Identification of Peptides from Ovine Milk Cheese Manufactured with Animal Rennet or Extracts of *Cynara cardunculus* as Coagulant

M. J. Sousa and F. X. Malcata*

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

Urea−PAGE of the water-insoluble extract (WISE) of ovine raw milk cheeses manufactured with proteinases of *Cynara cardunculus* or with commercial animal rennet indicated that the animal rennet acts more intensively, in quantitative terms, on ovine β-, α_{s1}-, and α_{s2}-caseins than the plant rennet. The water-soluble extract (WSE) from cheese produced by plant rennet was constituted by fragments of ovine β- and α_{s2}-caseins; peptides β-(f128−*), β-(f166−*), and β-(f191−*) were produced only by plant rennet, whereas peptides β-(f164−*) and β-(f191−*) were produced only by animal rennet. The peptide β-(f1−190) was identified as the primary product of ovine β-casein degradation in the WISE for both rennets. The complementary peptides α_{s1}-(f1−23) and α_{s1}-(f24−191) were produced by both rennets from ovine α_{s1}-casein; however, the bond Phe23−Val24 was cleaved by as early as 7 days in cheese manufactured with *C. cardunculus*, whereas 28 days had to elapse before that could be detected in the case of animal rennet. The peptide α_{s1}-(f24−165) was produced only by plant rennet, whereas the peptide α_{s1}-(f120−191) was produced only by animal rennet. The peptides produced from bovine α_{s2}-casein in cheese could not be traced as deriving from the action of proteinases from either rennet, so their existence is likely due to proteinases or peptidases released in cheese as a result of its indigenous microflora.

Keywords: *Thistle flower; cheese-making; cheese ripening; proteolysis; enzyme activity*

INTRODUCTION

Although the precise role of proteolysis in the development of cheese flavor has not yet been established beyond doubt, some patterns of proteolysis have been implicated with incidence of off-flavors in cheese. It is known that products of proteolysis per se (i.e., peptides and free amino acids) are significant in terms of cheese taste, especially in terms of "background" flavor, as well as in the occurence of bitterness, but are unlikely contributors to aroma; however, compounds arising from the catabolism of free amino acids do have a direct contribution to both cheese taste and aroma. The quantitative and qualitative composition of the amino acid pool in cheese has long been used as an index of cheese ripening (Fox et al., 1995), and at least in some instances these parameters correlate with flavor and body development; however, they provide little information about the mechanism of cheese ripening. In fact, any correct description of proteolysis requires identification of the peptide bonds cleaved, which in turn requires isolation of products of proteolysis and determination of their primary sequence.

The role of proteolytic enzymes, either individually or in combination, has been assessed using essentially three complementary approaches: (i) model cheese systems, deprived of one or more proteolytic agents, in which acidification is effected by acid-δ-lactone rather than a starter culture and in which coagulant and indigenous milk enzymes have been previously inactivated or inhibited; (ii) caseins (or casein-derived peptides) in solution, which provide a clue for the activity and specificity of the major proteinases and peptidases; and (iii) actual cheeses, from which peptides are isolated and in which case identification of the agent(s) responsible for their formation in cheese is rationally derived on the basis of known specificity of proteinases/peptidases to caseins in solution (Fox et al., 1994). Primary proteolysis of caseins (brought about by coagulant proteases and plasmin) produces a range of small- and intermediate-sized peptides, usually detected by PAGE, included in the water-soluble N (Visser, 1976, 1977a−c; Visser and de Groot- Mostert, 1977), which in turn act as substrates for proteinases and peptidases of starter and nonstarter bacteria, eventually leading to release of free amino acids.

This study was developed as a logical extension of previous work by Sousa and Malcata (1997) on a similar subject and has attempted to further the characterization of proteolysis via isolation and identification of major peptides in the water-insoluble and water-soluble extracts of ovine cheeses produced by proteinases from plant rennet (aqueous extracts of *C. cardunculus)* and animal rennet while addressing the issue of whether the results of studies on hydrolysis of caseins in solution can be extrapolated to hydrolysis in actual cheese.

MATERIALS AND METHODS

Cheese-Making and Sampling. Raw ovine milk from the Serra da Estrela region was collected from a sheep flock (selected in view of excellent health and uniform physiological

* Author to whom correspondence should be addressed (fax 351-2-590351; e-mail xmalcata@esb.ucp.pt).

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condition) on the morning of cheese-making and transported to the pilot plant. The milk was divided in two equal portions: 16 cheeses were manufactured using extracts of *C. cardunculus* as rennet at a level of 0.16 g of dry flowers/L of milk (stylets and stigmae of the dry flowers were ground for 1 min and soaked in tap water for 10 min with stirring), whereas another 16 cheeses were manufactured using animal rennet (1:10000 Stabo, Chris. Hansen's), following the slightly modified traditional technology described elsewhere by Sousa and Malcata (1997).

Two cheeses manufactured with each type of rennet were selected randomly for biochemical sampling and analysis after 0, 3, 7, 14, 28, 42, 56, and 68 days (i.e., 32 distinct cheeses were sampled in total), and the average for the two cheeses of every analytical determination was considered as a datum point.

Proteolysis Analyses. Water-soluble extract (WSE) of an aliquot of the cheese sample was prepared according to the procedure of Kuchroo and Fox (1982). Samples of water-insoluble extract (WISE) and WSE were freeze-dried before further analysis.

Urea−PAGE. Urea−polyacrylamide gel electrophoresis (urea−PAGE) (12.5% C, 4% T, pH 8.9) was performed on WISE and WSE using a Protean II XI vertical slab-gel unit (Bio-Rad Laboratories, Watford, U.K.) and the stacking gel system of Andrews (1983) with modifications (Shalabi and Fox, 1987); the gels were stained with Coomassie Blue G-250 (Bio-Rad) using the method of Blakesley and Boezi (1977). Quantitation of intact ovine β- and α_s-caseins was by densitometry using a model CD60 densitometer (Desaga, Heidelberg, Germany).

RP-HPLC. Peptide profiles of the WSE were obtained by reverse-phase high-performance liquid chromatography (RP-HPLC) using the method of Singh et al. (1995) in a Beckman system (San Ramon, CA) composed by an autosampler with temperature control for the column (autosampler 502), a solvent delivery system with two pumps (programmable solvent module 126), a programmable multiwavelength spectrophotometer (diode array detector 168), a personal computer with a software package for system control and data acquisition (Gold v6.01), a Lichrosorb 250 × 4 mm RP-C8 (5 μm) column, and a Lichrocart 4-4 guard column (Merck, Darmstadt, Germany). Elution was effected at 30 °C using a mobile phase of two solvents, A [0.1% trifluoroacetic acid (TFA) from Sigma (St. Louis, MO) in H_2O] and B [0.1% TFA in acetonitrile, from Romil (Leicester, U.K.)], starting with pure A for 5 min and continuing with a linear gradient to 50% B over 55 min, 50% B for 6 min, a linear gradient to 60% B over 4 min, and 60% B for 3 min; absorbance of the eluate was read at 214 nm. Samples (10 mg/mL) of freeze-dried WSE were dissolved in a mixture of solvents A and B (1:0.01, v/v) and filtered through a 0.22 μm cellulose acetate filter, and an aliquot (75 μL) of the filtrate was injected using an eluant flow rate of 1.0 mL/min.

Isolation of Peptides and Sequencing. Peptides were isolated by manual collection at the outlet of the RP-HPLC or by electroblotting from urea−PAGE gels using a mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA); in the latter case, transfer of peptides was at 100 V for 30 min in 10 mM 3-(cyclohexylamino)-l-propanesulfonic acid (CAPS) buffer with 10% methanol onto poly(vinylidene difluoride) membranes (PVDF) with pore size of 0.22 μm (Bio-Rad). Membranes were rinsed with water and stained in 0.2% Ponceau S (Sigma) in 1% acetic acid for 1 min, followed by destaining in water. Bands corresponding to peptides were excised from the membranes and sequenced (5−10 cycles) via Edman degradation on an automated pulsed liquid-phase protein−peptide sequencer (Applied Biosystems model 477A, Foster City, CA). Liberated amino acids were detected as their phenylthiohydantoin derivatives.

RESULTS AND DISCUSSION

Casein Degradation. The results of urea−PAGE of the WISE and WSE from ovine raw milk cheeses manufactured with proteinases of *C. cardunculus* or commercial animal rennet are shown in Figure 1. The percent degradation measured by densitometry (a measure of the overall activity of attack by the enzyme) of the combined ovine β_1- and β_2-caseins was 33% (or 50%), whereas that of the ovine α_s-casein region was 47% (or 88%), in cheese manufactured with extracts of *C. cardunculus* (or animal rennet), respectively (Sousa and Malcata, 1997). Only the WSE of cheese manufactured with plant rennet exhibited bands that could be clearly resolved by urea−PAGE; this observation is an indication of the relative rates of enzyme attack and is consistent with the fact that proteolysis by *C. cardunculus* proceeds to a high extent but a low degree (i.e., mainly down to caseins and polypeptides), whereas proteolysis by animal rennet proceeds to a high degree (i.e., down to compounds detected by TCASN/TN and PTASN/TN) (Sousa and Malcata, 1997).

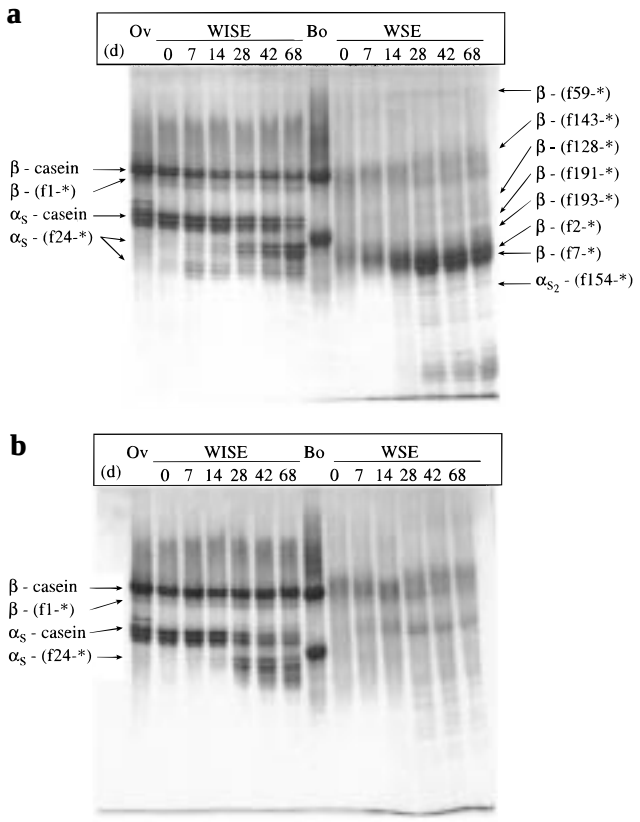

Figure 1. Urea−PAGE electrophoregrams (12.5% T, 4% C; pH 8.9) of WISE and WSE by 0, 7, 14, 28, 42, and 68 days of ripening, for ovine milk cheeses manufactured with extracts of *C. cardunculus* (a) and animal rennet (b). Bovine sodium caseinate (Bo) and ovine sodium caseinate (Ov) are included as references.

The electrophoretic bands corresponding to degradation products in WISE and WSE of cheeses were isolated and sequenced, and the sequence of the first 5−10 residues from the N terminus was compared with that of the known sequences of the caseins in question. The band β-(f1−*) in the WISE (see Figure 1) and the bands in the WSE (Figure 1a) were, with exception of fragment α_{s2}-(f154−*), produced from β-casein. The fragments of ovine β-(f191−*) and β-(f128−*) were possibly generated via action of proteinases of *C. cardunculus* because the corresponding cleavage sites Leu190−Tyr191 and Leu127−Thr128 were previously identified in solutions of ovine β-casein hydrolyzed by purified proteinases of *C. cardunculus* (Sousa and Malcata, 1998). Peptide

β-(f1−*) in the WISE (Figure 1a) of ovine cheese manufactured with proteinases from *C. cardunculus* is probably the complementary fragment of β-(f128−*) or β-(f191−*), considering that fragments β-(f2−*), β-(f7−*), β-(f59−*), β-(f143−*), and β-(f193−*) were not identified as released by *C. cardunculus* (Sousa and Malcata, 1998); they probably result from the catalytic action of indigenous proteinases from the milk and enzymes from the microorganisms in cheese. It should be noted here that, although the indigenous microflora of the milk may play a role in enzyme-mediated release of small, water-soluble peptides, their importance might be rather limited in view of the slow acidification that takes place during cheese manufacture. Peptides β-(f7−*) and β-(f143−*) were reported as products released by cell envelope proteinases of *Lactococcus* spp. acting on bovine β-casein (Reid et al., 1991). Peptide β-(f1−*) (see Figure 1b) in WISE is probably the complementary fragment of β-(f191−*); Whyte (1995) reported that chymosin-susceptible bonds in ovine β-casein were Leu190−Tyr191 and Ala187−Phe188, and the first peptide liberated was β-(f191−207), which is complementary to ovine β-I-casein. Chymosin plays a limited role on bovine β-casein in cheese; the most susceptible bonds in soluble bovine β-casein are Leu192−Tyr193 and Ala189−Phe190, although some activity is indicated by the presence of bovine β-(f1−192) (or β-I-casein) in the WISE of Cheddar cheese (McSweeney et al., 1994b) and a small C-terminal fragment, bovine β-(f193−209), which is claimed to be extremely bitter (Visser et al., 1983). However, when the salt content was increased, this peptide was not produced at all (Kelly, 1993).

Two bands with higher mobility than α_s-casein, that is, ovine α_s-(f24−*) (see Figure 1), were apparent by 28 days of ripening and increased in intensity with ripening time for cheese manufactured with *C. cardunculus* but remained virtually unchanged in the animal rennet counterpart. Ovine α_s-casein in solution hydrolyzed by proteinases from *C. cardunculus* leads to a set of bands (α_s-f24−*) of higher electrophoretic mobility than α_s-casein and comparable in electrophoretic mobility to bovine α_{s1}-I-casein (Sousa and Malcata, 1998). Another two bands with highest mobility in WISE, that is, α_s-(f24−*) (Figure 1a), had the same N terminus as their aforementioned counterparts but were produced from the very beginning of ripening in cheeses manufactured with *C. cardunculus* but not with animal rennet (see Figure 1b); this observation probably indicates that ovine α_s-casein is cleaved earlier and in a more unselective fashion by proteinases of *C. cardunculus* than by those of animal rennet. Two sets of bands with similar electrophoretic mobilities were found by Fernandez del Pozo et al. (1988) in cheese manufactured, and by Sousa (1993) in solutions of bovine α_{s1}-casein incubated, with extracts from flowers of *Cynara* spp. The fragment identified after 28 days of ripening in the WISE of ovine cheese manufactured with proteinases of *C. cardunculus* and animal rennet as ovine α_s-(f24−*) (see Figure 1) derives from hydrolysis of the most susceptible bond in ovine α_s-casein to proteinases of *C. cardunculus,* as proven in solution (Sousa and Malcata, 1998), and to chymosin (Whyte, 1995), that is, ovine Phe23−Val24, hydrolysis of which yields peptides f1−23 and f24−191. Richardson and Creamer (1973) reported that residual chymosin in cheese hydrolyzes rapidly bovine α_{s1}-casein at Phe23−Phe24 during the initial stages of ripening, thus resulting in formation of a large peptide, that is, α_{s1}-(f24−199), also termed α_{s1}-I-casein, and a small peptide, that is, α_{s1}-(f1−23). Hydrolysis of this single bond of α_{s1}-casein (coupled with potential changes in water binding) causes a rapid change in the rubbery texture of young curd into a smoother, more homogeneous product (Lawrence et al., 1987), and it may account for the fact that ovine cheeses manufactured with *C. cardunculus* possess a smoother texture than when animal rennet is employed; note that Phe23−Val24 is already hydrolyzed by 7 days of ripening in the former (see Figure 1a), but only after 28 days of ripening in the latter, and that the type of rennet has no significant effect on cheese composition (Sousa and Malcata, 1997). The peptide α_{s1}-(f24−199) is present, at least in the early stages of ripening, in all types of cheese, and increasing salt-in-moisture concentration in cheese does not influence the rate of initial hydrolysis of α_{s1}-casein but inhibits subsequent hydrolysis of α_{s1}-(f24−199) (Exterkate and Alting, 1995). McSweeney et al. (1994b) reported that concentration of bovine α_{s1}-(f24−199) increased initially during ripening, but this peptide was eventually hydrolyzed by chymosin with release of α_{s1}-(f24−*), α_{s1}-(f33−*), and α_{s1}-(f102−*), which could be detected in WSE of Cheddar cheese and which could be implicated with chymosin cleavage sites (McSweeney et al., 1993). The peptide α_{s2}-(f154−*) could not be proven as produced by proteinases of *C. cardunculus,* so it was probably a result of the catalytic action of proteinases or peptidases present in cheese as such or as result of microbial contamination.

Peptide Profile. The RP-HPLC peptide profiles of the WSE of fresh cheeses manufactured with plant and animal rennets were similar to one another; the number, and corresponding concentration, of the various peptides was rather low during the early stages of ripening, but both increased quickly thereafter (Sousa and Malcata, 1997).

Changes in the peak areas of the major peptides in the WSE of cheeses manufactured with plant and animal rennets are presented in Figure 2, which are a measure of the selectivity (or chemical mechanism) of attack by the enzyme. In general, the peptide profile of cheeses manufactured with plant rennet (see Figure 2a) exhibited a higher number of peaks than that of cheeses manufactured with animal rennet (see Figure 2b); these differences reflect differences in the mechanisms by which the two types of enzymes act, that is, differences in peptide linkages attacked, with corresponding differences in the fragments obtained. These data suggest a broader specificity of the proteinases of *C. cardunculus* than of those of animal rennet, which agrees with results found in experiments using soluble caseins (Sousa and Malcata, 1998); this is expected because the feedstock milk, the cheese-making protocol, the ripening conditions, and the native microflora were similar (Sousa and Malcata, 1997). The major primary peptides formed at the highest rates were those denoted 28, 42, 49, 55, and 60 in cheeses manufactured with plant rennet (see Figure 2a) and 31, 36, and 42 in cheeses manufactured with animal rennet (see Figure 2b). Qualitative differences between the peptide profiles of these cheeses include, but are not restricted to, the fact that peptides denoted 2, 7, 8, 15, 23, 35, 37, 39, 51,

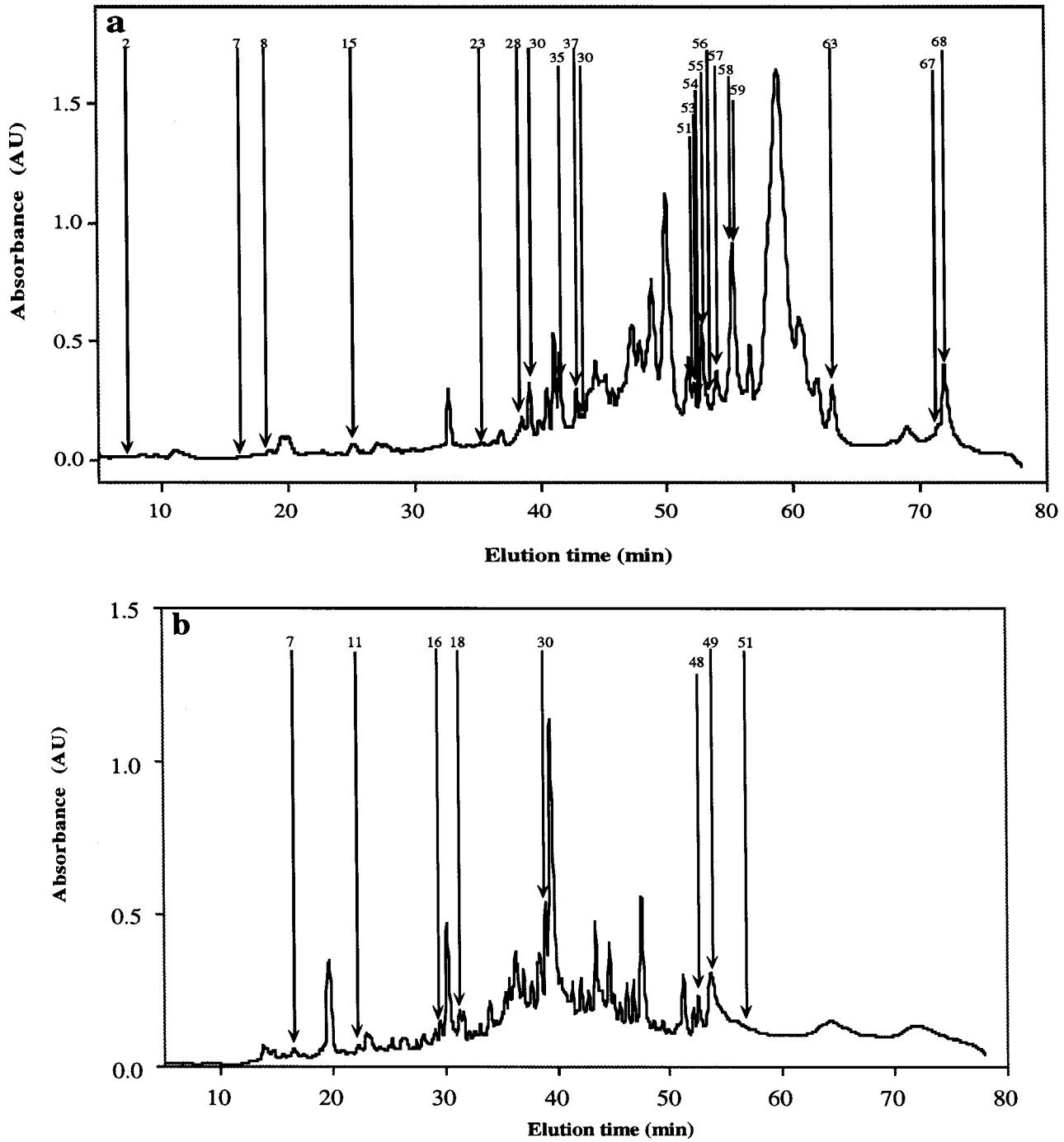

Figure 2. Chromatograms in a typical RP-HPLC run of the WSE of cheeses ripened for 68 days and manufactured from raw milk using plant rennet (a) or animal rennet (b). Only those peptides that differentiate the chromatograms from one another are labeled.

53−59, 63, and 65−68 (see Figure 3a) were present only in cheeses manufactured with plant rennet, whereas peptides denoted 7, 11, 16, 18, 30, 48, 49, 51, and 63 (see Figure 3b) were present only in cheeses manufactured with animal rennet.

Identification of Peptides. The peptides that had been previously isolated in this research effort via RP-HPLC were partially sequenced according to the Edman procedure from their N terminus in attempts to identify them; the sequences of the peptides in ovine cheeses manufactured with plant rennet or animal rennet are shown in Table 1. These results indicate the following in ovine cheese manufactured with proteinases of *C. cardunculus:* β-casein was cleaved at bonds Leu6−Asn7, Glu44−Leu45, Val82−Val83, Met93−Gly94, Leu127−Thr128, Thr128−Asp129, Val140−Gln141, Thr154−Val155, Leu165−Ser166, Lys176−Ala177, Asp182−Met183, Leu190−Tyr191, Leu196−Gly197, and Arg200−Gly201; α_{s1}-casein was cleaved at bonds Phe23−Val24, Phe28−Pro29, and Tyr165-Thr166; and α_{s2}-casein was cleaved at bonds Val73−Asp74, Ile85−Asn86, and Trp193−Thr194. Conversely, in ovine cheese manufactured with animal rennet the following was found: β-casein was cleaved at bonds His50−Pro51, Asn68−Ser69, Lys102−Lys103, Leu163−Ser164, and Leu190−Tyr191; α_{s1}-casein was cleaved at bonds Phe23−Val24, Val24−Val25, Val25−Ala26, and Gln119−Leu120; and α_{s2}-casein was cleaved at bonds Pro13−Ile14,

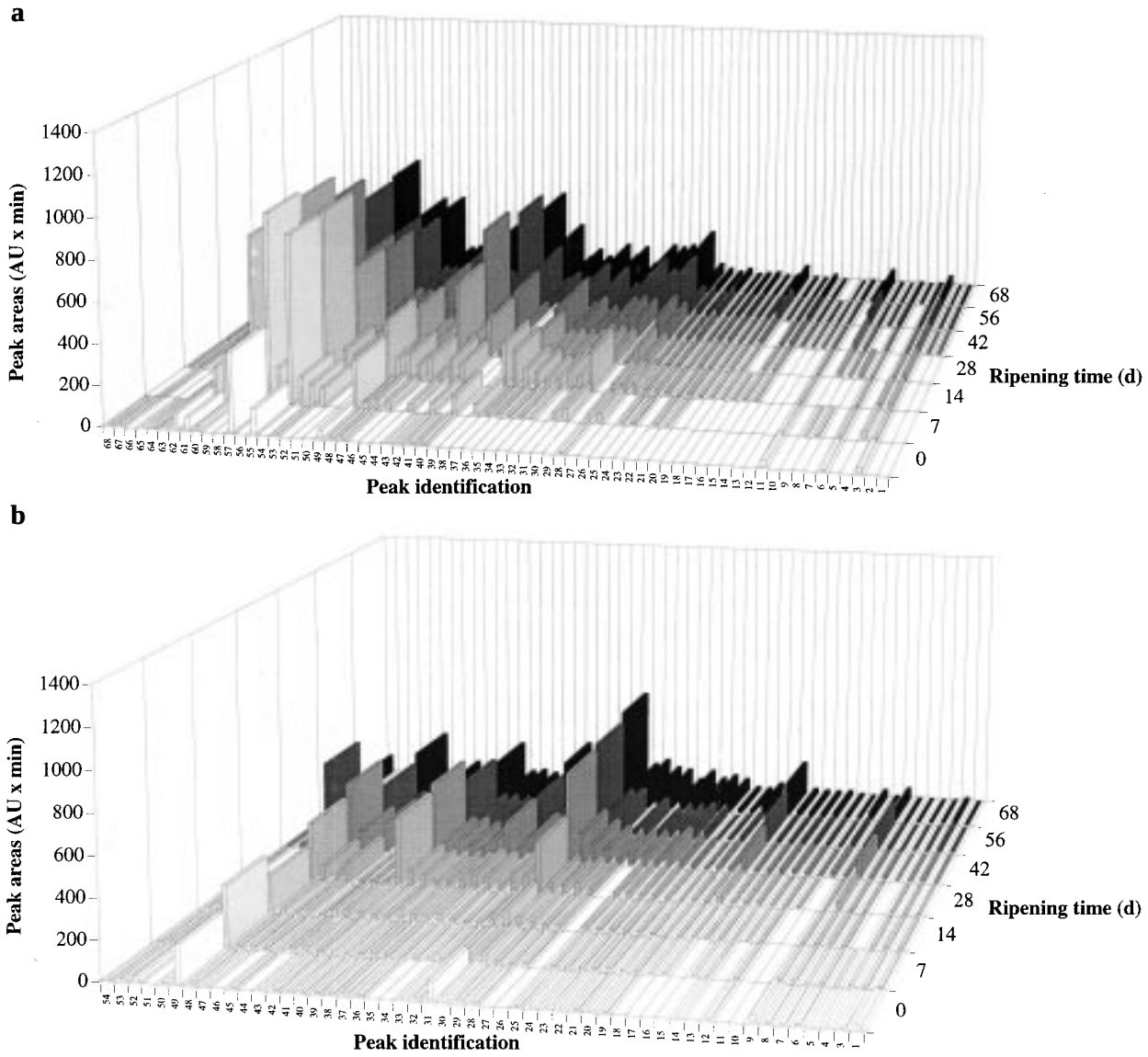

Figure 3. Changes throughout ripening in the area of the RP-HPLC peaks accounted for by peptides in cheeses manufactured from raw milk using extracts of *C. cardunculus* (a) or animal rennet (b).

Ile16−Ser17, Phe88−Tyr89, and Thr130−Ser131. This information is also graphically conveyed in Figure 4.

The major cleavage sites on ovine β-casein solutions by proteinases from *C. cardunculus* were Leu127−Thr128 and Leu190−Tyr191 (Sousa and Malcata, 1998); in the case of bovine β-casein, six major bonds were cleaved, which, in decreasing order of susceptibility, were Leu192−Tyr193, Leu191−Leu192, Leu165−Ser166, Phe190−Leu191, Ala189−Phe190, and Leu127−Thr128. The formation of peptide β-(f191−*) was catalyzed by proteinases from *C. cardunculus,* so the peptide found in WISE from ovine cheese was probably β-(f1−190). The peptides β-(f191−*) and β-(f164−*) seem to be produced by animal rennet because, as mentioned before, the bonds in ovine β-casein most susceptible to chymosin action were Leu190−Tyr191 and Ala187−Phe188 (Whyte, 1995); on bovine β-casein, the bonds cleaved were Leu192−Tyr193, Ala189−Phe190, Leu165−Ser166, Gln167−Ser168, Leu163−Ser164, Leu139−Leu140, and Leul27−Thr 128 (Visser and Slangen, 1977; Exterkate et al., 1997). Several fragments from ovine β-casein were found in both cheeses but could not be claimed to be produced by proteinases of *C. cardunculus* or animal rennet; however, a broader specificity of chymosin on ovine β-casein was observed in cheese than in solution, probably as a result of modification of the environment when going from a micellar solution to a proteinaceous network via interactions between enzyme(s) and casein substrate(s) (Exterkate et al., 1997). The bond Lys176−Ala177 was reported to be cleaved by plasmin in bovine β-casein, thus producing the peptide β-(f177−*) (Visser et al., 1989), whereas the bonds Leu6−Asn7, Thr128−Asp129, Val140−Gln141, Ser142−Trp143, and Leu165−Ser166 were found to be cleaved by cell envelope proteinases released by *Lactococcus* spp., thus producing the peptides β-(f7−*), β-(f129−*), β-(f141−*), β-(f143−*), and β-(f166−*), respectively (Visser et al., 1988, 1991; Reid et al., 1991); these cleavage sites are also apparent in Figure 4a.

The primary cleavage site of proteinases from *C. cardunculus* on ovine α_{s1}-casein solutions (Sousa and Malcata, 1998) and cheese (Sousa and Malcata, 1997) was Phe23−Val24. The corresponding peptide α_{s1}-(f24−*) was isolated from WISE by urea−PAGE and

Table 1. Peptide Fragments Identified in RP-HPLC Peaks of Water-Soluble Extract Obtained from Ovine Milk Cheeses by 68 Days of Ripening Manufactured with *C. cardunculus* or Animal Rennet

	peptide fragment sequenced	
peak	*C. cardunculus*	animal rennet
18		α_{s1}-(f1−*)
19	α_{s1}-(f1−*) + β-(f141−*)	α_{s1}-(f25−*) + β-(f69−*)
27		α_{s1}-(f1−*) + α_{s1}-(f120−*) + α_{s2}-(f14−*) + α_{s2}-(f89−*) + α_{s2}-(f131−*)
28		β-(f164−*)
30	α_{s1}-(f1−*) + β-(f166−*)	β-(f164−*)
31		α_{s1}-(f1−*) + β-(f164−*)
32	β-(f166−*)	α_{s1}-(f1−*)
33	α_{s1}-(f1−*) + β-(f166−*)	
35	α_{s1}-(f1−*) + β-(f166−*)	
36		α_{s1}-(f1−*) + α_{s1}-(f24−*) + α_{s1}-(f26−*) + β-(f51−*)
37	α_{s1}-(f1−*) + β-(f183−*)	
40	β-(f166−*) + β-(f183−*)	
42		α_{s1}-(f1−*)
43	α_{s1}-(f1−*) + β-(f166−*)	
44	β-(f83−*) + β-(f129−*)	
45	β-(f129−*)	
48	α_{s1}-(f29−*) + β-(f128−*) + β-(f129−*)	α_{s2}-(f17−*) + β-(f1−*) + β-(f191−*)
49	α_{s1}-(f1−*) + α_{s1}-(f174−*) + α_{s2}-(f74−*) + α_{s2}-(f86−*) + α_{s2}-(f194−*) + β-(f83−*) + β-(f94−*) + β-(f166−*) + β-(f201*)	α_{s2}-(f17−*) + β-(f1−*) + β-(f191−*)
53	β-(f197−*)	
55	β-(f166−*)	
56	β-(f141−*)	
57	α_{s1}-(f24−*) + β-(f143−*) + β-(f191−*)	
58	β-(f191−*)	
60	β-(f1−*) + β-(f7−*) + β-(f45−*) + β-(f128−*) + β-(f177−*)	
61	β-(f128−*)	
62	β-(f7−*)	
63	β-(f1−*)	

sequenced, and the complementary peptide α_{s1}-(f1−*) was isolated from WSE by RP-HPLC and also sequenced; thus, the primary peptides produced by proteinases from *C. cardunculus* were apparently α_{s1}-(f1−23) and α_{s1}-(f24−191). The peptide α_s-(f24−*) found in WISE (see Figure 1a) is probably α_{s1}-(f24−165) (see Figure 4b). The corresponding cleavage site of proteinases from *C. cardunculus* on bovine α_{s1}-casein in solution was Phe23−Phe24 (Macedo, 1993; Sousa, 1993); other susceptible bonds were also found (Macedo et al., 1996), but none of them were found in ovine cheeses. The peptide α_{s1}-(f29−*) was reported to be produced by chymosin on bovine (α_{s1}-casein in solution at pH 6.5 and 5.2 in the presence of 5% NaCl (McSweeney et al., 1993) but has not yet been implicated with the action of proteinases of the plant rennet in solution. The bond Phe23−Val24 was also found to be the primary cleavage site by chymosin in solutions of ovine α_{s1}-casein (Whyte, 1995); in bovine α_{s1}-casein solutions in the presence of 5% NaCl, chymosin cleaved several bonds, namely, Leu11−Pro12, Phe23−Phe24, Phe28−Pro29, Phe32−Gly33, Leu40−Ser41, Leu101−Lys102, Leu142−Ala143, Leu149−Phe150, Phe153−Tyr154, Leu156−Asp157, Tyr159−Pro160, Trp164−Tyr165, and Phe179−Ser180 (McSweeney et al., 1993). As mentioned previously, the concentration of bovine α_{s1}-(f24−199) was reported to increase initially during ripening, but this peptide was eventually hydrolyzed by chymosin with release of α_{s1}-(f24−*), α_{s1}-(f33−*), and α_{s1}-(f102−*), which could be detected in WSE of Cheddar cheese (McSweeney et al., 1994b) and which could be implicated with chymosin cleavage sites (McSweeney et al., 1993). However, none of the highly susceptible bonds toward the C terminus of the α_{s1}-casein in solution was cleaved by chymosin in cheese; Exterkate et al. (1997) reported that α_{s1}-casein is a poorly structured molecule in buffered solutions, so the specific structural arrangement believed to occur in cheese will reduce accessibility of the enzyme to the peptide segment that includes bonds Trp164−Tyr165, Leu156−Asp157, and Leu149−Phe150; instead, new cleavage sites (Leu109−Glu110, Glu110−Ile111, and Gln119−Leu120) could be detected in cheese. The peptides α_{s1}-(f24−*) and α_{s1}-(f120−*) were produced by animal rennet, so the corresponding band found in WISE from cheese (see Figure 1b) could be α_{s1}-(f24−119) (see Figure 4b). The peptides α_{s1}-(f25−*) and α_{s1}-(f26−*) (see Figure 4b) are likely degradation products from chymosin (Richardson and Creamer, 1973) or products from aminopeptidase action on peptide α_{s1}-(f24−*).

None of the fragments of α_{s2}-casein identified in cheeses manufactured with proteinases from either *C. cardunculus* or plant rennet corresponded to the susceptible peptide bonds of α_{s2}-casein (see Figure 4c). The bond of ovine α_{s2}-casein most susceptible to proteinases from *C. cardunculus* in solution is Phe88−Tyr89 (Sousa and Malcata, 1998), whereas in the case of bovine α_{s2}-casein in solution such plant proteinases catalyze hydrolysis of Phe88−Tyr99 and Tyr95−Leu96 (Macedo et al., 1996). The bonds on bovine α_{s2}-casein solutions most susceptible to chymosin action are, in turn, Phe88−Tyr89, Tyr95−Leu96, Gln97−Tyr98, Tyr98−Leu99, Leu99−Tyr100, Phe163−Leu164, Phe174−Ala175, and Tyr179−Leu180 (McSweeney et al., 1994a).

ABBREVIATIONS USED

RP-HPLC, reversed-phase high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride) membranes; WISE, water-insoluble extract; WSE, water-soluble extract.

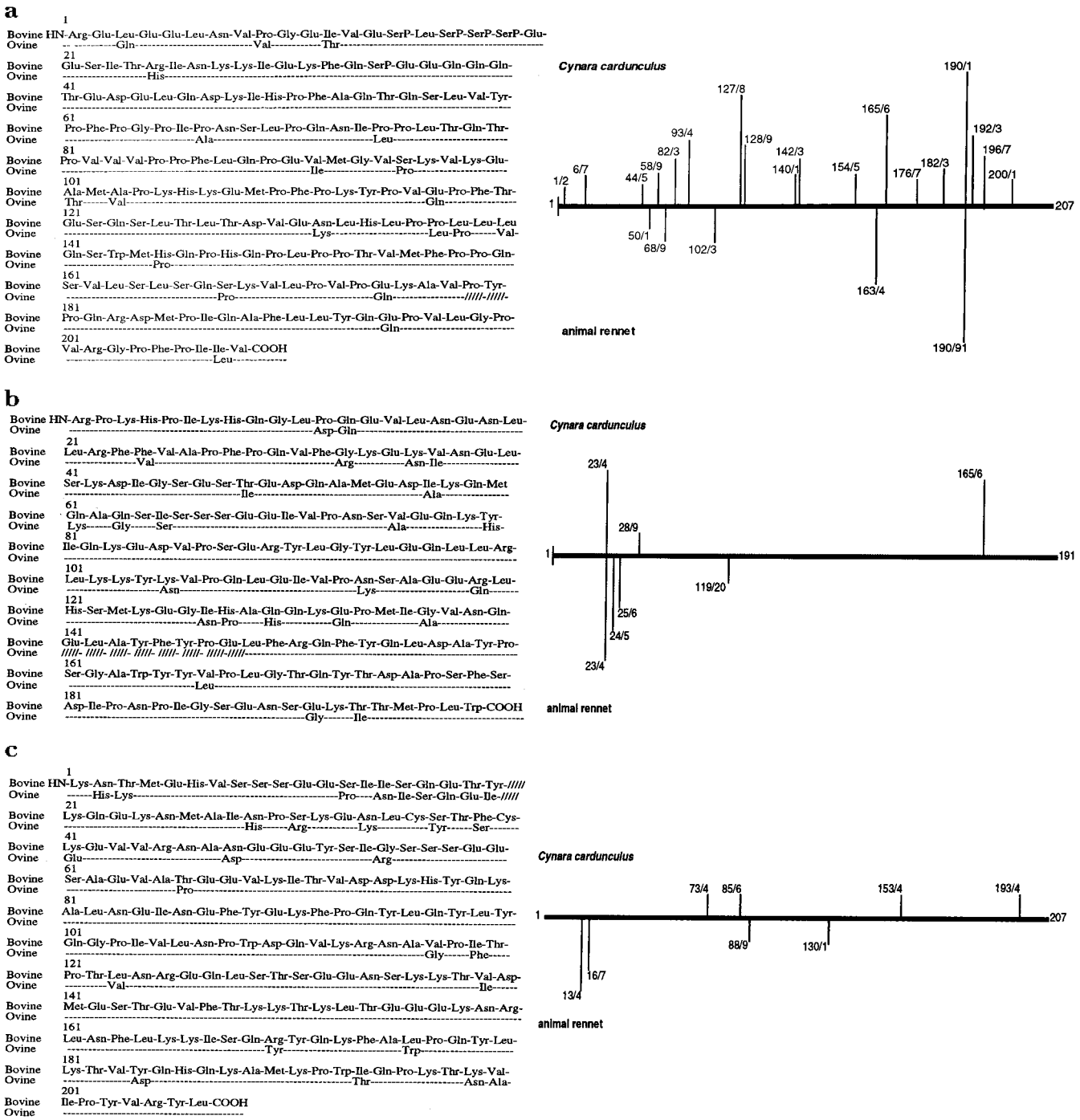

Figure 4. Comparison of primary structures of bovine β-casein (a), α_{s1}-casein (b), and α_{s2}-casein (c) [adapted from Richardson and Mercier (1979), Swaisgood (1982), Boisnard and Petrissant (1985), and Mercier et al. (1985); identical sequences are indicated by (- - -); amino acid residue deletions are indicated by (///)] and simplified primary structure of ovine counterparts with explicit indication of cleavage sites by extracts of *C. cardunculus* and by animal rennet.

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