Proteolytic Activities of Suparen and Rennilase on Buffalo, Cow, and Goat Whole Casein and β-Casein

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The proteolytic specificity and activity of *Mucor miehei* protease (Rennilase) and *Endothia parasitica* protease (Suparen) on buffalo, cow, and goat whole casein and β-casein (CN) were studied by analyzing the degradation products. The results suggest that Rennilase hydrolyzes casein of the three species in a manner similar to that of chymosin, resulting in the formation of α_{s1}-I and β-I, -II, -III as initial degradation fragments of α_{s1}- and β-CN. α_{s1}-I was also the initial breakdown product of α_{s1}-CN by Suparen. Contrary to Rennilase, Suparen showed a higher affinity toward β-CN and hydrolyzes β-CN, giving rise to degradation products characterized by mobility lower than that of β-CN. Increasing NaCl concentration (>3%) reduced the proteolysis of β-CN of the three species by Rennilase but not by Suparen. The hydrolysis of α_{s1}-CN and α_{s1}-I by the two enzymes was enhanced in the presence of NaCl.

Keywords: *Proteolysis; Suparen; Rennilase; buffalo, cow, goat caseins*

INTRODUCTION

Microbial milk clotting enzymes, obtained from *Mucor miehei* (Rennilase) and *Endothia parasitica* (Suparen), are widely used in cheese manufacture; their availability minimizes calf rennet supply fluctuations, and, in addition, cheeses made with microbial rennet meet particular religious dietary requirements. The microbial milk clotting enzymes, the proteolytic properties of which resemble closely those of chymosin and pepsin in cheese manufacture, differ in the rates at which they hydrolyze casein (CN) fractions. The specificity of *M. miehei* and *E. parasitica* proteases was shown with cow milk to be broader than that of chymosin and pepsin, and their preferences for individual caseins as substrate are different. *M. miehei* and *E. parasitica* proteases act first on κ-CN, which destabilizes the casein micelles resulting in aggregation, that is, coagulation of milk. The proteolytic activity on cow κ-CN has been shown to be in the order of *M. miehei* protease > calf rennet > *E. parasitica* protease. Calf rennet attacks mainly α_{s1}-CN and *E. parasitica* protease mainly β-CN, whereas *M. miehei* protease hydrolyzes both casein fractions to about the same degree (Vanderpoorten and Weckx, 1972; Tam and Whitaker, 1972; Edwards and Kosokowski, 1969; El-Shibiny and El-Salam, 1976).

Limited data are available on the comparison of proteolytic activity and specificity of microbial milk clotting enzymes on buffalo, cow, and goat casein. The objective of the present investigation was to compare the proteolytic actions of *M. miehei* and *E. parasitica* proteases on buffalo, cow, and goat whole casein and β-CN in model solution under the same conditions. It is the continuation of a previous work with chymosin and porcine pepsin (Awad et al., 1998).

MATERIALS AND METHODS

Milk Clotting Enzymes. Rennilase (*M. miehei* protease) (Novo Nordisk, Bagsvaerd Denmark) and Suparen (*E. parasitica* protease) (Pfizer AG, Zurich) were used without further purification.

Casein. Hammarsten cow casein was obtained from Merck. Whole buffalo and goat caseins were prepared by isoelectric precipitation (pH 4.6) of raw buffalo and goat skim milk from the herds of the Faculty of Agriculture, Alexandria University, Egypt. Pure β-CN of each species was obtained by further purifying the crude β-CN on reverse phase high-performance liquid chromatography (RP-HPLC) as described previously (Awad et al., 1998).

Incubation of Model Solutions. To compare the proteolytic properties of Suparen and Rennilase with those of chymosin and porcine pepsin on buffalo, cow, and goat casein, all of the experiments in the present study were carried out under the same conditions used in an earlier study in which the proteolytic activity of chymosin and pepsin on the caseins was investigated. The results of the study with chymosin and pepsin are summarized in a previous paper (Awad et al., 1998).

Briefly, the incubation mixture contained 2% casein (w/v) and milk clotting enzyme at the level of 0.5 rennet unit (RU)/mL in a 0.2 M sodium acetate buffer of pH 5.4. One RU is the amount of enzyme required to coagulate 1.2 g of low-heat skim milk powder in 10 mL of 10 mM $CaCl_2$ in 100 s at 30 °C (International Dairy Federation, 1987). The incubation was carried out at 30 °C for up to 8 h. Aliquots were withdrawn from the incubation mixtures at intervals for urea−polyacrylamide gel electrophoresis (urea−PAGE) analysis. After the pH had been adjusted to 4.6 with 1 N HCl, the aliquot was centrifuged at 15000*g* for 10 min at 4 °C, and the supernatant containing the peptides was lyophilized and stored at −28 °C until analyzed by HPLC. The reaction was terminated by heating at 90 °C for 10 min.

Urea−PAGE. Gel electrophoresis was performed according to the method of Andrews (1983).

RP-HPLC. The profile of the casein peptide extracts was visualized on a Merck-Hitachi HPLC system L-6200 with a

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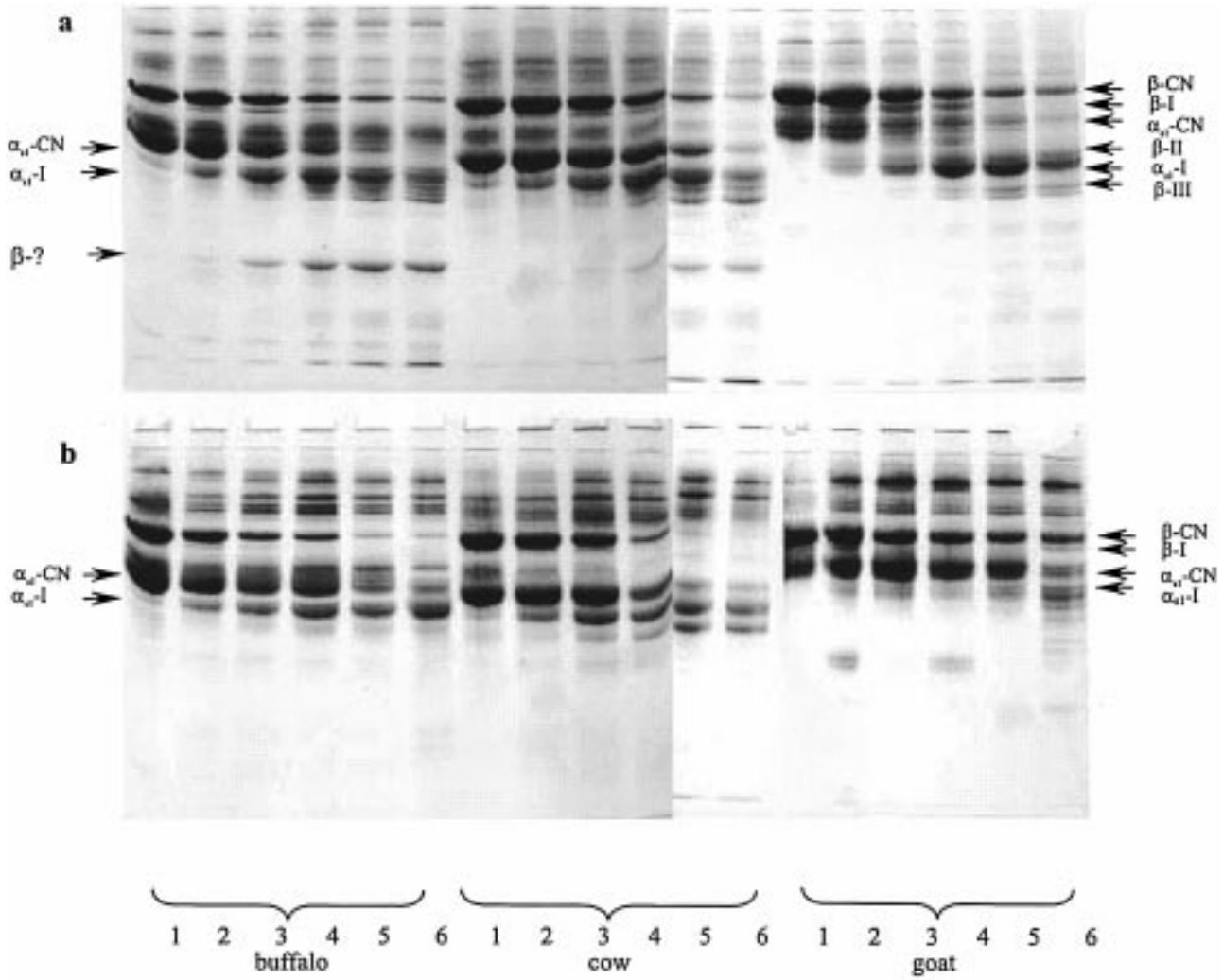

Figure 1. Urea−PAGE of buffalo, cow, and goat whole casein hydrolysis by Rennilase (a) and Suparen (b) at pH 5.4 and 30 °C: untreated controls (lane 1); samples hydrolyzed for 3, 30, 150, 240, and 480 min (lanes 2−6), respectively.

C_{18}-Lichrospher analytical column (250 × 4.6 mm) by using the conditions described previously (Awad et al., 1998).

RESULTS AND DISCUSSION

Proteolysis by Rennilase. The results of the hydrolysis of buffalo, cow, and goat whole caseins and *β*-CNs by Suparen and Rennilase are shown in Figures 1 and 2. By the action of Rennilase, large peptides were split off from whole caseins of all three species (Figure 1a). We assigned one fast-moving peptide to be α_{s1}-I, as compared with the electrophoretic mobility of cow α_{s1}-I generated by chymosin. Similar to the observations in the previous study with chymosin and porcine pepsin (Awad et al., 1998), buffalo α_{s1}-CN, as well as its breakdown product α_{s1}-I, had slightly lower mobility than cow α_{s1}-CN and α_{s1}-I. Goat α_{s1}-CN, as well as its α_{s1}-I, had the lowest electrophoretic mobility and both appeared as three bands, which might be the result of genetic polymorphism of α_{s1}-CN (Brignon et al., 1990). However, the difference in electrophoretic mobility is more pronounced for α_{s1}-CN than that for α_{s1}-I of the three species. α_{s1}-I could be found in all cases after 3 min of hydrolysis, and after 8 h of incubation, α_{s1}-CN disappeared completely to yield mainly α_{s1}-I and other smaller breakdown products. Buffalo α_{S1}-I was subsequently degraded into smaller peptides migrating closely to each other. Goat α_{s1}-I was also further degraded, but to a lesser extent compared to cow and buffalo α_{s1}-I, as judged by the staining intensity of the band.

Buffalo, cow, and goat *β*-CNs and their major degradation fragments by Rennilase, although formed in small quantities, had the same mobility (Figure 2a) as those produced by chymosin and pepsin shown in the previous study (Awad et al., 1998). Therefore, we designated these *β*-CN fragments *β*-I, -II, and -III. Goat *β*-CN migrated as two bands, according to Richardson and Creamer (1974); this is due to the defference in phosphorylation. Each of the goat *β*-I, -II, and -III appeared also as double bands, these observations being consistent with our previous observation in the study with chymosin and pepsin (Awad et al., 1998). *β*-I was already present in the 3 min casein hydrolysates. The band of goat *β*-I did not intensify with prolonged incubation, as was the case with chymosin, because goat *β*-I, like *β*-I of buffalo and of cow, was further degraded to *β*-II and *β*-III, as well as to other smaller degradation fragments. A band migrating much faster than α_{s1}-CN present in buffalo and cow casein hydrolysates after 30 min of incubation is likely to originate from *β*-CN because this band was also present in *β*-CN hydrolysates (Figure 2a, *β*-?). Because the staining intensity of the bands generally depends upon the protein concentration, it appeared that buffalo and cow α_{s1}-CNs are

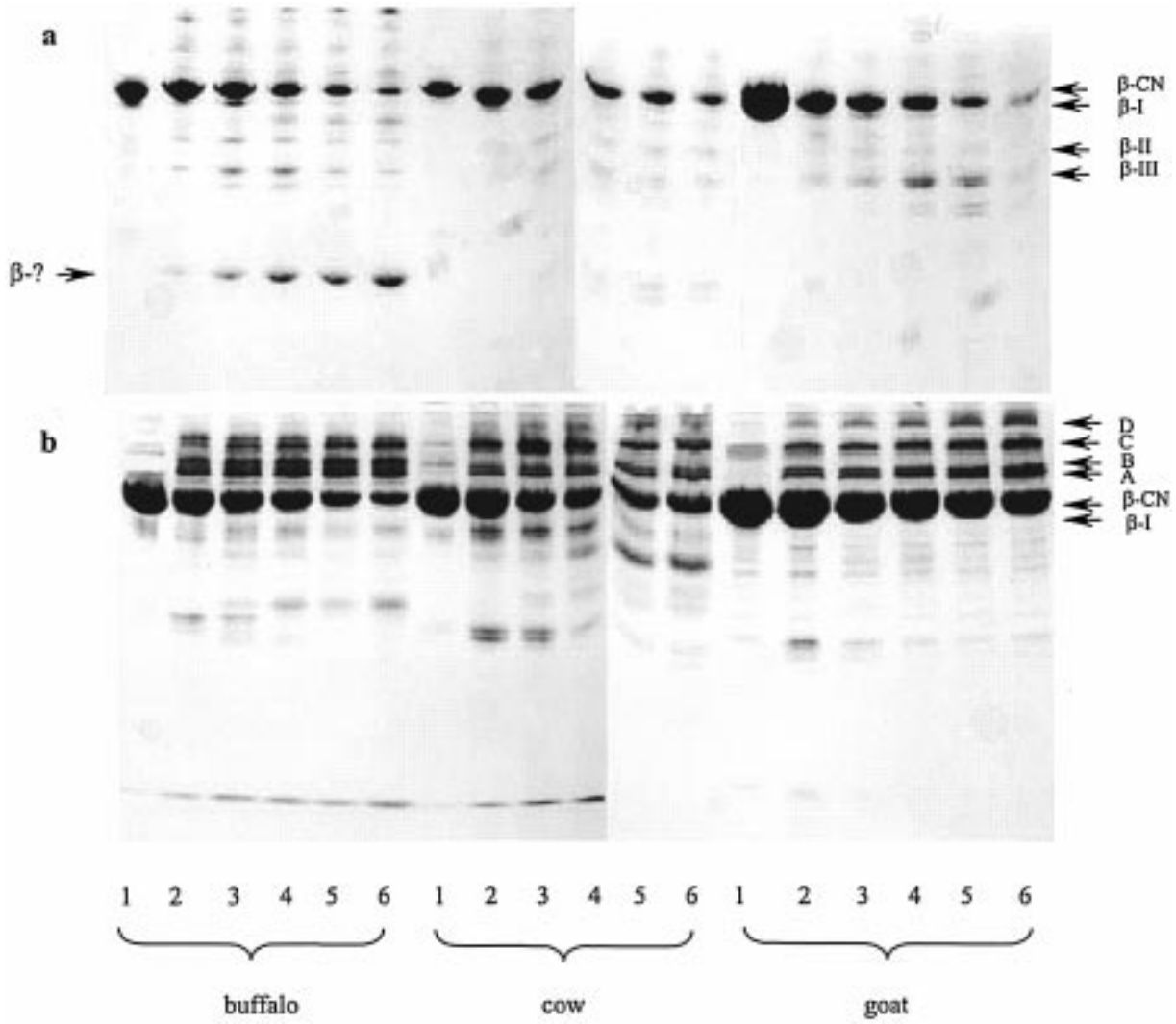

Figure 2. Urea−PAGE of buffalo, cow, and goat *β*-casein hydrolysis by Rennilase (a) and Suparen (b) at pH 5.4 and 30 °C; untreated controls (lane 1); samples hydrolyzed for 3, 30, 150, 240, and 480 min (lanes 2−6), respectively.

hydrolyzed to a greater extent than their *β*-CNs (Figure 1a). This result disagrees with the observation of Edwards and Kosokowski (1969), who reported that *M. miehei* protease hydrolyzes cow α_{s1}-CN and *β*-CN at equal rates. In the case of goat casein, it is difficult to draw any conclusion because of the smaller proportion of α_s-CN in whole casein.

Proteolysis by Suparen. The eletrophoretic pattern of Suparen-produced casein hydrolysates is obviously different from that obtained with Rennilase (Figure 1b). Characteristic for Suparen is that, in contrast to Rennilase and independent of species, the major *β*-CN degradation fragments migrated more slowly than *β*-CN. In addition, differences were found in the electrophoretic mobility of the *β*-CN degradation fragments among the three species (Figure 2b). In buffalo and goat casein hydrolysate, three major degradation components appeared (buffalo, bands A−C; goat, bands A, C, and D) versus only two (bands A and C) in cow casein hydrolysate.

α_{s1}-CN was hydrolyzed by Suparen mainly to α_{s1}-I in buffalo and goat caseins, whereas cow α_{s1}-I was further hydrolyzed. The assignment of the α_{s1}-CN degradation fragment to α_{s1}-I was also based on the comparison of the electrophoretic mobility of the band to cow α_{s1}-I produced by chymosin (Awad et al., 1998). After 30 min of incubation, a band migrating more rapidly than α_{s1}-I appeared in cow casein hydrolysates. If one takes the band intensity as an indicator for quantity, it seemed that buffalo and cow α_{s1}-CNs were more susceptible to Suparen than goat α_{s1}-CN. The degradation of α_{s1}-CN by Suparen proceeded at a lower rate than that of *β*-CN, that is, opposite to the results obtained with Rennilase (Figure 1a).

It has been reported that microbial rennets are more proteolytic than animal rennets (Itoh and Thomasow, 1971; Vanderpoorten and Weckx, 1972; El-Shibiny and El-Salam, 1976). This could be confirmed in the present study when the relative staining intensity was used as a measure as well as when the electrophoretic pattern of casein hydrolysates produced by Rennilase and Suparen was compared with the earlier results obtained with chymosin and pepsin (Awad et al., 1998).

Peptide Profiles of Whole Casein and *β*-CN Hydrolysates. The RP-HPLC chromatograms of the pH 4.6 soluble peptide extracts from whole casein and *β*-CN hydrolyzed by Suparen and Rennilase are shown in Figures 3 and 4. Major differences were found in the chromatographic patterns between the casein hydrolysates obtained with the two enzymes. Breakdown fragments produced by Suparen started to be eluted after 20 min, whereas most of the peptides produced by Rennilase eluted only after 40 min. Differences between Rennilase and Suparen action are also obvious in

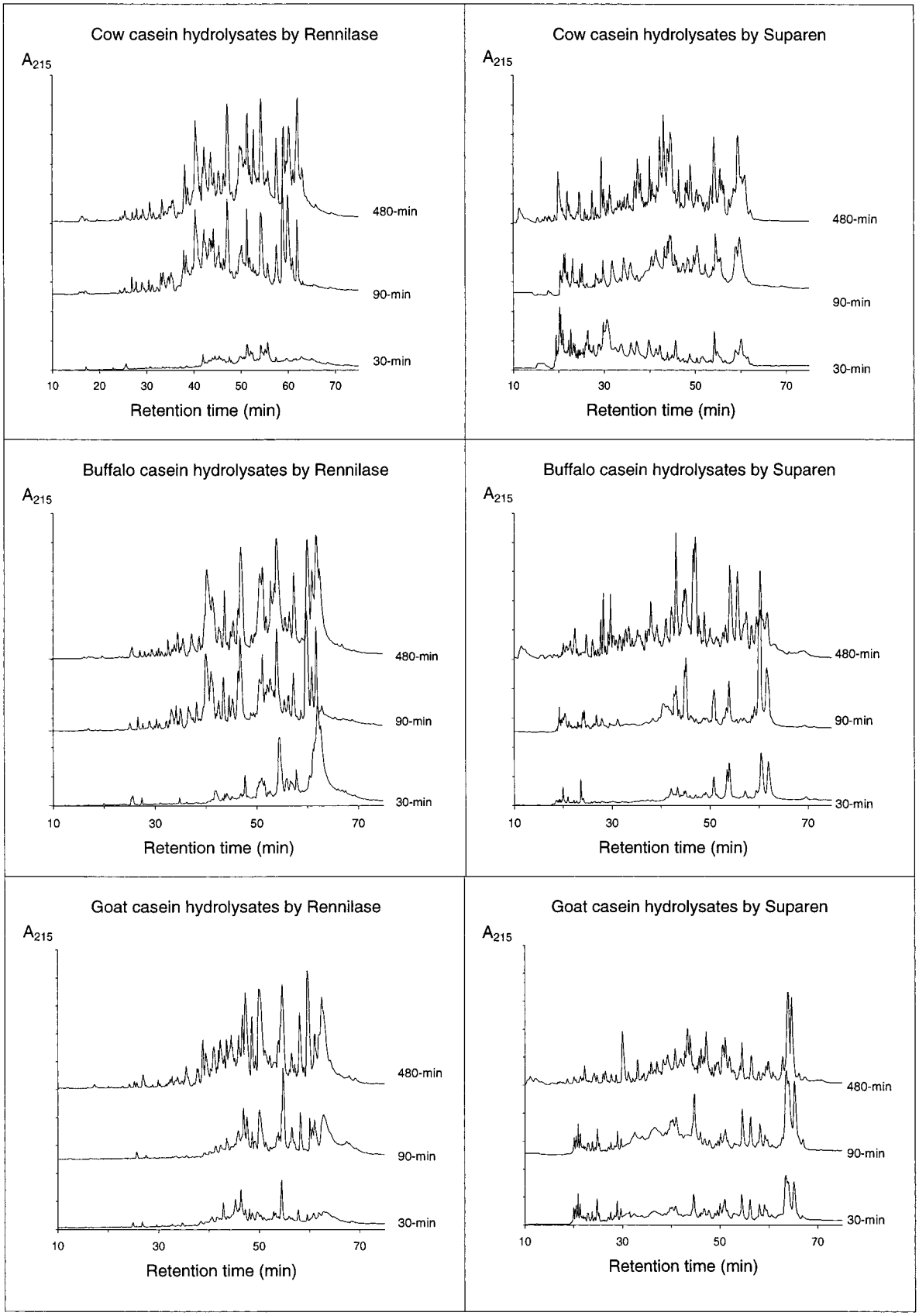

Figure 3. RP-HPLC pattern of hydrolysis products of cow, buffalo, and goat whole casein by Rennilase and Suparen at 30 °C and pH 5.4.

peptide profiles. Suparen was much more proteolytic than Rennilase, as can be concluded from the greater numbers of fractions found in the 30 min hydrolysates. The differences between the β-CN breakdown products by the two enzymes, which is clearly evident in the urea−PAGE, are also reflected in the peptide profiles (Figure 4), confirming the differences in their specificities of hydrolyzing β-CN.

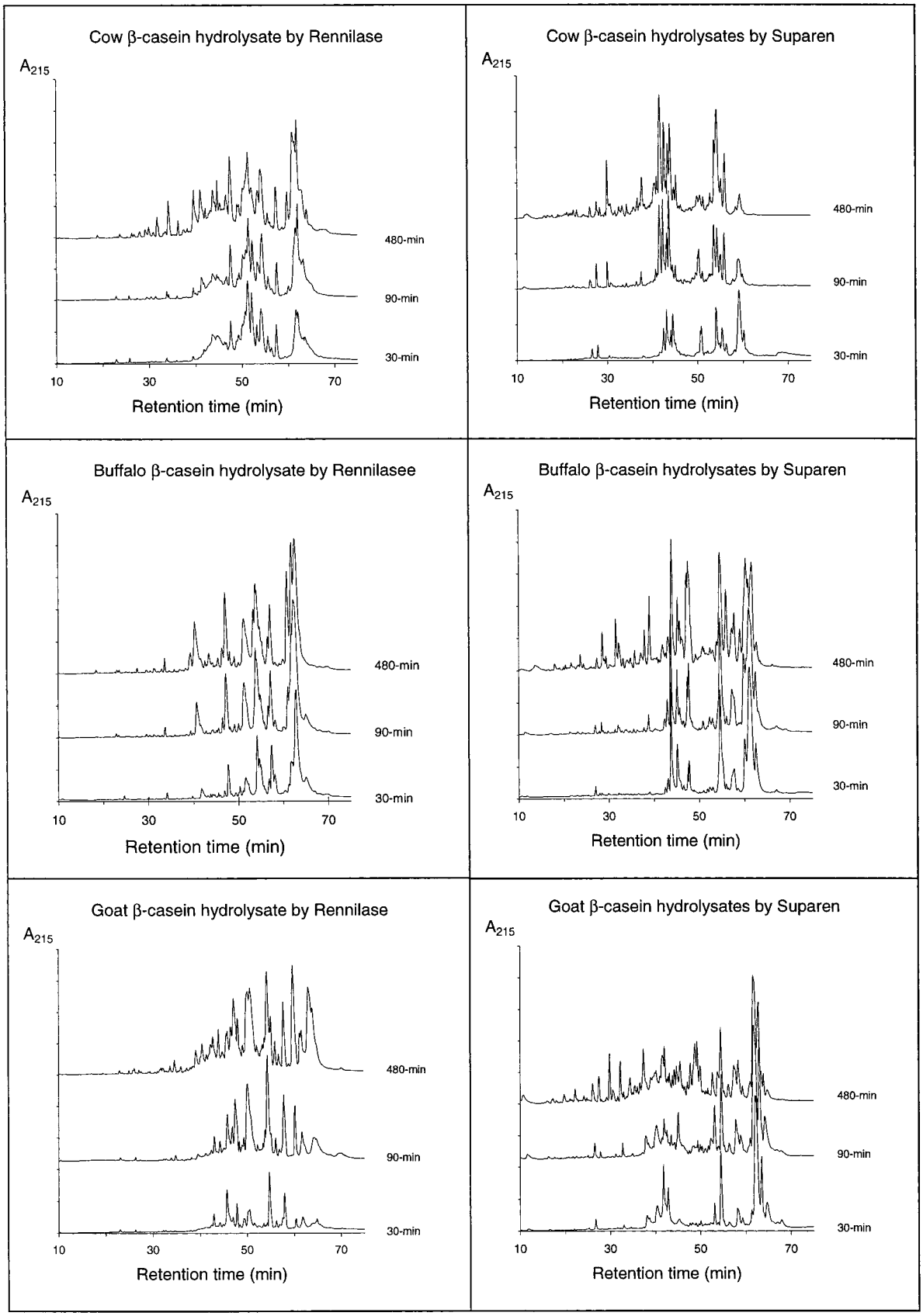

Figure 4. RP-HPLC pattern of hydrolysis products of cow, buffalo, and goat β-casein by Rennilase and Suparen at 30 °C and pH 5.4.

Characteristic chromatographic and electrophoretic patterns of the buffalo, cow, and goat casein hydrolysate obtained for Suparen and for Rennilase illustrate the differences in the proteolytic actions of the two enzymes, as well as differences in the composition of the individual casein (El-Salam and El-Shibiny, 1975; Addeo et al., 1977a,b, 1980; Brignon et al., 1989; Roberts et al., 1992). Taking the peak number in the chromato-

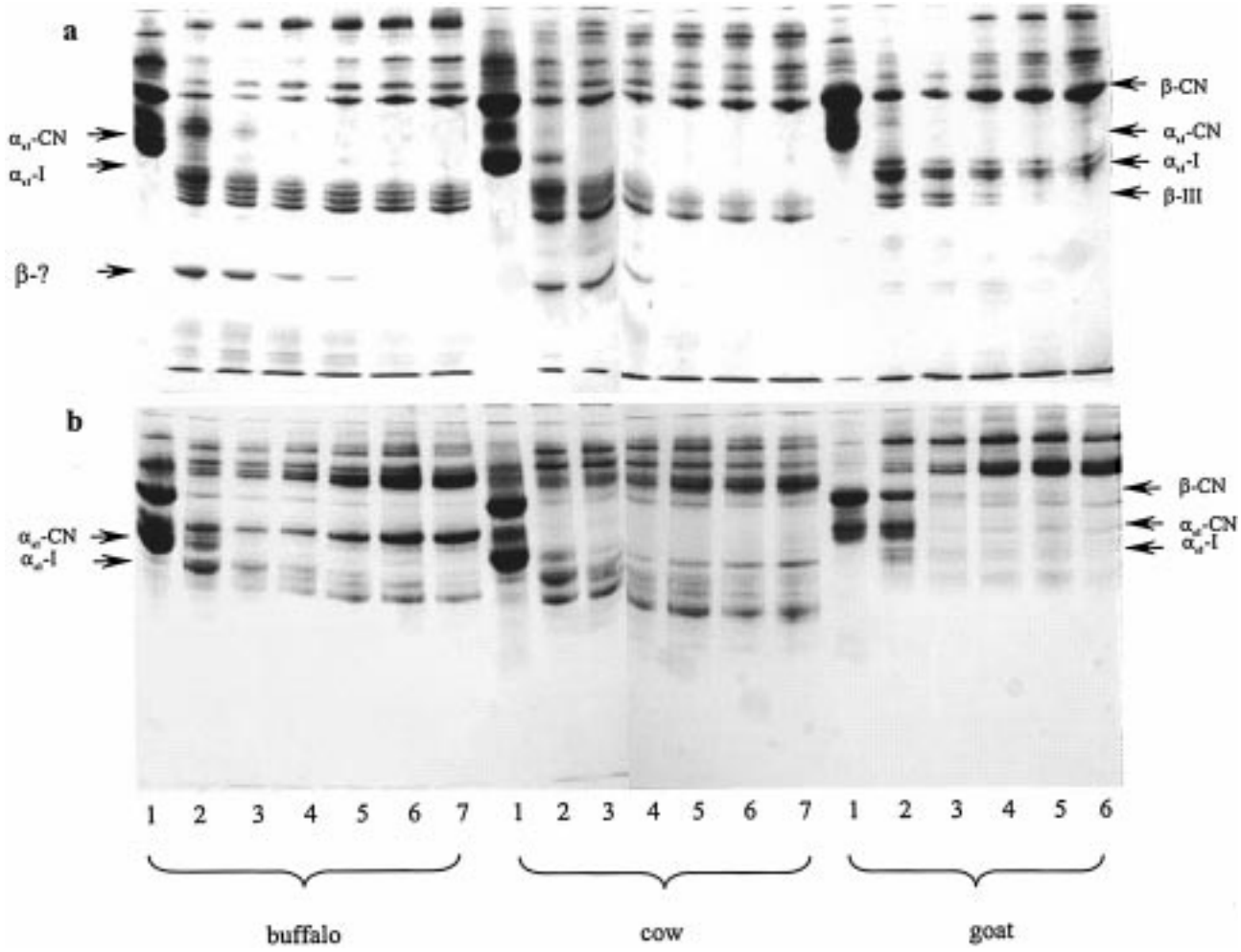

Figure 5. Urea−PAGE of NaCl effect on buffalo, cow, and goat whole casein hydrolysis by Rennilase (a) and Suparen (b) at pH 5.4 and 30 °C for 480 min: untreated controls (lane 1); samples hydrolyzed at 0, 1, 3, 5, 7, and 10% NaCl (lanes 2−7), respectively.

gram as an indicator, it can be concluded that coagulants of microbial origin, especially Suparen, are more proteolytic than coagulants of animal origin, as shown in an earlier work (Awad et al., 1998).

Influence of NaCl on the Proteolysis. The influence of NaCl on the hydrolysis of buffalo, cow, and goat whole casein by Rennilase and Suparen was investigated at pH 5.4 because this is the approximate pH of cheeses at the beginning of ripening (Figures 5 and 6). The hydrolysis of β-CN of the three species by Rennilase was more influenced by NaCl than the hydrolysis of α_{s1}-CN. With 3% of NaCl, the hydrolysis of β-CN was obviously inhibited, as shown in Figure 6. In the presence of 3% of NaCl, the area of the peaks eluted at 60−64 min was decreased. These peaks are most likely originating from β-CN, as compared with Figure 4. The influence of NaCl concentration on the peak area in the HPLC pattern had been investigated in a preliminary test by enriching the NaCl concentration of the pH 4.6 soluble nitrogen fraction of casein or β-CN hydrolyzed at 0% of NaCl to 10%. No influence was detected. Therefore, the decrease of peak area indicates the inhibitory effect of NaCl on casein hydrolysis. In urea−PAGE (Figure 5a), the inhibitory effect of NaCl on degrading β-CN became also evident at a concentration of 3%, as judged by band intensity. With increasing NaCl concentration, the assumed β-CN fragment (β-?) disappeared at the same time bands migrating behind β-CN appeared. In addition, the disappearance of β-III at NaCl concentration >3% could also be observed in goat casein model solution. The hydrolysis of α_{s1}-CN was not inhibited by the presence of up to 10% NaCl (Figure 5a). The fractions eluted before 50 min, most of which originated from α_{s1}-CN, were less affected by the NaCl, confirming the observation on the urea−PAGE. In addition, hydrolysis of α_{s1}-CN and α_{s1}-I by Rennilase was even enhanced in the presence of NaCl at concentrations >1%.

Unlike Rennilase, Suparen continued to degrade β-CN, independent of species, in the presence of NaCl of up to at least 7%. In all three cases, the intensity of the band migrating just behind β-CN became increasingly pronounced with increasing NaCl concentration (Figure 5b). This change was also reflected in the HPLC pattern. At NaCl concentration >3%, late-running fractions were inhibited to different extents. Fractions with retention times >60 min are likely to originate from β-CN, as compared with Figure 4. Hydrolysis of β-CN by Suparen seemed to be unaffected by NaCl (Figure 5), which indicates that the further degradation of β-CN fragments is more sensitive to NaCl. In buffalo casein model solution, the fragment migrating behind α_{s1}-CN was accumulated as the NaCl concentration was increased. Suparen showed also higher proteolytic activity on buffalo and goat α_{s1}-CN in the presence of NaCl. A slight inhibition could be observed on the hydrolysis of cow α_{s1}-CN in the presence of 10% NaCl. The staining intensity of cow α_{s1}-CN indicates that low concentra-

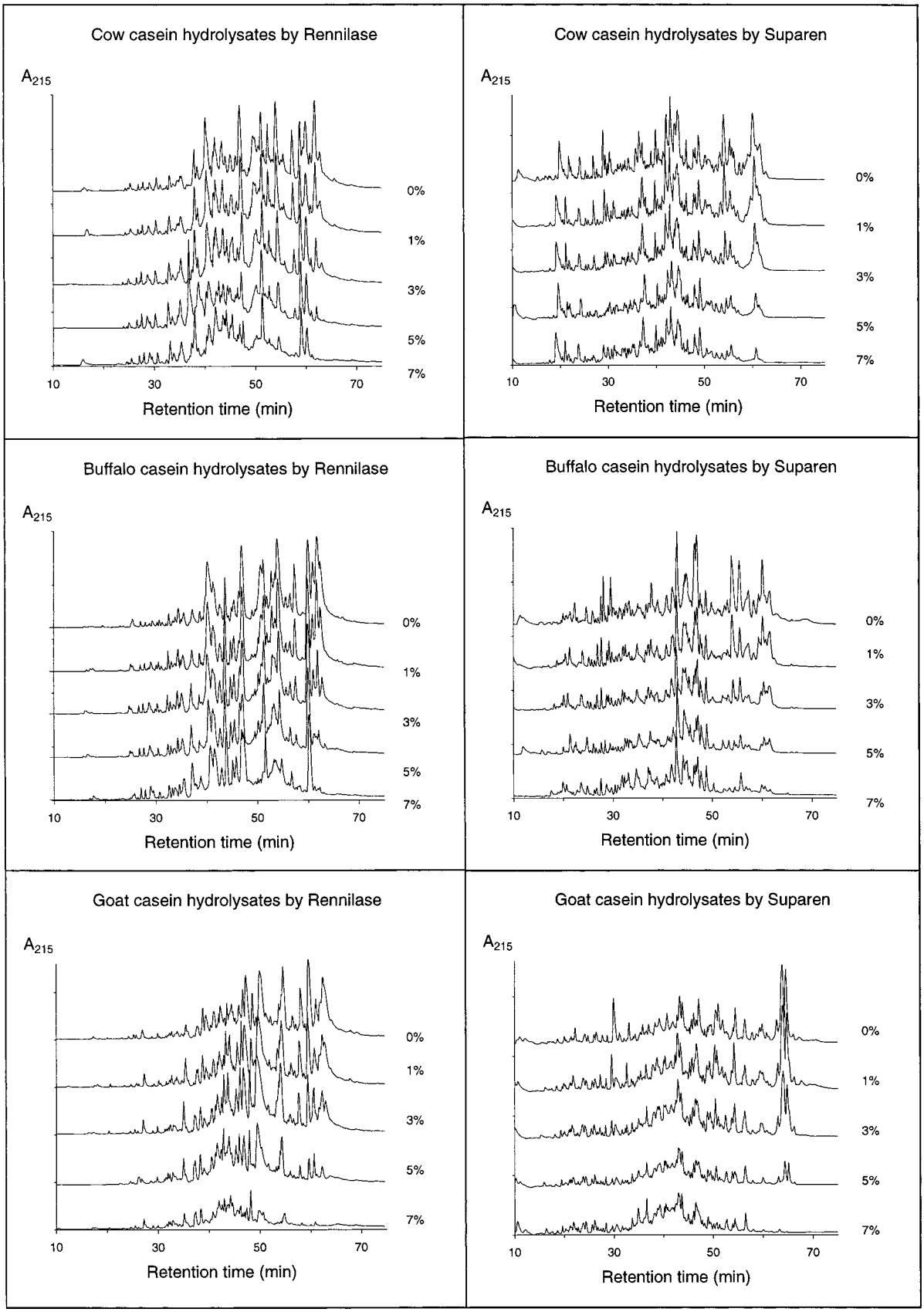

Figure 6. RP-HPLC pattern of hydrolysis products of cow, buffalo, and goat whole casein by Rennilase and Suparen at different NaCl concentrations at pH 5.4 and 30 °C for 480 min.

tions of NaCl (1−5%) promote the hydrolysis of α_{s1}-CN. Enhanced hydrolysis of α_{s1}-I of the three species by Suparen at NaCl concentrations >1% was clearly evident. With calf rennet and bovine pepsin, Fox and Walley (1971) showed that the proteolysis of cow β-CN was significantly reduced by 5% and completely inhibited by 10% NaCl. In contrast, the rate of hydrolysis of cow α_{s1}-CN was found to be maximal in the presence of

5−10% NaCl. Trujillo et al. (1995) demonstrated as well that the proteolysis of goat β-CN by calf rennet is reduced by the addition of 5% NaCl. We have also observed the inhibitory effect of NaCl at a concentration >3% on the hydrolysis of cow, buffalo, and goat β-CN by recombinant chymosin and porcine pepsin (Awad et al., 1998), whereas the hydrolysis of α_{s1}-CN of the three species was not reduced by NaCl. Until now, few results have been published regarding the influence of NaCl on the proteolytic activity of Rennilase and Suparen. In the present study, both PAGE and RP-HPLC results suggest that the hydrolysis of β-CN of the three species by Rennilase can be inhibited by the presence of NaCl at concentrations >3%. Contrary to Rennilase, the hydrolysis of β-CN by Suparen was unaffected in the presence of NaCl with a concentration of up to at least 7%, although further degradation of β-CN fragments by Suparen was shown to be sensitive to NaCl.

Conclusion. The results suggest that Rennilase hydrolyzes buffalo, cow, and goat casein in a manner similar to chymosin, resulting in the formation of α_{s1}-I and β-I, -II, and -III as initial degradation fragments of α_{s1}- and β-CN. α_{s1}-I is also the initial breakdown product of α_{s1}-CN by Suparen. Different from Rennilase, Suparen hydrolyzes β-CN, giving rise to degradation products, some of which are characterized by mobility lower than that of β-CN. Increasing NaCl concentration (>3%) reduces the proteolysis of β-CN of all three species by Rennilase, but not by Suparen. The hydrolysis of α_{s1}-CN and α_{s1}-I by these two enzymes was enhanced in the presence of NaCl. The results indicate that peptide bonds in α_{s1}-CN and β-CN, which are attacked by Rennilase, are also most susceptible to chymosin and pepsin. The characteristic proteolytic activity of Suparen is that it has a different specificity and higher affinity toward β-CN.

ABBREVIATIONS USED

CN, casein; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse phase high-performance liquid chromatography.

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