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Proteolytic activities of some milk clotting enzymes on ovine casein

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Abstract

Proteolytic activity of some milk clotting enzymes (calf and lamb rennets, bovine chymosin and pepsin, and proteases from *Rhizomucor miehei* and *Cryphonectria parasitica*) on ovine whole casein was determined by urea-PAGE and RP-HPLC. Microbial enzymes were more proteolytic than animal enzymes when acting on ovine whole casein. Lamb rennet and *C. parasitica* protease showed the lowest and the highest degree of proteolysis, respectively. Urea-PAGE results showed that all enzymes hydrolyzed ovine casein resulting in the formation of α_{s1}-I and β-I as initial breakdown products of α_{s1}-CN and β-CN. In addition to these products, *C. parasitica* protease produced a series of degradation products with lower mobilities than β-CN. Minor quantitative differences between coagulants from animal origin, but great quantitative and qualitative differences between microbial and animal coagulants (assessed throughout the study of the RP-HPLC peptide profiles), were observed.

Keywords: Ovine casein; Coagulants; Proteolysis

1. Introduction

While glycolysis and lypolysis are critical in certain varieties of cheese (e.g. blue, hard Italian and Swiss-type), proteolysis is essential in all varieties, especially internal-bacterial ripening and surface-ripening cheeses, in which it is probably the main biochemical event during ripening.

The main agents responsible for proteolysis during cheese ripening are: endogenous milk enzymes (e.g. plasmin), residual coagulant retained in the curd after manufacture and proteolytic enzymes of starter and non-starter bacteria and secondary innocula. These proteolytic agents act in a concerted fashion to hydrolyze paracasein to peptides and amino acids.

The principal pathway of proteolysis appears to involve a primary limited breakdown of the paracasein by residual coagulant and/or endogenous milk enzymes to polypeptides which are further degraded by the proteinase-peptidase system of starter and non-starter bacteria to peptides and amino acids (Fox, 1989).

The proteolytic activity and specificity of calf chymosin and the principal rennet substitutes on bovine caseins is fairly well established and these findings can, largely, be extended to cheese (McSweeney, Pochet, Fox & Healy, 1994).

Gastric proteinases from young calves, kids, and lambs have been traditionally used for the manufacture of most cheese varieties, with calf rennet being the most common type. Lamb and kid rennets are still used in the production of many cheese varieties in Italy, France and Spain. An increasing world cheese production, concomitant with a reduced supply of calf rennet, has led to a search for rennet substitutes from different origins.

Only a few studies have been performed with ovine casein and limited data are available on the proteolytic processes produced by milk coagulants other than calf rennet. Recently, the proteolytic activity of aspartic proteinases from *Cynara cardunculus* L. on ovine caseins has been studied (Silva & Malcata, 1999; Sousa & Malcata, 1998).

The objective of this work was to compare the proteolytic activities of different milk clotting enzymes (calf and lamb rennets, bovine chymosin and pepsin, and proteases from *Rhizomucor miehei* and *Cryphonectria parasitica*) on whole ovine casein in model systems.

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2. Materials and methods

2.1. Materials

Raw ewe's milk (Manchega breed) was obtained from a rural dairy farm (Can Gelats Nous, Riudarenes, Spain). Skimmed milk was prepared by centrifugation at 2500 *g* and 30°C for 30 min. Ovine whole casein was prepared by isoelectric precipitation (3.3 M Na-acetate buffer, pH 4.6) from skimmed milk, previously diluted with an equal volume of distilled water, and centrifuged for 20 min at 500 *g*. The precipitate was dispersed in distilled water and precipitated and washed three times successively. Finally, casein was freeze-dried.

Commercial calf rennet (Renifor-15/E) was obtained from Lamirsa (Laboratorios Miret S.A., Barcelona, Spain) containing 780 mg chymosin/l and 565 mg pepsin/l.

Pure chymosin (Aniren™ 880) was obtained from Sanofi Bio-Ind (21201 Beaune Cedex, France) containing 880 mg chymosin/l.

Pure bovine pepsin (Bovipep® 1700) was obtained from Sanofi Bio-Ind (21201 Beaune Cedex, France) containing 1700 mg pepsin/l.

Lamb rennet was obtained from Caglio Star España, S.A. (Cieza, Murcia) with a declared coagulant activity of 1:15000.

Rhizomucor miehei protease (Hannilase®) was obtained from CHL-Lacta S.L (Madrid, Spain) with a declared coagulant activity of 1:14000.

Cryphonectria parasitica protease (Suparen®) was obtained from Pfizer Inc. (Wisconsin, USA) with a declared coagulant activity of 1:15000.

Enzyme solutions were standardised to equal milk clotting activity (5 min) by a modification of Berridge's method [International Dairy Federation (IDF), 1987]. Enzyme solution (1 ml) was added to glass tubes containing 10 ml low-heat skimmed milk powder (INRA, Poligny, France) containing 10 mM $CaCl_2$ at 30°C. The clotting time was calculated by the observation of graininess or the appearance of flecks on the tube wall.

2.2. Hydrolysis conditions

Solutions of ovine whole casein (25 mg/ml) in 50 mM sodium acetate buffer at pH 6.2 containing 0.02% (w/v) thimerosal to prevent microbial activity, were heated (80°C, 30 min) to inactive milk protease during incubation and then the coagulant enzyme solution was added. The solutions were then incubated at 30°C during different incubation times (0.5, 1, 3, 6, 18, 24, 48 and 72 h). At the end of each period, coagulants were inactivated by heating (100°C, 5 min) and pH lowered to 4.6. After centrifugation (16,000 *g*, 5 min) the supernatants were filtered through 0.45 μm filters and analysed by RP-HPLC to separate the pH 4.6-soluble peptides. The pellets were redissolved in 7.0 M urea and samples were taken for electrophoretic separation using urea-PAGE.

Hydrolysis conditions and incubation times, and coagulant concentrations were taken based on preliminary experiments, to give a satisfactory hydrolysis.

2.3. Protein concentration

The protein content of the pH 4.6-soluble extracts from the hydrolyzates was determined by Lowry's method (Lowry, Roseberough, Farr & Randall, 1951). Ovine casein was used as the standard.

2.4. Electrophoresis

Alkaline urea-PAGE with 0.7 mm spacers was performed according to the method of Akroyd (1968) with a 8.8%T (grams of acrylamide plus grams of bisacrylamide/100 ml), 2.3%C (grams of bisacrylamide/%T) and 5*M* urea at pH 8.9 as described by Carretero, Trujillo, Mor-Mur, Pla and Guamis (1994).

Gels were stained with Coomassie Blue R-250 and were destained by repeated washing in an ethanol/acetic acid/glycerol/water (200/50/25/725; v/v/v/v) solution.

2.5. RP-HPLC profiles

Peptides soluble at pH 4.6 were separated by RP-HPLC on a DeltaPack C_{18} (5 μm) column (3.9×150 mm; Waters) using the following eluent system: solvent A [1.15 ml/l trifluoroacetic acid (TFA) in water] and solvent B (600 ml acetonitrile, 400 ml water and 1 ml TFA/l) with a linear gradient from 0 to 80% of solvent B for 35 min at 40°C. Flow rate was 1 ml/min and the eluted peaks were detected by UV-absorbance at 214 nm (Trujillo, Miranda, LeBars & Delacroix-Buchet, 1998).

3. Results and discussion

The proteolytic activity (monitored by quantifying the pH 4.6-soluble nitrogen by Lowry's method) of the milk-clotting enzyme preparations on ovine whole casein is shown in Fig. 1. Lamb rennet and *C. parasitica* showed the lowest and the highest degree of proteolysis, respectively on ovine casein. Bovine chymosin and pepsin, and *R. miehei* protease were more proteolytic than calf rennet when acting on ovine casein up to 24 h. From 24 h on, bovine chymosin and pepsin, and *R. miehei* protease raised a visible plateau, while calf rennet increased the breakdown products soluble at pH 4.6 during the incubation time. Although lamb rennet was not so proteolytic as calf rennet, it presented the same tendency on hydrolysing ovine casein. It seems to be that the concerted action of both chymosin and pepsin

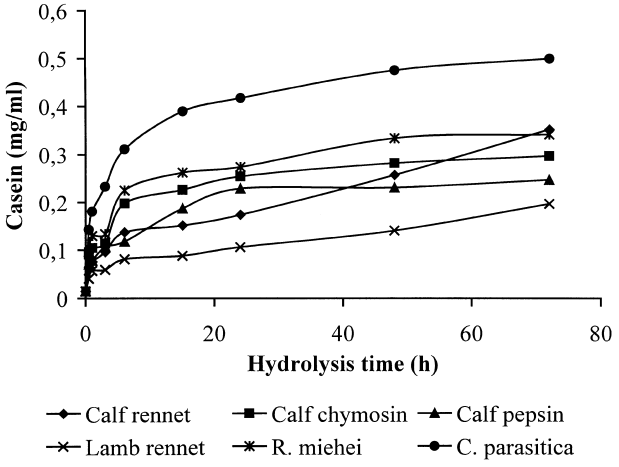

Fig. 1. Time course hydrolysis of whole ovine casein by the milk clotting enzymes.

(main enzymes in rennet preparations) would provide appropiate substrates for continuing the hydrolysis process.

A high ratio of milk clotting-to-proteolytic activity is an essential requirement for calf rennet substitutes. Indices of the latter ratio have been determined by both direct and indirect methods. Vanderpoorten and Weckx (1972) compared the proteolytic activities of calf rennet and microbial-derived coagulants (*R. miehei* and *C. parasitica*) on bovine whole casein and casein fractions at pH 6.7 and 30°C by monitoring the release of non-protein nitrogen over an incubation period of 120 min and by gel electrophoresis. On whole casein and on α_{s1}- and β-CNs, greatest proteolytic activity decreased in the following order: *C. parasitica* > *R. miehei* > calf rennet. Similar results were obtained by Tam and Whitaker (1972) and Ustunol and Zeckzer (1996) using the number of peptide bonds hydrolyzed at pH 5.5 and 6.0 and the TNBS (2,4,6-trinitrobenzenesulfonic acid) assay, respectively, as indices of proteolytic activity. Trends in proteolytic activity have been confirmed by measuring protein, non-protein nitrogen and/or fat levels in cheese wheys and by measuring yield losses (Emmons, Beckett & Binns, 1990).

3.1. Urea-PAGE

Urea-PAGE electrophoretogram of isoelectric ovine casein (Fig. 2, lanes 1 and 10) showed a group of bands with lower mobility containing β-CN and κ-CN. This is followed by the α_s-CN complex that migrated as three bands which have been identified as a mixture of α_{s1}-CN and α_{s2}-CN (Chianese, Mauriello, Moio, Intorcia & Addeo, 1992). Both ovine β-CN and α_{s1}-CN are characterized by post-translational modifications such as phosphorylation. The electrophoretic profile that was observed for β-CN consisted of two main bands of great intensity that correspond to β-CN 5P and 6P forms (Richardson & Mercier, 1979), although other minor bands with slow mobilities, corresponding to other phosphorylation levels, could be observed. Chianese et al. (1995) showed, by electrophoretic and electrospray-ionization mass spectrometry techniques, the presence of multiphosphorylated forms of β-CN, in addition to the main forms β-CN 5P and 6P containing two to seven phosphate groups per molecule. In the same way, α_{s1}-CN migrates as three bands with 8–10 phosphate residues per molecule.

The nomenclature given in Fig. 2 is according to Creamer (1976) for β-CN-derived peptides, and we have assigned the name of α_{s1}-I to the peptide produced from ovine α_{s1}-CN by rennet, as compared with the electrophoretic mobilities of bovine α_{s1}-I peptide generated by rennet. From the electrophoretograms, the following more general and significant changes were observed: (1) β-CN and α_{s1}-CN were hydrolyzed at different rates depending on the milk clotting preparation during the incubation times. (2) A slow electrophoretic band, which represents para-κ-CN, had already appeared from the first moments of hydrolysis (0.5 h) indicating that enzyme preparations hydrolyzed κ-CN. (3) Peptides with slightly higher mobilities than β-CN and α_s-CN, corresponding to the breakdown products β-I and α_{s1}-I respectively, were formed during the incubation time.

κ-CN is the principal casein fraction affected by chymosin in the primary phase of the milk clotting process. During this enzymatic reaction, the Phe_{105}-Met_{106} bond is specifically cleaved in bovine as well as in ovine κ-CNs (Jollès, Schoentgen, Hermann, Alais & Jollès, 1974), releasing para-κ-CN or κ-CN f (1-105) and κ-macropeptide or κ-CN f (106-171).

The cleavage of Phe_{23}-Phe_{24} in bovine α_{s1}-CN and Leu_{192}-Tyr_{193} in bovine β-CN by chymosin produced α_{s1}-I or α_{s1}-CN f (24-191) and β-I or β-CN f (1-192), respectively (Creamer, 1976; McSweeney, Olson, Fox, Healy & Hojrup, 1993a). The bond Phe_{23}-Phe_{24} has also been found to be the primary cleavage site by chymosin in a solution of ovine α_{s1}-CN. On the other hand, the first chymosin-susceptible bond in ovine β-CN is Leu_{190}-Tyr_{191} and the first peptide liberated is β-CN f (191-207), which is complementary to ovine β-I or β-CN f (1-190) (Whyte, 1995). The homology between the sequences of ovine and bovine β-CNs is very high (>90%), with similar amino acid sequences in the regions described as susceptible to cleavage by calf chymosin in bovine β-CN. However, the most significant difference is due to the deletion of the Pro_{179}-Tyr_{180} dipeptide in ovine β-CN (Richardson & Mercier, 1979), which explains why the first chymosin-susceptible bond in ovine β-CN was Leu_{190}-Tyr_{191}.

As can be seen in the electrophoretograms, the breakdown products formed from β-CN and α_{s1}-CN by the milk clotting enzymes also appeared as different bands according to the phosphorylation level of each

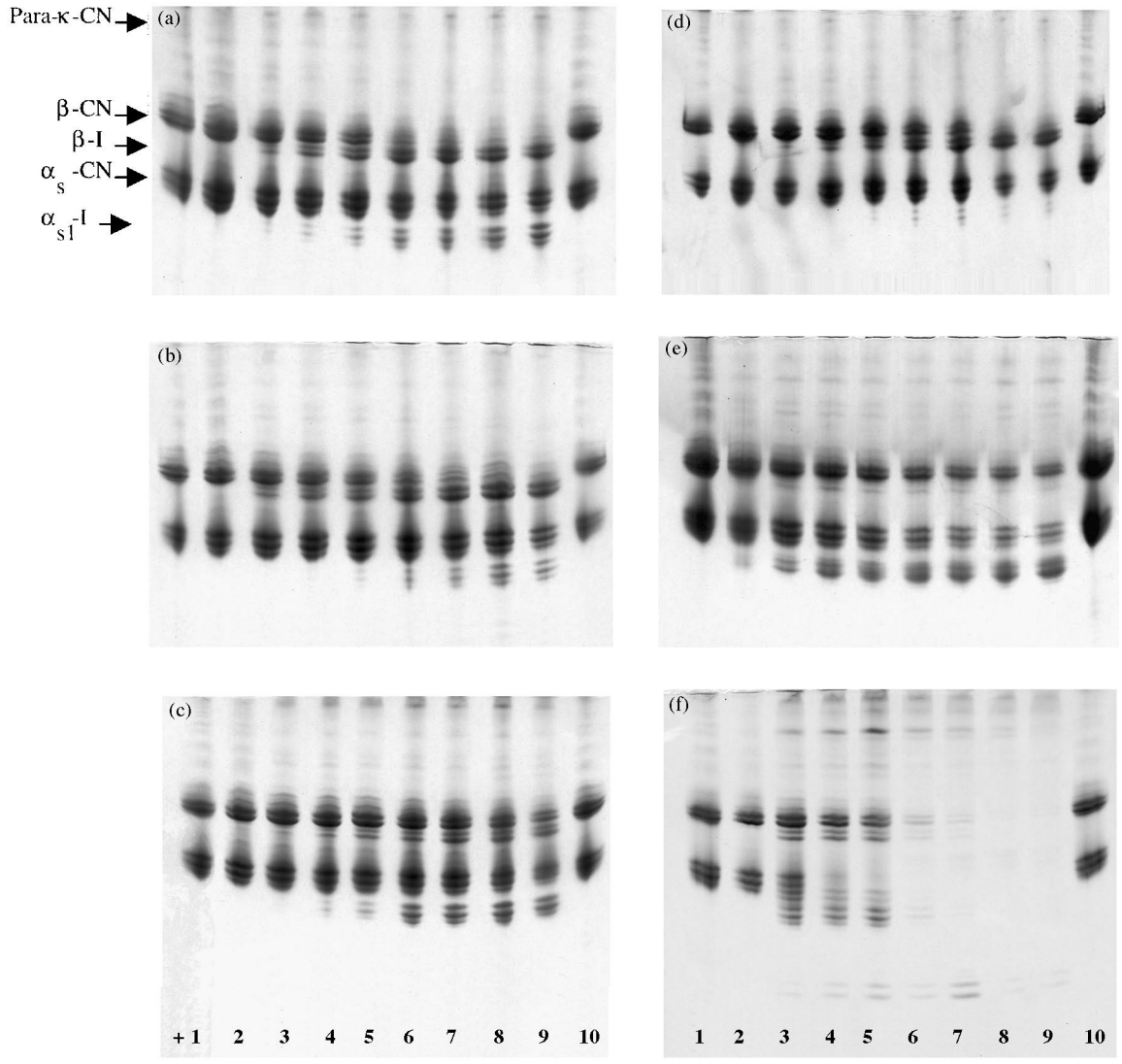

Fig. 2. Urea-PAGE electrophoretograms of whole ovine casein hydrolysis by calf rennet (a), bovine chymosin (b) and pepsin (c), lamb rennet (d), *R. miehei* protease (e) and *C. Parasitica* protease (f) at pH 6.2, 30°C for 0.5, 1, 3, 6, 15, 24, 48 and 72 h (lanes 2–9). Unrenneted controls (1 and 10).

protein. This observation indicates that the phosphorylation zones of these proteins were included in the breakdown products, and the poor activity of the milk clotting proteases in cleaving sites in the phosphorylated region of caseins. These findings agree with previous observations made for chymosin on caprine β-CN and α_{s1}-CN (Trujillo, Guamis & Carretero, 1995; Trujillo et al., 1998).

Apart from these general characteristics, substantial differences in proteolysis between the milk clotting preparations were observed. Calf and lamb rennets, and bovine chymosin showed similar ability on hydrolyzing ovine β-CN; however, bovine pepsin and protease from *R. miehei* showed low proteolytic activity on ovine β-CN with appreciable amounts of intact β-CN being visible after 72 h of hydrolysis [Fig. 2(c) and (e)]. Poor proteolytic activity of bovine pepsin and *R. miehei* protease on bovine and caprine β-CNs have been reported by Ustunol and Zeckzer (1996) and Awad, Lüthi-Peng and Puhan (1999), respectively. On the contrary, protease from *C. parasitica* was the most proteolytic enzyme when hydrolyzing β-CN [Fig. 2(f)].

Ovine β-CN was completely hydrolyzed to β-I by calf and lamb rennets, bovine chymosin and *C. parasitica* protease with an increased intensity with the incubation time. However, it was only partially hydrolyzed by bovine pepsin and protease from *R. miehei*. β-I was quite resistant to further hydrolysis by calf and lamb rennets, and to up to 24 h of hydrolysis by bovine chymosin and pepsin (visible from the electrophoretograms because β-I intensified with prolonged incubation time), whereas it was readily hydrolyzed by microbial enzymes. Bovine β-I peptide is further hydrolyzed by calf rennet into β-II, and β-III peptides, corresponding, respectively, to β-CN f (1-163) and β-CN f (1-139) (Creamer, 1976). In accordance with these previous

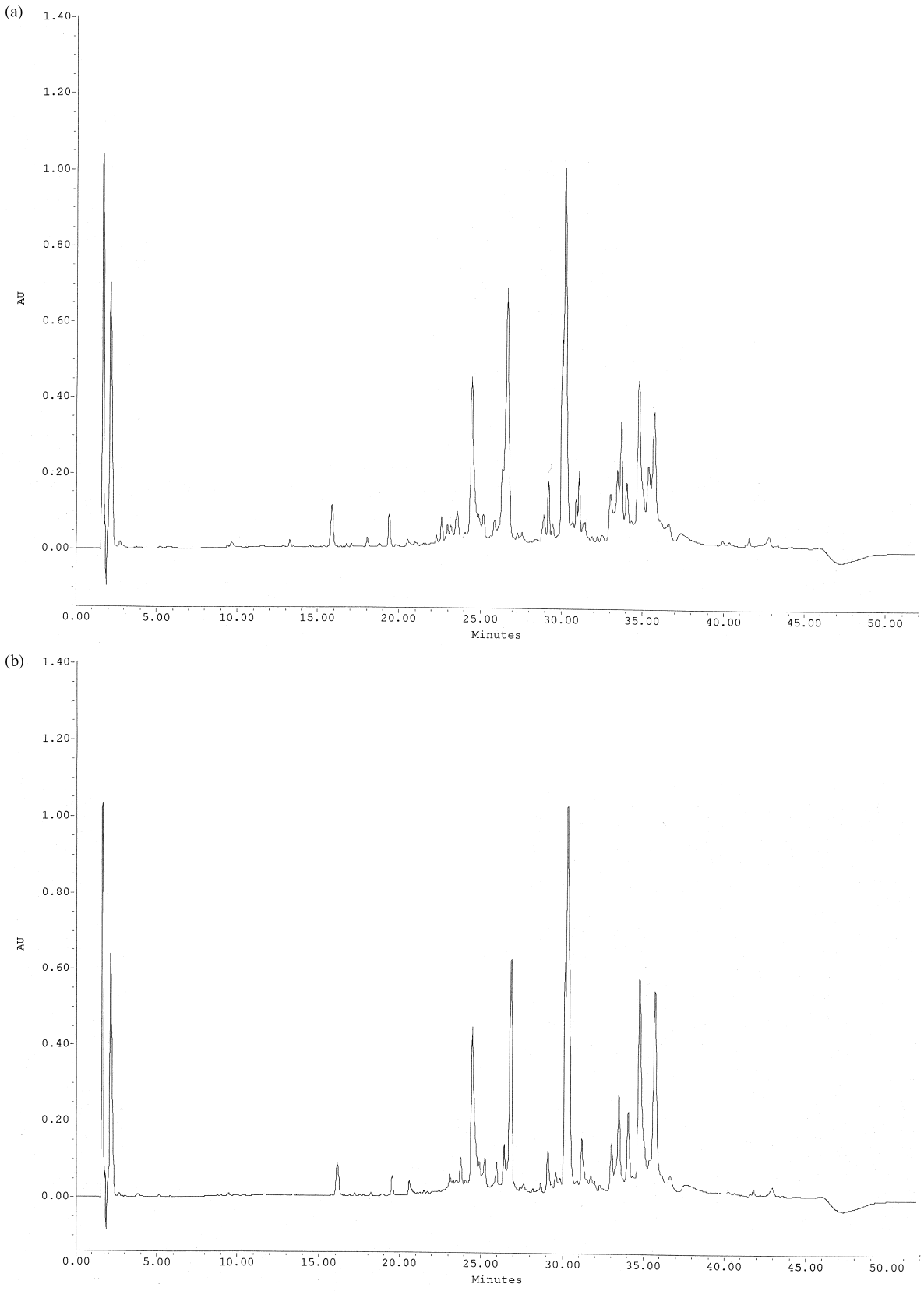

Fig. 3. RP-HPLC chromatograms of pH 4.6-soluble extracts from whole ovine casein hydrolysed by calf rennet (a), bovine chymosin (b) and pepsin (c), lamb rennet (d), *R. miehei* (e) and *C. parasitica* (f) proteases (*continued on next page*).

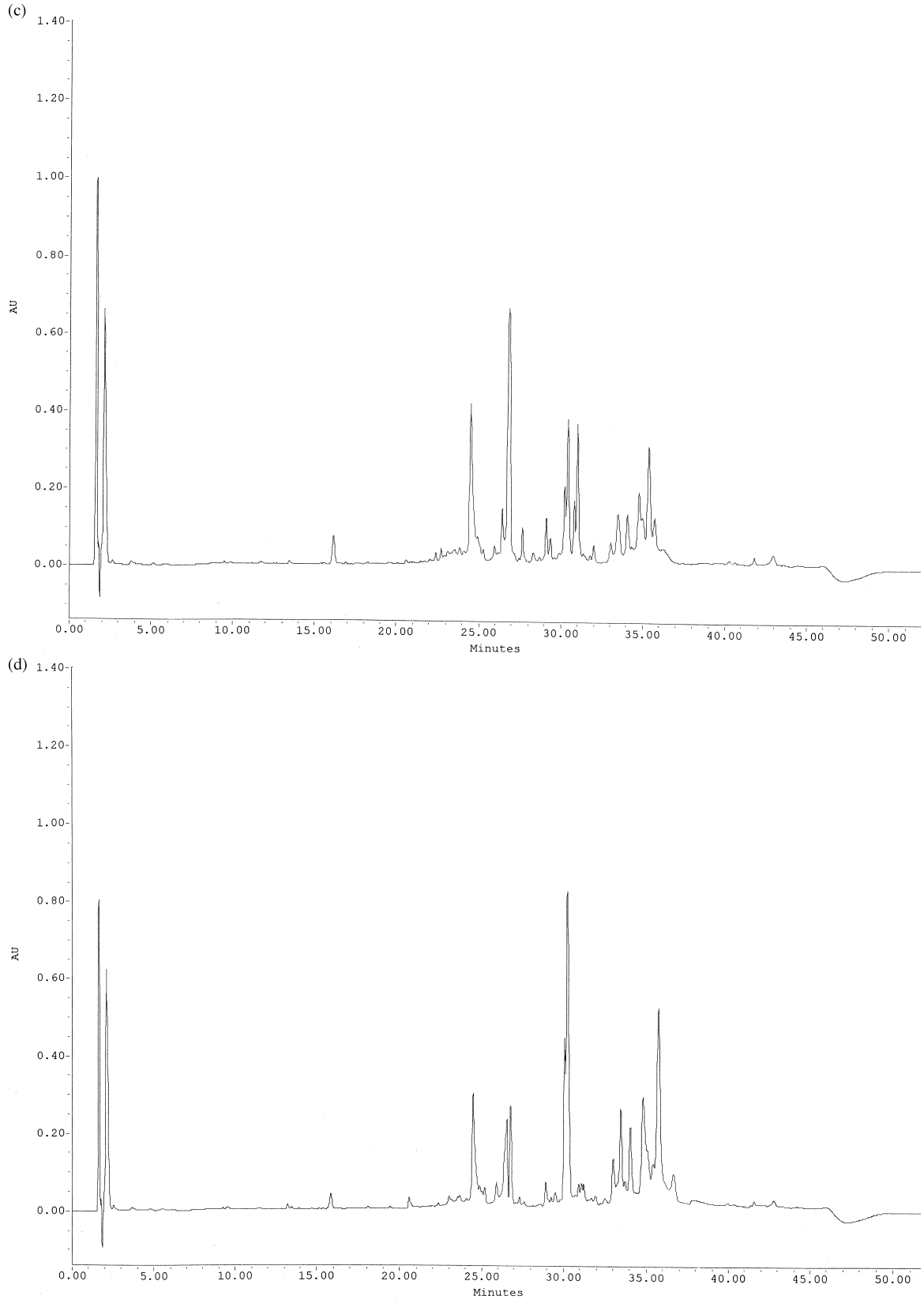

Fig. 3. (*continued*)

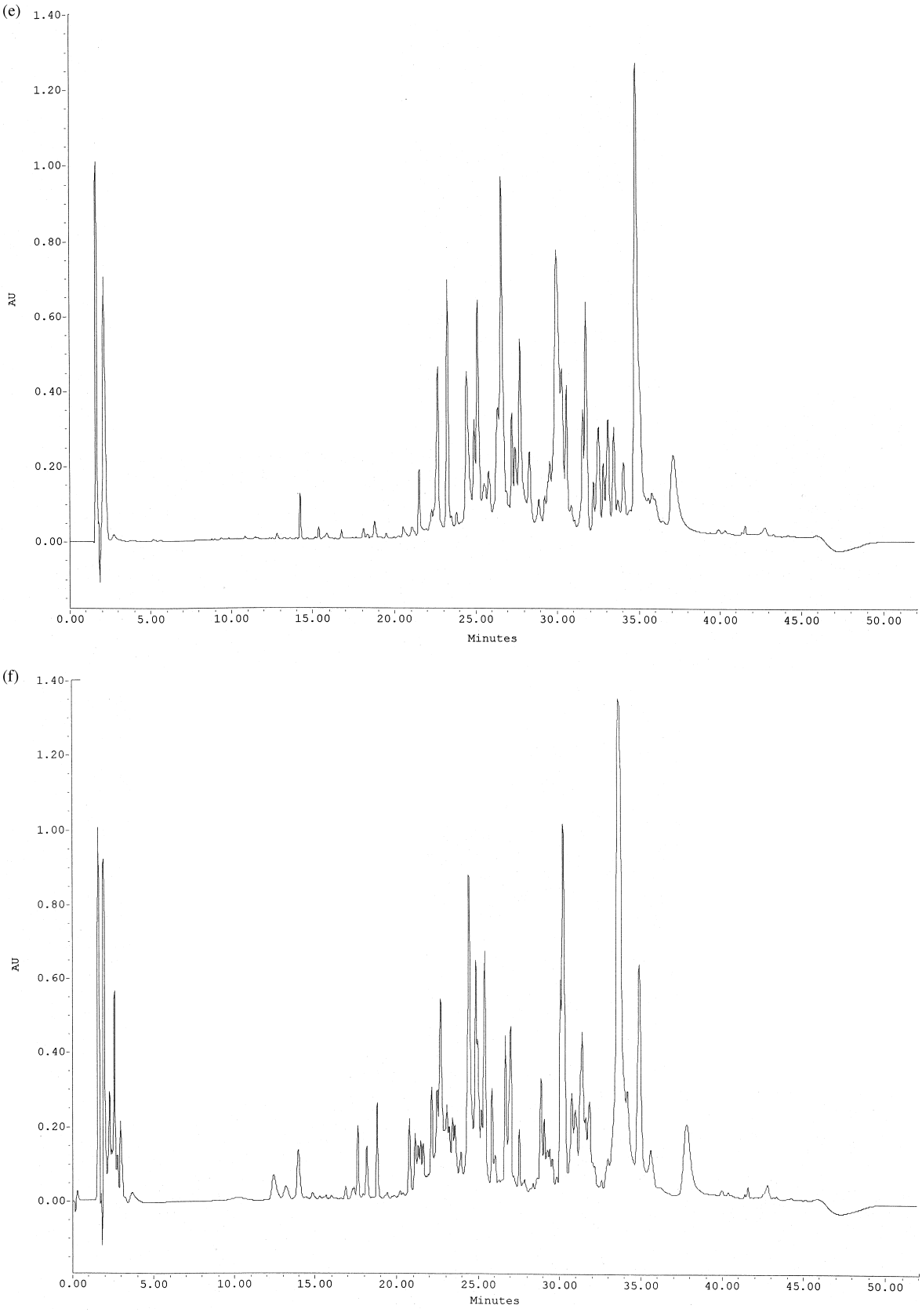

Fig. 3. (*continued*)

studies, different authors found that the proteolytic activity of bovine chymosin and pepsin on ovine and caprine β-CNs was similar to that in the bovine counterpart, with peptides β-I, β-II and β-III being formed from ovine and caprine β-CNs (Mulvihill & Fox, 1979; Trujillo et al., 1995).

Hydrolyzates obtained at 48–72 h from calf and lamb rennets, and bovine chymosin and pepsin showed that bands of β-I peptides were further degraded probably to β-II and β-III, as well as to other minor breakdown products. However, these breakdown products were not clearly visible on the gels, probably due to the partial overlapping with the intact caseins. Trujillo, Guamis and Carretero (1997) showed that caprine β-II and β-III peptides have the same mobility on urea-PAGE as the fastest bands from caprine α_s-CN and α_{s1}-I, respectively.

From the hydrolyzates obtained by the action of *C. parasitica* protease, the most proteolytic coagulant used [Fig. 2(f)], a series of bands (doublet) was visible which partially overlapped with the fastest α_s-CN bands. These bands may correspond to β-II peptides. On the same hydrolyzates, other proteinaceus material with very high electrophoretic mobility was observed. As these compounds appeared as doublets, they may have originated from β-CN, and may correspond to β-III peptides.

α_s-Casein were also degraded during the hydrolysis times with an extent depending on the milk clotting preparation used. Lamb rennet had the lowest and protease from *C. parasitica* the highest proteolytic activities on hydrolyzing α_s-CNs. α_{s1}-Casein was degraded to its primary hydrolysis product α_s-I, which intensified during the incubation time, except in *C. parasitica* hydrolyzates, where it was rapidly hydrolyzed after formation. The hydrolysis pathway of bovine and caprine α_{s1}-CN by the chymosin action is well known. Peptide α_{s1}-I or α_{s1}-CN f (24-199), the first degradation product from bovine and caprine α_{s1}-CNs, is further degraded at pH 5.2 in the presence of 3–5% NaCl (i.e. the conditions in many young cheeses), cleaving Phe_{179}-Ser_{180}, Trp_{164}-Tyr_{165}, Leu_{156}-Asp_{157}, Leu_{149}-Phe_{150} and Leu_{142}-Ala_{143} bonds on the C-terminal, and Phe_{28}-Pro_{29}, Phe_{32}-Gly_{33} and Leu_{101}-Lys_{102} bonds on the N-terminal sequence of bovine and caprine α_{s1}-CNs (McSweeney et al., 1993a; Trujillo et al., 1998).

Although α_{s2}-CN is susceptible to proteolysis by the chymosin action as McSweeney, Olson, Fox, Healy and Hojrup (1993b) have shown in bovine casein, the quantity of enzyme necessary to achieve equivalent rates of hydrolysis is approximately twice that required for α_{s1}-CN. The fate of α_{s2}-CN during cheese ripening is unclear. In Cheddar cheese, bands in the α_{s2}-CN zone of electrophoretograms decrease slowly during ripening, but none of the major bands visible on urea-PAGE originated from α_{s2}-CN (McSweeney et al., 1994). In the electrophoretic system used, α_{s2}-CN overlaps with α_{s1}-CN bands, making α_{s2}-CN degradation difficult to quantify. In the case of hydrolyzates from *C. parasitica* protease, both α_{s1}- and α_{s2}-CN were completely hydrolyzed after 24 h of incubation. Numerous breakdown products with very low electrophoretic mobility were observed on the same hydrolyzates. The origin of these products is uncertain. The ability of *C. parasitica* protease on hydrolyzing β-CN and the electrophoretic location of these breakdown products (γ-CN zone) would indicate that they originated from β-CN. However, similar electrophoretic behaviour has been shown by chymosin when acting on isolated caprine α_{s2}-CN (Trujillo, 1996). These products were also susceptible to being hydrolyzed by *C. parasitica* protease during the incubation times.

3.2. RP-HPLC profiles

The pH 4.6-soluble peptide profiles of the hydrolyzates obtained at pH 6.2 and 30°C for 48 h by the milk clotting preparations on ovine casein are shown in Fig. 3. Milk clotting preparation from animal sources had all the major pH 4.6-soluble peptides in common, and only quantitative differences were detected. However, both microbial coagulants had qualitative and quantitative differences with respect to the animal coagulants. *C. parasitica* protease was more proteolytic than *R. miehei* protease, agreeing with the urea-PAGE results.

In agreement with other studies, the proteolytic activities of microbial-derived coagulants, especially *C. parasitica* protease, were excessive compared to those of animal rennets, and hence may have undesirable side-effects such as weak gel structures, high protein/fat losses in the whey and reduced cheese yields during cheesemaking (Emmons et al., 1990), or the development of bitter flavour associated with an excessive casein proteolysis, especially of β-CN (Sullivan & Jago, 1972).

4. Conclusion

From the pH 4.6-soluble protein content obtained by Lowry's method, pH 4.6-soluble peptide profile by RP-HPLC, and pH 4.6-insoluble peptides by urea-PAGE, microbial coagulants, especially *C. parasitica* protease, were more proteolytic than animal coagulants when acting on ovine whole casein.

All the coagulants hydrolyzed ovine casein, producing α_{s1}-I and β-I as initial breakdown products detected by urea-PAGE from α_{s1}-CN and β-CN, respectively, at different rates depending on the coagulant used. Lamb rennet had the lowest and *C. parasitica* protease the highest proteolytic activities on hydrolyzing ovine casein.

RP-HPLC profiles from pH 4.6-soluble hydrolyzates showed only minor quantitative differences between coagulants from animal origin, while great quantitative

and qualitative differences between microbial (especially from *C. parasitica* preparation) and animal coagulants were observed.

As microbial coagulants are less specific than animal rennets, they could have an important impact upon cheese quality and yield.

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