Influence of the Coagulant Level on Early Proteolysis in Ovine Cheese-like Systems Made with Sterilized Milk and *Cynara cardunculus*

S.V. Silva and F.X. Malcata

ABSTRACT: The effect of coagulant level on the quality and quantity of protein breakdown during the first 24 h of ripening of cheese-like systems, manufactured with sterilized ovine milk using crude aqueous extracts of *Cynara cardunculus* as coagulant, was experimentally assessed. Urea-polyacrylamide gel electrophoresis was performed on both water-soluble and water-insoluble cheese extracts to monitor the casein degradation pattern; the ripening extension index and the ripening depth index were thus calculated. Peptides from the water-soluble fraction were isolated by reverse-phase, high-performance liquid chromatography and partially sequenced by Edman degradation. Higher residual coagulant levels in curdled milk led to earlier breakdown of caseins, as expected. The primary cleavage sites were Phe105-Met106 in κ-casein, Phe23-Val24 in α_{s1}-casein, and Leu127-Thr128, Ser142-Trp143, Leu165-Ser166, and Leu190-Tyr191 in β-casein.

Keywords: plant rennet, ripening, dairy food, HPLC, electrophoresis

Introduction

Heat treatments applied to cheesemaking milk pursue one main goal: to ensure the safety of the feedstock via elimination of the adventitious pathogenic microflora and via reduction of the risk of manufacturing accidents through destruction of undesirable microflora (Hermier and Cerf 2000). Pasteurization is a relatively mild heat treatment, usually performed at 63 °C to 65 °C for 30 to 32 min or alternatively at 72 °C to 75 °C for 15 to 30 s—which is used to extend the shelf-life of milk for several days; it inactivates a major fraction of pathogenic bacteria and destroys more than 99% of non-spore-forming microorganisms in raw milk. When bacterial spores may be present, heat treatments using temperatures above 100 °C, for example, conventional sterilization (110 °C for 10 to 20 min) or ultra-high temperature (UHT) treatment (135 °C to 150 °C for 2 to 5 s) are required. Heating milk before cheese manufacture, for a certain time and to a temperature above that prevailing during regular pasteurization, is also sometimes used by cheesemakers to increase cheese yield (Calvo and others 1992). However, the interactions of whey proteins with casein micelles brought about by heating milk interfere with the rennet coagulation process, hence leading to longer coagulation times and weaker curd structures (Dalgleish 1987; Banks 1990; Singh and Waungana 2001). It is known that the resulting cheese tends to develop atypical flavor and textural characteristics during ripening afterward, which in turn depend mainly on the quantitative extent and the qualitative profile of proteolysis. The coagulant retained in the curd, and to a lesser extent the indigenous milk enzymes, are responsible for the initial steps of casein breakdown (usually termed *primary proteolysis* as a whole).

Bitter peptides, derived from the hydrophobic fragments of caseins, are often responsible for off-tastes in the final cheese; however, such defects cannot be detected unless their concentration exceeds a certain threshold (Lemieux and Simard 1991). Rennet itself has the ability to catalyze release of bitter peptides; hence, if it is retained in the curd to high levels, it may eventually cause substantial bitterness (Stadhouders and Hup 1975; Visser 1977a). Therefore, the amount of coagulant used to manufacture cheese is of nuclear importance to the organoleptic characteristics of the final product.

The first 24 h after coagulation are the most important for the biochemistry of ripening, namely for peptide formation (Picon and others 1995), especially if model cheese systems, characterized by high specific surface areas and high concentrations of rennet, are considered. During this period, the temperature of the cheese declines from the typical relatively high coagulating to the typical relatively low ripening temperature; the optimum temperatures for proteolytic activity brought about by coagulating enzymes are known to lie in between. Casein aggregation and whey expression continue taking place throughout the early stages of ripening; casein is compacted within the curd, water is lost, and fat globules are entrapped and compressed, all of which are determinant of the final cheese structure and composition (Green and Grandison 1999).

In the Iberian Peninsula, traditional cheeses manufactured from raw ewe's and/or goat's milk and highly appreciated for their unique organoleptic features, are manufactured on the farm level using aqueous extracts of the wild thistle (*Cynara cardunculus*) as coagulant. After collection from the mature plants, and before use, the flowers are dried on the shade in the open air, stored in a dry place, and sold as such at local markets. Two aspartic proteinases, tentatively called cardosins A and B—which resemble chymosin and pepsin, respectively, in their catalytic performance (Veríssimo

and others 1995), are responsible for the clotting activity of that plant.

Proteolysis of ovine and caprine cheeses manufactured with raw milk, or milk pasteurized at 72 °C for 15 s, has been studied to some degree (Sousa and Malcata 1996, 1997a, 1997b, 1998a); however, few data are available concerning sterilized milk, and scarcer information encompassing the initial 24 h of ripening has been reported in the literature, especially in the presence of a coagulant from plant origin. Moreover, the existing information pertaining to the biochemistry of cheese ripening manufactured from highly heated bovine milk is rather limited and sometimes contradictory (Lau and others 1991; Calvo and others 1992; Benfeldt and others 1997; Beuvier and others 1997; Benfeldt and Sørensen 2001).

The goal of this work was thus to study the influence of a plant coagulant level on protein breakdown, during the initial stages of ripening of cheese-like systems manufactured from ovine sterilized milk, to better understand the magnitude of the influence of coagulating enzymes upon proteolysis in cheese.

Materials and Methods

Cheesemaking and sampling

Milk produced by animals of the *Bordaleira* ovine breed was heated at 110 °C in an autoclave for 10 min and then cooled down to room temperature within 15 min. Portions of 100 mL of milk were placed in 250-mL sterilized flasks, in a total of 22 flasks. Milk sterility was checked as absence of microorganisms of every of those flasks after cooling down to room temperature, on plate count agar, incubated at 30 °C for 48 h (Tavaria and Malcata 1998). Under sterile conditions, 0.250 mL (that is, 1.74×10^{-6} $kg_{protein}/L$) of aqueous thistle extract (obtained from 0.6 g of dry flowers macerated in 10 mL of water; enzymes were extracted with water from *Cynara* flowers only once and kept refrigerated between experiments; no filtration was needed, as the solution was limpid) was then added to each of the 8 flasks containing milk; 0.500 mL (that is, 3.48×10^{-6} $kg_{protein}/L$) of the same coagulant solution was then added to another 8 flasks also containing milk. The remaining 4 flasks were used as control, so no coagulant solution was added to the milk therein. The resulting mixtures were incubated at 28 °C until coagulation occurred (approximately 45 min) because previous experience indicated this is the temperature usually prevailing in traditional cheesemaking using ewe's milk and this plant coagulant. At this time, the curd was carefully cut with a sterile spatula, stirred, and allowed to set to permit syneresis; draining was achieved, again under sterile conditions, and whey was removed by opening the flasks every 15 min for 1.5 h (the yield was approximately 40% on weight basis); the flasks were placed in a chamber maintained at 10 °C. Two flasks (or 2 control samples, when appropriate) were taken randomly at 2, 4, 8, and 24 h for analysis, and the average of every set of replicated analytical determinations for each set of 2 cheeses was considered as a datum point.

Microbiological analysis

Total viable counts of the cheese-like systems were determined according to the procedure described in detail by Tavaria and Malcata (1998).

pH measurement

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

Table 1—pH (average of 2 cheeses ± standard deviation) in cheese-like systems produced with 2 different levels of coagulant

Coagulant level	Ripening time (h)			
($kg_{protein}/L$)	2	4	8	24
1.74×10^{-6}	6.41 ± 0.03	6.48 ± 0.14	6.37 ± 0.10	6.37 ± 0.07
3.48×10^{-6}	6.39 ± 0.01	6.45 ± 0.16	6.36 ± 0.01	6.36 ± 0.13

Proteolysis monitoring

Proteolysis indices. The water-soluble extract (WSE) and the water-insoluble extract (WISE) were obtained by the procedure of Kuchroo and Fox (1982); cheese was homogenized in a stomacher at 20 °C for 10 min, with twice its weight of water. The slurry was held at 40 °C for 1 h, centrifuged, and duly filtered. The filtrate (WSE) and the retentate (WISE) were freeze-dried before analysis was in order. The 12% trichloroacetic acid–soluble nitrogen (TCASN) was prepared by adding x mL of 48% (w/v) TCA to 4x mL of WSN; the mixture was allowed to stand for 30 min at room temperature and then filtered through nr 542 filter paper Whatman (Maidstone, U.K.); the micro-kjeldahl procedure was used to determine the total nitrogen (TN), water-soluble nitrogen (WSN), and TCASN. The ripening extension index (WSN/TN) and the ripening depth index (TCASN/TN), pertaining to the experimental cheese-like systems, were then calculated.

Urea-PAGE. Urea-polyacrylamide gel electrophoresis (Urea-PAGE) (12.5% T, total monomer [acrylamide plus bisacrylamide] concentration; 4% C, weight percentage of crosslinker [bisacrylamide]; and pH 8.9) was performed on samples of freeze-dried WSE (0.025 $kg/L_{sample\ buffer}$) and WISE (0.007 $kg/L_{sample\ buffer}$) (Shalabi and Fox 1987), using a Protean II XI vertical slab-gel unit (Bio-Rad Laboratoires, Watford, U.K.) and the stacking gel system of Andrews (1983) with modifications (Blakesley and Boezi 1977). The gels were stained with Coomassie Blue G250 (Bio-Rad), following the method of Blakesley and Boezi (1977).

RP-HPLC. Reverse-phase high-performance liquid chromatography (RP-HPLC) of WSE was performed in an Alliance 2690 HPLC-system using a 996 Photodiode Array Detector (Waters, Milford, Mass., U.S.A.) with the Millenium software. A Purospher® STAR RP-18e (5 μm) column (Merck, Darmstadt, Germany) was used with a Lichrocart® 250-4 guard-column (Merck). Elution was at 40 °C via a mobile phase of 2 solvents: A, 0.1% (v/v) trifluoroacetic acid in water; and B, 0.1% (v/v) trifluoroacetic acid in acetonitrile:water (60:40) at a flow rate of 1.0 mL/min, starting with 100% A for 10 min, 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 and others 1995). Detection was by spectrophotometry at 214 nm.

Samples (10 mg/mL) of freeze-dried WSE were dissolved in water and filtered through a 0.22-μm cellulose acetate filter, and an aliquot (100 μL) of the filtrate was injected. Peptides were manually collected at the outlet of the RP-HPLC.

Sequencing. Isolated peptides were sequenced using a model 491 automated, pulsed liquid-phase protein-peptide sequencer (Applied Biosystems, Foster City, Calif., U.S.A.). 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 identify the peptide bonds cleaved by the enzymes.

Statistical analysis

Statistical analyses were performed based on ANOVA, with $P < 0.05$ as level of significance, using Microsoft Excel 2000 (C.P.C., Porto, Portugal).

Results and Discussion

The curd pH was not influenced by proteolysis, since no significant differences ($P > 0.05$) between pH values in cheese-like systems were observed when using 2 different levels of coagulant (Table 1). No microbial counts (<10 colony-forming units [CFU]/mL) were recorded in either cheese-like system or in the control for 24 h. Therefore, the proteolysis observed was solely because of the action of coagulating and/or indigenous milk enzymes.

Urea-PAGE electrophoretograms of cheese-like systems, obtained from ovine milk using 2 different levels of coagulant, throughout the first 24 h of ripening are depicted in Figure 1. Based on earlier experience in this analytical area, and recalling Richardson and Creamer (1976), 2 major groups of electrophoretic bands were identified; a group with the higher and another group with the lower electrophoretic mobility, corresponding to α_s- and β-caseins, respectively (which are denoted in Figure 1 as an α_s-casein region and a β-casein region, as appropriate). The patterns of casein breakdown at both coagulant concentrations were, as expected, qualitatively similar. By 24 h, a noticeable decrease in the α_s-casein level was observed, and 1 set of bands characterized by higher electrophoretic mobility appeared, which may be accounted for by α_{s1}-(f24-*) (Sousa and Malcata 1998a).

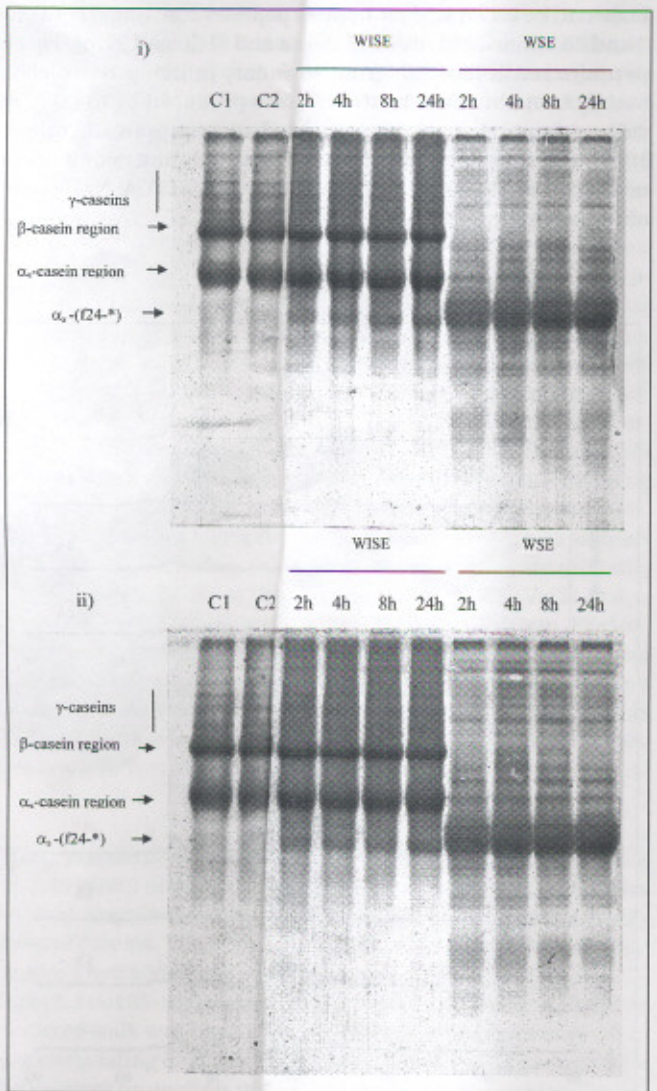

Figure 1—Urea-polyacrylamide gel electrophoresis (urea-PAGE) electrophoretograms of the water-insoluble extract (WISE) and the water-soluble extract (WSE) of ovine cheese-like systems, produced with 2 levels of coagulant: (i) 1.74×10^{-6} and (ii) 3.48×10^{-6} $kg_{protein}/L$. C1 and C2 represent ovine sodium caseinate, by 2 h and 24 h, respectively, used as controls.

It is known that residual chymosin in cheese cleaves bovine α_{s1}-casein at Phe23-Phe24 during the initial stages of ripening, hence leading to formation of a large peptide: α_{s1}-(f24-199), also termed α_{s1}-I-casein, and a small peptide: α_{s1}-(f1-23) (Richardson and Creamer 1973). β-Casein remains more stable, even though some β-casein–derived peptides (once termed γ-caseins) become already visible, as a result of cleavage of the C-terminal residues of β-casein (Irigoyen and others 2002). The coagulating enzymes are believed not to be directly affected by the heat treatment of the cheese milk. However, the accessibility of their substrates, primarily κ- and α_{s1}-caseins (Fox 1989), may be significantly affected because whey proteins (mainly β-lactoglobulin) attach covalently to the surface of casein micelles through thiol-disulphide interchange with κ-casein (Singh 1992). Recall that β- and α_{s2}-caseins are cleaved mainly by plasmin, which, together with its precursor plasminogen, is stable at high temperatures. However, plasmin activity is directly affected by the heat treatment of milk, owing to its effect on β- and α_{s2}-casein conformations (Snoeren and others 1979; Alichanidis and others 1986; Rollema and Poll 1986; Benfeldt and others 1997). Our results are similar to those reported by Calvo and others (1992) after 24 h of ripening, in studies pertaining to ripening of Cheddar-type cheese manufactured from bovine overheated milk.

The products of degradation of α_s-casein were visible earlier in cheese-like systems manufactured with the higher concentration of coagulant (Figure 1). Johnston and others (1994), who studied the influence of rennet concentration upon manufacture of Cheddar cheese, observed a significant increase in the rate of degradation of α_{s1}-casein with increasing rennet concentration, but no measurable effect upon β-casein degradation was noticed. A similar study was performed by de Jong (1977) using Meshanger cheese, who reported that, once the cheese had reached pH 5.4, the decrease in the content of intact α_{s1}-casein was proportional to the concentration of rennet retained in the cheese.

The degree of formation of soluble nitrogen compounds throughout cheese ripening is an index of the extent of proteolysis because it may serve as an indicator of casein hydrolysis brought about by rennet and milk proteases present at the beginning of ripening (Visser 1977b).

The ripening extension index (WSN/TN) has been used to follow cheese aging (Sousa and Malcata 1997a). The WSN/TN values increased throughout ripening when using either of the 2 levels of coagulant, 1.74 and 3.48×10^{-6} $kg_{protein}/L$, but those values were, as expected, higher in the latter case (Figure 2). The ripening index was significantly influenced by the ripening time ($P < 0.0001$) and the level of coagulant ($P < 0.0001$), but no significant interaction between those 2 parameters was found ($P > 0.05$). The *C. cardunculus* extract used as coagulant is mainly constituted by cardosin A and cardosin B. The first acts in a way similar to chymosin, a proteolytic enzyme specific toward the Phe105-Met106 bond of κ-casein. Cardosin B acts in a way similar to pepsin, a nonspecific and highly proteolytic enzyme. This dual composition of the rennet might explain the relatively more extensive hydrolysis of caseins than when animal rennets are used after as little as 24 h of ripening.

Table 2—Peptides accounting for reverse-phase, high-performance liquid chromatography (RP-HPLC) peaks of the water-soluble extract of ovine cheese-like systems ripened for 24 h

HPLC peak number[a]	N-terminal sequence	Cleavage site (in casein)
1	H_2N-Ser-Gln-Pro-Lys-Val-Leu-?	Leu165-Ser166 (β-CN)
2	H_2N-Met-Ala-Ile-Pro-Pro-Lys-?	Phe105-Met106 (κ-CN)
3	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)
4	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)
5	H_2N-Trp-Met-His-Gln-Pro-Pro-Gln-?	Ser142-Trp143 (β-CN)
6	H_2N-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-?	Leu190-Tyr191(β-CN)
7	H_2N-Tyr-Gln-Glu-Pro-Val-Leu-Gly-?	Leu190-Tyr191(β-CN)
8	H_2N-Trp-Met-His-Gln-Pro-Pro-GLn-?	Ser142-Trp143 (β-CN)
9	H_2N-Arg-Glu-Gln-Glu-Glu-Leu-Asn-Val-?	—
10	H_2N-Thr-Asp-Val-Glu-Lys-Leu-His-Leu-?	Leu127-Thr128 (β-CN)

[a]According to the numbering followed in Figure 4.

In what concerns TCASN/TN, ANOVA indicated that there is a significant ($P < 0.05$) interaction between ripening time and coagulation level; none of those parameters can thus be evaluated independently until 24 h have elapsed. Quantification of the peptides soluble in TCA, at levels between 2% and 12% (w/v), is one of the most expeditious methods to assess proteolysis in cheese; the higher the TCA concentration, the shorter the average size of the soluble peptides (Yvon and others 1989). The 12%-TCASN is known to be accounted for by small peptides containing between 2 and 20 amino acid residues (Sousa and Malcata 1997a). Those peptides result basically from secondary proteolysis, which is mainly brought about by the enzymes produced by the starter cultures and released thereby upon lysis (Grappin and others 1985). Once no starter cultures were added during manufacture of our cheese-like system, relatively low levels of TCASN/TN were obtained (Figure 3).

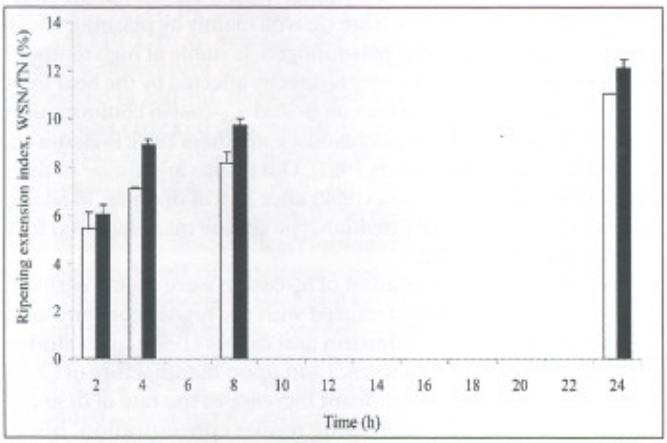

Figure 2—Comparison of the water-soluble nitrogen (WSN) fractional content of ovine cheese-like systems produced with 2 levels of coagulant: (□) 1.74 × 10^{-6} and (■) 3.48 × 10^{-6} $kg_{protein}$/L (average ± standard deviation).

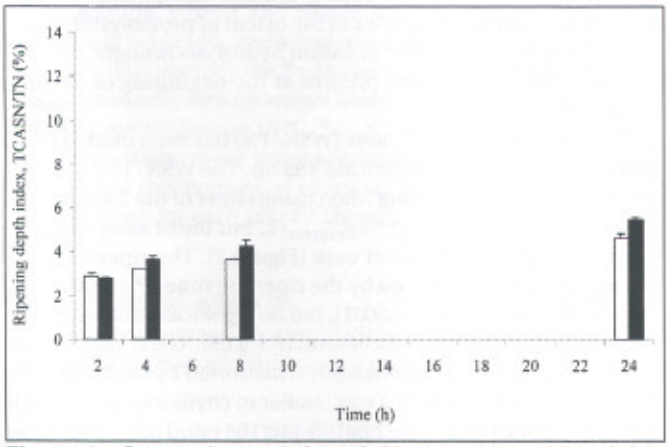

Figure 3—Comparison of the trichloroacetic acid–soluble nitrogen (TCASN) fractional content of ovine cheese-like systems produced with 2 levels of coagulant: (□) 1.74 × 10^{-6} and (■) 3.48 × 10^{-6} $kg_{protein}$/L (average ± standard deviation).

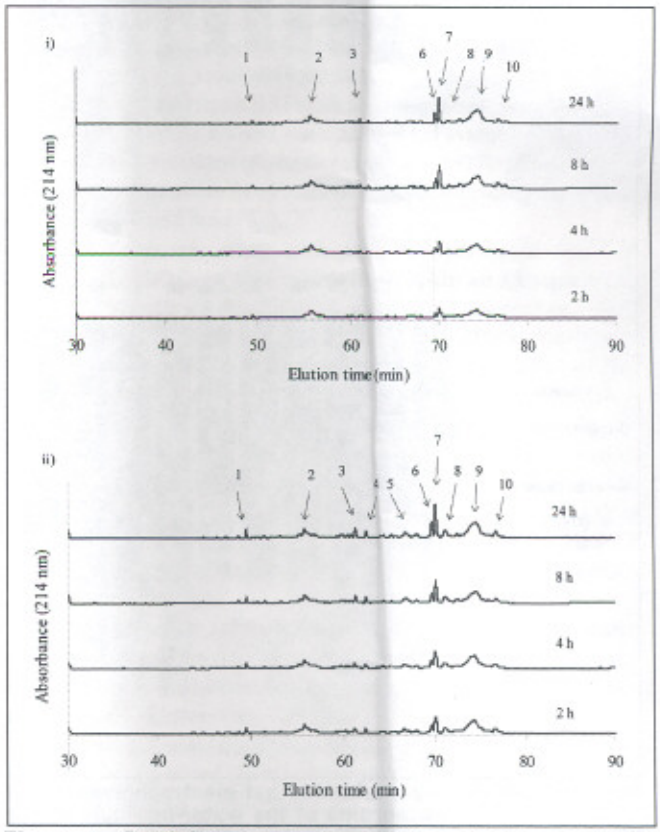

Figure 4—Partial reverse-phase high-performance liquid chromatography (RP-HPLC) profiles of the water-soluble extract (WSE) of ovine cheese-like systems produced with 2 levels of plant coagulant: (i) 1.74 × 10^{-6} and (ii) 3.48 × 10^{-6} $kg_{protein}$/L.

The peptide profiles of cheese-like systems, as obtained by RP-HPLC, are shown in Figure 4. It is apparent that these profiles are, in both situations, very similar to each other at all maturation times considered. In every case, the number and concentrations of the various peptides were rather low. Sousa and Malcata (1997b) reached a similar result at the early stages of ripening of cheeses manufactured with either plant or animal rennet. However, the peptide peak areas in the WSE, which are a measure of the selectivity of attack by the enzymes (Sousa and Malcata 1998a) are, in the case of cheese-like systems manufactured with the higher concentration of coagulant, above those corresponding to its lower counterpart; this result is logical because a higher amount of enzyme added leads, in principle, also to a higher amount of enzyme retained in the curd during syneresis.

The major primary peptides formed after as little as 2 h of maturation were those denoted as 2, 6, and 7 (Figure 4i) in the WSE of cheese-like systems made with 1.74×10^{-6} $kg_{protein}/L$ as coagulant, and as 1 to 4 and 6 to 8 (Figure 4ii) in the WSE of cheese-like systems made with 3.48×10^{-6} $kg_{protein}/L$ as coagulant. By 24 h, the peaks denoted as 1 to 10 were noticed in both cases. Those peptides, isolated via RP-HPLC, were partially sequenced from their N-terminus through Edman degradation, in attempts to identify their origin. From the data depicted in Table 2, the major peptide bonds cleaved were Phe105-Met106 in κ-casein (peak 2), and Leu127-Thr128, Ser142-Trp143, Leu165-Ser166, and Leu190-Tyr191 in β-casein (peaks 3, 4, and 10; peaks 5 and 8; peak 1; and peaks 6 and 7, respectively). Sousa and Malcata (1998a) also reported that the WSE from 68-d-old raw ovine milk cheeses produced by extracts of *C. cardunculus* was constituted by fragments of β-casein (and α_{s2}-casein), mainly β-(f128-*), β-(f166-*), and β-(f191-*); all of those are in agreement with our results, despite putative differences between the milk (sterilized/raw) for cheese production. Possible differences between the final cheeses would thus arise mainly because of the microflora that normally exists, and which is primarily responsible for secondary proteolysis. Furthermore, our results provide evidence that those peptide bonds are cleaved very early during cheese manufacture, thus releasing peptides that will serve as substrates for secondary proteolysis. Other works concerning fundamental studies can also support this statement because, in solution, ovine caseins are primarily cleaved by cardosins at the peptide bonds Phe23-Val24 in α_{s1}-casein, Leu127-Thr128, and Leu190-Tyr191 in β-casein, Phe105-Met106 in κ-casein, and Phe88-Tyr89 in α_{s2}-casein (Sousa and Malcata 1998b). Isolated cardosins A and B, when acting upon ovine Na-caseinate, were able to cleave the peptide bonds Trp164-Tyr165 in α_{s1}-casein, and Leu127-Thr128, Leu165-Ser166, and Leu190-Tyr191 in β-casein (Silva 1999).

Conclusions

The protein concentration of the coagulant influences the rate and extent of degradation of caseins (as expected), with higher residual coagulant levels in curdled milk leading to earlier breakdown of caseins. The major cleavage sites are Phe105-Met106 in κ-casein, Phe23-Val24 in α_{s1}-casein, and Leu127-Thr128, Ser142-Trp143, Leu165-Ser166, and Leu190-Tyr191 in β-casein. Because sterilized milk was used throughout, this emphasizes once more that coagulating enzymes are the main substances responsible for the primary proteolysis that occurs during cheesemaking.

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