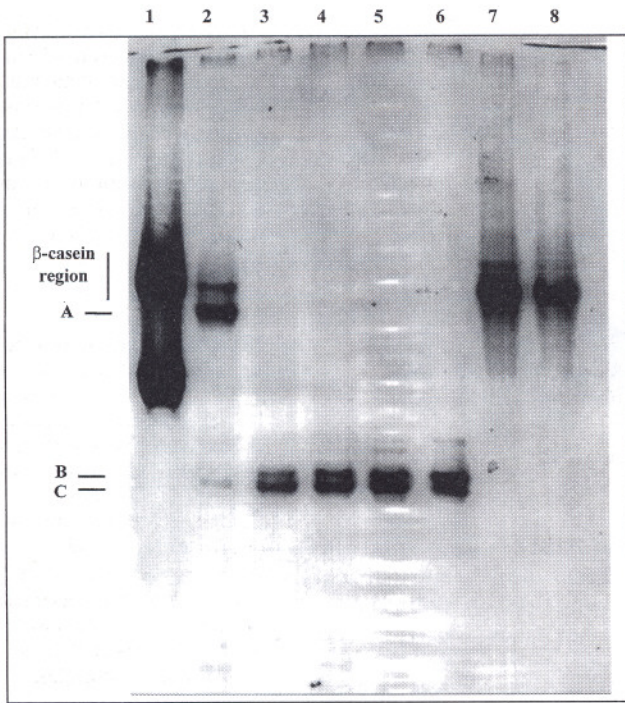


Figure 3. Urea-PAGE electrophoregram of separated β -caseins after incubation for 1 min, 1 h, 3 h, 6 h and 10 h (lanes 2–6, respectively) with cardosin A; lane 1 contains plain ovine Na-caseinate, and lanes 7 and 8 contain plain ovine β -casein, after incubation for 1 min and 10 h, all included as controls.

Figure 3. Diagramme électrophorétique urée-EGPA de caséines β après incubation pendant 1 min, 1 h, 3 h, 6 h et 10 h (lignes 2–6, respectivement) avec cardosine A; la ligne 1 contient du caséinate de sodium de brebis pur, et les lignes 7 et 8 contiennent des caséines β après incubation pendant 1 min et 10 h; utilisés comme contrôles.

with higher electrophoretic mobility than the α_s -casein region can be observed in *figure 2*. Two bands were apparent just after 1 min of hydrolysis but eventually disappeared as incubation progressed (see bands A and B of lane 6 in *figure 2*), whereas bands displaying higher mobilities became thicker and thicker. These results are comparable with those obtained by Ramalho-Santos et al. [10] pertaining to the action of cardosin A on bovine α_{s1} -casein; according to these authors, Phe23-Phe24 is the most susceptible peptide bond and leads to production of fragments f1-23 and f24-199. The Trp164-Tyr166 portion of the latter fragment is then attacked and four polypeptides originate, viz f24-164, f24-165, f165-199 and f166-199; then the Phe153-Tyr154 bond is broken, thus producing f24-153, f154-165 and f154-166, and finally the Phe150-Arg151 bond is hydrolyzed, thus releasing f24-150 and f151-154. The appearance of the polypeptide Tyr154-Trp199

was noticed only due to the slower action of cardosin A on the region Trp164-Tyr166. Inspection of *figure 4* indicates that α_s -caseins were degraded up to 67.3 % by 10 h of hydrolysis.

When in separated form, β -caseins were degraded quickly and extensively and reached a degradation degree of 75.7 % by 10 h of hydrolysis (see *figure 4*). Hydrolysis of ovine β -casein (*figure 3*) led to the production of one band with higher electrophoretic mobility than that of the β -casein region (see band A of lane 2 in *figure 3*) by 1 min of incubation, which disappeared gradually after 1 h. Such peptide was apparently equivalent to bovine β -I-casein [13]; in fact, the bonds Leu192-Tyr193 and Ala189-Phe190 of bovine β -casein are the most susceptible to the catalytic action by either chymosin or proteinases from *C. cardunculus* [13, 18], and β -I-casein, which results from cleavage of those bonds, is thus a mixture of f1-189 and f1-192; in ovine

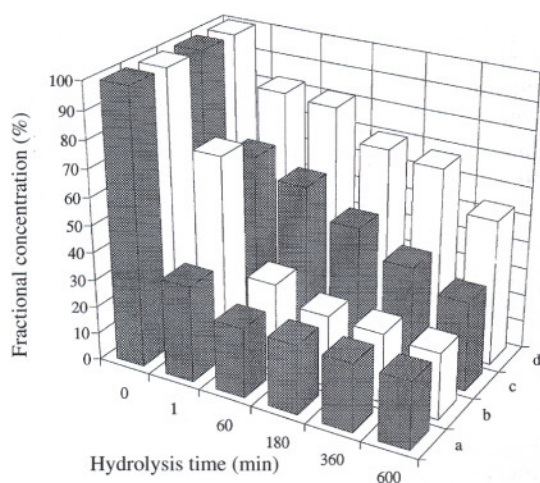


Figure 4. Fractional disappearance of pure caseins in separated α_s -caseins or β -caseins (a: β -casein; c: α_s -casein) and mixed in Na-caseinate form (b: β -casein; d: α_s -casein) via hydrolysis effected by cardosin A.

Figure 4. Disparition fractionnée des caséines en caséines α_s ou β séparées (a: caséine β ; c: caséine α_s) et sous la forme d'un mélange en Na-caséinate (b: caséine β ; d: caséine α_s) à travers l'hydrolyse catalysée par cardosine A.

β -casein, Leu190-Tyr191 and Ala187-Phe188 are the corresponding bonds cleaved [14]. Two bands possessing higher electrophoretic mobility than band A (see bands B and C of lane 3 in figure 3) could be noticed by 1 h of hydrolysis and became more intense as time elapsed.

There is a noticeable difference between the action of this aspartic proteinase upon ovine caseins in their isolated forms and in their mixed (or Na-caseinate) counterparts regarding the rates of proteolysis and degrees of degradation. Proteolysis of β -caseins occurred to similar extents (up to 76%), but separated ones were hydrolyzed more quickly than when mixed with α_s -caseins in Na-caseinate. With regard to α_s -caseins, these proteins were as a whole more extensively degraded in separated form than in Na-caseinate form. Although it might be claimed that the rate of hydrolysis of either α_s - and β -caseins is higher in separated form than in Na-caseinate form due to a dilution factor (recall that α_s - and β -caseins exist at the mass ratio of 3:4.7 in Na-caseinate), it should be noted that the variation of either protein concentration was in both cases normalized by its initial concentration; hence, significant differences in proteolysis rates are likely due to the state of aggregation of the caseins.

From the results presented above, it is apparent that β -caseins, either in separated form or mixed with α_s -caseins in Na-caseinate, are more susceptible to proteolysis than α_s -caseins. Enzymes from *C. cardunculus* act on bovine α_{s1} -, α_{s2} - and β -caseins [9] to a higher extent than the enzymes present in commercial rennets. Sousa and Malcata [14] reported that crude aqueous extracts of flowers of *C. cardunculus* attack both ovine α_s - and β -caseins in cheese, although β -caseins were reported to be less susceptible to proteolysis than α_s -caseins.

Although our results were produced in vitro, their extrapolation to cheese ripening can be relatively backed up because 6% of the coagulant used in cheese manufacture is retained in the curd [13]. However, such extrapolation should be cautious because two factors constrain casein proteolysis: the accessibility of the peptide bonds to the enzyme and the actual enzyme specificity. In milk, most caseins exist naturally as micelles; the putative inaccessibility of the cleavage sites to the enzyme when caseins are in micellar form may lead to lower rates of degradation than in separated, soluble form [4]. Furthermore, the complexity of the three dimensional structure of the casein network in cheese will also interfere with accessibility. On the other hand, the speci-

ficity of the enzymes in pure form may be different from that of the enzymes in crude extracts, although this point requires further experimentation in order to become clear.

As major conclusions, it can be said that significantly different proteolysis patterns exist for the action of cardosin A upon ovine caseins, and that α_s -caseins, either in separated form or mixed with other caseins in Na-caseinate, are as a whole less susceptible to proteolysis than β -caseins.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. José Empis (*Instituto Superior Técnico*, Lisbon, Portugal) for cosupervision within the scope of the graduate program where the author S.V. Silva was enrolled. Financial support for S.V. Silva was provided by an MSc fellowship (BM/8824/96) issued by PRAXIS XXI (JNICT, Portugal). Partial financial support for this work was provided by grants through projects PROTOLACTIS: *PROdução, por Tecnologias Optimizadas, de LACTicínios Tradicionais* (PAMAF, Portugal) and IMPACTO: *Investigação dirigida ao Melhoramento do queijo serra Por integração de abordagens Científicas e Tecnológicas* (PRAXIS XXI, Portugal).

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