Proteolytic Activities of Chymosin and Porcine Pepsin on Buffalo, Cow, and Goat Whole and β-Casein Fractions

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The proteolytic specificity and activity of a recombinant chymosin (Maxiren) and porcine pepsin on buffalo, cow, and goat whole casein (CN) and β-CN were studied by analyzing the degradation products. The results suggest that the hydrolysis of whole casein of buffalo and goat by chymosin was similar to that of cow casein resulting in α_{s1}-I and β-I, -II, and -III as degradation fragments of α_{s1}- and β-CN. The exception was goat β-I which was resistant to further hydrolysis by chymosin but not to porcine pepsin at pH 5.4−6.2. Increasing NaCl concentration to ≥5% reduced the proteolysis of β-CN in all three species, but not that of α_{s1}-CN. The fragments of β-I, -II, and -III produced from β-CN of the three species gave identical results with PAGE. α_{s1}-I and its degradation fragments had in all three species, regardless of the different electrophoretic mobilities on PAGE, the same sequence of appearance. The results indicate that chymosin and porcine pepsin attacked in buffalo and goat caseins the same regions as known for cow α_{s1}- and β-CN.

Keywords: *Proteolysis; chymosin; porcine pepsin; buffalo, cow, goat caseins*

INTRODUCTION

Gastric proteases are traditionally used for the manufacture of most cheese varieties, with calf and bovine rennet, the extract from the abomasum being the most common. Both contain chymosin and pepsin at different levels depending on the age of the animal (Andren and Collin, 1988). Chymosin, the main enzyme of calf rennet, acts first on κ-CN, this destabilizes the casein (CN) micelles and results in coagulation of casein. During cheese ripening, α_{s1}-CN and β-CN are further degraded by chymosin, plasmin, and proteases of microorganism retained in the cheese curd. Proteolysis of α_{s1}-CN and β-CN is the most important change during cheese ripening and is responsible for cheese texture and flavor. The hydrolysis of cow α_{s1}-CN and β-CN by chymosin has been extensively studied, and the most sensitive peptide bonds proved to be Phe_{23}-Phe_{24} in α_{s1}-CN and Leu_{192}-Tyr_{193} in β-CN. The subsequent proteolysis of fragment α_{s1} -I (f24-199) occurs at bonds Trp_{164}-Tyr_{165} followed by Leu_{149}-Phe_{150} (Visser, 1993). In fragment β-I (1−192) bonds Leu_{163}-Ser_{164}, or Leu_{165}-Ser_{166} and Leu_{139}-Leu_{140} are hydrolyzed (Creamer, 1976). Chymosin is active on both cow α_{s1}-CN and β-CN in model solution; in cheese, however, it appears to hydrolyze mainly α_{s1}-CN, because the hydrophobic aggregation of β-CN masks its susceptible sites to chymosin (Fox and Stepaniak, 1993).

Although pepsin resembles calf chymosin in some properties, its use as a 100% replacement for animal rennet in cheese making is limited due to its tendency of forming bitter peptides. In addition, the flavor of cheese produced with pepsin is generally bland (Garg and Johri, 1994). Bovine pepsin has been shown to hydrolyze the bonds Phe_{23}-Phe_{24} of α_{s1}-CN and Leu_{192}-

Tyr_{193} of β-CN at first and is compared to chymosin less substrate specific with cow casein. The intensity of proteolytic activity of chymosin and bovine pepsin on α_{s1}-CN and β-CN of cow and goat depends on pH and is sensitive to NaCl (Shimizu et al., 1985; Guillou et al., 1991; Trujillo et al., 1995). It has been reported that the proteolysis of β-CN by chymosin or bovine pepsin was significantly reduced by 5% and completely inhibited by 10% NaCl, while the rate of proteolysis of α_{s1}-CN was found maximal in the presence of 5−10% NaCl (Fox and Walley, 1971).

Only a few studies have been performed with buffalo and goat casein, although buffalo and goat milk production account for approximately 10% and 2%, respectively, of the world milk production (Sørensen, 1997), and more than one-third of buffalo milk and most of the goat milk are processed to cheese (Kalantzopoulos, 1993). Limited data is available on comparing proteolytic activity and specificity of chymosin and pepsin on buffalo, cow, and goat casein under same conditions.

The objective of the present investigation was to compare the proteolytic action of a recombinant chymosin (Maxiren) and porcine pepsin on buffalo, cow, and goat whole casein and β-CN in model solution under the same condition. This may also provide insight into the possible role of chymosin and pepsin in development of goat cheese flavor, e.g. bitterness, which is usually not found in goat cheese (Pélissier and Manchon, 1976).

MATERIALS AND METHODS

Milk Clotting Enzymes. Recombinant chymosin, Maxiren 15L, was a product of Gist-brocades (Seclin Cedex France); porcine pepsin (EC 3.4.23.1) was obtained from Fluka, Switzerland.

Casein. Hammarsten cow casein was obtained from Merck, Switzerland. Whole buffalo and goat casein were prepared by isoelectric precipitation (pH 4.6) of raw

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buffalo and goat skim-milk from the herd of the Faculty of Agriculture, Alexandria University, Egypt.

Pure β-CN was obtained from precipitated crude β-CN (Payens and Heremans, 1969) on a Merck-Hitachi HPLC system L-6200. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. A preparative column of Grom-Sil 300 ODS-5-ST, C-18, 250 × 20 mm (Stagroma, Germany) was used. Crude β-CN was dissolved in 8 M urea and 1% 2-mercaptoethanol and filtered through a 0.2 μ membrane filter (Millipore Corp., Bedford, MA), and 1 mL of sample was injected onto the column. A linear gradient from 0 to 40% solvent B at a flow rate of 9.5 mL/min was used for 5 min, followed by 40% solvent B for 45 min. Separation was conducted at 30 °C, and proteins were monitored at 215 and 280 nm. β-CN fractions were pooled and freeze-dried. The purified β-CN was homogeneous on SDS−PAGE.

Incubation of Model Solutions. After the casein stock solution (2.5%, w/v, in H₂O) had been heated at 66 °C for 20 min to prevent microbial and milk protease activity, buffer and milk clotting enzyme were added. The final 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 CaCl₂ in 100 s at 30 °C (IDF 110A, 1987).

The incubation was carried out at 30 °C for up to 8 h for HPLC and urea−PAGE. For SDS−PAGE, the same incubation mixture was further incubated at 15 °C for 14 days under aseptic and anaerobic conditions. Aliqouts were withdrawn from the incubation mixtures at intervals for further analysis. The reaction was terminated by heating at 90 °C for 10 min.

Extraction of peptides from the incubation mixture for RP-HPLC and SDS−PAGE was accomplished by lowering the pH to 4.6 with 1 N HCl, followed by centrifugation at 15000g for 10 min at 4 °C. The supernatant containing the peptides was lyophilized.

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

SDS−PAGE. ExcelGel SDS Homogeneous 15 (T = 15%, C = 3%, Pharmacia Biotech, Uppsala Sweden) with SDS buffer strips were used. Twenty microliters of 2% freeze-dried peptides extract in H₂O was diluted with 250 μL sample buffer containing 0.4 M Tris-HCl, pH 8.0, 1% SDS, 0.5% DTT, and a trace of bromphenol blue. The sample was heated at 80 °C for 5 min, and 15 μL was applied to the gel, which was run horizontal at 30 mA. The gel was stained with silver nitrate and preserved according to the method described by Pharmacia Biotech (1995). Band scanning was carried out with Video Copy Processor, Appligene, P65E and quantitative determination of the fragments was made by Molecular Dynamic Image Quant v 3.3 Program.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). The profile of the peptide extracts was visualized by using the same HPLC system as mentioned above. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. A Lichrospher analytical column (WP 300 RP-18, 5 μm, 250 × 4.6 mm) from Merck was used for analysis. Samples were filtered through a 0.2 μ membrane filter (Millipore Corp., Bedford, MA), and 50 μL (2% lyophilyzed peptide extract in water) was

injected onto the column. The sample was eluted with a four-step linear gradient over a period of 75 min, starting with solvent A for 5 min, then linear increase from 0 to 50% solvent B at a flow rate of 1.0 mL/min for 60 min, followed by 50% solvent B for 5 min and by a linear gradient from 50 to 100% B developed over 5 min to clean the column. Before the next injection, the column was allowed to equilibrate with solvent A for 15 min. Separation was conducted at 30 °C and peptides monitored at 215 and 280 nm.

RESULTS AND DISCUSSION

Proteolysis of Buffalo, Cow, and Goat Whole and β-CN by Chymosin and Porcine Pepsin. The hydrolysis of buffalo, cow, and goat whole and β-CN by the recombinant chymosin and porcine pepsin is shown in Figures 1 and 2. By the action of chymosin, from whole casein of all three origins, large peptides, α_{s1}-I and β I, were split-off (Figure 1a). Buffalo α_{s1}-CN, as well as its breakdown product α_{s1}-I, had slightly slower mobility than cow α_{s1}-CN and α_{s1}-I. Goat α_{s1}-CN, as well as α_{s1}-I, had the lowest electrophoretic mobility and both appeared as three bands, which might be a result of genetic polymorphism of α_{s1}-CN (Brignon et al., 1990). α_{s1}-I was visible in the 3 min hydrolysate of buffalo and cow casein, while in goat casein hydrolysate it appeared only after 30 min. After 4 h of incubation, in whole casein of all three species, α_{s1}-CN disappeared completely to yield mainly α_{s1}-I. The degradation of α_{s1}-CN proceeded at a higher rate than that of β-CN.

Buffalo, cow, and goat β-CNs and their chymosin degradation products β-I, -II, and -III had the similar mobilities. Goat β-CN migrated as two bands, β_1-CN and β_2,-CN, which differ in level of phosphorylation (Richardson and Creamer, 1974). The hydrolysis products β-I, -II, and -III appeared also as double bands, and are consistent with the previous observation of Trujillo et al. (1995). The higher proportion of β-CN than α_s-CN in goat casein was also evident. β-I was already present in the 3 min buffalo and cow casein hydrolyzed by chymosin, and the band intensified with prolonged incubation. β-I of buffalo and cow was further degraded to β-II and β-III. A band migrating slower than β-CN appeared after 150 min in all three caseins hydrolyzed by chymosin (Figure 1a and 2a). This band is unlikely a chymosin product, since it has been reported that β-CN fragments produced by chymosin migrate faster than β-CN (Creamer, 1976). Whether this band presents γ-CN, the β-CN degradation product by plasmin, needs to be further investigated. Goat β-CN completely disappeared after 8 h, but goat β-I was resistant to further breakdown. After 8 h incubation, a larger amount of buffalo and cow β-CN than goat β-CN remained intact, indicating that goat β-CN was more susceptible to hydrolysis by chymosin.

The hydrolysis of whole casein by porcine pepsin is shown in Figure 1b. The cleavage of the presumable Phe₂₃-Phe₂₄ in cow α_{s1}-CN and Leu₁₉₂-Tyr₁₉₃ in cow β-CN led to products α_{s1}-I and β-I, the latter being further hydrolyzed to β-II and β-III, according to the classification given by Creamer (1976). Similar degradation occurs with buffalo and goat β-CN (Figure 2b). After only 3 min of incubation with pepsin, was β-I already hydrolyzed to β-II in all three species. After 30 min incubation β-III appeared and the amount increased after 8 h incubation. β-IV was formed in smaller quantity than β-V in the 8 h hydrolysate of goat β-CN.
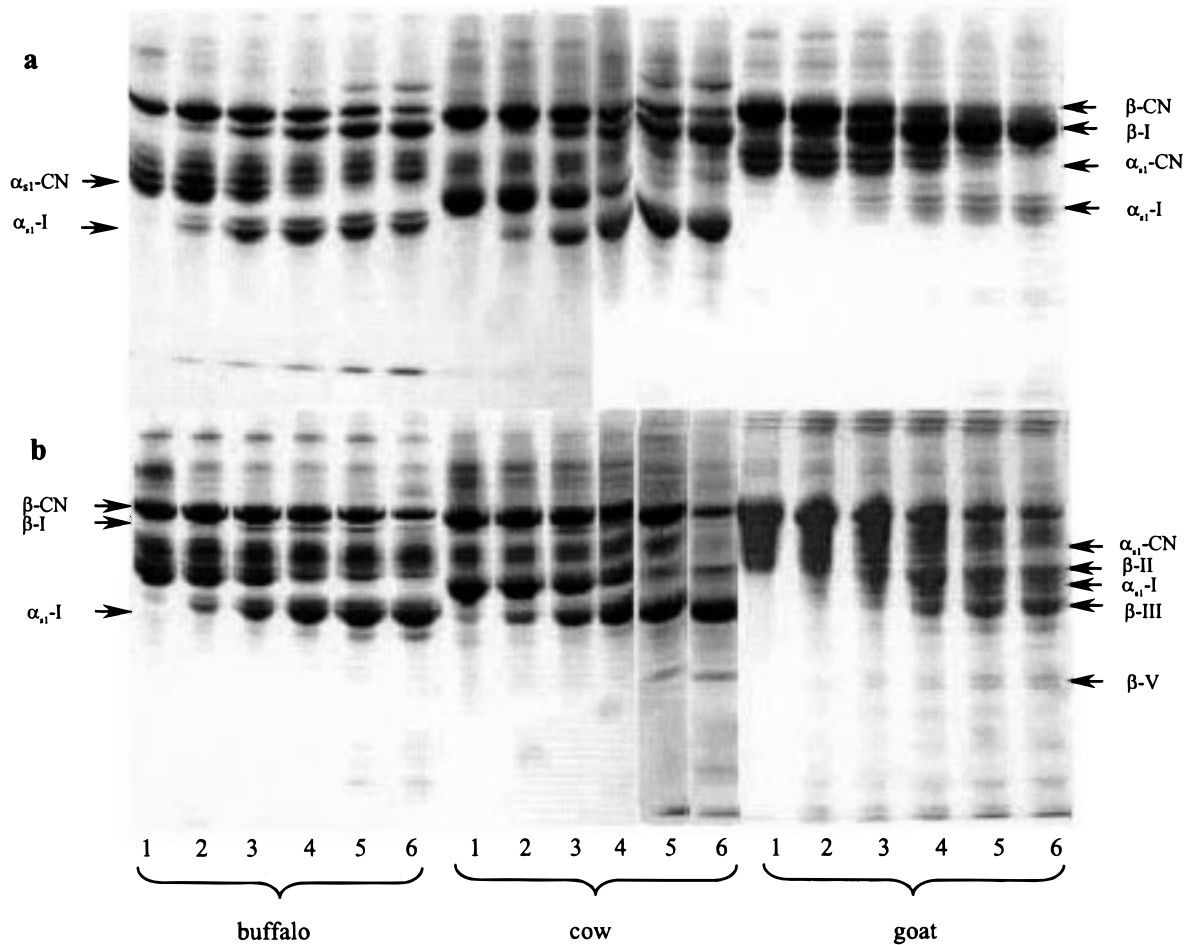
Figure 1. Urea−PAGE of buffalo, cow, and goat whole casein hydrolysis by chymosin (a) and porcine pepsin (b) at pH 5.4 and 30 °C: Untreated controls (lane 1), and samples hydrolyzed for 3, 30, 150, 240, and 480 min (lanes 2−6), respectively.

β-CNs were not entirely hydrolyzed even after 8 h of incubation. It can be concluded that porcine pepsin shows lower affinity than chymosin toward all three β-CNs under the conditions of this study.

The hydrolysis pathway of cow whole casein by chymosin is well established. The peptide α_{s1}-I (f24-199), the first degradation product from α_{s1}-CN, is further hydrolyzed to yield α_{s1}-II (f24-164), α_{s1}-III (f24-153), and α_{s1}-IV (f24-158) at pH 5.2−5.8 (Mulvihill and Fox, 1977). Cow β-CN is hydrolyzed by calf rennet into β-I, -II, and -III, corresponding respectively to f1-192, f1-163, and f1-139 (Creamer, 1976). In accordance with these previous studies, we found that the proteolytic activity and specificity of chymosin on buffalo and goat casein were similar to that in cow casein. The fragments of β-I, -II, and -III produced from β-CN of the three species gave identical eletrophoretic mobilities in urea−PAGE. For all three species, α_{s1}-I and its degradation fragments had the same sequence of appearance. These results might indicate that chymosin has similar specificities toward buffalo, cow, and goat casein, as there is relatively high homogeneity in the primary structure of casein of the three species (Addeo et al., 1977a,b and 1980; Brignon et al., 1989; Roberts et al., 1992). Although there are 14% amino acid substitutions in goat α_{s1}-CN compared with cow α_{s1}-CN, the regions susceptible to chymosin are very conserved.

Hydrolysis of buffalo and cow caseins by porcine pepsin showed the same pattern, regardless of the extent of degradation as judged by the intensity of the bands formed. Differences were observed in the hy-

drolysis of goat β-CN by chymosin and pepsin. Goat β-CN was more susceptible to chymosin than to porcine pepsin, but the reverse was the case with β-I. α_{s1}-CN of the three species was more susceptible to both chymosin and porcine pepsin than the β-CNs. Porcine pepsin showed in general higher proteolytic action on casein than chymosin, especially on goat casein under conditions of the study.

Peptide Profiles of Whole and β-CN Hydrolysates. The RP-HPLC chromatograms of the pH 4.6 soluble peptide extracts from casein hydrolyzed by chymosin and porcine pepsin are shown in Figures 3 and 4. In 30 min of chymosin hydrolysis of buffalo casein, three dominant peaks appeared at retention time (Rt) 50, 54.3, and 61.5 min. The first two peaks were also present in cow and the second in goat casein hydrolyzed by chymosin. The fraction with Rt 54.3 min was found in all three chymosin casein hydrolysates (Figure 3). In cow and buffalo, it could well be the β-CN fragment f193-209, and in the case of goat casein, it is β-CN f191-207. The reason for this assumption is that the peptide Leu-Tyr, cleaved by chymosin, is in cow and buffalo milk at position 192−193, whereas the same bond is in goat β-CN at position 190−191, because of the deletion of Pro$_{179}$-Tyr$_{180}$ in goat β-CN. The sequence of the complementary fragment of β-I is identical for cow, buffalo, and goat casein except a substitution of Leu to Ile at position 206 of goat β-CN. The peptide Rt 54.3 min eluted with acetonitrile concentration of 44.1% has been identified by Exterkate (1997) on RP-HPLC as β-CN f193-209. In a similar way, the fragment at Rt 50 min in buffalo and
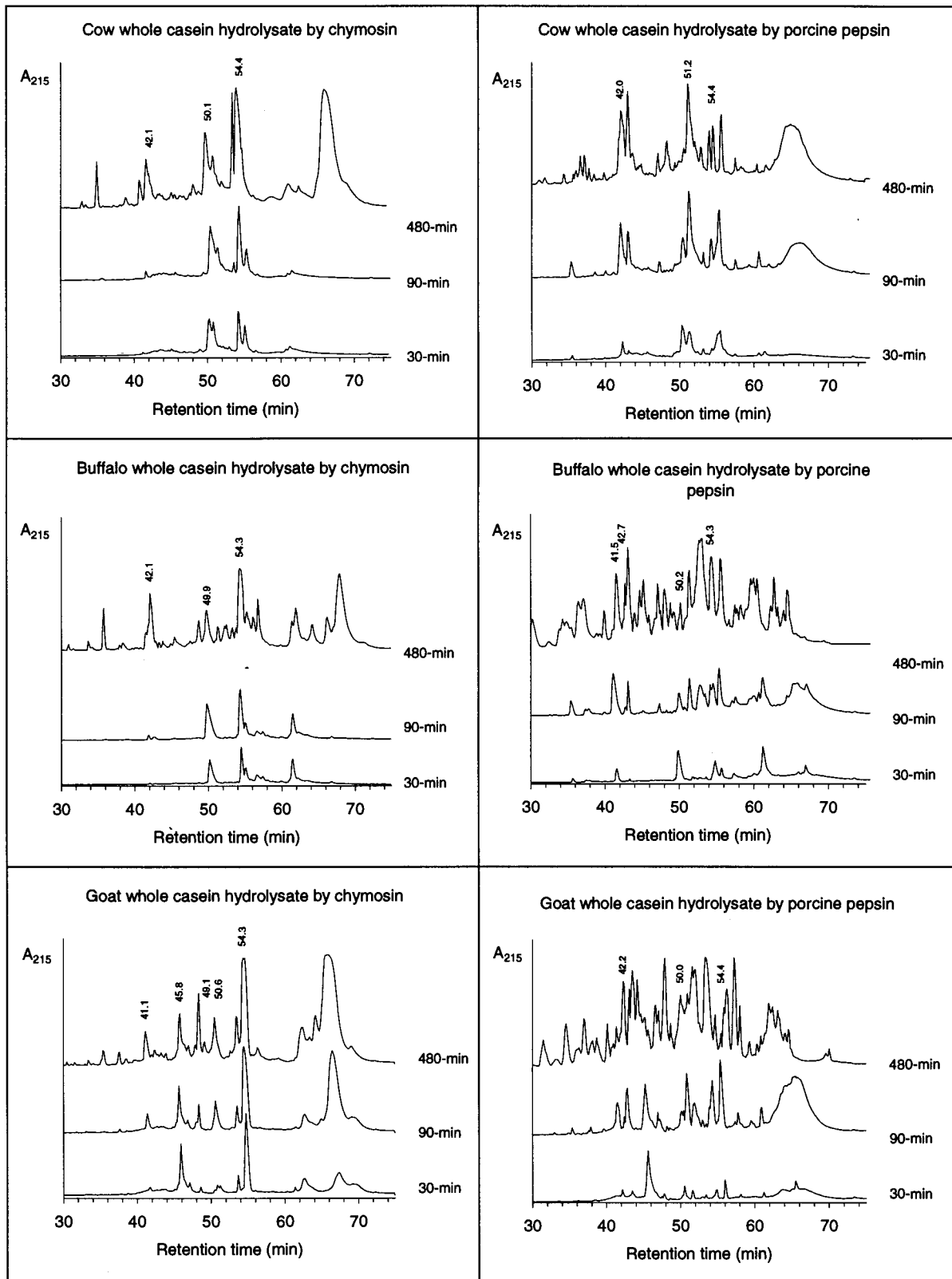
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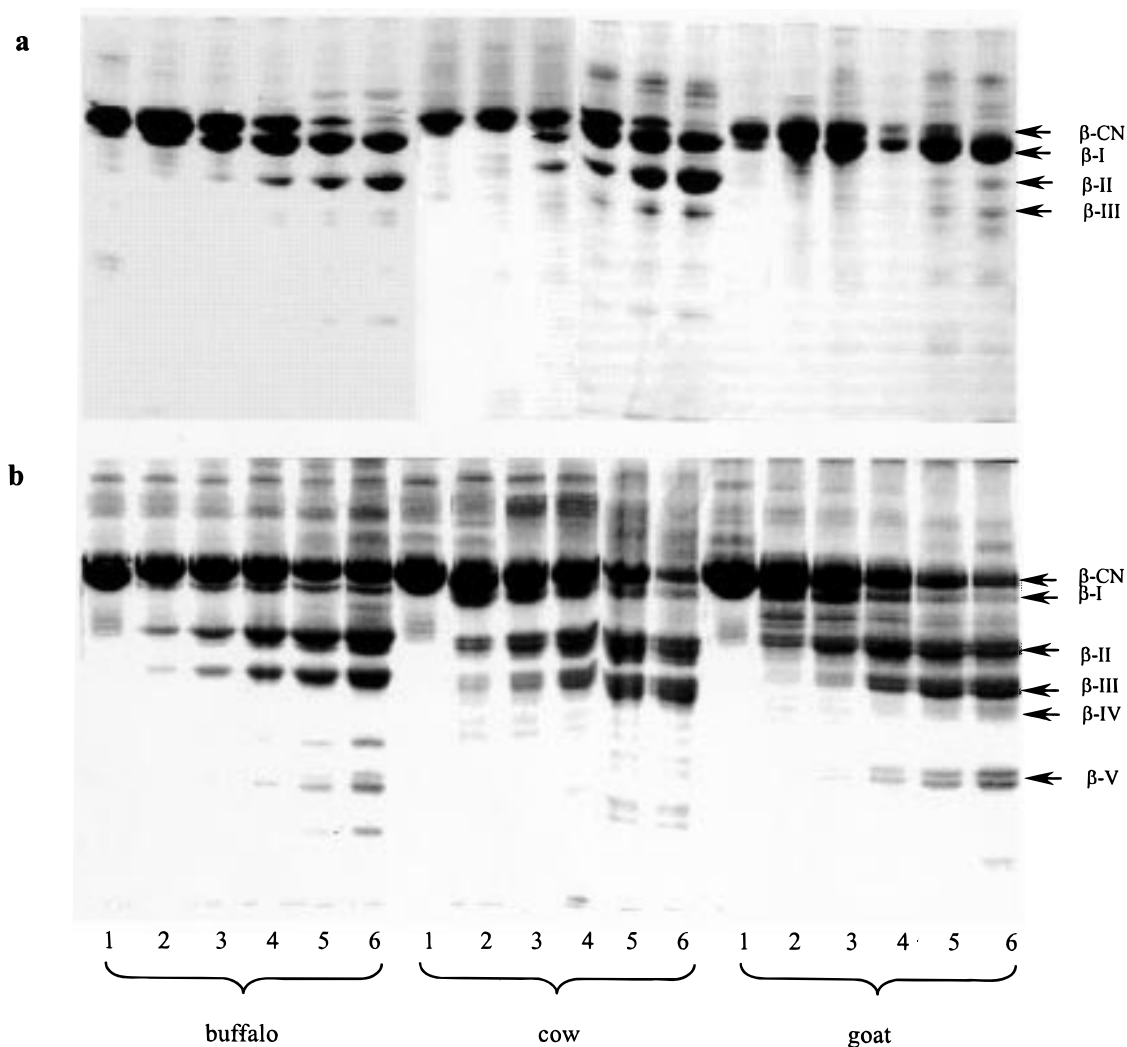
Figure 2. Urea−PAGE of buffalo, cow, and goat β-CN hydrolysis by chymosin (a) and porcine pepsin (b) at pH 5.4 and 30 °C: Untreated controls (lane 1), and samples hydrolyzed for 3, 30, 150, 240, and 480 min (lanes 2−6), respectively.

cow casein hydrolysates could be assigned to α_{s1} (f1-23) according to Exterkate et al. (1997). Additional evidence supporting the assignments of peak Rt 50 min and Rt 54.3 min is that the former, corresponding to α_{s1}- (f1-23), was not found in the peptide map of β-CN hydrolysates produced by chymosin. In the case of using chymosin, the fraction Rt 50 min was neither present after 30 min nor after 480 min in goat casein hydrolysate. The reason could be the relatively low content of α_{s1}-CN in goat casein, which is usually about one-fifth of that of cow α_{s1}-CN (Remeuf and Lenoir, 1985). In addition, the five amino acid substitutions in the fragment of f1-23 of goat α_{s1}-CN (Brignon et al., 1989) may affect the Rt value of the fragment as well. The peak at Rt 45.8 min in goat casein 30 min hydrolysate was neither found in buffalo's nor cow's hydrolysates even after 8 h incubation.

The Rt 42 min fraction present in 480 min hydrolysates of buffalo and cow whole and β-CNs by chymosin, eluted in 33.1% acetonitrile, could be assigned to β (f166-189), which is the complementary fragment of β-II from β-I (Exterkate et al., 1997). The increase of this peak with increasing incubation time confirmed the observation with urea−PAGE that β-I was further degraded to β-II by both enzymes in cow and buffalo whole and β-CN. That absence of the Rt 42 min peak in 8 h goat β-CN hydrolyzed by chymosin confirms the observation that β-I is resistant to further hydrolysis. The expected

fraction of β (f166−189) at Rt 42 min present in goat β-CN hydrolyzed by porcine pepsin confirms that goat β-I was further degraded by pepsin to β-II and β-III. Peptide profiles of 8 h casein hydrolysates of the three species showed significant differences in peak number, peak area, and their retention time, but certain peaks remained identical. The peaks eluted from buffalo and goat whole casein hydrolysates at Rt 42−51 min were not observed in cow casein hydrolysate. Figure 4 clearly indicated that the proteolytic activity and specificity of chymosin on cow β-CN is similar to that on buffalo β-CN, but to a lesser extent to goat β-CN, which confirms the observation made with SDS− and urea− PAGE (Figure 2). The results shown in Figures 3 and 4 are in agreement with those from SDS− and urea− PAGE that the porcine pepsin was more proteolytic on casein under conditions of study than was chymosin, especially on goat casein since, at all incubation intervals, pepsin digests contained more fractions. The peak at Rt 50 min in the β-CN hydrolyzed by porcine pepsin indicates that a porcine pepsin derived β-CN fragment from the three species is also eluted at the same retention time as α_{s1} (f1-23). This reflects the differences in the specificity of chymosin and porcine pepsin toward the casein fractions.

Effect of pH on the Degradation of β-CN by Chymosin and Pepsin. Figure 5a shows the influence of pH on the hydrolysis of β-CN by chymosin. The

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Figure 3. RP-HPLC pattern of hydrolysis products of cow, buffalo, and goat whole casein by chymosin and porcine pepsin.

degradation of β-CN was pH-dependent and the activity decreased at pH ≥ 5.8. With increasing pH, the staining intensity of band β-I remained almost unchanged, indicating that no further hydrolysis of β-I occurred. At pH 5.0–5.8, an intensive band of β-II was visible in cow and buffalo, while in goat casein hydrolysate only traces of β-II appeared at pH 5.0. Cow β-CN was completely hydrolyzed at pH 5.0 and 5.4, and further degradation
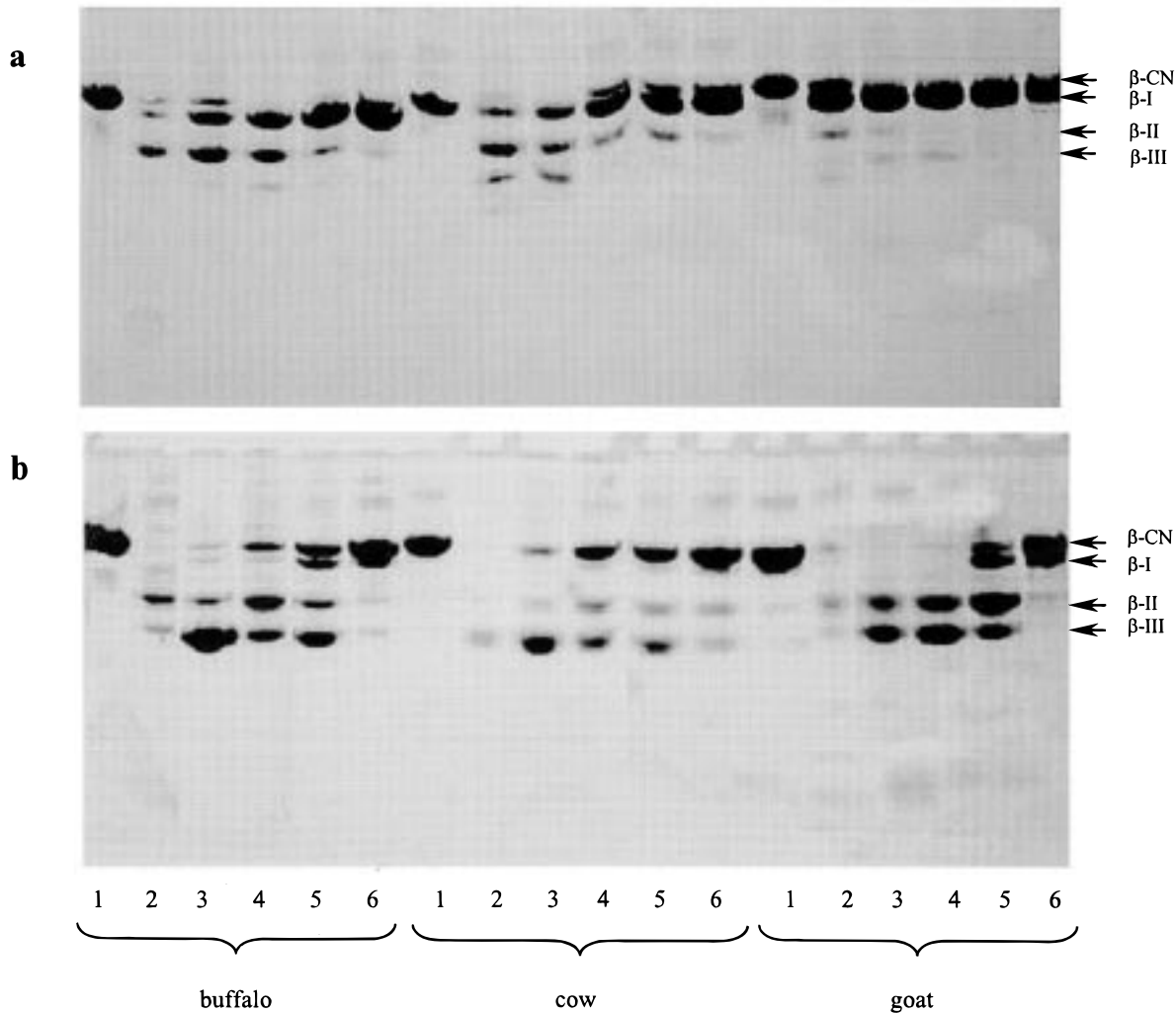
of β-I was more intensive on cow β-CN. Goat β-CN, compared with cow and buffalo, was in the entire pH range completely hydrolyzed by chymosin, mainly to β-I. The fact that further hydrolysis of goat β-I by chymosin is very limited may explain the observation why bitterness is less developed in goat cheeses, since it is known that bitter peptides are often short peptides of β-CN degradation (Fox and Walley, 1971).

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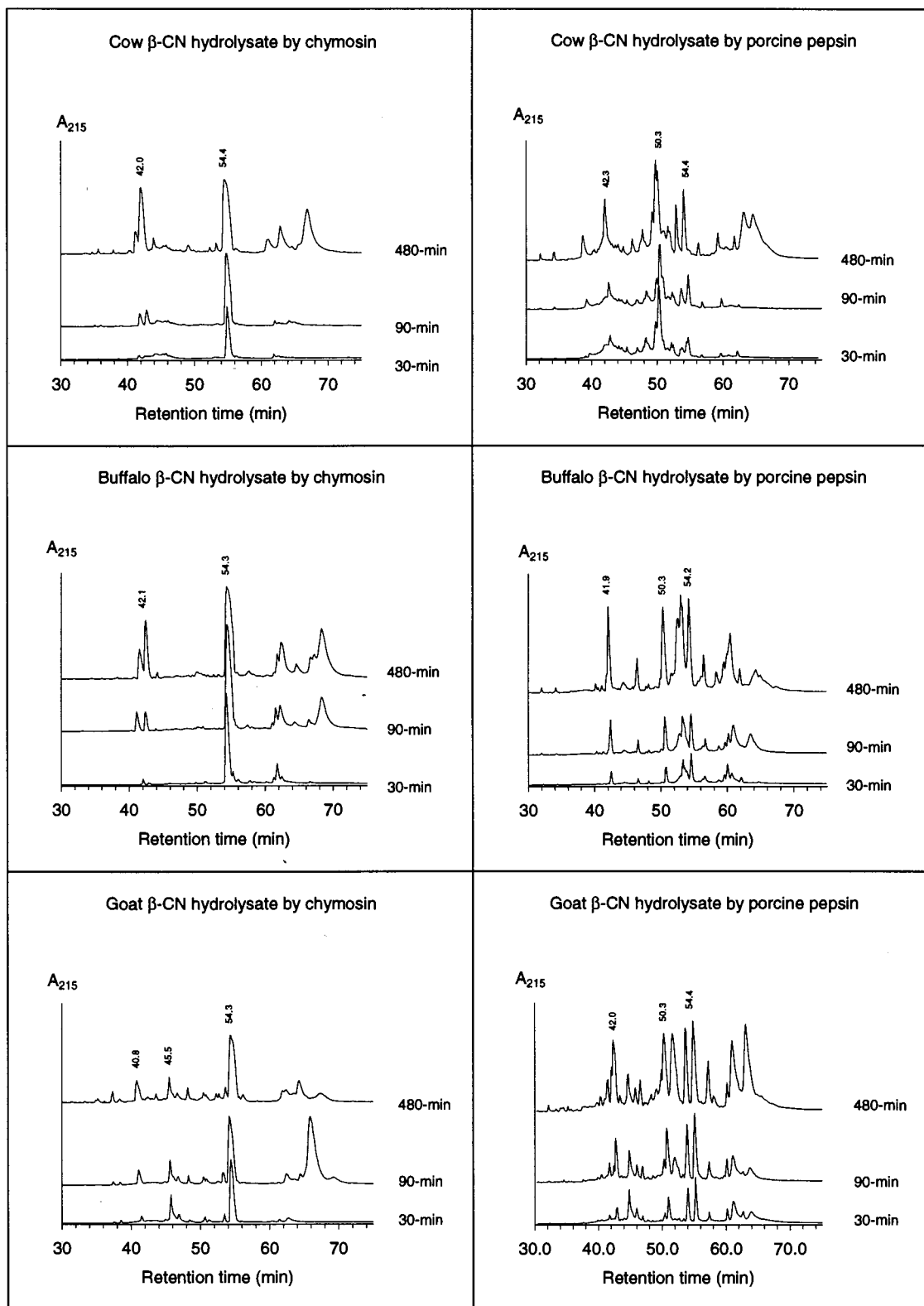
Figure 4. RP-HPLC pattern of hydrolysis products of cow, buffalo, and goat β-CN by chymosin and porcine pepsin.

The influence of pH on the hydrolysis of all three β-CNs by porcine pepsin is much different from that by chymosin (Figure 5b). At pH 5, the β-CNs were hydrolyzed extensively by porcine pepsin and a decreasing pepsin activity could be already observed at pH > 5.4. The further degradation of β-I and β-II seems to be less pH dependent in the range of pH 5.0–6.2. At pH 6.6, only traces β-I were formed. At pH 5.0–6.2, porcine pepsin cleaved cow, buffalo, and goat β-CN to β-I, β-II,

and β-III. It can be assumed that bovine pepsin present in calf rennet acts similarly to porcine pepsin and is responsible for the formation of goat β-II and β-III at pH 5.4–6.2. Similar results have been obtained also by Trujillo et al. (1995), who demonstrated that goat β-I was resistant to further hydrolysis by pure bovine chymosin at pH $\geq$ 5.4, whereas it was hydrolyzed by bovine pepsin to β-II, β-III, and β-IV in the pH range of 3.8–6.6.

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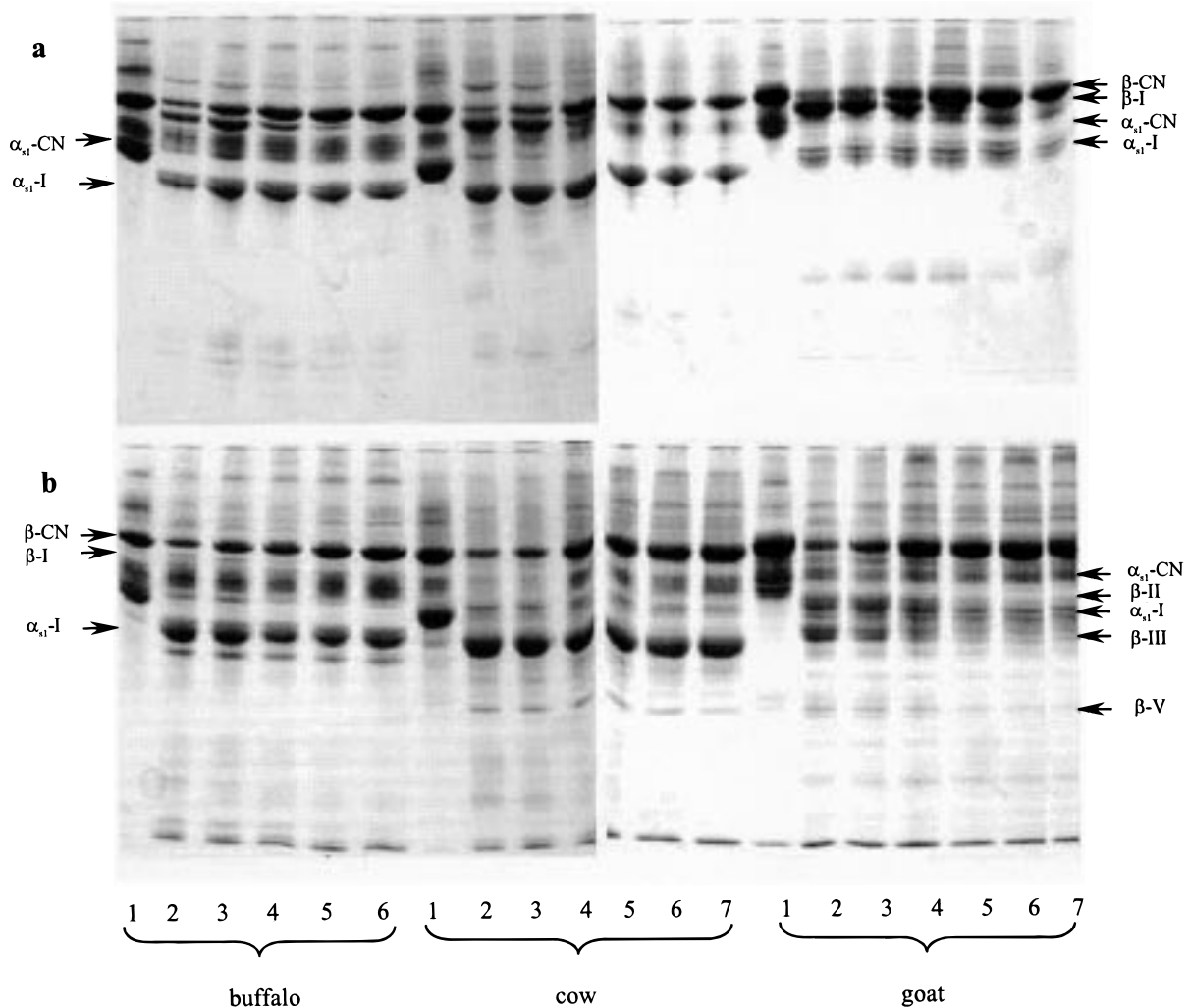

Figure 5. Urea−PAGE of pH effect on buffalo, cow, and goat β-CN hydrolysis by chymosin (a) and porcine pepsin (b) at 30 °C for 480 min: Untreated controls (lane 1), and samples hydrolyzed at pH 5.0, 5.4, 5.8, 6.2, and 6.6 (lanes 2−6), respectively.

Influence of NaCl on the Hydrolysis of Whole Buffalo, Cow, and Goat Caseins by Chymosin and Porcine Pepsin. For the investigation of the influence of NaCl on the hydrolysis of buffalo, cow, and goat whole casein by chymosin and porcine pepsin only pH 5.4 was chosen because this is the approximate pH of cheese at the beginning of ripening (Figures 6 and 7). The hydrolysis of β-CN of the three species by both enzymes was more sensitive to the presence of NaCl than the hydrolysis of α_{s1}-CN. The inhibitory effect of NaCl on the hydrolysis of β-CN was observed at 1% NaCl. As obvious from Figure 7, low concentration of NaCl (1%) influenced the peaks eluted at Rt 64−67 min, which were formed during hydrolysis by chymosin and pepsin in 8 h in absence of NaCl. A concentration of 3% NaCl inhibited to some extent the formation of peptide β (f194−209), in whole casein of all three species. The hydrolysis of β-CN by chymosin was in general strongly inhibited in the presence of 5% NaCl. From urea−PAGE it can be concluded that the degradation of β-CN to β-I by chymosin was reduced with increasing NaCl concentration, while hydrolysis of α_{s1}-CN was not affected by the presence of 5−10% NaCl. The inhibitory effect of NaCl on β-CN degradation is shown by the reduced peaks at Rt 42 and 54.3 min, which represent, respectively, the complementary fragments of β-II (f1−165) and β-I (f1−192). In contrast, the peak Rt 50 min, the α_{s1} (f1−23) fragment, remained almost unchanged in

the presence of up to 5% of NaCl. The fractions eluted before Rt 50 min, most of which originate from α_{s1}-CN, were less affected by the NaCl, supporting the observation on the urea−PAGE (Figure 6). Both PAGE and RP-HPLC results together suggest that the hydrolysis of the bond Leu$_{192}$-Tyr$_{193}$ in cow and buffalo β-CN, and Leu$_{190}$-Tyr$_{191}$ in goat β-CN was particularly sensitive to NaCl concentration ≥5%. In a previous study, Fox and Walley (1971) showed that the proteolysis of β-CN by calf rennet or bovine pepsin was significantly reduced by 5%, and completely inhibited by 10% NaCl. In contrast, the rate of hydolysis of α_{s1}-CN was found 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.

The inhibitory effect of NaCl on chymosin action on β-CN in solution or in cheese was explained by Kelly et al. (1996), suggesting that it is due to the aggregation of β-CN at elevated ionic strength, with the consequence that the cleavage sites in the hydrophobic part of the molecule are inaccessible. Thus, the effect of NaCl was rather on the substrate than on the activity of chymosin itself. This is likely to be the case also with porcine pepsin, as its hydrolysis of β-CN was retarded by NaCl, while the hydolysis of α_{s1}-CN by porcine pepsin was not affected by the presence of up to 5% NaCl, although

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Figure 6. Urea−PAGE of NaCl effect on buffalo, cow and goat whole casein hydrolysis by chymosin (a) and porcine pepsin (b) at pH 5.4 and 30 °C for 480 min: Untreated controls (lane 1), and samples hydrolyzed at 0, 1, 3, 5, 7, and 10% NaCl (lanes 2−7), respectively.

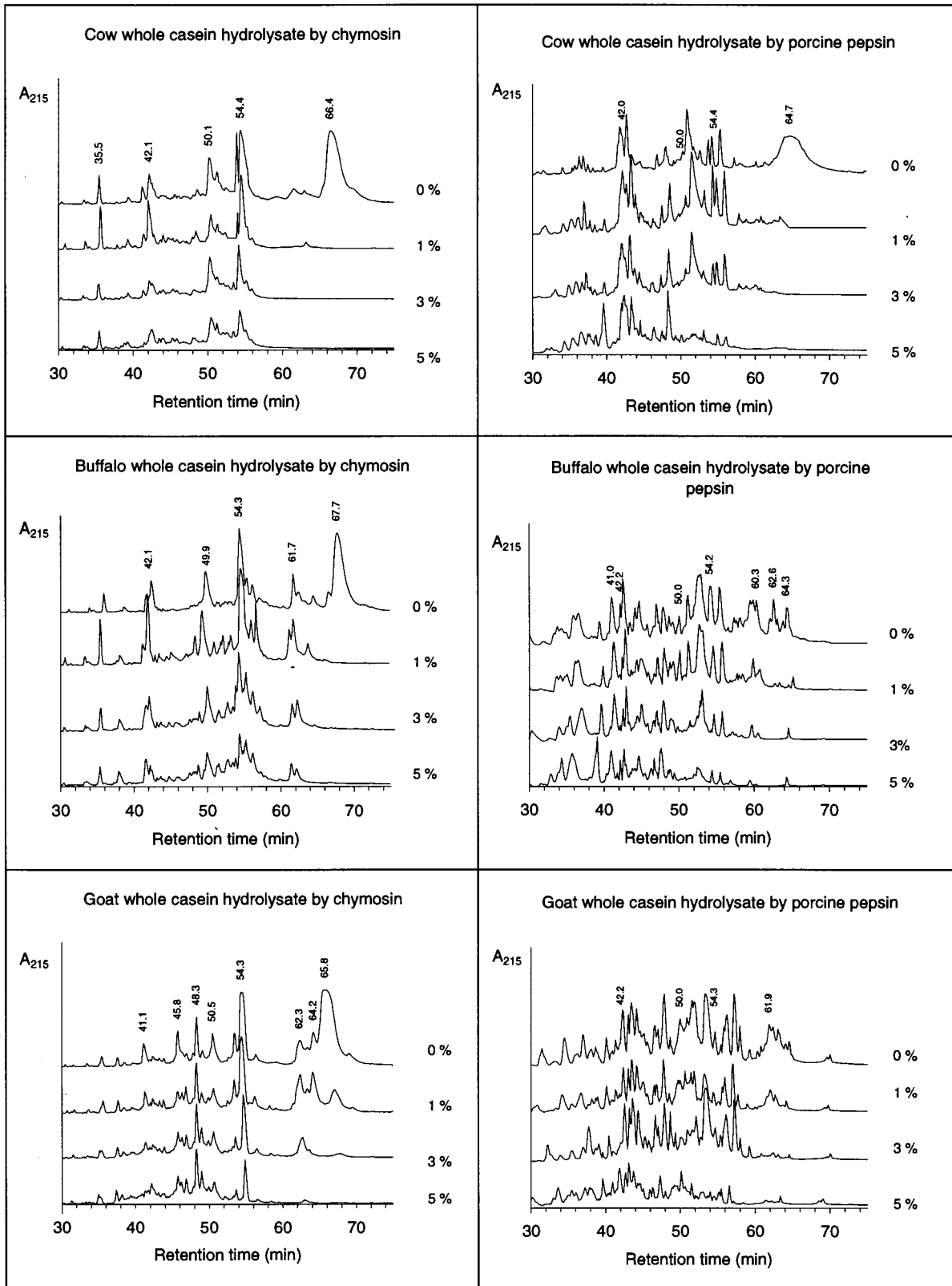pepsin has a broader specificity than chymosin on the α_{s1}- and β-CN.

It has been also reported by several authors that NaCl plays an important role in preventing the development of bitterness in cheese (Fox and Walley, 1971; Jago, 1974; Stadhouders and Hup, 1975; Visser et al., 1983). If the hypothesis that bitter peptides mainly originate from β-CN is correct, then the reduced β-CN hydrolysis would mean less chance for development of bitterness during maturation of cheeses with higher NaCl content. The presented results may support the possible explanation that the resistance of goat β-I to chymosin may be one of the reasons why bitterness is usually not observed in goat cheeses.

Size Distribution of pH 4.6 Soluble Peptides from Casein Hydrolysate. The rennet retained in the cheese curd makes a major and essential contribution to proteolysis during cheese ripening and, consequently, to flavor and texture development (Fox, 1989). The breakdown products of α_{s1}-CN, and to a lesser extent of β-CN by rennet and plasmin, are suitable substrates for proteases and peptidases of starter bacteria and milk flora in cheese. To gain insight into the sizes of the casein fragments generated by chymosin and porcine pepsin, 2% casein solutions, adjusted to pH 5.4 were

first incubated at 30 °C for 8 h, followed by additional incubation for 14 d at 15 °C.

The pH 4.6 soluble fragments of the incubation mixtures before and after being further incubated were analyzed with SDS−PAGE and the bands were scanned and quantified (Figure 8) The degradation products of the three caseins had apparent molecular weight (MW) in the range between 8200 and 27 000 Da determined with SDS−PAGE. As shown in Figure 8, porcine pepsin tended to produce from whole casein of all three species middle size (10−15 kDa) fragments, whereas with chymosin over 70% of the degradation fragments were of MW > 15 kDa. In the casein hydrolysates produced by porcine pepsin, middle size fragments counted for about 50%, whereas high MW peptides (>15 kDa) amounted for less than 30%. Changes could be observed in goat β-CN hydrolysates. As observed in urea−PAGE, chymosin hydrolyzed goat β-CN mainly to β-I and further degradation was limited. SDS−PAGE also showed that over 90% of goat β-CN fragments degraded by chymosin were of high MW. This size distribution was reached after 8 h and remained unchanged until the end of 14 d incubation. Contrary to that, degradation products of β-CN by pepsin contained less than 10% of high MW fragments. Middle size peptides of goat β-CN

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Figure 7. RP-HPLC pattern of hydrolysis products of cow, buffalo, and goat whole casein by chymosin and porcine pepsin at different NaCl concentrations at pH 5.4 and 30 °C for 480 min.

produced by pepsin counted for more than 50% and 40% for low MW (<10 kDa) peptides.

Fragment size profiles of buffalo and cow β-CN by pepsin were very similar. These results indicate that porcine pepsin generates medium and small size fragments from whole casein, which are likely to originate from α_{s1}-CN. The products of proteolysis can be further

fragmented to small peptides and free amino acids by the enzymes of starter bacteria and milk flora.

CONCLUSION

The results in this study suggest that the hydrolysis of buffalo and goat casein by recombinant chymosin was

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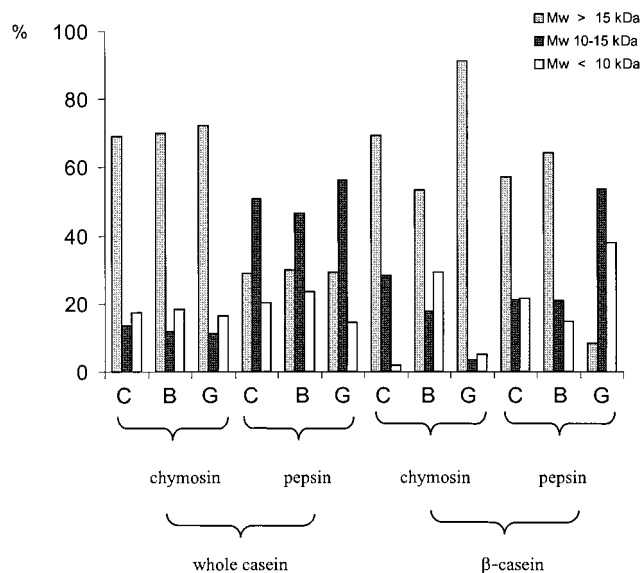
Figure 8. Size distribution of pH 4.6 soluble fragments extracted from buffalo (B), cow (C), and goat (G) whole and β-CN hydrolysates by chymosin and porcine pepsin. Incubation at 15 °C for 14 days.

similar to that of cow casein, producing α_{s1}-I and β-I, -II, and -III as the degradation fragments from α_{s1}- and β-CN. The exception is that goat β-I is resistant to chymosin but not to porcine pepsin. Although porcine pepsin first splits the bond of Phe$_{23}$-Phe$_{24}$ in α_{s1}-CN, and Leu$_{192}$-Tyr$_{193}$ in β-CN to yield α_{s1}-I and β-I, its specificity and proteolytic activity on casein was broader and higher than those of chymosin, especially on goat casein. For equal enzyme activity, porcine pepsin was more proteolytic on casein than chymosin. Buffalo, cow, and goat α_{s1}-CNs were more susceptible to chymosin and pepsin than their β-CNs.

Hydrolysis of β-CN by chymosin and porcine pepsin was pH-dependent. Above pH 5.8, the hydrolysis of buffalo and cow β-CNs by chymosin was retarded significantly, and a decreased activity of porcine pepsin on β-CN also became evident above pH 6.2. Chymosin produced mainly β-I from goat β-CN at pH 5.0–6.2. while β-I was readily further degraded to β-II and β-III in this pH range by pepsin. Consequently, β-II and β-III are likely to be the degradation products of pepsin contained in the animal rennet. The resistance of goat β-I to chymosin might be one reason for the less bitterness in goat cheeses.

NaCl concentration had marked effect on the proteolysis of β-CN, but not on that of α_{s1}-CN of the three species. PAGE and RP-HPLC showed that the bond of Leu$_{192}$-Tyr$_{193}$ in β-CN of the three species was particularly sensitive to NaCl concentration of 5%.

The size distribution of the pH 4.6 soluble peptides produced by chymosin and porcine pepsin was similar for peptides extracted from buffalo, cow, and goat whole casein. Pepsin generated more middle and low MW peptides than did chymosin. When β-CN was used as substrate, the size distribution differed in the origin of β-CN, with goat β-CN being the extreme case, confirming that goat β-I was resistant to chymosin but very susceptible to porcine pepsin.

ABBREVATIONS USED

CN, casein; PAGE, polyacrylamide gel electrophoresis; Rt, retention time.

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