Hydrolysis of Bovine and Caprine Caseins by Rennet and Plasmin in Model Systems

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The proteolytic activity of both calf rennet and plasmin combined on bovine and caprine caseins was studied under various pH conditions by electrophoresis. Electrophoretic studies of the pH 4.6-insoluble fraction showed that both enzyme preparations (rennet and plasmin) hydrolyzed caseins, giving the typical breakdown products derived from individual caseins (CN): α_{s1}-I-CN and β-I to β-III from α_{s1}-CN and β-CN, respectively, by rennet action and γ-CNs from β-CN by plasmin action. These breakdown products were more or less evident in the hydrolysates depending on the pH conditions used, and they were subsequently hydrolyzed during the different hydrolysis times. Isolated γ-CNs were resistant to the rennet action, showing that although these breakdown products contain the chymosin-susceptible bonds of β-CN, presumably these bonds are inaccessible in γ-CNs. However, β-CN-derived products by the rennet action (i.e., β-I, etc.) were largely hydrolyzed by the plasmin action to yield peptides in the electrophoretic zone of γ-CNs. Bovine α_{s1}-I-CN and para-κ-CN, the first proteolytic products from α_{s1}-CN and κ-CN, respectively, by rennet, were degraded by plasmin, indicating that these breakdown products are susceptible to further proteolysis by plasmin in solution.

Keywords: *Caseins; rennet; plasmin; hydrolysis*

INTRODUCTION

Cheese ripening involves several biochemical processes including proteolysis, lipolysis, and glycolysis. Proteolysis is the most complex and perhaps important biochemical event during the ripening of most cheese varieties (Fox, 1989).

The rate and extent of proteolysis that occurs during cheese ripening are determined by chemical composition (salt, pH, and moisture) and by proteolytic enzymes present: residual milk-clotting enzymes, milk proteinases (particularly plasmin), and proteases and peptidases from starter and nonstarter bacteria. The concerted action of residual milk-clotting enzymes, milk proteinases, and starter proteinases provides suitable materials for the starter peptidases, which produce small peptides and free amino acids for development of cheese flavor.

Milk-clotting enzymes and plasmin are the major proteolytic enzymes in cheese (Fox et al., 1993). Plasmin is a serine proteinase with trypsin-like properties with a high specificity for peptide bonds involving lysyl residues; it can affect the properties of milk and dairy products during storage and also has a significant effect on many cheese varieties during ripening (Grufferty and Fox, 1988). The role of milk-clotting enzymes in cheese manufacture is well-known, and their mechanism of action is now clearly established (Fox, 1988). Only about 6−10% of the calf rennet added to cheese milk is retained in the cheese curd, but this makes a major, perhaps essential, contribution to proteolysis during ripening and consequently to flavor and texture development.

The proteolytic activity and specificity of rennet and plasmin on isolated bovine and caprine α_{s1}-casein (CN) and β-CN have been investigated (McSweeney et al., 1993a,b; Visser and Slangen, 1977; Groves et al., 1972; Trujillo et al., 1995, 1997, 1998b). In cheese, hydrolysis of casein by rennet differs from that in model protein systems because the pH is reduced to 5.2, the temperature is reduced to 7−15 °C, salt is added to achieve 3−5% salt-in-moisture, and casein is in an aggregated state. However, results obtained by different authors (McSweeney et al., 1994; Trujillo et al., 1996) indicate that most of the breakdown products in cheese detectable by urea−PAGE can be explained by the action of chymosin, plasmin, and pepsin and also indicate that the main cleavage sites (in solution) of chymosin on α_{s1}-CN and of plasmin on β-CN are hydrolyzed in cheese.

The combined action of both rennet and plasmin on casein has received little attention, although it represents a model enzyme system which brings us closer to the complex enzyme system that we find in cheese. A better knowledge of the proteolytic action of rennet enzymes and plasmin on casein and on high molecular weight peptides may help us to understand more clearly the hydrolysis processes that occur in cheese.

The objectives of this study were to investigate the proteolysis of bovine and caprine caseins in solution by rennet and plasmin under different proteolytic conditions with observations on cheese ripening and to determine whether the primary breakdown products

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arising from β-, α_{s1}-, and κ-CNs by rennet (i.e., β-I, α_{s1}-I-CN and para-κ-CN, respectively) and plasmin (γ-CN and N-terminal fragments of β-CN) are subsequently hydrolyzed by plasmin and rennet, respectively.

MATERIALS AND METHODS

Preparation of Caseins. Skim milks were separated by centrifugation at 2500*g* at 30 °C for 30 min. Whole bovine and caprine caseins were prepared by isoelectric precipitation (3.3 M acetate buffer, pH 4.6) from samples of bulk goat and cow skim milks (Experimental Farm of Universitat Autònoma of Barcelona), previously diluted with an equal volume of distilled water, and centrifuged for 20 min at 500*g*. The precipitates were dispersed in distilled water, and three successive precipitation and washes were performed.

Caprine β-CN and bovine α_{s1}-, β-, and κ-CNs were obtained from whole casein according to the batch fractionation method of Wei and Whitney (1985) with diethylaminoethylcellulose fast flow (Sigma, St. Louis, MO).

Enzymes. Commercial calf rennet (Renifor-15/E) containing 780 mg of chymosin (EC 3.4.23.4)/L and 565 mg of pepsin (EC 3.4.23.1)/L was obtained from Lamirsa (Laboratorios Miret S.A., Barcelona, Spain). Calf rennet used in the experiments contained 100 rennet units (RU)/mL. One RU is the amount of enzyme required to coagulate 10 mL of low-heat skim milk powder dissolved in 10 mM $CaCl_2$ in 100 s at 30 °C under the conditions specified by the International Dairy Federation (1987).

Plasmin (fibrinolysin, EC 3.4.21.7) from bovine plasma was obtained from Sigma. One plasmin unit (U) will produce a ΔA_{275} of 1.0 from α-CN in 20 min at pH 7.5 at 37 °C, when perchloric acid soluble products are measured in a volume of 5 mL.

Hydrolysis Conditions. Solutions of whole bovine and caprine caseins (10 mg/mL) in 50 mM sodium acetate buffer, pH 6.6, containing thimerosal (0.2 g/L) to prevent microbial activity were treated with calf rennet and plasmin at levels of 0.1 RU/mL and 0.02 U/mL, respectively. The solutions were then individually adjusted to a range of pH values (3.8, 4.2, 4.6, 5.0, 5.4, 5.8, 6.2, and 6.6) and incubated at 30 °C for 15 h. Other casein solutions were adjusted to pH 6.2 and incubated at 30 °C during different times (1, 2, 4, 6, 15, and 30 h). At the end of each incubation, rennet and plasmin were inactivated by heating (100 °C, 5 min) and the pH was adjusted to 4.6 by adding 3.33 M acetate buffer. After centrifugation (15000*g*, 10 min), the pellets were redissolved in 7.0 M urea, and samples were taken for electrophoresis by urea−PAGE.

Both hydrolysis conditions and incubation times and rennet and plasmin concentrations used were based on preliminary experiments to give a satisfactory hydrolysis. Other special hydrolysis conditions used in the experiments are explained under Results and Discussion.

Electrophoresis. Alkaline urea−PAGE with 0.7 mm spacers was performed with 8.8% T (T = grams of acrylamide plus grams of bisacrylamide/100 mL), 2.3% C (C = grams of bisacrylamide/% T), and 5 M urea at pH 8.9, as described by Carretero et al. (1994). SDS−PAGE was performed following the Laemmli (1970) technique with a resolving gel of 15% T/2.7% C and a stacking gel of 4% T/2.7% C. A low molecular weight calibration kit (Sigma) was used for molecular weight measurements.

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.

Band scanning was carried out with a laser densitometer (LKB 2202 Ultroscan) at $\lambda = 633$ nm, connected to a Hewlett-Packard 3390A integrator used for densitometric readings.

RESULTS AND DISCUSSION

Hydrolyis of Whole Bovine and Caprine Caseins by Rennet and Plasmin. Figure 1A shows the electrophoretic patterns in 15 h of pH 4.6-insoluble fractions

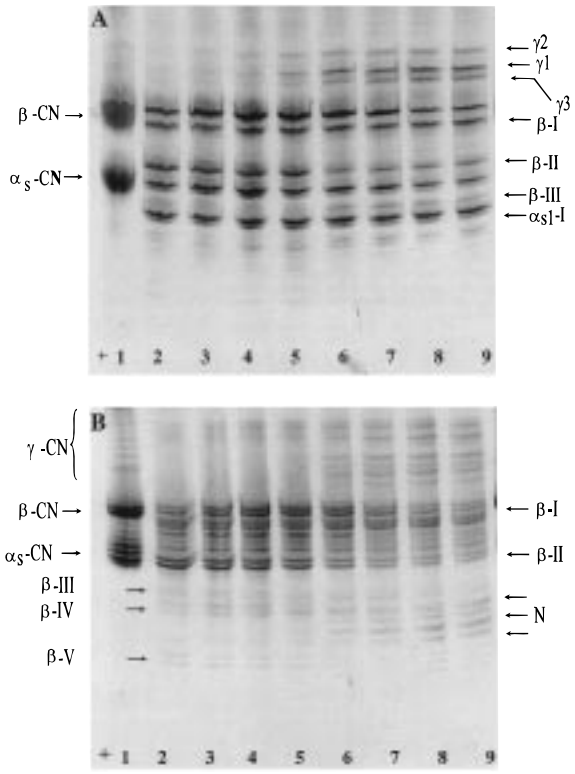

Figure 1. Urea−PAGE electrophoretograms of whole bovine (A) and caprine (B) caseins hydrolyzed by both calf rennet and plasmin at different pH values and 30 °C for 15 h: untreated controls (lane 1) and pH 4.6-insoluble fractions obtained from samples hydrolyzed at pH 3.8, 4.2, 4.6, 5.0, 5.4, 5.8, 6.2, and 6.6 (lanes 2−9), respectively. N, N-terminal fragments from caprine β-casein by the plasmin action.

from hydrolysates obtained by the combined action of both rennet and plasmin on bovine casein at different pH values. The hydrolysis of bovine α_{s1}- and β-CNs by chymosin in solution has been extensively studied (McSweeney et al., 1993a; Visser and Slangen, 1977). In solution, chymosin hydrolyzes bovine α_{s1}-CN at various sites. The primary site of chymosin action on α_{s1}-CN is $Phe_{23}-Phe_{24}$ to produce the α_{s1}-I-CN or α_{s1}-CN (f24−199) and the small peptide α_{s1}-CN (f1−23) (McSweeney et al., 1993a). The preferential breakdown of α_{s1}-CN can be clearly observed by the appearance of a prominent band representing α_{s1}-I-CN on the gel. The small complementary peptide α_{s1}-CN (f1−23) is soluble under the conditions used and, therefore, is not detected on the gel. The α_{s1}-I-CN product has also been identified as primary degradation product in many cow's milk cheeses, for example, Cheddar (Creamer and Richardson, 1974). Cleavage at this site has significance in producing a softening of cheese texture (Creamer and Olson, 1982). As is evident from the electrophoretograms, this α_{s1}-I-CN product can be further degraded by the added enzymes. Subsequent hydrolysis of the α_{s1}-I-CN by rennet in solution leads to the formation of α_{s1}-II-CN, α_{s1}-III-CN, etc. (Mulvihill and Fox, 1977). However, the plasmin action on α_{s1}-I-CN has not been reported.

Calf rennet contains ~10% bovine pepsin, and it has been shown to contribute to proteolysis in Cheddar cheese (McSweeney et al., 1994). Peptides produced from casein by bovine pepsin appear to be generally similar to those produced by chymosin (Fox, 1969), although there are some differences in the specificity

of bovine chymosin and pepsin A on isolated bovine β-CN (Guillou et al., 1991).

In the electrophoretic patterns shown in Figure 1A, breakdown products arising from β-CN by rennet were also visible. Hydrolysis of isolated bovine β-CN by chymosin results in the production of three peptides, namely β-I, β-II, and β-III representing the β-CN (f1−192/189), (f1−163/165/167), and (f1−139/127) fragments, respectively (Visser and Slangen, 1977). In Cheddar-type cheese made with rennet, proteolysis of β-CN lagged behind that of α_{s1}-CN, >50% of the initial amount of β-CN remaining intact at the end of ripening (Fox, 1989). Although most of the proteolytic products of β-CN observed from cheese gel electrophoresis studies are due to plasmin activity, the band correponding to β-I has been described by different authors in many cheese varieties (Carretero et al., 1994; McSweeney et al., 1994).

Plasmin action on bovine β-CN results in the formation of the γ-CN and part of the proteose peptone fraction of milk nitrogen (Gordon et al., 1972). The three well-established cleavage sites in bovine β-CN are Lys_{28}−Lys_{29}, Lys_{105}−His_{106}, and Lys_{107}-Glu_{108} to give γ_1-CN or β-CN (f29−209), γ_2-CN or β-CN (f106−209), and γ_3-CN or β-CN (f108−209), respectively, and their complementary peptides known as proteose peptone components 8-fast or β-CN (f1−28) and 5 or β-CN (f1−105/107), and other fragments as proteose peptone 8-slow or β-CN (f29−105/107). Most recently, other peptides resulting from plasmin action on bovine β-CN have been described (Fox et al., 1995; Trujillo et al., 1997). In Figure 1A, the urea−PAGE patterns showed that hydrolysis of bovine β-CN by plasmin resulted in a number of bands that were identified as γ-CN. The identity of these bands is generally recognized, but the order in which they migrate (γ_3-, γ_1-, and γ_2-CN) has been misinterpreted, as McSweeney et al. (1994) have shown.

The pH of the reaction strongly influenced the rate of casein hydrolysis as the differences of band intensities showed. Typical hydrolysis products from β-CN and α_{s1}-CN by the rennet action (β-I and β-II and α_{s1}-I-CN, respectively) appeared at pH ≤5.0. At pH 5.0 there was evidence of a small quantity of products arising from the hydrolysis of β-CN by plasmin (γ-CN components) too. At pH ≥5.4 all of the previously mentioned products were produced; also formed in a more pronounced manner were the γ-CN components, as was β-III peptide, which was nearly nonexistent at pH ≤5.0. Although plasmin is stable between pH 4 and 9 (Kaminogawa et al., 1972), it had a noticeable activity at pH ≥5; however, rennet had an important activity at all pH levels studied. The β-I and β-II peptides were formed more intensely at pH ≤5.0, whereas β-III was formed at pH ≥5.4. The α_{s1}-I-CN appeared in similar proportions throughout the range of pH values. β- and α_{s1}-CNs reached maximum hydrolysis at pH 6.6 as both enzymes are active at this pH.

In Figure 1B the hydrolysates obtained from caprine casein in identical proteolytic conditions are shown. In general, caprine casein presented electrophoretic patterns that were more complex than those described for bovine casein, with the following characteristics:

1. The zone belonging to the γ-CN was more complex than in the case of the bovine casein, appearing in no less than seven bands, some of which were identified with the homologous components of bovine γ-CN (Trujillo et al., 1997). However, there appeared a series of bands with slower electrophoretic mobility than γ-CN that do not appear in the bovine hydrolysates. The caprine γ-CN components became visible at pH ≥5.4, whereas the bands with low mobility were visible for all pH values, although in a more pronounced form at pH ≥5.4. The fact that these bands with slow electrophoretic mobility also appear at extremely acid pH suggests that they are formed by the action of the rennet enzymes and not by plasmin.

2. β-CN was degraded maximally at pH 3.8 and at pH ≥6.2, whereas it had minimum hydrolysis at pH 4.6.

3. β-I peptide was formed in considerable quantities for all values of pH studied.

4. Below the β-I bands there appeared a series of bands that were clearly observable at pH ≤5.4 and which corresponded to α_{s2}-CN at different phosphorylation levels. These bands appeared with low intensity at pH ≥5.8, suggesting that they are preferentially hydrolyzed by the plasmin action. However, chymosin produces several peptides from caprine α_{s2}-CN in solution, but it appears to be relatively resistant to proteolysis in bacterially ripened cheeses (Trujillo et al., 1996).

5. β-II peptide was formed intensely at pH ≤5.0, although it was also found at the other pH values.

6. A series of electrophoretic bands were observed that could be identified as β-III, β-IV, and β-V (Trujillo et al., 1995); these were perfectly visible at pH ≤5.0. At pH ≥5.4 the electrophoretic pattern that followed the β-II peptides was more complex, due to the partial overlapping of β-III, β-IV, and β-V peptides with the N-terminal fragments arising from the hydrolysis of β-CN by the plasmin action.

At pH ≥5.8, the breakdown products arising fom β-CN by the rennet action were formed less intensely than at the other pH values. It is precisely at this value of pH (≥5.8) that plasmin had a more pronounced action visible because of the presence of caprine γ-CNs. The combined action of rennet and plasmin at this pH value in the hydrolysis of casein would be the cause of the intense hydrolysis observed.

In Figure 2 we can see the electrophoretograms that correspond to the hydrolysis of bovine and caprine caseins, respectively, by the combined action of rennet and plasmin enzymes at pH 6.2 and 30 °C, during different times (1, 2, 4, 6, 15, and 30 h). At this pH value both enzymes show a considerable activity toward the casein. In both cases it was possible to observe the appearance of the typical breakdown products from α_{s1}- and β-CNs by the rennet and plasmin action. The bovine hydrolysates showed the following products: α_{s1}-I, β-I, β-II, β-III, and γ-CNs. These were produced in a progressive manner as the hydrolysis time increased, but they were also hydrolyzed subsequently in other products as was seen in the last period (30 h). In this hydrolysis time both γ-CNs and α_{s1}-I-CN, β-I, β-II, and β-III were decreasing in optical intensity, and some of them were absent, showing that these hydrolysis products are susceptible to being hydrolyzed again by the same enzymes.

The breakdown products β-I, β-II, and γ-CNs visibly appeared in the caprine hydrolysates. β-III and β-IV peptides appeared partially overlapped with the N-terminal fragments arising from β-CN by the plasmin action, as is shown in Figure 1B.

It should be emphasized that in equal proteolytic

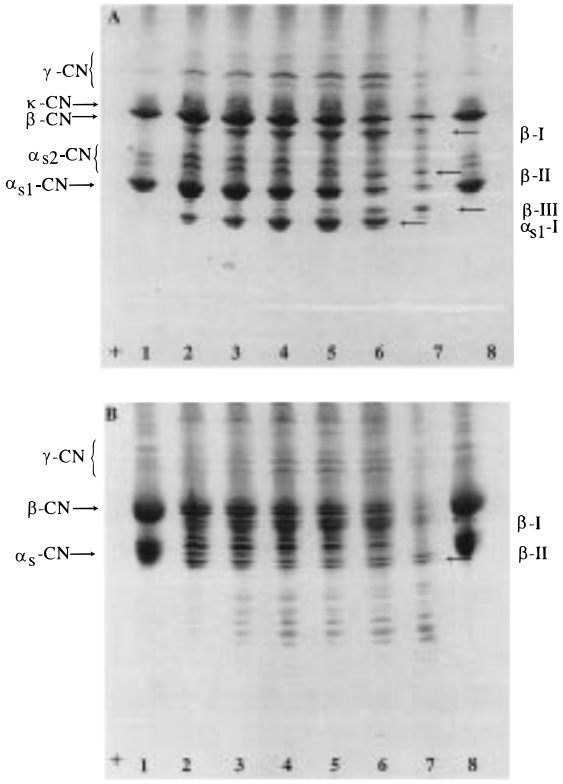

Figure 2. Urea−PAGE electrophoretograms of whole bovine (A) and caprine (B) caseins hydrolyzed by both calf rennet and plasmin at pH 6.2 and 30 °C during different times: untreated control (lanes 1 and 8) and pH 4.6-insoluble fractions obtained from samples hydrolyzed for 1, 2, 4, 6, 15, and 30 h (lanes 2−7), respectively.

conditions caprine α_{s1}-CN disappeared almost completely after 6 h of hydrolysis (which is somewhat confusing to appreciate because of the overlapping of this protein with β-II), whereas in its bovine counterpart traces were observed after 30 h, showing the greater susceptibility of caprine α_{s1}-CN to proteolysis. Moreover, the primary proteolytic product from caprine α_{s1}-CN by the rennet action could not be perceived in the electrophoregrams because it was rapidly hydrolyzed after its formation, whereas in the bovine electrophoretograms α_{s1}-I-CN was visible at all hydrolysis times, with noticeable quantities appearing after 15 h of hydrolysis. It seems that both caprine α_{s1}-CN and its primary breakdown product are more susceptible to the rennet action than their bovine counterparts (Trujillo et al., 1997, 1998a).

Bovine and caprine α_{s2}-CNs proved to be more resistant to hydrolysis, compared to the rest of the casein components, but they were hydrolyzed gradually throughout all time periods, leaving only traces after 30 h. An interesting fact to note is the overlapping of bovine β-II peptide with one of the principal components of α_{s2}-CN, perfectly visible in the electrophoregrams (Figure 2A, lanes 5 and 6).

Hydrolysis of Bovine and Caprine β-CN by the Consecutive Action of Plasmin and Rennet and Vice Versa. Neither the action of rennet on γ-CNs nor the action of plasmin on the breakdown products obtained from β-CN by the rennet action in solution appears to have been studied. γ-CNs contain the chymosin-susceptible bonds of β-CN, and the hydrolysis products from β-CN by the chymosin action (i.e., β-I, β-II, etc.) contain the plasmin-susceptible bonds of β-CN; therefore, these peptides may be hydrolyzed further.

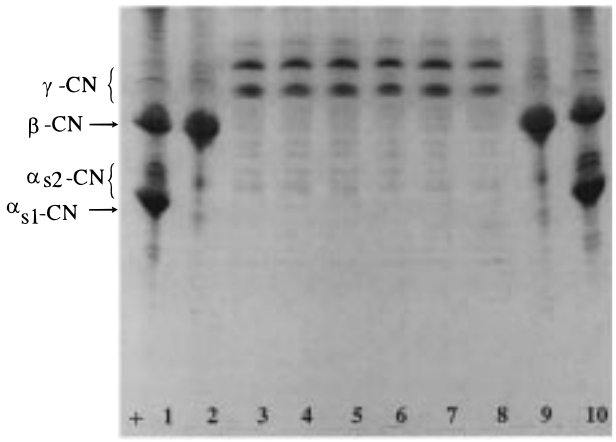

Figure 3. Urea−PAGE electrophoretograms of bovine β-casein hydrolyzed by the consecutive action of plasmin during 3 h (lane 3) at pH 8.0 and subsequently by rennet at pH 6.2 during 1, 2, 4, 6, and 24 h (lanes 4−8), respectively. Also shown are bovine whole casein (lanes 1 and 10) and bovine β-casein (lanes 2 and 9).

To verify this hypothesis, two experiments were carried out. In one, we hydrolyzed solutions of bovine and caprine β-CN (10 mg/mL) with plasmin (0.02 U/mL) at pH 8.0 and 37 °C for 3 h. This hydrolysis time assured the γ-CNs formation from β-CN. When this time was completed, the enzyme was inactivated by heating (100 °C, 10 min) and the pH 4.6-soluble and -insoluble components were separated. The pH 4.6-insoluble fraction was collected by centrifugation and redissolved in 50 mM acetate buffer (pH 6.2), and a rennet solution (0.1 RU/mL) was then added. This was kept at 30 °C during different time periods (0, 1, 2, 4, and 6 h). When these times were completed, the enzyme was inactivated by heating (100 °C, 5 min), and the pH 4.6-insoluble components were submitted to urea−PAGE analysis.

Solutions of bovine and caprine β-CNs (10 mg/mL) were hydrolyzed in the same way with rennet (0.1 RU/mL) at pH 6.2 for 30 h at 30 °C, ensuring the complete hydrolysis of β-CN. After this hydrolysis time, rennet was inactivated by heating and the pH 4.6-insoluble fraction was obtained by centrifugation. Immediately, the precipitate was redissolved in 50 mM Tris-HCl buffer (pH 8.0) and plasmin (0.02 U/mL) was added. Samples were taken after 0, 0.5, 1, 2, and 6 h at 37 °C, plasmin was inactivated, the pH 4.6-insoluble components were separated, and a portion of the sample was analyzed by urea−PAGE.

The results of the first experiments (Figure 3) showed that bovine γ-CNs did not suffer noticeable subsequent hydrolysis by the rennet action in the tested time periods because no new breakdown products appeared. Identical findings were obtained for goat γ-CNs (results not shown). These results are comparable to those obtained in several cheeses (i.e., Gouda, Emmental, Romano, and Gruyère), in which the concentration of γ-CNs increases during the ripening due to the hydrolysis of β-CN by plasmin and remains fairly stable, especially in the final periods of ripening (Farkye and Fox, 1991). Presumably the rennet-susceptible bonds of β-CN are inaccessible in γ-CNs.

Figure 4 show the results of the second experiment. After 30 h of hydrolysis, bovine and caprine β-CNs were degraded by the rennet enzymes (lanes 3). The breakdown products that appeared were essentially β-I,

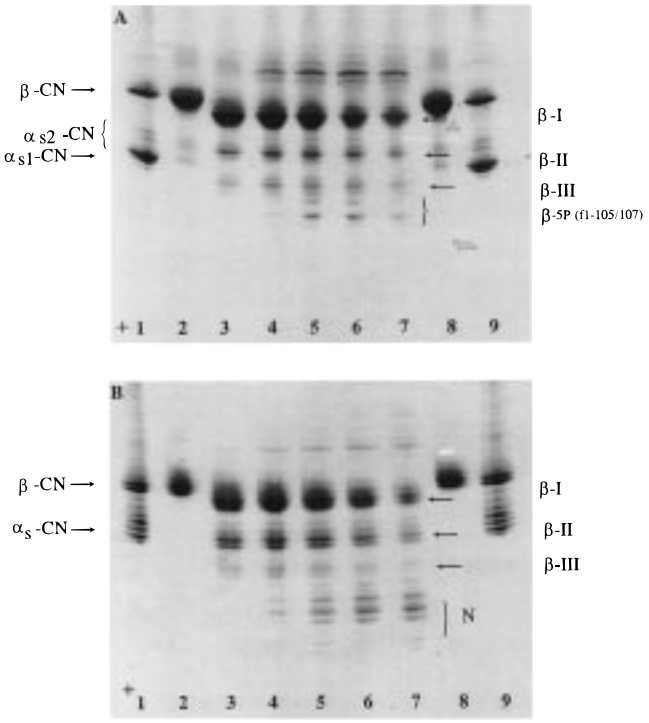

Figure 4. Urea−PAGE electrophoretograms of bovine (A) and caprine (B) *β*-caseins hydrolyzed by the consecutive action of rennet during 30 h (lane 3) at pH 6.2 and subsequently by plasmin at pH 8.0 during 0.5, 1, 2, and 6 h (lanes 4−7), respectively. Also shown are whole casein (lanes 1 and 9) and *β*-casein (lanes 2 and 8). N, N-terminal fragments from caprine *β*-casein by the plasmin action.

although noticeable quantities of *β*-II and *β*-III were also formed in both species. These products were submitted to hydrolysis with plasmin. During the successive times and by the plasmin action, these products arising from *β*-CN were degraded in turn in other products, as shown by the decrease of their respective optical intensities and by the appearance of different new products that were situated in the electrophoretic zone of the *γ*-CNs. In the electrophoregrams pertaining to bovine *β*-CN, no less than six new products appeared, whereas three appeared in caprine hydrolysates. In both cases the majority product that formed had electrophoretic characteristics similar to those of the γ_1-CN component. The higher electrophoretic bands showed a pattern that was almost identical to that of the N-terminal fragments obtained in the hydrolysis of bovine and caprine *β*-CNs by the plasmin action.

Hydrolysis of Bovine α_{s1}- and *κ*-CNs by the Consecutive Action of Rennet and Plasmin. Plasmin is active on all caseins, especially α_{s2}- and *β*-CNs, and α_{s1}- and *κ*-CN are attacked slowly (Grufferty and Fox, 1988). Proteolytic specificity of plasmin on bovine α_{s1}-CN in solution has been determined by McSweeney et al. (1993b) and LeBars et al. (1993). However, although there are a number of potential plasmin cleavage sites in *κ*-CN, plasmin has low activity on this protein and the specificity does not appear to have been determined (Grufferty and Fox, 1988).

In cheese, the primary breakdown product arising from α_{s1}-CN by the rennet action (α_{s1}-I-CN) remains more or less stable until the advanced stages of ripening to be later degraded. The same happens with the primary breakdown product arising from *κ*-CN (para-*κ*-CN). However, there are discrepancies between authors with respect to the resistance or not to para-*κ*-CN hydrolysis during cheese ripening [see Trujillo et al. (1997)]. The proteolytic pathways in cheese involved in these hydrolysis are not known for sure in either case, although the subsequent hydrolysis of the high molecular weight peptides is believed to be primarily the result of proteolytic enzymes from lactic acid bacteria (Fox et al., 1993).

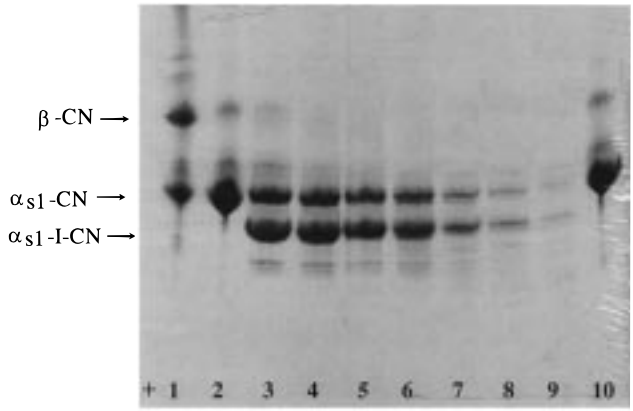

Figure 5. Urea−PAGE electrophoretograms of bovine α_{s1}-casein hydrolyzed by the consecutive action of rennet during 24 h (lane 3) at pH 6.2 and subsequently by plasmin at pH 8.0 during 0.5, 1, 2, 4, 6, and 8 h (lanes 4−9). Also shown are whole bovine casein (lane 1) and α_{s1}-casein (lanes 2 and 10).

Because these two breakdown products contain the majority of the bonds that are susceptible to the plasmin action, they could be hydrolyzed further by this enzyme. However, the action of plasmin on α_{s1}-I-CN and para-*κ*-CN in solution does not appear to have been reported.

Thus, we decided to obtain the primary products α_{s1}-I-CN and para-*κ*-CNs from α_{s1}- and *κ*-CNs, respectively, by rennet action so as to then hydrolyze them with plasmin as follows. Solutions of bovine α_{s1}- and *κ*-CNs (10 mg/mL) in 50 mM sodium acetate buffer (pH 6.2), containing thimerosal (0.2 g/L) to prevent microbial activity, were treated with calf rennet at a level of 0.1 RU/mL for 24 h and 15 min, respectively, at 30 °C. At the end of each incubation, rennet was inactivated by heating (100 °C, 5 min) and the pH adjusted to 4.6. After centrifugation (15000*g*, 10 min), the pellets were redissolved in 50 mM Tris-HCl buffer at pH 8.0 and the solutions were treated with plasmin (0.02 U/mL). Samples were taken periodically (0.5−24 h) and heated (100 °C, 10 min), and the pH was lowered to 4.6. After centrifugation (15000*g*, 10 min), the pellets were taken for electrophoretic separation by SDS−PAGE.

Figure 5 shows the pH 4.6-insoluble fractions from the hydrolysis of bovine α_{s1}-I-CN by the plasmin action. After 24 h of α_{s1}-CN hydrolysis by rennet, there remained noticeable quantities of intact protein (lane 3). In the subsequent periods of hydrolysis, both the residual α_{s1}-CN and α_{s1}-I-CN were hydrolyzed by plasmin and only traces were left after 24 h, indicating that both substrates were susceptible to hydrolysis by plasmin. Primary plasmin cleavage sites found in bovine α_{s1}-CN suggest that it is cleaved initially toward its center (e.g., Lys_{102}−Lys_{103}, Lys_{103}−Tyr_{104}, or Lys_{105}−Val_{106}) and at the Arg_{22}−Phe_{23} bond (McSweeney et al., 1993b). Therefore, most of the primary cleavage sites of α_{s1}-CN could be hydrolyzed in α_{s1}-I-CN by plasmin.

Incubation of *κ*-CN with rennet for 15 min yielded incomplete hydrolysis of *κ*-CN in para-*κ*-CN (Figure 6,

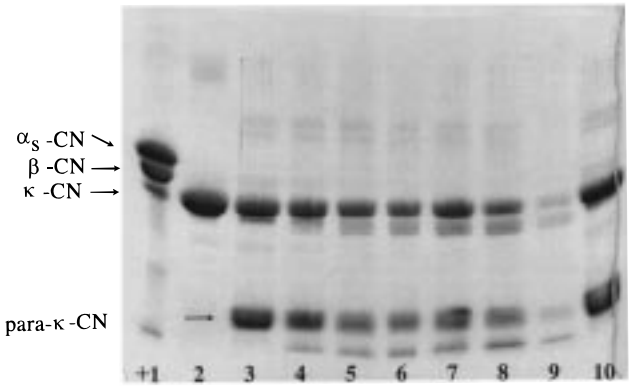

Figure 6. SDS−PAGE electrophoretograms of bovine *κ*-casein hydrolyzed by the consecutive action of rennet during 15 min (lanes 3 and 10) at pH 6.2 and subsequently by plasmin at pH 8.0 during 1, 2, 4, 6, 8, and 24 h (lanes 4−9). Also shown are whole bovine casein (lane 1) and *κ*-casein (lane 2).

lane 3). The complementary peptide *κ*-CN (f106−169) or caseinmacropeptide is soluble under the conditions used and, therefore, is not detected on the gel. When plasmin was added, both substrates (*κ*- and para-*κ*-CNs) were degraded in the following time periods until some of them were absent at 24 h. In this hydrolysis two proteolytic products were observed. One of them had an electrophoretic mobility between those of *κ*- and para-*κ*-CNs and the other had higher mobility than para-*κ*-CN. Both products were formed from isolated *κ*-CN by plasmin, too, and did not originate from para-*κ*-CN. The molecular weights determined for these peptides by SDS−PAGE were 18500 and 10600, respectively. These results show not only that *κ*-CN is susceptible to the plasmin action but also that para-*κ*-CN can be hydrolyzed by plasmin in small peptides, which in the electrophoretic system used were not detected on the gel.

Although plasmin is not as important as chymosin for ripening in many cheese types, it is still a factor to be considered. It appears that the ripening of high-cooked hard cheeses is greatly dependent on the action of this enzyme as well as the proteolytic enzymes of the starter. Plasmin makes a significant contribution to proteolysis in these cheeses in which the coagulant is extensively denatured by the high cooking temperatures.

Results obtained in this work suggest that the majority of primary hydrolysis products observed in PAGE are further hydrolyzed in solution by rennet and/or plasmin. However, this area has been largely unexplored in cheese and should make an interesting area of study.

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