J. Dairy Sci. 87:565–573

Influence of Residual Milk-Clotting Enzyme on α_{s1} Casein Hydrolysis During Ripening of Reggianito Argentino Cheese

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ABSTRACT

Milk-clotting enzyme is considered largely denatured after the cooking step in hard cheeses. Nevertheless, typical hydrolysis products derived from rennet action on α_{s1}-casein have been detected during the ripening of hard cheeses. The aim of the present work was to investigate the influence of residual milk-clotting enzyme on α_{s1}-casein hydrolysis in Reggianito cheeses. For that purpose, we studied the influence of cooking temperature (45, 52, and 60°C) on milk-clotting enzyme residual activity and α_{s1}-casein hydrolysis during ripening. Milk-clotting enzyme residual activity in cheeses was assessed using a chromatographic method, and the hydrolysis of α_{s1}-casein was determined by electrophoresis and high performance liquid chromatography. Milk-clotting enzyme activity was very low or undetectable in 60°C- and 52°C-cooked cheeses at the beginning of the ripening, but it increased afterwards, particularly in 52°C-cooked cheeses. Cheese curds that were cooked at 45°C had higher initial milk clotting activity, but also in this case, there was a later increase. Hydrolysis of α_{s1}-casein was detected early in cheeses made at 45°C, and later in those made at higher temperatures. The peptide α_{s1}-I was not detected in 60°C-cooked cheeses. The results suggest that residual milk-clotting enzyme can contribute to proteolysis during ripening of hard cheeses, because it probably renatures partially after the cooking step. Consequently, the production of peptides derived from α_{s1}-casein in hard cheeses may be at least, partially due to this proteolytic agent.
(**Key words:** milk-clotting enzyme, hard cooked cheese, proteolysis, α_{s1}-casein)

Abbreviation key: CMP = casein macro peptide, **CT** = cheeses in which the cooking step was performed at control temperature (52°C), **HT** = cheeses in which the cooking step was performed at high temperature (60°C), **LT** = cheeses in which the cooking step was performed at low temperature (45°C), **PC** = principal component, **PCA** = principal component analysis.

INTRODUCTION

Proteolysis in cheese has been widely studied and is considered one of the most important biochemical events during the ripening process (Sousa et al., 2001). Protein hydrolysis contributes to cheese final quality both directly and indirectly, because it modifies texture and releases some taste and aroma compounds and most of their precursors (McSweeney and Sousa, 2000).

Milk-clotting enzyme is an important proteolytic agent during cheese ripening. After cheese making, only 0 to 15% of the rennet activity added to the milk is retained in the curd (Sousa et al., 2001). However, these minor proportions of residual milk-clotting enzyme can cause extensive proteolysis in cheese, as has been evidenced in soft and semi-hard cheese varieties (O'Keefe et al., 1978; Noomen et al., 1978; Hynes et al., 2001, among others). In hard cheeses, this proportion is usually considered very low because the enzyme is largely denatured during the cooking stage (Gaiaschi et al., 2000; Sousa et al., 2001; Gagnaire et al., 2001), although exact extension and degree of reversibility of denaturation is still unknown.

Casein α_{s1} is hydrolyzed by chymosin at the primary site Phe_{23}-Phe_{24} in two peptides: α_{s1}(f1–23) and α_{s1}(f24–199), also called α_{s1}-I (Carles and Ribadeau Dumas, 1985; McSweeney et al., 1993). This cleavage has been detected early during ripening of soft and semi-hard cheeses, which is consistent with the relatively high activity of milk-clotting enzyme in these types of cheese (de Jong, 1978). However, the peptide α_{s1}-I has also been detected in hard cheeses, where its presence is more difficult to explain.

There are several hypotheses to explain the production of peptide α_{s1}-I in hard cheeses, although conclusive evidence is still needed. Delacroix-Buchet and Fournier (1992) have shown that an increase from 52 to 56°C in cooking temperature decreased the hydroly-

Received May 9, 2003.
Accepted June 27, 2003.
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sis of α_{s1}-CN in Gruyère cheeses, suggesting that residual milk-clotting enzyme contributes to α_{s1}-I production in this variety. Kindstedt et al. (1995) found that proteolysis was significantly slowed down in Mozzarella cheese when a reduced dose of chymosin was used, which indicates that rennet was active during ripening in spite of high cooking temperatures. Other researchers have postulated that α_{s1}-I is produced by chymosin, but early during cheese making, i.e., before the heating of the curd (Chianese et al., 1996; Gaiaschi et al., 2000).

On the other hand, Visser and De Groot Moster (1977) have found α_{s1}-I formation in Gouda cheese with inactivated rennet after 6 mo of ripening. It has been suggested that α_{s1}-I formation could be the result of either cathepsin D, an indigenous aspartic protease whose specificity on CN is similar to that of chymosin, or a small amount of residual rennet (Larsen et al., 2000). Larsen et al. (2000) detected α_{s1}-I in 39-wk-old feta cheese manufactured without rennet, which was probably the result of cathepsin D activity on α_{s1}-CN. Hurley et al. (2000a) have also reported that α_{s1}-CN proteolysis in starter-free rennet-free quarg cheeses can be attributed to cathepsin D; however, the authors have not detected the peptide α_{s1}-I in 12-wk-old quarg (Hurley et al., 2000a). Gagnaire et al. (2001) suggested that α_{s1}(f1–23) and α_{s1}-I presence in Emmental juice and CN fraction, respectively, may be caused by cell envelope proteases of starter bacteria and cathepsin D, although Visser (1993) have reported α_{s1}(f1–23) is not formed as such by starter proteinases. The authors used rennet by heating *E. parasitica* in their experiments and considered it completely inactivated *Cryphonectria* during cheese making.

Finally, it has been reported that plasmin activity is elevated in hard cheese varieties, due to the thermal inactivation of inhibitors of plasminogen activators, resulting in the increased conversion of the precursor to the active enzyme (Sousa et al., 2001). Nevertheless, plasmin action is mainly directed towards β- and α_{s2}-CN and to a lesser extent to a α_{s1}, with splitting at different peptide bonds than Phe_{23}-Phe_{24} (Gaiaschi et al., 2000).

As can be seen, although different authors have detected the products of α_{s1}-CN hydrolysis at the peptide bond Phe_{23}-Phe_{24} in hard cheeses, there is little agreement about the proteolytic agent or agents that can cause the breakdown.

The objective of the present study was to investigate the influence of milk-clotting enzyme on the hydrolysis of α_{s1}-CN in hard cheeses. For that purpose, we studied the influence of cooking temperature in milk-clotting enzyme activity and in α_{s1}-CN hydrolysis.

MATERIALS AND METHODS

Cheese Making

Reggianito Argentino cheeses were manufactured according to the standard process adapted to laboratory-scale vats (Gallino, 1994; Meinardi et al., 2002), with different final cooking temperatures. An ensemble of three 5-L vats, equipped with a system for heating and cooling vats simultaneously, was used for cheese making. Raw bulk milk, which was supplied by a near dairy plant, was standardized to 2.50% of fat and batch pasteurized at 65°C for 20 min. After cooling to 33°C, $CaCl_2$ was added to a final concentration of 0.02% (wt/vol). Starter consisted of selected strains of *Lactobacillus helveticus* cultured in sterile whey (Candioti et al., 2002). A volume of whey starter culture enough to increase the initial milk acidity by 400 mg of lactic acid L^{-1} was added (25 to 30 ml of whey culture L^{-1} of milk). After 10 min of manual stirring, milk-clotting enzyme was added (0.29 mL L^{-1} of adult bovine rennet, 230 international milk clotting units mL^{-1}, Naturen, Chr. Hansen, Quilmes, Argentina). After 18 to 20 min, the curd was cut to the adequate grain size (approximately half a rice grain), and the mixture of the curd particles and whey was gently stirred and heated at 0.5°C min^{-1}, until it reached 45°C, to reduce humidity in curd grains. At this point, one of the vats was removed from the ensemble, whey was drained and discarded, and the curd was molded to obtain a low-temperature cooked cheese (**LT**). Meanwhile, heating continued more rapidly in the 2 other vats (1°C min^{-1}). When temperature reached 52°C, the second vat was taken out, and we proceeded as before to obtain control temperature cooked cheese (**CT**). The cooking step continued in the third vat until temperature reached 60°C. At this time we proceeded as before to obtain high-temperature cooked cheese (**HT**). The curd was pressed during 24 h, after which it was brined in 20%, wt/vol, pH 5.40 brine at 12°C for 8 h. Ripening was carried out at 12°C and 80% relative humidity for 3 mo.

Two cheese-making experiments were performed on 2 different cheese-making days, with different milk.

Activity of Residual Coagulant

The residual activity of milk-clotting enzyme was determined as described by the method of Zoon et al. (1994) modified by Rampilli et al. (1998). Extracts of the cheeses were obtained by blending 10 g of ground cheese and 20 ml of phosphate buffer with mortar and pestle. The mixture of cheese and buffer was incubated 4 h at 30°C, and then centrifuged at 3000 × *g* for 30 min. Insoluble residue was discarded, while aqueous phase was recovered and constituted the cheese extract.

The final volume of the aqueous phase was measured, and its pH was adjusted to 6.20. In this extracting procedure, we replaced piperazina-N,Nbis[2 etanosolfonato] (PIPES) buffer pH 6.7, used in previous works, by $NaPO_4H_2/Na_2PO_4H$ buffer (pH 6.5).

An aliquot of 2.5 ml of cheese extract was seeded on 10 ml of standard milk substrate, which consisted of low-heat skim milk powder reconstituted in $CaCl_2$ 0.01 *M* at 12%, wt/vol, and adjusted to pH 6.2. The mixture of standard milk-cheese extract was then incubated 30 min at 30°C. After that, proteins were precipitated with TCA 24% with continued stirring to obtain soluble fraction in 8% TCA (Ollieman and Van Riel, 1989; López-Fandiño and Ramos, 1992). The suspension was held under magnetic stirring by 30 min. The supernatant was filtered through fast flow rate filter paper and preserved. Residual milk-clotting enzyme in cheese extracts produced CN macro peptide (**CMP**) from κ-CN in the milk standard substrate, in proportional extent to its activity.

In parallel, 100 μL of a solution 1% (vol/vol) of milk-clotting enzyme was added to other aliquot of 2.5 ml of cheese extract. Rennet was the same used in cheese-making experiments. This extract plus milk-clotting enzyme was seeded in standard milk, incubated at 30°C for 4 h, and treated as described above. The supernatant contained the maximum amount of CMP that could be obtained from the hydrolysis of κ-CN in the standard milk, expressed as CMP_∞.

A third aliquot of 2.5 ml of cheese extract was seeded in the standard milk, but, in this case, the reaction was immediately stopped by the addition of TCA, without incubation, and then the procedure was the same as described above. The supernatant constituted the blank.

Chromatographic separation of CMP was performed according to Ollieman and van Riel (1989), under conditions of a linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid by using HPLC. The mobile phases A and B consisted of water, acetonitrile, and trifluoroacetic acid in the following proportions: 85:15:0.1 and 55:45:0.1%, vol/vol, respectively. Column temperature was set at 30°C, UV detection was performed at 210 nm, and the flow rate maintained at 1 ml min^{-1}. An aliquot of the supernatant was filtered by 0.45μm disposable filter (Millex, Millipore, Sao Paulo, Brazil), and 60 μL was injected into the column, which was a 250 mm × 4.6 mm Aquapore OD-300 C18, 5 nm – 300 A° analytical column (Perkin Elmer, Norwalk, CT).

The proportion of solvent B was modified as follows: starting from 27% B, it increased at 0.18% min^{-1} (27 min), 13.5% min^{-1} (5 min), 0% min^{-1} (5 min) and then returned to starting conditions, which took 5 min. These last setting conditions were maintained for 5 min. The HPLC equipment consisted of a quaternary pump, an on-line degasser and UV/VIS detector, all Series 200, purchased from Perkin Elmer (Perkin Elmer, Norwalk, CT). An interface module connected to a computer was used for acquisition of chromatographic data with the software Turbochrom (Perkin Elmer). With the absolute area of CMP peak in chromatograms, expressed in arbitrary units, the following ratio was obtained:

$$x = \frac{[CMP]_\infty - [CMP]_t}{[CMP]_\infty},$$

where $[CMP]_t$ is represented by the area of the CMP peak in milk seeded with the sample, and $[CMP]_\infty$ is the area of the CMP peak in milk seeded with the sample plus added milk-clotting enzyme. In both cases, the area of CMP originally present in the cheese extract was subtracted, using the blank.

A calibration curve was previously obtained relating –ln x and microliters of milk-clotting enzyme per kilogram of cheese, or international milk clotting units per kilogram of cheese. Every point in calibration curve was obtained by calculating –ln x as described above, but in this case the standard milk was seeded with known amounts of milk-clotting enzyme. The rennet used for calibration curve was the same that in cheese-making experiments (Rampilli et al., 1998).

Retention time of CMP was determined by running a standard, which consisted of 10 mg of standard κ-CN (Sigma, St. Louis, MO) dissolved in 500 μL of phosphate buffer (pH 6.5) and seeded with 500 μL of adult bovine rennet solution (0.1%, vol/vol) (Naturen, Chr. Hansen). The mixture was incubated 1 h at 30°C, then 500 μL of TCA 24% was added to obtain TCA 8% soluble fraction. The mixture was centrifuged at 12,000 × *g* and filtered by 0.45-μm disposable filters (Millex, Millipore); 60 μL of supernatant was injected into the column.

We assessed milk-clotting enzyme activity in cheeses at 1, 45, and 90 d of ripening.

Proteolysis Assessment

The hydrolysis of α_{s1}-CN was assessed by liquid chromatography of soluble extract of cheeses at pH 4.6 and electrophoresis of the insoluble CN fraction. Cheese samples (5 g) were homogenized with 15 ml of deionized water using a pestle. The mixture was held at 40°C for 1 h, then 15 min at room temperature. After that, the pH of the mixture was adjusted slowly (20 min) to 4.6 with HCl (1 *M*) under magnetic stirring. The mixture was then centrifuged at 4000 × *g* for 20 min. Supernatant was filtered through fast-flow rate filter paper, and its volume was adjusted to 25 ml. Insoluble fraction was also recovered.

Electrophoresis. The insoluble residue at pH 4.6 was analyzed by electrophoresis on polyacrylamide gel with urea (Urea-PAGE) in a Mini Protean II cube (Bio-Rad Laboratories, CA) by the Andrews method (Andrews, 1983), with a concentration of acrilamide of 7.5% (Hynes et al., 1999). Proteins were stained with Coomasie blue G-250.

Liquid chromatography. The HPLC system was the same used for milk-clotting enzyme activity analysis. Mobile phases were also alike, but acetonitrile gradient was modified as follows: starting from 100% A and 0% B for 5 min, a linear increment of B proportion was started (4% B min^{-1}) until 80% B, this condition was then maintained for 1 min. After that, B proportion decreased linearly to 0% in 1 min, and these last setting conditions were maintained 9 min before new injection. Temperature column was set on 40°C and UV detection was performed at 214 nm.

To identify soluble peptides resultant from the hydrolysis of α_{s1}-CN by milk-clotting enzyme, 10 mg of α_{s1} standard CN (Sigma, St. Louis) were dissolved in 500 μL of phosphate buffer (pH 6.5), and 500 μL of adult bovine rennet solution was added (0.1%, vol/vol). The rennet was the same used throughout all the study (Naturen, Chr. Hansen). The mixture was incubated 2 h at 30°C, then reaction was stopped by immersion in a boiling water bath by 10 min. After cooling at room temperature, the pH was brought to 4.6 by adding 33 μL of acetic acid 33.3%, vol/vol, and 33 μL of sodium acetate 3.33 *M*, and the sample was centrifuged at 12,000 $\times$ *g*. Soluble fraction was filtered by 0.45-μm disposable filters (Millex, Millipore), and 60 μL was injected into the column (Hynes et al., 1999).

To assess α_{s1}-CN hydrolysis, we analyze the following samples: milk; coagulum; curd after cutting and before cooking step; and 1-, 15-, 45- and 90-d-old cheeses.

Principal component analysis (**PCA**) was applied to data obtained from RP-HPLC profiles; PCA was performed with Statgraphic plus 3.0 (Rockville, MD).

RESULTS

Figure 1 shows chromatographic profiles of soluble peptides in TCA 8%, resulting from standard milk incubation with extract of LT, CT, and HT cheeses at 1, 45 and 90 d of ripening (first cheese making, second cheese making pattern was similar and is not shown). Not surprisingly, CMP peak area was higher in LT than in the other cheeses at the beginning of the ripening because the heating of the curd was not strong enough to largely denature coagulant enzyme. However, the most remarkable feature is that CMP peak area increases with ripening time, even in HT cheeses where CMP peak was very small or did not appear at all at the beginning of the ripening.

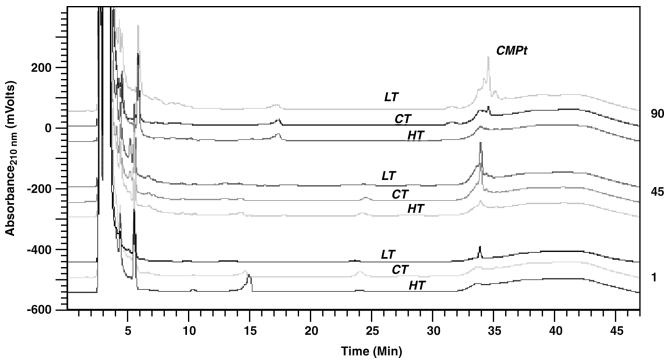

Figure 1. Chromatographic profiles of TCA 8% soluble fraction of standard milk incubated with cheese extract. CMP_t: casein macropeptide released after 30 min of reaction. **LT**, **CT**, and **HT**: cheeses in which cooking step was performed at low (45°C), standard (52°C), or high (60°C) temperature, respectively. 1, 45, 90 d or ripening.

Milk-clotting enzyme activities in cheeses of 1, 45, and 90 d of ripening were calculated from the chromatograms and are shown in Figure 2. As milk clotting activity in different cheese-making days showed some differences, we did not perform statistical analysis. Nevertheless, both experiments were considered reproducible to the extent that a similar pattern was found in both of them (Figure 2a and b). Differences in milk-clotting residual activity between cheese-making days may be the result of small variations in cheese technology, such as heating rate, moisture in the curd grain, and rate of pH decreasing. Even if cheese makings were comparable from a technological point of view, and no differences were detected by proximate analysis of cheeses (results not shown), small variations could result in the observed changes in the enzyme retention and activity.

Milk-clotting enzyme activity was very low or undetectable in cheeses in which curd was cooked at 60°C (HT) at the beginning of the ripening process. However, it showed a considerable increase during ripening, until final values of about 60 μL of milk-clotting enzyme per kilogram. One-day-old control (CT) cheeses also showed low values of residual rennet activity that dramatically increase during the first stage of ripening. The milk clotting activity decreased after 45 d, but the final value was at least 4 times higher than the initial one. Cheeses treated at low cooking temperatures (LT) had higher residual milk clotting activity than HT and CT at the beginning of the ripening, and in this case there also was an increase during the first stage of ripening followed by a decrease. As in the other cheeses, the final rennet activity was much higher than that detected in the unripened curd, although the increase was not as

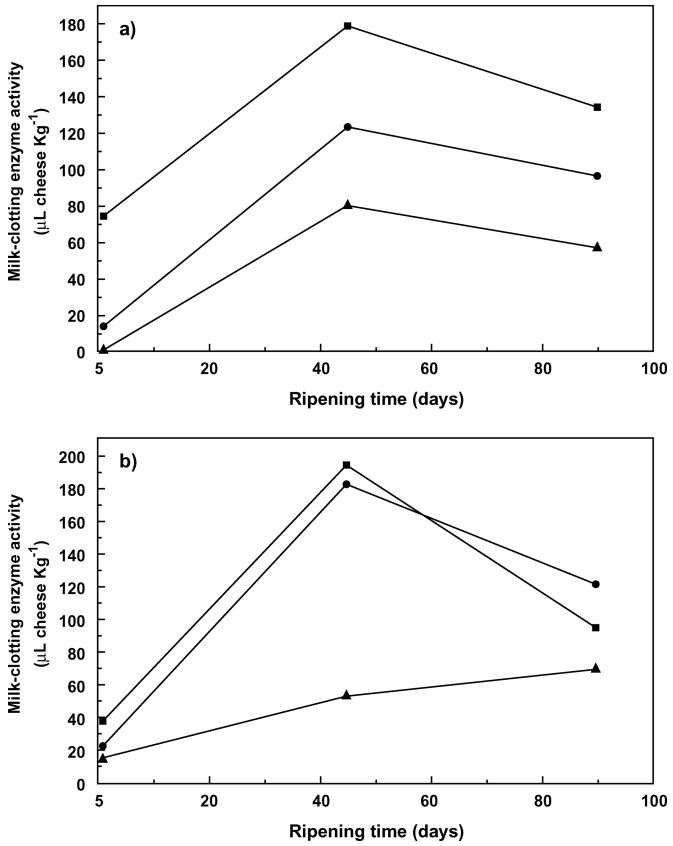

Figure 2. Evolution of residual milk-clotting enzyme activity during ripening of cheeses, expressed as microliters of adult bovine coagulant per kilogram of cheese. Cheeses in which cooking step was performed at low temperature (45°C) –·–,: standard temperature (52°C) –•–, or high temperature (60°C) –▲–.

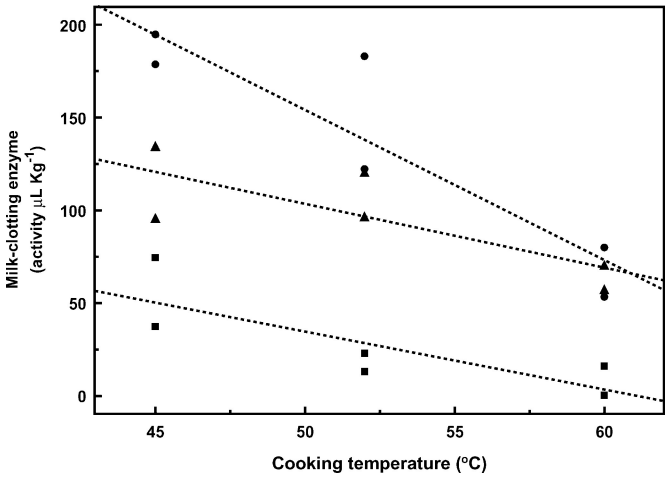

Figure 3. Correlation of milk-clotting enzyme activity and curd cooking temperature, for all cheeses at 1 (–·–), 45 (–•–), and 90 (–▲–) d of ripening. Dotted lines: linear correlation fit. Correlation coefficient R = –0.80701, P = 0.05227 (1-d-old cheeses); R = –0.79793, P = 0.05712 (45-d-old cheeses); R = –0.91013, P = 0.01175 (90-d-old cheeses).

pronounced. Control cheeses showed the highest increase in milk-clotting activity, so that in the second cheese making we found very similar values for CT and LT cheeses at both 45 and 90 d of ripening, even though rennet activity in LT cheese had initially doubled that of CT cheese (Figure 2b). In the first cheese making, however, milk-clotting activity in CT cheese was always lower than in LT cheese, although the increase was slightly steeper during the first phase of ripening and the decrease was slower during the second step, resulting in similar final values for CT and LT (Figure 2a).

These results clearly showed a net increase in residual milk clotting activity during ripening, which has been detected for the three types of cheese, but seems to be most important in control ones.

Taking into account this tendency, we correlated residual milk-clotting activity to cooking temperature for 1-, 45- and 90-d-old cheeses separately. Fair linear correlation functions were obtained, which moved to higher values of residual rennet activity with ripening time, suggesting the reactivation of the enzyme during ripening (Figure 3).

Urea-PAGE patterns of CN fractions of cheeses at 1, 15, 45 and 90 d of ripening are shown in Figure 4 for first cheese making (second cheese-making pattern was similar and is not shown). Hydrolysis of α_{s1}-CN and consequent appearance of peptide α_{s1}-I were detected in 1-d-old LT cheeses, but were not evidenced in CT and HT cheeses. After 15 d of ripening, α_{s1}-I band increased in LT cheeses and it was first detected in CT cheeses, whereas in 45-d-old cheeses α_{s1}-I band intensity was similar both in LT and CT. At 90 d of ripening, α_{s1}-CN band seemed weaker in LT cheeses, although no further increase in α_{s1}-I band was noticeable, probably because this peptide was degraded in turn. We did not detect the presence of α_{s1}-I in HT cheeses.

Peptide profiles of soluble fraction at pH 4.6 were rather simple at the beginning of the ripening process, but they become more complex later. We run the standard of hydrolyzed α_{s1}-CN all the 6 chromatograms to check retention time, which was repeatable as long as the same mobile phases were used and column temperature was standardized. Nevertheless, it should be taken into account that soluble cheese extract are complex mixtures and several peptides have probably coeluted with α_{s1}-related products. Figure 5 shows peptide profiles of soluble cheese extract and the standard for first cheese making (second cheese-making pattern was similar and is not shown).

We did not detect α_{s1} (f1–23) in milk, coagulum, or curd after cut (results not shown), but a peak with the

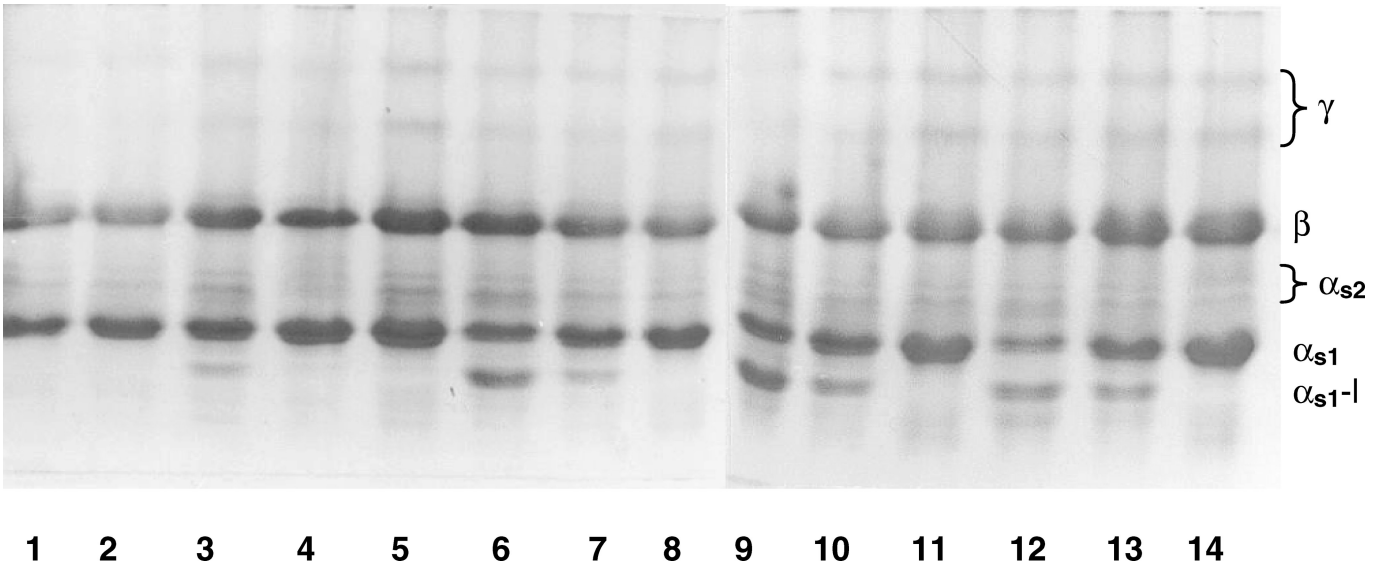

Figure 4. Electrophoresis of insoluble cheese fractions at pH 4.6. Lane 1: coagulum; 2: curd after cut and before cooking, 3–5: 1-d-old cheeses in which cooking step was performed at low temperature (45°C), standard temperature (52°C), or high temperature (60°C), respectively; 6–8: the same cheeses in identical order, at 15 d of ripening; 9–11: the same cheeses in identical order, at 45 d of ripening; 12–14: the same cheeses in identical order, at 90 d of ripening.

same retention time appeared in 1-d-old LT cheeses and in all cheeses later during ripening. Figure 6 shows the evolution of absolute area in arbitrary units for this peak. It increased in the first stage of ripening in LT and CT cheeses, and then decreased. This tendency seems opposite to electrophoresis results; nevertheless the chromatographic technique was more sensitive and detected better the hydrolysis of α_{s1}-CN early in ripening. Afterwards, α_{s1}(f1–23) was probably hydrolyzed and did not cumulate in cheese as α_{s1}-I did. The HT cheeses showed a small peak at the same retention time during all ripening.

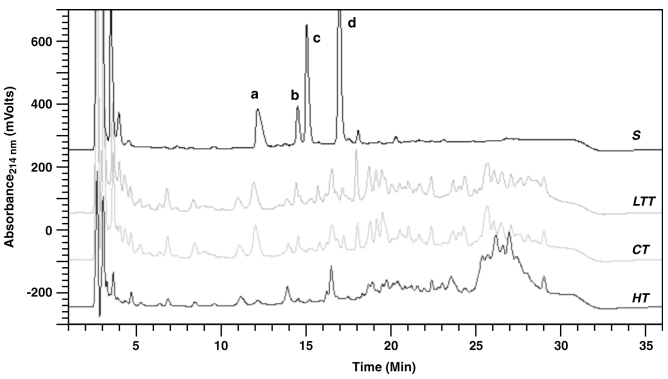

Figure 5. Chromatographic profiles of 15-d-old cheeses soluble fraction at pH 4.6. **LT**, **CT**, and **HT**: cheeses in which cooking step was performed at low (45°C), standard (52°C), or high (60°) temperature, respectively. **a**, **b**, **c**, **d**: peaks whose retention times coincide with those resultant from hydrolysis of standard α_{s1} casein by chymosin (***S***).

To minimize the error that may be caused for a mistaken identification of α_{s1} (f1–23), we pick up 4 peaks from the chromatograms, whose retention times coincide with the peptides in the standard, and used them as variables of a PCA. We performed PCA with standardization of the variables to a mean of zero, and their original variances (covariance matrix), and we retained the first 2 principal components (**PC**) (Pripp et al., 2000). The PC1 represented mostly peaks identified as ***a*** and ***c*** (See Figure 5), whereas PC2 represented ***c*** and ***d***. The biplot shows a group of cheeses with low levels of these peptides, including 1-d-old cheeses and CT cheeses of 45 and 90 d of ripening. The LT cheeses showed high levels of the peptides at the beginning, and then decreased. The proteolysis in HT cheeses seems to have increased during ripening (Figure 7).

Because only 2 trials were taken, we consider the results obtained in the present study as preliminary. Further investigation both on cheese slurries and cheese-making experiments is scheduled in our laboratory to confirm our findings.

DISCUSSION

This study shows that residual milk-clotting enzyme can contribute to proteolysis during ripening of hard cheeses, apparently to a higher extent than it was believed so far.

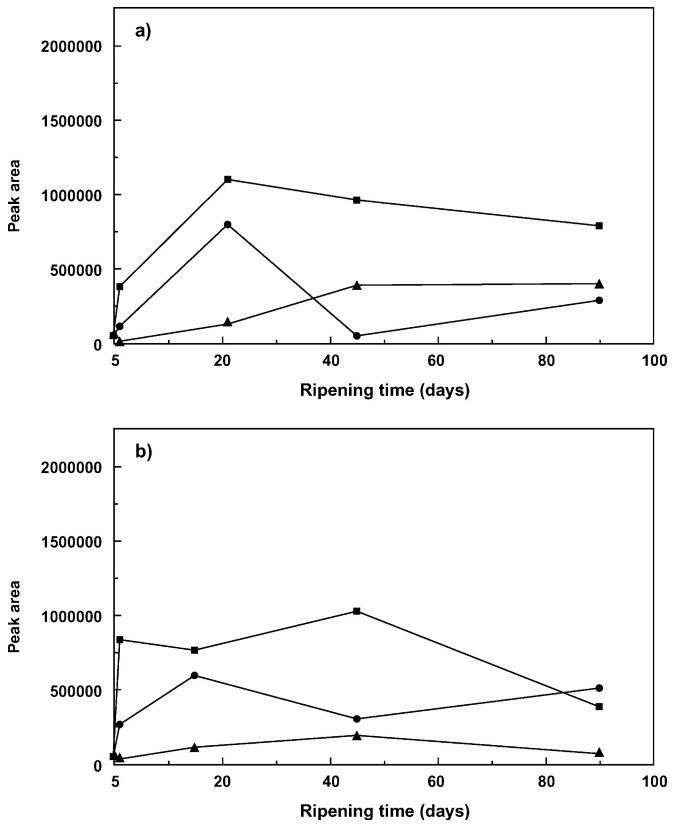

Figure 6. Absolute area of a peak, presumptively identified as α_{s1}(f1–23), in chromatographic profiles of cheese fraction soluble at pH 4.6. Cheeses in which cooking step was performed at low temperature (45°C) –•–,: standard temperature (52°C) –●–, or high temperature (60°C) –▲–.

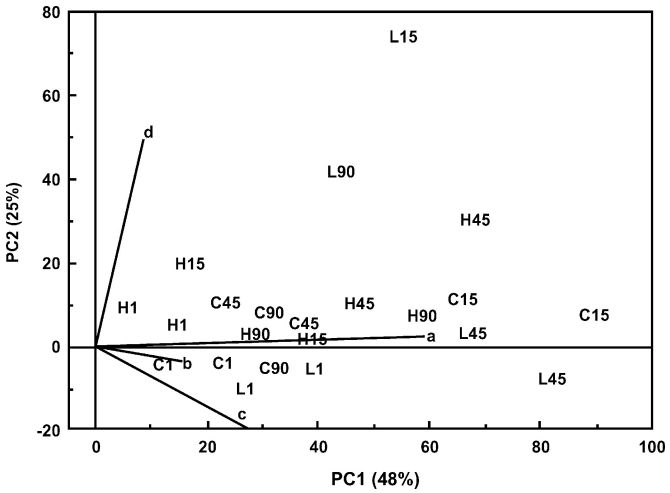

Figure 7. Biplot representation of peptide profiles data after a principal component analysis (PCA). **a**, **b**, **c**, and **d**: Peaks on chromatographic profiles selected as PCA variables. **L1**, **L15**, **L45**, and **L90**: cheeses in which cooking step was performed at low temperature (45°C) at 1, 45 and 90 d or ripening, respectively. **C1**, **C15**, **C45**, and **C90**: cheeses in which cooking step was performed at control temperature (52°C) at 1, 45, and 90 d or ripening, respectively. **H1**, **H15**, **H45** and **H90**: cheeses in which cooking step was performed at control temperature (60°C) at 1, 45, and 90 d or ripening, respectively.

Prolonged heating can inactivate all milk-clotting enzymes, and a coagulant containing chymosin and pepsin has been reported to be inactivated in skim milk in 23.6 min at 60°C (Walsh and Xioshan Li, 2000). Nevertheless, such strong heat treatments are rare in cheese technology, where the cooking step may be prolonged (30 min to 1 h in Swiss and Parmigiano Reggiano cheeses), but it is usually performed at lower temperatures (56°C maximum).

Even though none or very weak milk clotting activity in hard cheese have been reported (Rampilli et al., 1998), our results showed that residual rennet activity can increase during ripening from undetectable levels to considerable values. This is probably because the unfolding of the enzyme is a reversible process, and it slowly renatures when stressing environmental conditions disappear after the cooking step is over (Lehninger, 1995).

It may be opposed to that hypothesis that the differences in rennet activity among differently aged cheeses could be caused by the extraction procedure, which would be more efficient in ripened cheeses than in young ones. Nevertheless, we found it difficult that ripening can modify the release of rennet into the cheese extract to such a great extent. Even if that were true, the fact still remains that higher amounts of rennet than initially believed are present in the cheese. In a study about a new method to determine residual coagulant activity in cheese, Hurley et al. (1999) reported values that were on average 3 times higher in commercial Cheddar cheeses than in the Cheddar curd made at the laboratory and used to develop the method. The authors did not discuss the difference, but their results showed the same tendency as ours and may indicate that residual rennet regain activity even in semi-hard cheeses. Conversely, Rampilli et al. (1998) found an important decrease in milk clotting activity during the first days of ripening of Pecorino cheese, that they attributed to whey drawing off and salting effect. The difference with our results may be because we took our first sample when cheese was already salted and whey did not draw off any more, so that we did not detect any decrease in rennet activity. After few days of ripening, Rampilli et al. (1998) noticed an increase in milk clotting activity, but slighter than that observed in our results.

Provided that the method we used to determine residual milk-clotting enzyme is actually a test for aspartic protease activity, it could involve cathepsin D as well. However, it has been reported that cathepsin D has

very poor milk clotting activity because it hydrolyzes κ-CN slowly (McSweeney and Sousa, 2000; Hurley et al., 2000b). On the other hand, this indigenous aspartic protease is mainly present in whey (Hurley et al., 2000b) so it is probably lost during cheese making. It is reasonable to think that cathepsin D made little contribution, if any, to the protease activity detected in cheese in the present conditions.

The hydrolysis of α_{s1}-CN evidenced by electrophoresis was well correlated with residual rennet activity: in young cheeses α_{s1}-I was only detected in LT cheeses, but equivalent band intensities were found in 45-d-old cheeses LT and CT, which had similar milk-clotting enzyme activities. In 90-d-old L cheeses, it was evidenced a weakening of α_{s1}-I band, probably because it was available earlier during ripening and underwent further breakdown. Residual milk clotting activity in HT cheeses probably was not enough to produce detectable amounts of α_{s1}-I because it was not found in 90 d of ripening.

Chromatograms of cheese-soluble fraction represent a "picture" of a dynamic system of peptide production and degradation, not only by residual rennet but also by the other present enzymes. The soluble peptide α_{s1}(f1–23), for example, has been signaled as the primary target for lactococcal starter proteinases (Visser, 1993). Principal component analysis showed that soluble peptides in cheese extract were related both to type of cheese and ripening time.

We conclude that residual milk-clotting enzyme is at least partially responsible for the attack of α_{s1}-CN at the peptide bond Phe_{23}-Phe_{24} to give peptides α_{s1}(f1–23) and α_{s1}-I in Reggianito cheeses. This breakdown, that is early produced during the ripening of soft cheeses, seems to be, on the contrary, quite delayed in hard cheese varieties. This may be a consequence of renaturation of milk-clotting enzyme, which may occur during the first stage of cheese ripening.

ACKNOWLEDGMENTS

The authors wish to thank Carlos Zalazar for advising in experimental design and critical reading of the manuscript.

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