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Partial Identification of Water-Soluble Peptides Released at Early Stages of Proteolysis in Sterilized Ovine Cheese-Like Systems: Influence of Type of Coagulant and Starter

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ABSTRACT

Cheese-like systems were manufactured from sterilized ovine milk, using crude aqueous extracts of *Cynara cardunculus* or cardosin A isolated therefrom as clotting agent. The effect of adding a commercial starter culture was also assessed. The impact of the type of coagulant used during the initial 24 h of proteolysis was evaluated via separation of peptides in the water-soluble extracts by reverse-phase HPLC, followed by partial sequencing via Edman degradation. Cardosin A accounted for most events of primary proteolysis. The major cleavage sites were Phe105-Met106 in κ-casein, and Leu127-Thr128, Ser142-Trp143, Leu165-Ser166, and Leu190-Tyr191 in β-casein. The starter culture did not play an active role during the initial stages of ripening.
(**Key words:** casein hydrolysis, *Cynara cardunculus*, rennet, RP-HPLC)

Abbreviation key: RP-HPLC = reverse-phase high-performance liquid chromatography, **WISE** = water-insoluble extract, **WSE** = water-soluble extract.

INTRODUCTION

Rennet substitutes of plant origin are not often used in cheese making. However, the proteinases present in the flowers of *Cynara cardunculus*, viz. cardosins A and B, have been successfully employed for centuries in the Iberian Peninsula for cheese making at the farm level (Vieira de Sá and Barbosa, 1972; Pires et al., 1994; Trujillo et al., 1994). Cardosin A is similar, in terms of specificity and activity, to chymosin, whereas cardosin B resembles pepsin (Veríssimo et al., 1995). The use of this plant rennet often entails problems regarding curd formation and final cheese characteristics, probably because the coagulant activities are difficult to standardize.

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Whereas most of the rennet added to milk during cheese making is eventually lost in whey, a minor fraction that depends on the type of cheese (Choisy et al., 2000) is retained within the curd; this fraction is responsible for its initial proteolysis. During this early stage, caseins are hydrolyzed into large, well-defined peptides via residual rennet, which are subsequently digested into smaller ones and even free AA by enzymes contributed by starter or nonstarter microorganisms (Benfeldt and Sørensen, 2001). Primary proteolysis is possibly the most important set of events toward the development of flavor and texture in ripened cheeses (Fox et al., 1999).

The initial 24 h following coagulation are particularly important in terms of peptide release (Picon et al., 1995). Casein aggregation and whey expression continue to take place throughout that period, and afterwards, casein is compacted within the curd and water is concomitantly lost, while fat globules are entrapped and compressed (all of which determine final cheese structure and composition) (Green and Grandison, 1999).

Proteinase activities are often assessed using skim milk or solutions of casein (Hill and Gasson, 1986; Khalid et al., 1991), whereas peptidase activities usually are studied with chromogenic substrates or sodium caseinate in solution (Hickey et al., 1983; Peterson et al., 1990). However, extrapolation of results from the aforementioned liquid media to an actual solid cheese system is risky, owing to differences between their nature. Furthermore, there are studies (McSweeney et al., 1993) indicating that some of the enzyme-mediated events that occur rapidly in solution fail to do so in cheese. On the other hand, true cheese matrices are too complex and heterogeneous in nature to permit fundamental validation of the performance of proteases as they relate to proteolysis. Hence, a compromise is urged, as provided by cheese-like systems.

Considerable effort has been expended recently to characterize the process of proteolysis affected by enzymes from *C. cardunculus* during the ripening of raw ovine milk cheeses, as well as during hydrolysis of caseins in solution (Silva and Malcata, 1998, 2004, 2005; Sousa and Malcata, 1998a,b; Silva, 1999; Silva et al.,

2002). However, very little data are available in the literature regarding the use of sterilized milk as substrate, and information is even scarcer regarding the initial 24 h of ripening, especially when a coagulant of plant origin is concerned (Silva and Malcata, 2004). On the other hand, the effect of the presence (or absence) of a starter culture is important because it typically is present in relatively higher numbers during the early stages of ripening, either adventitiously in milk or deliberately added during cheese making, and it possesses significant activity that may contribute to proteolysis.

Overall bulk characterization of nitrogen in water-soluble extracts of cheese is a commonly used index of cheese ripening (Fox et al., 1999); reverse-phase HPLC (**RP-HPLC**) has proven suitable to resolve and analyze the peptides and AA therein, which sets the basis for a more refined and meaningful characterization.

Therefore, the aim of this work was to isolate the peptides (and identify the cleavage sites within their percursor caseins) in the water-soluble fraction of cheese-like systems, manufactured with crude aqueous extracts of *C. cardunculus* or isolated cardosin A during the initial stages of ripening, in the presence or absence of starter culture, in an attempt to gain a deeper understanding of the unique features of said coagulant.

MATERIALS AND METHODS

Cheese Making and Sampling

Milk produced by selected ewes of the Bordaleira breed was heated to 110°C for 10 min. Milk sterility was defined as the absence of microorganisms on plate count agar incubated at 30°C for 48 h (Tavaria and Malcata, 1998). Milk aliquots (100 mL) were placed in 250-mL sterilized flasks. One half of the milk aliquots were treated with commercial starter Flora danica DRI/vac (Chr. Hansen's Lab, Denmark A/S, Copenhagen, Denmark), at the recommended level. Both types of aliquots were heated to 28°C; 0.250 mL of aqueous thistle extract (or solution of cardosin A) was added under sterile conditions and left to coagulate for 45 min. The curd was then cut, stirred, and allowed to set so as to permit draining (under sterile conditions). The whey produced was removed (again under sterile conditions) by opening the flasks every 15 min for 1.5 h. The flasks were placed in a chamber maintained at 10°C. Two cheese-like systems manufactured with each type of coagulant were randomly selected for analysis by 2, 8, and 24 h. The average of every set of replicated analytical determinations for each set of 2 cheeses was considered as a datum point.

pH Measurement

The pH was measured by directly probing the curd with a glass electrode connected to a potentiometer (MicropH 2002, Crison Instruments, Barcelona, Spain).

Microbiological Enumeration

The numbers of total viable microorganisms (on plate count agar) and lactic acid bacteria (on M17 agar) in the cheese-like systems were determined according to Sousa and Malcata (1996).

Extract Characterization

The water-soluble extract (**WSE**) and the water-insoluble extract (**WISE**) were obtained by the procedure of Kuchroo and Fox (1982); the cheese-like system was homogenized in a stomacher at 20°C for 10 min with twice its weight of water; the slurry was then held at 40°C for 1 h, centrifuged, and filtered. The filtrate (WSE) and the retentate (WISE) were finally freeze-dried. Before analysis, samples (10 mg/mL) were dissolved in 0.1% (vol/vol) trifluoroacetic acid solution, and filtered through a 0.45-μm cellulose acetate filter.

Peptide Resolution

Reverse-phase HPLC of WSE was performed in a Waters Alliance 2690 HPLC system, using Millenium software (Waters, Milford, MA) for system control and data acquisition. A Purospher STAR RP-18e (5 μm) column (Merck, Darmstadt, Germany) was used, along with a Lichrocart 250-4 guard-column (Merck). Elution was at 40°C via a mobile phase of 2 solvents: A was 0.1% (vol/vol) trifluoroacetic acid in water, and B was 0.1% (vol/vol) trifluoroacetic acid in acetonitrile:water (60:40); the flow rate was 1.0 mL/min; the solvent profile started with 100% A for 10 min, with a linear gradient from 0 to 80% B over 80 min, and a mixture of 20% A and 80% B for 10 min (González de Llano et al., 1995); detection was by spectrophotometry at 214 nm. Results were expressed as absorbance units.

Peptide Identification

Peptides isolated by RP-HPLC (as described above) were sequenced using an automated, pulsed liquid-phase protein-peptide sequencer (model 491, Applied Biosystems, Foster City, CA). Amino acids liberated were detected as their phenylthiohydantoin derivatives. The partial sequence thus obtained was checked against the (known) sequence of caseins, so as to determine the most likely cleavage site.

Statistical Analysis

Multivariate analysis of the peptide peak areas associated with WSE of the 4 different cheese-like systems was performed with SPSS software, v. 12.0 (SPSS Inc., Chicago, IL). Tukey's and post hoc tests were used as appropriate.

RESULTS AND DISCUSSION

The most representative portions of the RP-HPLC chromatograms of the WSE of the cheese-like systems are shown in Figure 1. Changes with ripening time in the relative peak area of the major water-soluble peptides are represented in Figure 2. Each peak represents at least one peptide, and the area of the peak is, in principle, proportional to the concentration of the peptide(s) at stake.

Inspection of Figure 1 indicates that there are few qualitative differences between the WSE of the 4 types of cheese-like systems studied. In all cases, it is apparent that most peptides were eluted between 55 and 80 min. During the initial stages of ripening, proteolysis is due mainly to the action of deliberately added coagulating enzymes retained in the curd or indigenous milk enzymes (mainly plasmin); caseins are hydrolyzed to large- to medium-sized peptides, which may be further degraded to smaller peptides. This explains why the first portion of the chromatograms is essentially void, as that portion is normally accounted for by small (hence hydrophilic) peptides (Kaiser et al., 1992; Belitz and Kaiser, 1993).

The peptide profiles of the WSE of all cheese-like systems were essentially identical. The peptides were sequenced from their N-termini in order to identify them; these sequences are depicted in Table 1. In all cases, ovine κ-casein was cleaved by the coagulant enzyme(s) at Phe105-Met106 (peptide peak denoted as 3 in Figure 1). It was reported elsewhere (Sousa and Malcata, 1998b; Silva, 1999) that, in solution, κ-casein is hydrolyzed by enzymatic extracts from flowers of *C. cardunculus*, as well as by cardosin A isolated at the same peptide bond; in bovine κ-casein, Phe105-Met106 also is the most susceptible peptide bond. It is well established that such a peptide bond is the primary responsible for coagulation of the casein micelles during cheese making (Fox, 1999). Sousa and Malcata (1998a) have further demonstrated that such a peptide bond in κ-casein is the most labile to cleavage, not only in raw, but also in pasteurized ovine milk cheeses made with crude aqueous extracts of *C. cardunculus* (with or without added starters).

In addition to the aforementioned peak 3, when a crude aqueous extract of *C. cardunculus* is used as the coagulant, the primary peptide peaks apparent by as soon as within 2 h of ripening are those denoted as 1, 2, 12, 13, 15, 16, and 17. When no starter culture was used, peptide 14 became surprisingly also visible by that time. The peptide peaks that account for the highest peak areas were those denoted as 3 [κ-(f106-*)], 13 [β-(f191-*)] and 16 [β-(f1-*)]; even though these areas were lower when no starter culture was employed (Figures 2A and B), differences were not significant ($P > 0.05$) between the various kinds of cheese-like systems (Table 2). After 24 h of maturation, peptide peaks denoted as 1, 2, 3, 7, 12, 13, 14, 15, 16, and 17 were present, irrespective of the presence of starter culture; peptide peaks 3, 13, 16, and 17 [β-(f128-*)] were those showing the highest peak areas, but significant differences between the various kinds of cheese-like systems were found only in the case of peptide 17 [β-(f128-*)] (Table 2).

Some of the products of proteolysis of β-casein, detected by 2 h in WSE of cheese-like systems manufactured with extracts of *C. cardunculus*, were also detected by the same time of hydrolysis when β-casein was tested in solution (Sousa and Malcata, 1998b), namely β-(f191-*) and β-(f128-*).

The peptide profiles produced via the action of cardosin A (with or without added starter culture) were similar, except in that peptide peak denoted as 18 was only present by 8 h of ripening in cheeses with starter addition (Figures 1C and D). In both cases, the primary peptides denoted as 1, 2, 3, 4, 5, 6, 7, 12, 13, 14, 15, 16, and 17 appeared as early as by 2 h of ripening. The peptides denoted as 3 [κ-(f106-*)], 13 [β-(f191-*)], and 16 [β-(f1-*)] entertained the highest peak areas; however, the presence or absence of starter culture had no significant effect on their areas. Besides peptides denoted as 3, 13, and 16, peptides 17 [β-(f128-*)] and 18 [β-(f128-*)] exhibited the highest peak areas by 24 h of ripening (Figures 2C and D). By this time, however, significant differences were found between cheese-like systems manufactured in the presence or in the absence of starter culture (Table 2), except for peptide 13.

When a crude aqueous extract of *C. cardunculus* is used as coagulant together with a starter culture, four additional peptide peaks are visible: 8 [β-(f128-*)], 9 [β-(f191-*)], 10 [β-(f191-*)], and 11 [β-(f192-*)]. These peaks are not present in any of the other three situations.

Most products of proteolysis of isolated β-casein in solution, or of sodium-caseinate, by aqueous extracts of *C. cardunculus* (or cardosin A isolated therefrom) were detected in the WSE of sterilized ovine cheese-like systems manufactured with or without starter, using again aqueous extracts of *C. cardunculus* (Sousa and Malcata, 1998b; Silva, 1999); this indicates that extensive degradation of such a casein actually occurs in those cheeses.

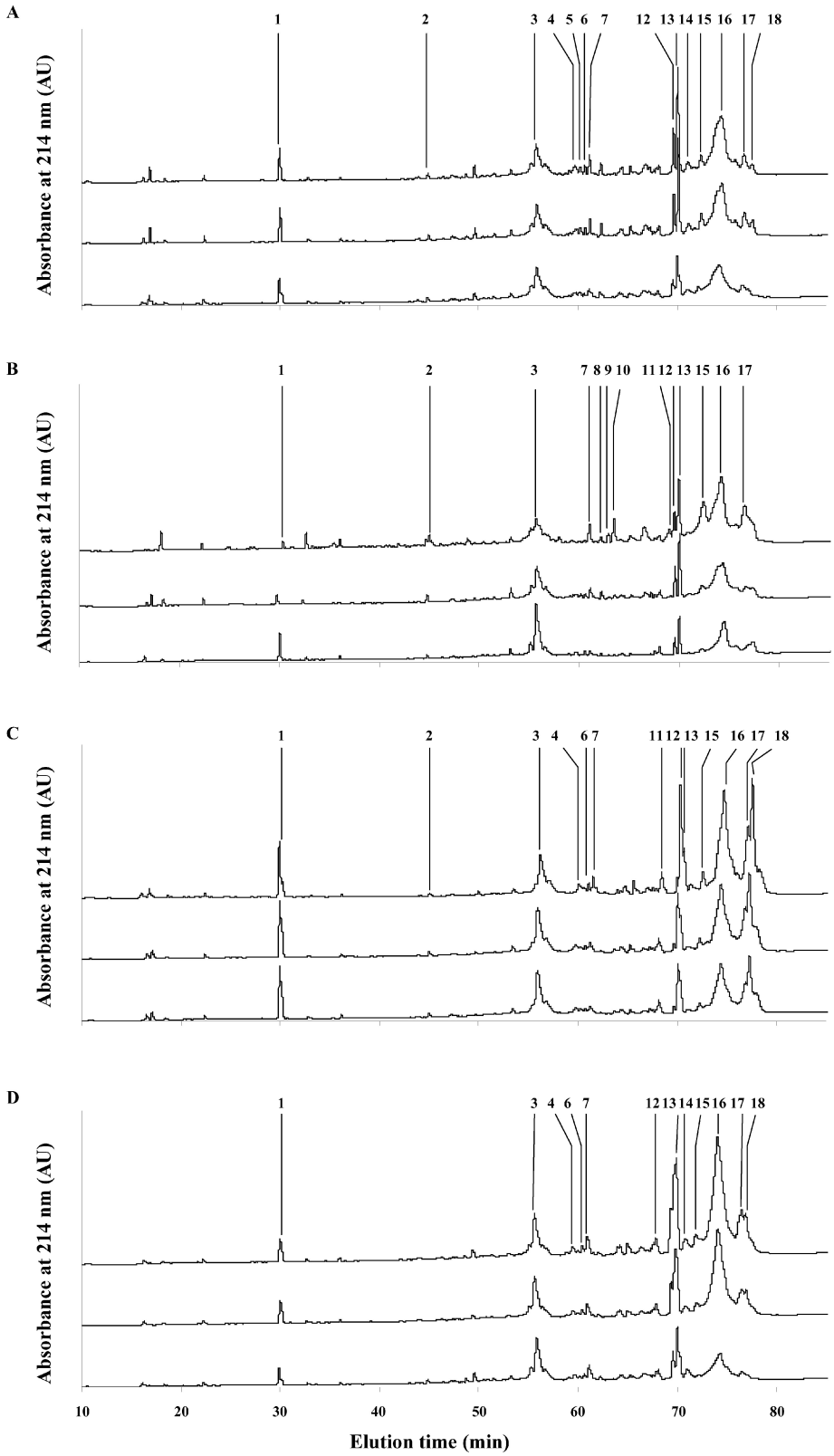

Figure 1. Reverse-phase HPLC qualitative profiles (peak locations) of the water-soluble extract of ovine cheese-like systems produced with crude aqueous extracts of *Cynara cardunculus* (A and B) and cardosin A (C and D), with (B and D) and without (A and C) added starters, at three different ripening times.

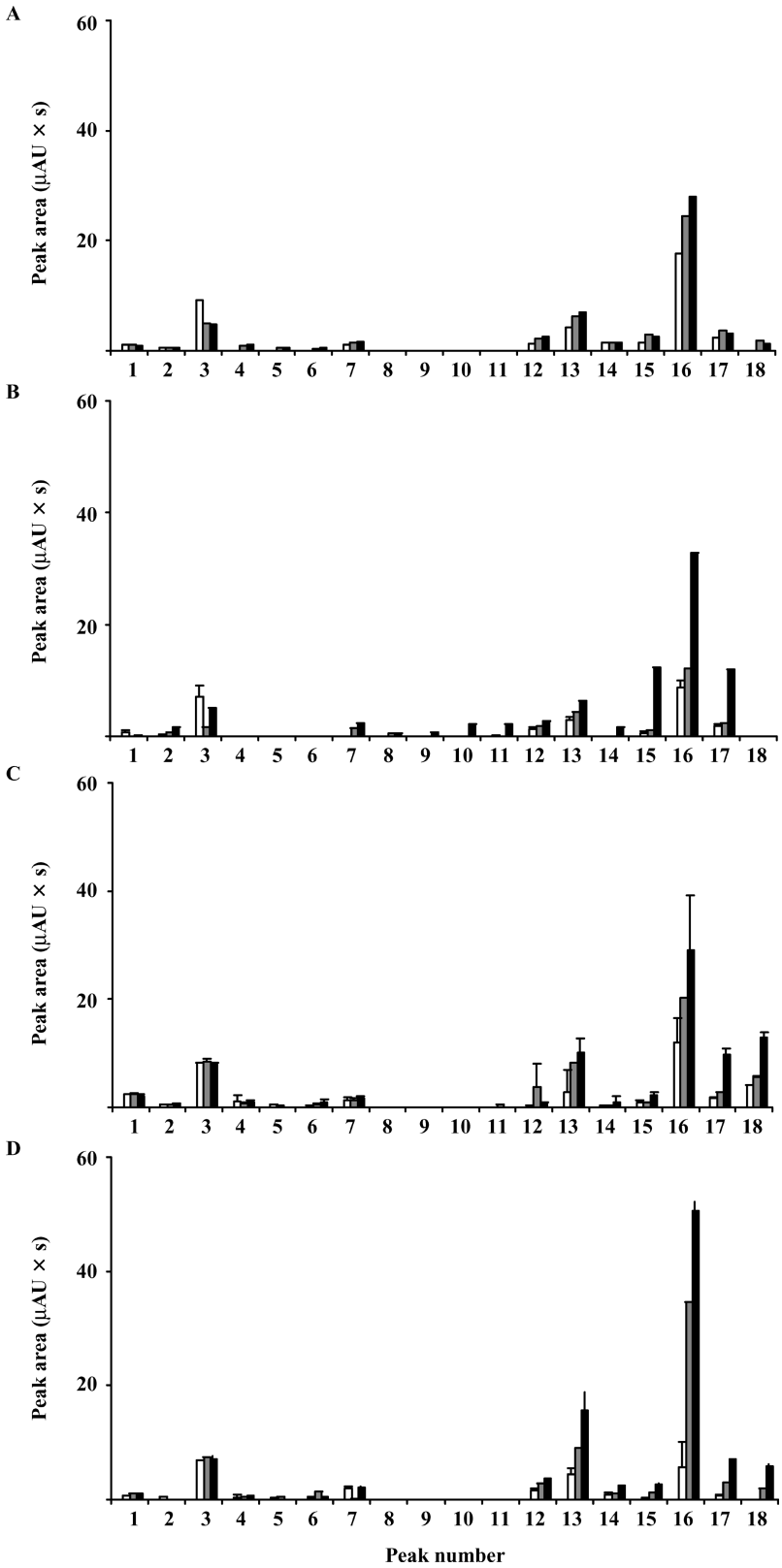

Figure 2. Reverse-phase HPLC quantitative profiles (peak areas with SD) of the water-soluble extract of ovine cheese-like systems produced with crude aqueous extracts of *Cynara cardunculus* (A and B) and cardosin A (C and D), with (B and D) and without (A and C) added starters, at 3 ripening times (white box, 2 h; gray box, 8 h; black box, 24 h).

Table 1. Identification of the major peptides present in the water-soluble fraction of ovine cheese-like systems ripened for 24 h.

HPLC peak no.[1]	N-terminal sequence[2]	Cleavage site
1	ND	—
2	H_2N-Ser-Gln-Pro-Lys-Val-Leu-?	Leu165-Ser166 (β-CN)
3	H_2N-Met-Ala-Ile-Pro-Pro-Lys-?	Phe105-Met106 (κ-CN)
4	ND	—
5	ND	—
6	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)
7	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)
8	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)
9	H_2N-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-?	Leu190-Tyr191 (β-CN)
10	H_2N-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-?	Leu190-Tyr191 (β-CN)
11	H_2N-Gln-Glu-Pro-Val-Leu-Gly-Pro-?	Tyr191-Gln192 (β-CN)
12	H_2N-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-?	Leu190-Tyr191 (β-CN)
13	H_2N-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-?	Leu190-Tyr191 (β-CN)
14	H_2N-Trp-Met-His-Gln-Pro-Pro-?	Ser142-Trp143 (β-CN)
15	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)
16	H_2N-Arg-Glu-Gln-Glu-Glu-Leu-Asn-Val-?	—
17	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)
18	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)

[1]According to the numbering followed in Figure 1.

[2]ND = Determination not successful.

Experimental hydrolysis of β-casein in solution by enzymes present in crude aqueous extracts of *C. cardunculus* indicated that the most susceptible peptide bonds are Leu127-Thr128 and Leu190-Tyr191 (Sousa and Malcata, 1998b). Besides these, another 2 cleavage sites were found to exist in cheese-like systems, viz. Ser142-Trp143 and Leu165-Ser166. Moreover, when a starter culture was added, the peptide bond Tyr191-Gln192 was also cleaved (Table 1).

The specificity of cardosin A in cheese-like systems (made with or without starter) was similar to that observed in solution, insofar as the same peptide bonds in the C-terminal part of β-casein are cleaved (Silva, 1999). However, additional cleavage sites were observed, namely the peptide bond Ser142-Trp143 (Table 1).

In the case of α_{s1}-casein, none of its highly susceptible peptide bonds when in solution was cleaved, by cardosin A or B in the crude aqueous extracts of *C. cardunculus* (or by purified cardosin A), in cheese-like systems during the initial 24 h of ripening. Recall that ovine α_{s1}-casein in solution or in sodium caseinate is hy-

Table 2. Average value of peak areas, in absorbance units × elution time (μAU × s), of the major peaks from the water-soluble extract of ovine cheese-like systems.

Ripening time (h)	Cheese[1]	Peak Area						
		Peak 3	Peak 12	Peak 13	Peak 15	Peak 16	Peak 17	Peak 18
2	A	9,127,483[a]	9,127,483[a]	4,318,291[a]	1,397,124[a]	17,715,425[a]	2,435,212[a]	0[a]
	B	7,092,341[a]	7,092,341[a,c]	2,866,206[a]	555,126[a,b]	8,635,490[a]	1,877,520[a]	0[a]
	C	8,209,041[a]	8,209,041[a]	2,893,874[a]	952,643[a,c]	12,074,410[a]	1,610,077[a,b]	2,021,479[b]
	D	6,875,584[a]	6,875,584[b,c]	4,459,888[a]	134,143[b,c]	5,703,686[a]	765,392[b]	0[a]
8	A	5,044,034[a]	2,294,366[a]	6,217,821[a]	2,881,447[a]	24,560,027[a]	3,709,148[a]	1,852,620[a]
	B	1,657,683[b]	1,856,584[a]	4,285,053[a]	1,147,830[b]	12,133,853[b]	2,319,226[b]	0[b]
	C	8,390,034[c]	3,728,128[a]	4,125,397[a]	949,952[c]	20,170,985[c]	2,861,584[c]	5,549,991[c]
	D	7,418,800[c]	2,755,896[a]	8,972,711[a]	1,231,587[d]	34,568,195[d]	2,954,546[d]	1,894,389[a]
24	A	4,853,610[a]	2,622,633[a]	6,978,346[a]	2,530,234[a]	27,937,737[a]	3,176,243[a]	1,206,396[a]
	B	5,013,444[a]	2,675,679[a]	6,407,786[a]	12,356,823[b]	32,769,704[a,b]	11,936,098[b]	0[b]
	C	8,179,780[b]	863,415[b]	10,175,431[b]	2,279,579[a]	29,039,688[a]	9,800,479[b]	13,006,585[c]
	D	7,017,301[c]	3,726,998[c]	15,598,252[b]	2,646,328[a]	50,532,724[b,c]	7,125,094[c]	5,864,947[d]

[a,b,c,d]Means followed by the same superscript are not statistically different from each other ($P < 0.05$).

[1]A = Ovine cheese-like systems, produced with crude aqueous extracts of *Cynara cardunculus* without added starter; B = ovine cheese-like systems, produced with crude aqueous extracts of *C. cardunculus* with added starter; C = ovine cheese-like systems, produced with cardosin A without added starter; D = ovine cheese-like systems, produced with cardosin A with added starter.

drolyzed at peptide bonds Trp164-Tyr165 and Phe153-Tyr154, respectively, by cardosin A (Silva, 1999); however, its major cleavage site in ovine caseinate by aqueous extract of *C. cardunculus* is Phe23-Val24 (Sousa and Malcata, 1998b). The cheese environment possesses a restricted water content (measured by water activity, a_w below unity) that influences the availability of water molecules to contribute to the conformation of enzyme and/or of substrate; this might help explain the differences between enzyme activity in solution and in cheese-like systems. Hence, a specific rearrangement of the α_{s1}-casein molecule induced by a typical cheese environment may have caused the loss of lability of said peptide bonds. Moreover, the milk used in this study was sterilized ovine milk; it is known that thermal treatment of cheese milk does not directly affect the coagulating enzymes, but rather the accessibility of their substrates due to covalent attachment of whey proteins (particularly β-lactoglobulin) to the surface of the casein micelles via thiol-disulfide interchange with κ-casein (Benfeldt and Sørensen, 2001).

Statistical analysis of the peptide areas of major peaks, namely peptides denoted as 3, 12 [β-(f191-*)], 13, 15 [β-(f128-*)], 16, 17, and 18, permitted knowledge of the influence of the cheese-like system on each individual peptide throughout ripening (Table 2). After 24 h, significant differences were found for peptide 3 between cheeses manufactured with cardosin A, irrespective of starter addition. This is understandable since cardosin A is the main enzyme responsible for the specific cleavage of the Phe105-Met106 peptide bond, whereas cardosin B is much less specific in its proteolytic action.

Concerning peptide 12 [β-(f191-*)], significant differences were found only between the cheese-like systems manufactured with cardosin A after 2 h of maturation, whereas no significant differences were found between cheese-like systems manufactured with extracts of *C. cardunculus* by 24 h.

For peptide 13, no significant differences between the various kinds of cheese-like systems were found ($P > 0.05$) by 2 h of ripening; however, significant differences were found between cheese-like systems manufactured with cardosin A with added starter vs. no starter added ($P < 0.05$), which suggests that cardosin A is likely responsible for the appearance of this peptide.

With respect to peptide 15, the extracts of *C. cardunculus* coupled with starter contributed significantly ($P < 0.05$) to the peptide's formation by 24 h of maturation, whereas a significant effect was found for peptide 16 in cheese-like systems manufactured with extracts of *C. cardunculus* (or with cardosin A).

Significant differences were found between cheese-like systems, except for cheeses manufactured with extracts of *C. cardunculus* (or with cardosin A) with starter and cardosin A without starter, regarding peptide 17 by 24 h of ripening.

In the case of peptide 18, significant differences were found between all kinds of cheese-like systems.

The pathways of degradation of α_{s1}- and β-caseins by enzymes from *C. cardunculus*, as derived from solution experiments, do not necessarily mimic those in cheese; hence, straightforward extrapolation of conclusions obtained regarding caseins in solution to cheese systems is risky (Exterkate et al., 1997). Since assessment of performance of an entity in cheese ripening is difficult and time-consuming, the use of model systems appears promising, provided that actual conditions prevailing in cheese can be sufficiently approximated.

CONCLUSIONS

During the 24 h of ripening considered, no significant differences were found between the peptide profiles of cheese-like systems made with crude aqueous extracts of *C. cardunculus* or with cardosin A isolated therefrom. Even though the peak areas were higher in the former case, cardosin A seems to be the main actor in primary proteolysis (with starter cultures playing a negligible role during this period). The major cleavage sites of the whole set of caseins are Phe105-Met106 in κ-casein, and Leu127-Thr128, Ser142-Trp143, Leu165-Ser166, and Leu190-Tyr191 in β-casein. The starter apparently does not contribute to initial proteolysis to a significant extent.

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