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Camel and Bovine Chymosin Hydrolysis of Bovine α_{S1}- and β-Caseins Studied by Comparative Peptide Mapping

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ABSTRACT: In many cheese varieties, the general proteolytic activity of the coagulant is of great importance to the development of flavor and texture during ripening. This study used capillary electrophoresis and LC-MS/MS to compare the in vitro proteolytic behavior of camel and bovine chymosin (CC/BC) on bovine α_{S1}- and β-casein (CN) at pH 6.5 and 30 °C. β-CN hydrolysis was also studied at pH 5.2 and in the presence of 0, 2, and 5% (w/v) NaCl. A total of 25 α_{S1}- and 80 β-CN peptides were identified, and initial rates of early peptide formation were determined. The modes of proteolytic action of CC and BC shared a high degree of similarity generally. However, except for a few peptide bonds, CC was markedly less active, the magnitude of which varied widely with cleavage site. Preferential α_{S1}-CN (Phe23−Phe24) and β-CN (Leu192−Tyr193) hydrolysis by CC proceeded at an estimated 36 and 7% of the initial rate of BC, respectively. The latter rate difference was largely pH and NaCl independent. Several cleavage sites appeared to be unique to CC and especially BC action, but qualitative differences were often predetermined by quantitative effects. In particular, negligible CC affinity to α_{S1}-$CN_{164/165}$ and β-$CN_{189/190}$ prevented further exposure of the N-terminal products. β-CN hydrolysis by either enzyme was always stimulated at the lower pH, yet either inhibited or stimulated by the presence of NaCl, depending mainly on the predominating type of molecular substrate interactions involved at the specific site of cleavage. The potential impact of this proteolytic behavior on cheese quality is discussed.

KEYWORDS: *camel chymosin, bovine chymosin, α_{S1}-casein hydrolysis, β-casein hydrolysis, enzyme specificity*

INTRODUCTION

Proteinases are the single class of enzymes that occupy a pivotal position with respect to their functionality in both physiological and commercial fields, of which the aspartic proteinase chymosin (EC 3.4.23.4) represents one of the best known examples.[1,2] Chymosin is nature's own catalyst for milk coagulation, occurring in the fourth stomach of suckling ruminants. To date, this specific function makes chymosin of bovine origin (*Bos taurus*) the principal coagulant used in the cheese industry, owing primarily to its superiority in combining a high milk-clotting activity (*C*), expressed in international milk-clotting units (IMCU), with a low general proteolytic activity (*P*) in comparison to other milk-clotting enzymes.[1]

Chymosin is added to the cheesemilk to specifically cleave the Phe105−Met106 bond of κ-casein (CN) and thereby induce coagulation. A certain fraction of the added chymosin is retained in the curd, which, in most cheese types, catalyzes the slower hydrolysis of other peptide bonds in casein. Such activity is of great importance to textural changes and for microbial substrate availability during early cheese ripening (e.g., α_{S1}-CN(f1−23)). This biphasic proteolysis of casein by the coagulant was first described by Nitschmann and Bohren.[3] On the other hand, extensive general proteolysis of the coagulant is associated with a risk of excessive accumulation of bitter peptides during ripening (e.g., β-CN(f193−209)), which may account for a notable flavor defect in Cheddar and other cheese varieties.[4]

The mechanism responsible for the highly specific mode of action of chymosin (i.e., high *C*) compared to other aspartic proteinases has been the subject of many kinetic[5−7] and crystallographic studies.[8−10] Moreover, the general proteolytic behavior of bovine chymosin (BC) toward isolates and mixtures of bovine casein has been widely investigated in vitro to gain a better understanding of the origin of an array of contributions to proteolysis during cheese ripening and the effect of environmental parameters including temperature, ionic strength, and pH.[11−16] Much of the proteolytic behavior revealed has been rationalized in view of the spatial arrangement of the caseins and, hence, their accessibility to enzymatic hydrolysis. In contrast to other water-soluble proteins, casein molecules appear to adopt peculiar tensegrity-analogue structures characterized by being open and flexible, yet rigid at the same time, and well-suited for the access of chymosin to substrate binding. Despite being the subject of much controversy, solid experimental evidence renders the existence of persistent secondary structures in the caseins probable.[17] Such structural motifs combined with their distinct amphiphilic properties imply a high propensity to self-association and structural adaptation to environmental changes driven by hydrophobic interaction.[18] Casein self-association is, in turn, counterbalanced by electrostatic and hydrogen bonding to the solvent, enabling substrate hydration.

For the reasons considered above, the recent finding of a 7-fold higher *C*/*P* ratio of chymosin of camel origin (*Camelus dromedarius*) as compared to its bovine counterpart (~70% higher *C* and only ~25% of *P*) using bovine milk as substrate appears to be of significant interest to the cheese industry.[19] The

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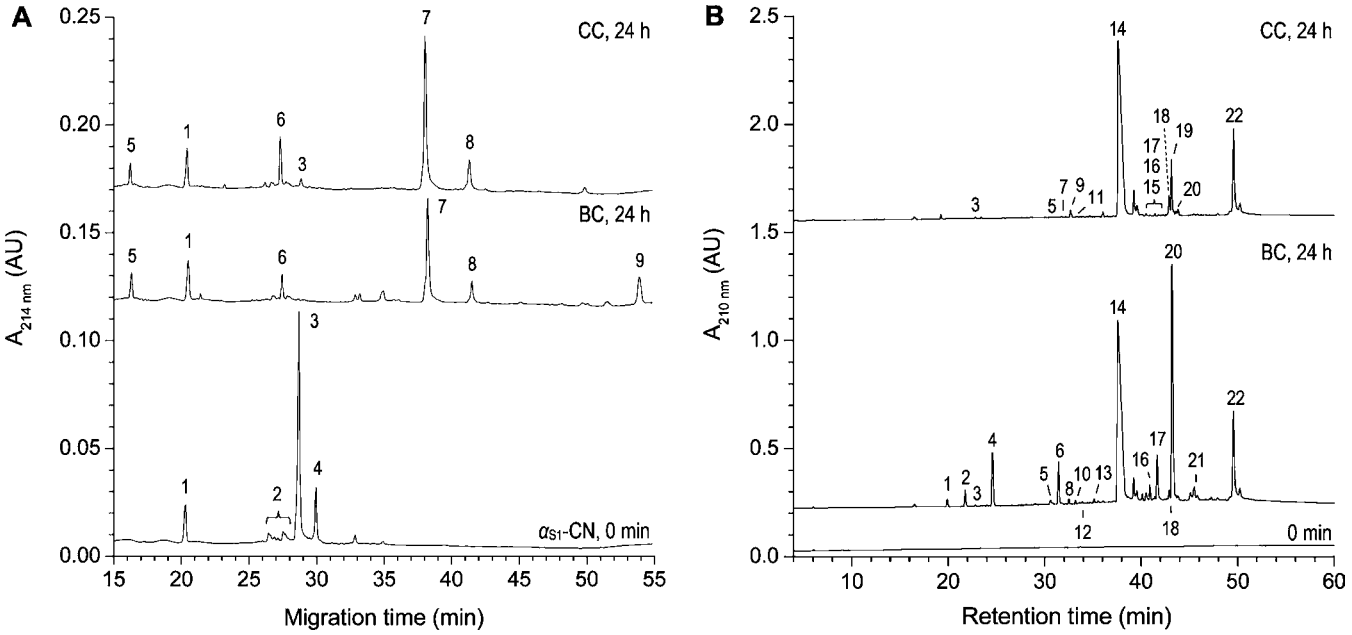

Figure 1. CE (A) and RP-HPLC (B) of the urea- and pH 4.6-soluble fractions, respectively, from the 24 h hydrolysates of α_{S1}-casein (CN) produced by camel chymosin (CC) and bovine chymosin (BC). Samples of the initial α_{S1}-CN substrate are included as a control (0 min). (A) Peaks: 1, Tyr-Ala (internal standard); 2, α_{S2}-CN(n*P*); 3, α_{S1}-CN(8*P*)^{B}; 4, α_{S1}-CN(9*P*)^{B}; 5, f1−23; 6, f102−199 (tentative); 7, f24−199(8*P*); 8, f24−199(9*P*); 9, f24−164(8*P*) (tentative). (B) Numbered peaks were identified by MS/MS as specified in Table 1.

impact of this difference was demonstrated in Cheddar cheese trials in which similar curd strengths and renneting times were obtained during manufacture with almost 30% less IMCU of camel chymosin (CC) as compared to BC, and significantly lower levels of primary proteolysis were measured during ripening of cheeses made with the former coagulant.[20]

Whereas the mode of action of BC is still far from entirely understood, investigations elucidating the differences responsible for the markedly improved performance of CC have only just been initiated. Sharing 85% amino acid sequence identity, the two enzymes adopt similar overall spatial foldings[21] and exhibit identical specificity toward the Phe105−Met106 bond of κ-CN.[22] Nevertheless, on the basis of distinct structural differences, the impact of several local enzyme−substrate interactions has been proposed including specific electrostatic attractive forces previously suggested to play a role in allosteric-like activation of BC.[10,21,23] Significant differences revealed in the kinetic parameters of CC and BC were in turn rationalized in view of the impact of the very same electrostatic interactions.[22]

Due to the biphasic role of the coagulant in casein breakdown during the manufacture and ripening of cheese, it is of importance to elucidate in detail not only the milk-clotting but also the general proteolytic properties of CC and, thus, understand its full potential for use in the cheese industry. The present research maps for the first time the in vitro proteolytic behavior of CC as compared to BC using isolated bovine α_{S1}- and β-CN as substrates. Furthermore, benefitting from recent advances in peptide identification, this investigation verified and extended the existing knowledge of α_{S1}- and β-CN hydrolysis by BC. Model conditions were chosen to resemble Cheddar cheesemaking and included an evaluation of the effects of pH and NaCl (at levels mimicking non-, low-, and normal-salted Cheddar) on β-CN hydrolysis. The enzymes were compared at equivalent IMCU concentrations in order for the results to extrapolate directly to a given cheese application.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade, and water was purified by deionization (18.2 MΩ·cm) through a Milli-Q Plus water system (Millipore, Bedford, MA, USA).

Enzymes. Fermentation-produced camel chymosin (CC) (CHY-MAX M, 1011 IMCU/mL) and fermentation-produced bovine chymosin (BC) (CHY-MAX Extra, 585 IMCU/mL) (Chr. Hansen A/S, Hørsholm, Denmark) were used. The activity of the enzyme preparations was determined according to ISO11815:IDF157[24] prior to use.

Purification of α_{S1}- and β-CN. Fresh, raw milk was collected from a single Holstein-Friesian cow, whole casein was prepared by acid precipitation, and α_{S1}- and β-CN were isolated by anion-exchange chromatography, as described previously.[22] The purity of the protein preparations was assessed by capillary electrophoresis (CE).[22]

Enzymatic Hydrolysis. Lyophilized α_{S1}-CN was dissolved in 0.1 M di/trisodium citrate, pH 6.5, and lyophilized β-CN in 0.1 M di/trisodium citrate, pH 6.5 or 5.2, containing 0, 2, or 5% (w/v) NaCl. Both caseins were dissolved to a concentration of 10 mg/mL (∼0.4 mM). Hydrolysis was initiated by adding 50 μL of freshly diluted CC or BC to 2.3 mL of protein solution to give a final activity of 1 IMCU/mL of either enzyme. The mixtures were incubated at 30 °C, and two aliquots were removed at 0 (before enzyme addition), 5, and 30 min and at 1, 3, 6, 12, and 24 h, one for CE (60 μL) and one for reversed phase (RP) HPLC analysis (200 μL). The enzymatic reaction was terminated by mixing the sample for CE analysis with 140 μL of CE sample buffer (10 M urea, 167 mM Tris, 67 mM EDTA, 42 mM 3-morpholinopropanesulphonic acid, 17 mM DL-DTT, 0.83 mg/mL methyl hydroxypropyl cellulose, and 1.6 mM Tyr-Ala (internal standard)),[25] and by heating the sample for RP-HPLC analysis at 85 °C for 10 min.[26] CE samples were incubated for 1 h at room temperature, after which 4 μL of DL-lactic acid (18.0% (v/v)) was added. RP-HPLC samples were adjusted to pH 4.6 by adding 12 μL of acetic acid (33.3% (v/v)), followed (after 10 min of incubation at room temperature) by 12 μL of sodium acetate (3.33 M), as adapted from McGann et al.[27] The pH 4.6-soluble fraction was collected after centrifugation (16000*g*, 10 min) and filtration (0.20 μm). The experiment included six control samples: two consisting of casein incubated for 24 h without added enzyme and prepared for CE and RP-HPLC analyses, and four consisting of casein and CC/BC immediately inactivated according to CE and RP-HPLC analyses, as described above.

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CE. Casein breakdown and the formation of large casein fragments were analyzed by CE (Agilent Technologies ApS, Hørsholm, Denmark and Hewlett-Packard International Sarl, Allerød, Denmark) according to the method of Recio and Olieman with some modifications, as detailed previously.[22] Peak assignment was based on published work[28] and a priori knowledge of the method and primary casein structure. UV peaks ($A_{214\ nm}$) were integrated and the peak areas normalized by dividing with the migration time and standardized according to the normalized peak area of Tyr−Ala for semiquantitative comparison across samples. Semiquantitative comparison across casein fragments was enabled by dividing the normalized, standardized peak areas by the molar extinction coefficient (ε) of the corresponding peptide calculated from

$$\varepsilon_{peptide}\ (M^{-1}\ cm^{-1}) = (\varepsilon_{peptide\ bond} \times n_{peptide\ bond}) + \left(\sum_{20}^{i=1} \varepsilon_{amino\ acid(i)} \times n_{amino\ acid(i)}\right) \quad (1)$$

using the values of $\varepsilon_{peptide\ bond}$ and $\varepsilon_{amino\ acid}$ measured by Kuipers and Gruppen.[29] The slope of the linear increase in corrected peak area (M cm) during hydrolysis ($R > 0.99$) was used for comparison of initial formation rates of the earliest large casein fragments.

RP-HPLC Coupled to Tandem Mass Spectrometry (MS/MS). The formation of pH 4.6-soluble peptides was analyzed by RP-HPLC ESI MS/MS (1100 series LC/MSD Trap, Agilent Technologies ApS) using linear gradient elution at 0.25 mL/min and a MS scan range of m/z 100−2200 according to the procedures described previously,[30] with the exception that an injection volume of 10 μL was used. Quantitative comparison across peptides was enabled by using the integrated peak areas ($A_{210\ nm}$) and calculated molar extinction coefficients (ε) (eq 1) in the Beer−Lambert law. The slope of the linear increase in molar peptide concentration during hydrolysis ($R > 0.99$) was used for comparison of initial formation rates of the earliest peptides.

RESULTS AND DISCUSSION

α_{S1}- and β-CN Preparations. Electrophoretic separation of the purified α_{S1}- and β-CN preparations is illustrated in the lower curves of Figures 1A and 2, respectively. An estimated purity of >90% was obtained for α_{S1}-CN, and only genetic variant B, which generally occurs at a highly dominating frequency in Holstein-Frisian breeds,[31] was present in the preparation. In further accord with typical milk composition, a smaller fraction (∼15%) corresponding to α_{S1}-CN with nine rather than eight phosphorylated Ser residues was observed. The preparation contained minute traces of α_{S2}-CN and, apart from that, no other milk proteins. The estimated purity of β-CN was >95% distributed almost equally between genetic variants A2 and B, although β-CN^{B} generally occurs at a much lower frequency than β-CN^{A2} in Holstein-Frisians.[31] No other protein components were identified in this preparation.

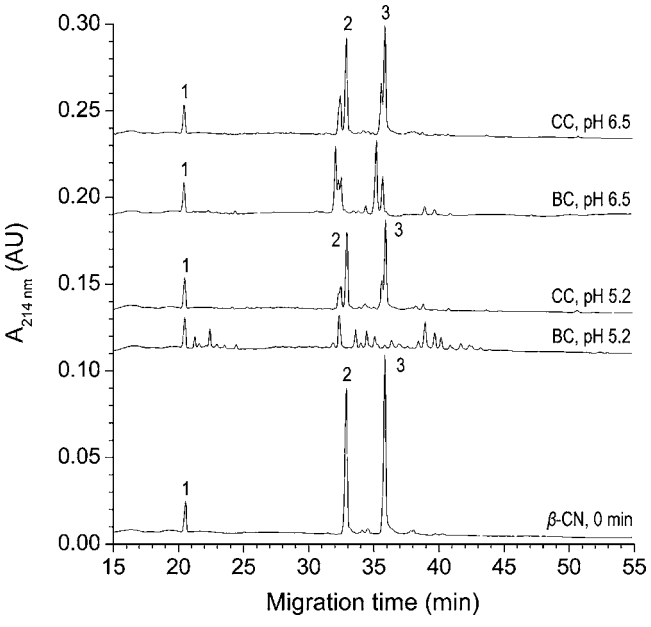

Figure 2. CE of 24 h hydrolysates of β-casein (CN) produced by camel chymosin (CC) and bovine chymosin (BC) at pH 6.5 and 5.2 (0% NaCl). The profile of the initial β-CN substrate is included as a control (0 min). Peaks: 1, Tyr-Ala (internal standard); 2, β-CN^{B}; 3, β-CN^{A2}.

Hydrolysis of α_{S1}- and β-CN at pH 6.5. The breakdown of intact α_{S1}- and β-CN by CC proceeded significantly more slowly, that is, at around 30 and 15% of the initial rate of BC, respectively (Figure 3). In comparison, using whole casein and a reaction pH of 5.8, Kappeler et al.[19] found that CC possessed only ∼25% of the P of BC at equal molar enzyme concentrations (corresponding to an approximate 1:2 ratio of BC/CC on an IMCU basis). With the aim to focus the following discussion on differences relating to the specificity and activity of CC and BC, exhaustive peptide mapping was sought to distill down to a qualitative and quantitative mapping of the particular sites of cleavage, as far as to the point where parallel formation and breakdown reactions blurred this approach.

α_{S1}-CN. Hydrolysis of α_{S1}-CN produced four CE peaks (peaks 5−8) common to both enzymes, whereas a fifth, slow-migrating peptide was observed only in the BC hydrolysates (peak 9) (Figure 1A). All of these peaks could be assigned to major casein fragments and, hence, quantitatively compared over the course of the hydrolysis (Figure 4A). Using LC-MS/MS, totals of 12 and 20 pH 4.6-soluble peptides were identified in the 24 h hydrolysates produced by CC and BC, respectively (Figure 1B; Table 1). Among these, 3 and 11 peptides were strictly specific to the action of CC and BC, respectively. The kinetics of early pH 4.6-soluble peptides is shown in Figure 4B, and initial formation rates derived thereof are listed in Table 2.

Above all, the results of CE and LC-MS/MS analyses concurrently showed a strong preferential cleavage of Phe23−Phe24 by both chymosins (Figure 4). The derived N-terminal f1−23 accumulated throughout the duration of the experiment, eventually reaching a plateau due to substrate exhaustion in the BC hydrolysate. This plateau corresponded to the initial substrate concentration (0.38 mM), reflecting a high resistance of f1−23 to further hydrolysis by chymosin. Only a few sparsely hydrolyzed bonds were indeed identified within this fragment and primarily in the CC hydrolysate (f1−20/22, f9−23). On the basis of the rate of initial f1−23 accumulation, we therefore infer that Phe23-Phe24 was hydrolyzed by CC at only 36% of the initial rate of BC, which corresponded roughly to the rate difference of overall α_{S1}-CN breakdown (∼30%; Figure 3A).

The most remarkable difference between the enzymatic modes of α_{S1}-CN hydrolysis was observed in the further conversion of the complementary C-terminal fragment (f24−199). CC showed the next largest preference for Leu101−Lys102, and this bond was also very susceptible to BC cleavage. However, the next most labile bond to BC activity was Trp164−Tyr165, which was in turn hardly touched by CC at all (Table 2). Interestingly, the f164−166 tripeptide was previously suggested to constitute the source of conformational rearrangements within the α_{S1}-CN molecule, induced by modifying the environment.[32,33] Other aspartic proteinases such as cathepsin D[34] and cardosin[35] also showed a high affinity to Trp164−Tyr165 in vitro.

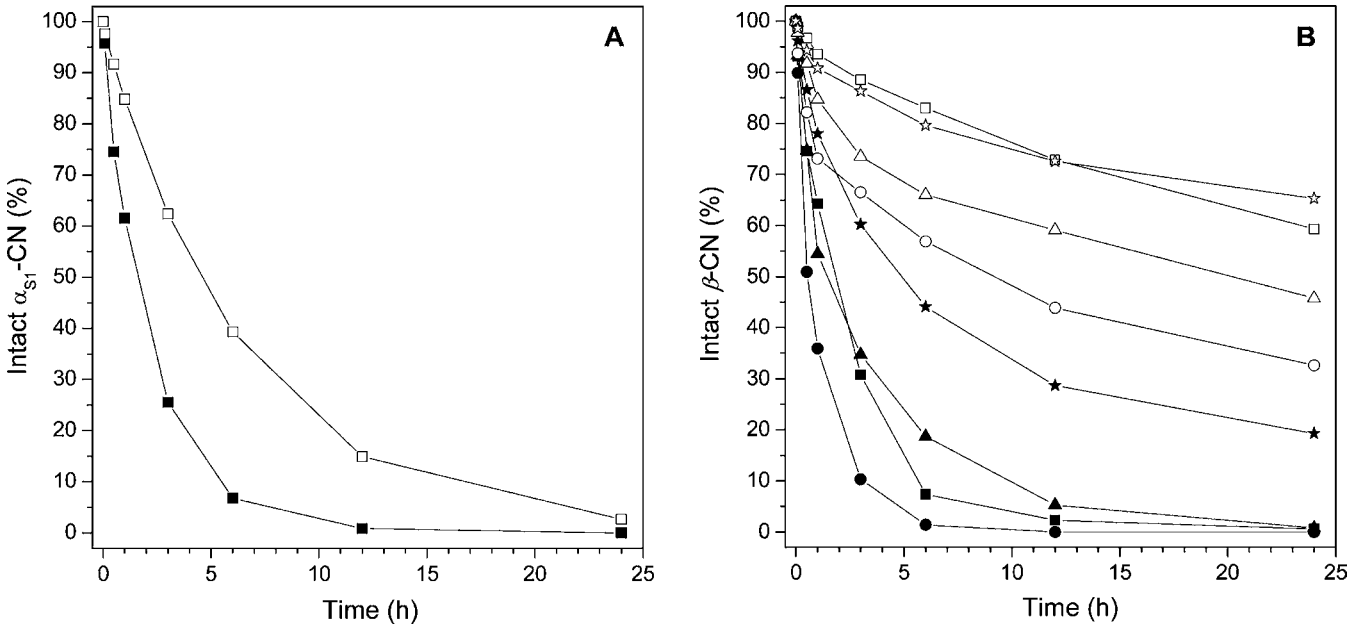

Figure 3. Breakdown of α_{S1}-casein (A) and β-casein (B) (expressed as % of the peak area at $A_{214\ nm}$ in t_0 samples) over 24 h of hydrolysis with camel chymosin (open symbols) and bovine chymosin (solid symbols): (□, ■) pH 6.5, 0% NaCl; (○, ●) pH 5.2, 0% NaCl; (△, ▲) pH 5.2, 2% NaCl; (☆, ★) pH 5.2, 5% NaCl.

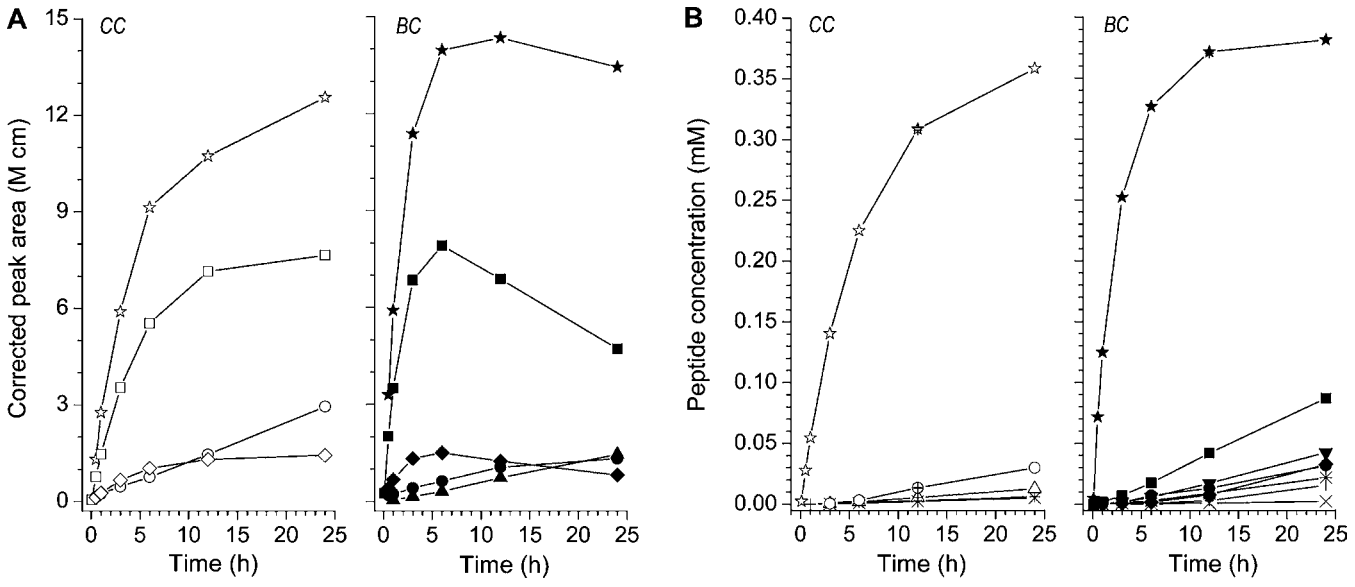

Figure 4. Peptide kinetics over 24 h of hydrolysis of α_{S1}-casein by camel chymosin (CC) (open symbols) and bovine chymosin (BC) (solid symbols) as estimated from CE (A) and RP-HPLC (B) analysis. (A) (☆, ★) f1−23 (5); (○, ●) f102−199 (6); (□, ■) f24−199(8*P*) (7); (◇, ◆) f24−199(9*P*) (8); (▲) f24−164(8*P*) (9). (B) (◆) f150−153 (2); (▼) f157−164 (4); (∗) f154−164/f150−156 (6); (+) f1−22 (9); (☆, ★) f1−23 (14); (l) f102−149 (17); (×) f24−98(8*P*) (18); (△) f24−98(7*P*) (19); (■) f165−199 (20); (○, ●) f24−101(7 + 8*P*) (22). Numbers in parentheses after peptides in preceding details for panels A and B refer to Figure 1, panels A and B, respectively. Data points of 12 h samples in panel B represent the mean ± standard deviation of quadruplicate sample injections.

The release of f24−101 exposed its C-terminal end, which was cleaved by both enzymes at Gln97−Leu98−Leu99 (Table 1). Initial accumulation of f24−98(8*P*) suggested CC to hydrolyze Leu98−Leu99 at about twice the rate of BC (Table 2), whereas this ratio was reversed in the case of Gln97−Leu98 (not shown). In the absence of further hydrolysis of f24−101, we may therefore reasonably infer comparable cleavage rates for Leu101−Lys102. According to Malin and Brown,[36] solvation of f24−101 causes little conformational change within the internal phosphoserine-rich region, whereas the more hydrophobic peptide termini undergo significant changes. The complementary f102−199 accumulated in the BC hydrolysate at only ∼60% of the rate of CC, presumably reflecting its further conversion via Trp164−Tyr165 cleavage, rather than a preference difference between the enzymes at Leu101−Lys102 (Table 2). It is therefore likely that BC hydrolyzed Trp164−Tyr165 using both f24−199 and f102−199 as substrates. Identification of a set of f102−*x* peptides especially in the BC hydrolysate likewise suggested f24−164 as substrate for Leu101−Lys102 hydrolysis (Table 1). Thus, hydrolysis of

Table 1. Identity of pH 4.6-Soluble Peptides Produced from α_{S1}-Casein over 24 h of Hydrolysis with Camel Chymosin (CC) and Bovine Chymosin (BC)

				pH 6.5	
				0% NaCl	
peak[a]	RI[a] (min)	amino acid sequence[b]	suggested peptide	CC	BC
5	30.5−30.8	RPKHPIKHQGLPQEVLNENL	f1−20	X	X
9	32.6−32.9	RPKHPIKHQGLPQEVLNENLLR	f1−22	X	–
14	37.5−38.4	RPKHPIKHQGLPQEVLNENLLRF	f1−23	X	X
15	40.7−40.9	QGLPQEVLNENLLRF	f9−23	(X)[c]	–
16	40.8−41.1	FVAP...S_{41}...YLEQ	f24−97(7*P*)	(X)	X
18	42.8−43.0	FVAP...s_{41}...LEQL	f24−98(8*P*)	X	X
19	43.1−43.3	FVAP...S_{41}...LEQL	f24−98(7*P*)	X	(X)
22	49.5−49.8	FVAP...s_{41}...LLRL	f24−101(8*P*)	(X)	(X)
22	49.5−49.8	FVAP...S_{41}...LLRL	f24−101(7*P*)	X	X
3	22.8−23.0	LRL	f99−101	X	X
11	33.2−33.4	KKYKVPQLEIVPNsAEERLHSM	f102−123(1*P*)	(X)	–
7	31.7−31.9	KKYKVPQLEIVPNsAEERLHSMK	f102−124(1*P*)	X	(X)
8	32.4−32.7	KKYKVPQLEIVPNsAEERLHSMKEGIH	f102−128(1*P*)	–	X
10	33.1−33.4	KKYKVPQLEIVPNsAEERLHSMKEGIHA	f102−129(1*P*)	–	X
13	35.1−35.3	KKYKVPQLEIVPNsAEERLHSMKEGIHAQQKEPMIGVNQEL	f102−142(1*P*)	–	X
17	41.6−41.9	KKYKVPQLEIVPNsAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPEL	f102−149(1*P*)	X	X
21	45.4−45.7	KKYKVPQLEIVPNsAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQF	f102−153(1*P*)	–	(X)
21	45.4−45.7	KKYKVPQLEIVPNsAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQL	f102−156(1*P*)	–	(X)
12	33.8−34.0	AYFYPEL	f143−149	–	X
2	21.7−22.0	FRQF	f150−153	–	X
6	31.3−31.7	FRQFYQL	f150−156	–	(X)
1	19.8−20.0	YQL	f154−156	–	X
6	31.3−31.7	YQLDAYPSGAW	f154−164	–	X
4	24.5−24.8	DAYPSGAW	f157−164	–	X
20	43.1−43.5	YYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW	f165−199	(X)	X
total no. of peptides produced[d]				12	20
no. of unique peptides produced				3	11 (12)[e]

[a]Peaks are numbered according to ascending retention interval (RI) (Figure 1B). [b]Amino acids given in single capital letter symbols. Phosphorylated serine is indicated by lower case "s". [c]Parentheses indicate that the MS signal(s) of the peptide did not dominate the MS spectra. [d]Corresponding peptides with different numbers of phosphorylations are counted as one. [e]Including f165−199.

Trp164−Tyr165 exposed several cleavage sites at the C-terminal end of f102−164, resulting in accumulation of f150−153/156, f154−156/164, and f157−164 only in the BC hydrolysate and at rates comparable to f24−101 (Table 2). Parallel accumulation of f102−149 suggested multiple substrates to contribute to the rich peptide release within residues 149−164. F143−164 was previously predicted to adopt an accessible conformation upon solvation.[36] Apart from a few minor cleavage sites within f102−149, this peptide and the C-terminal peptide of α_{S1}-CN, f165−199, were resistant to hydrolysis by BC. In comparison, trace activity to Leu149−Phe150 and two other bonds in the N-terminal region of f102−199 left this large casein fragment almost completely resistant to further hydrolysis by CC even after 24 h (Table 1).

The specificity of BC on α_{S1}-CN outlined above was in broad agreement with earlier studies undertaken at similar conditions,[15] except that we did not observe any activity to the N-terminal end of large f24−*x* peptides, nor to Tyr159−Pro160. On the other hand, Leu101−Lys102 was recognized as a major cleavage site in the present study. The high preference of BC to Leu101−Lys102 is well-recognized at low pH in solution as well as in cheese.[12,37] Apart from that, we identified only a few additional cleavage sites of rather low susceptibility (Table 1).

In summary, the preference of both enzymes toward Phe23−Phe24 was vastly superior to that of any other peptide bond within α_{S1}-CN. The next most susceptible bond to CC and BC activity was Leu101−Lys102 and Trp164−Tyr165, respectively, which were cleaved at only 3% of the rate of Phe23−Phe24 (Table 2). The third most CC susceptible bond was Leu98−Leu99 beyond which only minor cleavage sites were revealed. Hence, CC converted α_{S1}-CN into five major peptides including, in order of decreasing accumulation, f1−23 > f24−199 > f102−199 > f24−101 > f24−98. The third most BC susceptible bond could not be further differentiated between Leu101−Lys102, Leu149−Phe150, Phe153−Tyr154, and Leu156−Asp157, which were hydrolyzed at rates comparable to that of Leu101−Lys102 by CC (Table 2). Apart from Trp164−Tyr165 and activities exposed by its cleavage by BC, the proteolytic patterns thus revealed much qualitative resemblance, whereas CC was much less active than BC overall (Figure 1B).

β-CN. CC and BC hydrolysis of *β*-CN produced one and two to three major CE peaks, respectively (Figure 2). These peaks were poorly resolved and, consequently, no quantitative information could be extracted. A total of 6 and 15 pH 4.6-soluble peptides were identified by LC-MS/MS in the 24 h hydrolysates of CC and BC, respectively (Figure 5; Table 3). Among these, 0 CC- and 9 BC-derived peptides were solely produced by the respective chymosins. The kinetics and initial formation rates of the earliest peptides are shown in Figure 6A and Table 4.

Table 2. Initial Rate for the Formation of Early Peptides during the Hydrolysis of α_{S1}-Casein by Camel Chymosin (CC) and Bovine Chymosin (BC)

					initial rate of peptide formation[d] (nM/s)	
					pH 6.5	
					0% NaCl	
peak[a]	source[a]	α_{S1}-casein peptide	cleavage sites involved (P_1–P'_1)[b]	secondary structure[c]	CC	BC
9	LC-MS/MS	f1–22	Arg_{22}–*Phe_{23}*	*β*	0.072	0
14	LC-MS/MS	f1–23	*Phe_{23}*–*Phe_{24}*	*β*	12.9	36.0
18	LC-MS/MS	f24–98(8*P*)	*Phe_{23}*–*Phe_{24}*; *Leu_{98}*–*Leu_{99}*	*β*; *α*	0.064	0.031
19	LC-MS/MS	f24–98(7*P*)	*Phe_{23}*–*Phe_{24}*; *Leu_{98}*–*Leu_{99}*	*β*; *α*	0.16	ND[e]
22	LC-MS/MS	f24–101(7+8*P*)	*Phe_{23}*–*Phe_{24}*; *Leu_{101}*–Lys_{102}	*β*; r	0.40	0.39
7	CE	f24–199(8*P*)	*Phe_{23}*–*Phe_{24}*	*β*	1.7	4.2
8	CE	f24–199(9*P*)	*Phe_{23}*–*Phe_{24}*	*β*	0.25	0.54
17	LC-MS/MS	f102–149	*Leu_{101}*–Lys_{102}; *Leu_{149}*–*Phe_{150}*	r; *β*	0	0.21
6	CE	f102–199	*Leu_{101}*–Lys_{102}	r	0.12	0.075
2	LC-MS/MS	f150–153	*Leu_{149}*–*Phe_{150}*; *Phe_{153}*–*Tyr_{154}*	*β*; *β*	0	0.44
6	LC-MS/MS	f154–164 +	*Phe_{153}*–*Tyr_{154}*; *Trp_{164}*–*Tyr_{165}*	*β*; *β*	0	0.30
		f150–156	*Leu_{149}*–*Phe_{150}*; *Leu_{156}*–Asp_{157}	*β*; *β*		
4	LC-MS/MS	f157–164	*Leu_{156}*–Asp_{157}; *Trp_{164}*–*Tyr_{165}*	*β*; *β*	0	0.52
20	LC-MS/MS	f165–199	*Trp_{164}*–*Tyr_{165}*	*β*	0	1.0

[a]Number of peaks derived from CE and LC-MS/MS refers to Figure 1, panels A and B, respectively. [b]Hydrophobic amino acids are indicated in italics. [c]Secondary structural assignments are based on Kumosinski et al.[47] *α*, *α*-helix; *β*, *β*-sheet; r, reverse turn/random coil. [d]Initial rates derived from CE analysis are given in (M cm)/h and, hence, do not compare to rates derived from LC-MS/MS analysis (nM/s). [e]Not determined due to coeluted with f165–199.

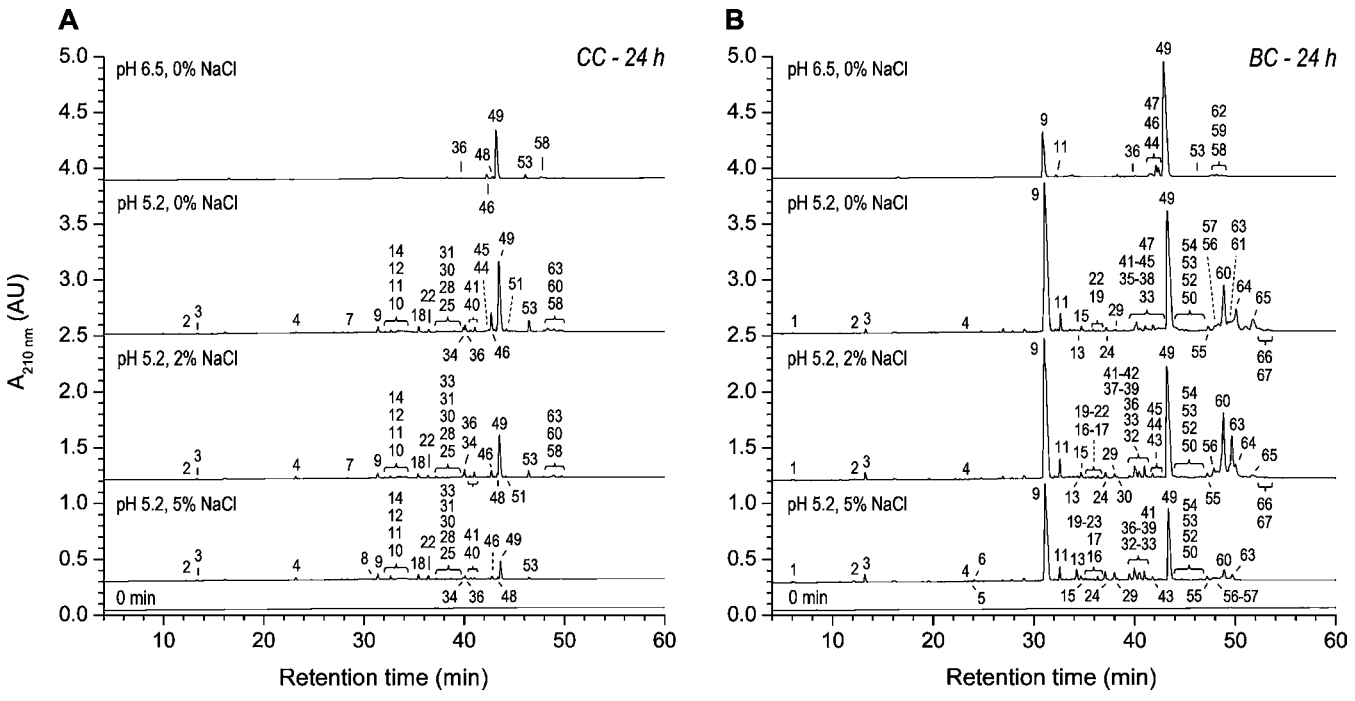

Figure 5. RP-HPLC profiles of pH 4.6-soluble peptides of *β*-casein produced by camel chymosin (A) and bovine chymosin (B) over 24 h of hydrolysis. Sample prepared of the initial *β*-casein substrate is included as a control (0 min). Numbered peaks were identified by MS/MS as specified in Table 3.

Irrespective of the type of chymosin, LC-MS/MS analysis revealed principal preference to Leu192–Tyr193, resulting in predominant accumulation of the very hydrophobic C-terminal f193–209 of *β*-CN (Figure 6A). Whereas CC experienced substrate abundance to Leu192–Tyr193 hydrolysis throughout the entire incubation period, intact *β*-CN was exhausted by BC hydrolysis after 12 h, and f193–209 plateaued at the level of the initial substrate concentration (0.43 mM). In accord with the fact that no other susceptible bonds were identified and its high tendency to associate in the hydrophilic environment,[16] we thus suggest that f193–209 was fully resistant to further conversion. Hence, CC exhibited only about 7% of the cleavage rate of BC

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Table 3. Identity of pH 4.6-Soluble Peptides Produced from β-Casein over 24 h of Hydrolysis with Camel Chymosin (CC) and Bovine Chymosin (BC)

				pH 6.5		pH 5.2					
				0% NaCl		0% NaCl		2% NaCl		5% NaCl	
peak[a]	RI[a] (min)	amino acid sequence[b]	suggested peptide	CC	BC	CC	BC	CC	BC	CC	BC
2	12.0−12.4	REL	f1−3	–	–	X	X	X	X	X	X
3	13.1−13.5	RELEE	f1−5	–	–	(X)[c]	X	(X)	X	X	X
4	23.1−23.4	RELEEL	f1−6	–	–	(X)	(X)	X	(X)	X	(X)
12	33.5−33.7	RELEELNVPGEIVEsLsssEESITRINK	f1−28(4*P*)	–	–	X	–	X	–	X	–
15	34.5−34.9	RELEELNVPGEIVEsLsssEESITRINKKIE	f1−31(4*P*)	–	–	–	X	–	X	–	X
14	34.2−34.4	RELEELNVPGEIVEsLsssEESITRINKKIEK	f1−32(4*P*)	–	–	X	–	X	–	X	–
25	37.1−37.4	RELEELNVPGEIVEsLsssEESITRINKKIEKF	f1−33(4*P*)	–	–	X	–	X	–	X	–
19	35.8−35.9	RELEE...QsEEQ	f1−38(5*P*)	–	–	–	X	–	X	–	(X)
20	35.9−36.1	RELEE...sEEQQ	f1−39(5*P*)	–	–	–	–	–	X	–	X
21	36.3−36.5	RELEE...QQQTE	f1−42(5*P*)	–	–	–	–	–	X	–	X
22	36.3−36.6	RELEE...QQTED	f1−43(5*P*)	–	–	(X)	(X)	(X)	(X)	(X)	(X)
30	38.2−38.5	RELEE...TEDEL	f1−45(5*P*)	–	–	(X)	–	(X)	X	(X)	X
28	37.7−38.0	RELEE...DELQD	f1−47(5*P*)	–	–	(X)	(X)	X	X	X	X
38	40.4−40.5	RELEE...HPFAQ	f1−54(5*P*)	–	–	–	X	–	X	–	X
37	40.1−40.4	RELEE...FAQTQ	f1−56(5*P*)	–	–	–	(X)	–	X	–	X
36	39.8−40.3	RELEE...AQTQS	f1−57(5*P*)	X	(X)	X	X	X	X	X	X
41	40.9−41.2	RELEE...QTQSL	f1−58(5*P*)	–	–	X	X	X	X	X	X
60	48.6−49.2	RELEE...H_{67}...QPEVM	f1−93(5*P*)[B]	–	–	X	(X)	X	X	–	(X)
63	49.6−50.0	RELEE...P_{67}...QPEVM	f1−93(5*P*)[A2]	–	–	X	X	X	X	–	(X)
60	48.6−49.2	RELEE...H_{67}...R_{122}...QSLTL	f1−127(5*P*)[B]	–	–	–	X	–	(X)	–	–
64	49.9−50.4	RELEE...P_{67}...S_{122}...QSLTL	f1−127(5*P*)[A2]	–	–	–	(X)	–	(X)	–	–
17	35.2−35.5	EELNV...QQQTE	f4−42(5*P*)	–	–	–	–	–	(X)	–	X
27	37.5−37.6	EELNV...TEDEL	f4−45(5*P*)	–	–	–	–	–	–	–	X
33	39.6−39.8	EELNV...AQTQS	f4−57(5*P*)	–	–	–	–	X	(X)	X	(X)
39	40.5−40.7	EELNV...QTQSL	f4−58(5*P*)	–	–	–	–	–	(X)	–	(X)
16	35.0−35.2	LNVPG...QQQTE	f6−42(5*P*)	–	–	–	–	–	(X)	–	X
26	37.3−37.5	LNVPG...TEDEL	f6−45(5*P*)	–	–	–	–	–	(X)	–	X
23	36.8−37.0	LNVPG...DELQD	f6−47(5*P*)	–	–	–	–	–	–	–	X
36	39.8−40.3	LNVPG...HPFAQ	f6−54(5*P*)	–	–	–	–	–	(X)	–	(X)
33	39.6−39.8	LNVPG...FAQTQ	f6−56(5*P*)	–	–	–	X	–	(X)	–	(X)
32	39.4−39.6	LNVPG...AQTQS	f6−57(5*P*)	–	–	–	–	–	X	–	X
39	40.5−40.7	LNVPG...QTQSL	f6−58(5*P*)	–	–	–	–	–	X	–	X
60	48.6−49.2	LNVPG...H_{67}...QPEVM	f6−93(5*P*)[B]	–	–	–	–	–	(X)	–	(X)
63	49.6−50.0	LNVPG...P_{67}...QPEVM	f6−93(5*P*)[A2]	–	–	–	–	–	(X)	–	(X)
31	38.6−38.8	NVPGE...AQTQS	f7−57(5*P*)	–	–	(X)	–	X	–	X	–
57	48.1−48.5	VYPFP...H_{67}...QPEVM	f59−93[B]	–	–	–	(X)	–	(X)	–	X
13	34.1−34.6	GVSKVKEAMAPKHKEMPFPKYPVQPFTE	f94−121	–	–	–	X	–	X	–	X
24	37.0−37.3	GVSKV...R_{122}...QSLTL	f94−127[B]	–	–	–	X	–	X	–	X
29	37.9−38.2	GVSKV...S_{122}...QSLTL	f94−127[A2]	–	–	–	X	–	X	–	X
54	46.5−46.8	GVSKV...R_{122}...LLLQS	f94−142[B]	–	–	–	X	–	X	–	X
56	47.7−48.1	GVSKV...S_{122}...LLLQS	f94−142[A2]	–	–	–	X	–	(X)	–	(X)
66	52.3−52.7	GVSKV...R_{122}...SVLSL	f94−165[B]	–	–	–	X	–	(X)	–	–
67	53.1−53.6	GVSKV...S_{122}...SVLSL	f94−165[A2]	–	–	–	X	–	(X)	–	–
5	23.6−23.8	SQSLTL	f122−127[A2]	–	–	–	–	–	–	–	X
6	24.0−24.2	RQSLTL	f122−127[B]	–	–	–	(X)	–	(X)	–	X
52	44.6−44.9	TDVENLHLPPLLL	f128−140	–	–	–	(X)	–	(X)	–	(X)
43	41.7−42.0	TDVENLHLPPLLLQS	f128−142	–	–	–	X	–	X	–	X
55	47.1−47.5	TDVENLHLPPLLLQSW	f128−143	–	–	–	X	–	X	–	X
64	49.9−50.4	TDVEN...PQSVL	f128−163	–	–	–	(X)	–	(X)	–	–
65	51.5−52.2	TDVEN...SVLSL	f128−165	–	–	–	X	–	X	–	–
64	49.9−50.4	TDVEN...LSLSQ	f128−167	–	–	–	X	–	X	–	–
61	49.2−49.4	TDVEN...MPIQA	f128−189	–	–	–	X	–	X	–	–
50	43.9−44.2	WMHQPHQPLPPTVMFPPQSVLSL	f143−165	–	–	–	X	–	(X)	–	(X)
42	41.2−41.4	WMHQPHQPLPPTVMFPPQSVLSLSQ	f143−167	–	–	–	X	–	(X)	–	–
47	42.8−43.1	WMHQP...MPIQA	f143−189	–	(X)	–	X	–	–	–	–
59	48.6−49.0	WMHQP...QAFLL	f143−192	–	X	–	–	–	–	–	–
31	38.6−38.8	MHQPHQPLPPTVMFPPQSVL	f144−163	–	–	(X)	–	(X)	–	(X)	–

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Table 3. continued

				pH 6.5		pH 5.2					
				0% NaCl		0% NaCl		2% NaCl		5% NaCl	
peak[a]	RI[a] (min)	amino acid sequence[b]	suggested peptide	CC	BC	CC	BC	CC	BC	CC	BC
45	42.2−42.6	MHQPHQPLPPTVMFPPQSVLSL	f144−165	−	−	(X)	X	(X)	X	−	X
35	39.8−40.0	MHQPHQPLPPTVMFPPQSVLSLSQ	f144−167	−	−	−	X	−	−	−	−
44	42.0−42.3	MHQPH...MPIQA	f144−189	−	X	X	X	X	X	−	(X)
51	44.1−44.4	MHQPH...PIQAF	f144−190	−	−	X	−	X	−	−	−
58	48.2−48.6	MHQPH...QAFLL	f144−192	X	X	X	−	(X)	−	−	−
1	5.9−6.4	SLSQ	f164−167	−	−	−	X	−	X	−	X
10	32.0−32.3	SLSQSKVLPVPQKAVPYPQRDMPIQ	f164−188	−	−	(X)	−	(X)	−	(X)	−
11	32.5−32.8	SLSQSKVLPVPQKAVPYPQRDMPIQA	f164−189	−	X	X	X	X	X	X	X
22	36.3−36.6	SLSQSKVLPVPQKAVPYPQRDMPIQAF	f164−190	−	−	X	−	X	−	X	−
40	40.5−40.8	SLSQSKVLPVPQKAVPYPQRDMPIQAFL	f164−191	−	−	X	−	X	−	X	−
48[d]	43.2−43.5	SLSQSKVLPVPQKAVPYPQRDMPIQAFLL	f164−192	X	X	X	−	X	−	X	−
8	30.7−31.0	SQSKVLPVPQKAVPYPQRDMPIQ	f166−188	−	−	−	−	−	−	(X)	−
9	30.8−31.6	SQSKVLPVPQKAVPYPQRDMPIQA	f166−189	−	X	X	X	X	X	X	X
18	35.3−35.6	SQSKVLPVPQKAVPYPQRDMPIQAF	f166−190	−	−	X	−	X	−	X	−
34	39.8−40.0	SQSKVLPVPQKAVPYPQRDMPIQAFL	f166−191	−	−	X	−	X	−	X	−
46	42.6−43.0	SQSKVLPVPQKAVPYPQRDMPIQAFLL	f166−192	X	X	X	−	X	−	X	−
9	30.8−31.6	SKVLPVPQKAVPYPQRDMPIQA	f168−189	−	X	X	X	(X)	X	−	X
47	42.8−43.1	SKVLPVPQKAVPYPQRDMPIQAFLL	f168−192	−	X	−	−	−	−	−	−
9	30.8−31.6	FLL	f190−192	−	X	X	X	X	X	X	X
62[e]	49.5−49.8	FLLYQEPVLGPVRGPFPIIV	f190−209	−	(X)	−	−	−	−	−	−
53	46.3−46.6	LLYQEPVLGPVRGPFPIIV	f191−209	X	(X)	X	(X)	X	(X)	X	(X)
7	28.2−28.5	YQEPVLGPVRGP	f193−204	−	−	(X)	−	(X)	−	−	−
49	43.1−43.8	YQEPVLGPVRGPFPIIV	f193−209	X	X	X	X	X	X	X	X
total no. of peptides produced[f]				6	15	32	40	33	49	27	45
total no. of phosphopeptides produced[f]				1	1	10	11	11	23	10	24
no. of unique peptides produced at equal levels of pH and NaCl[f]				0	9	16	24	15	31	13	31
no. of peptides unique to pH level (0% NaCl)[f] (comparison within enzyme)				0	6	26	31				
no. of peptides unique to NaCl level (pH 5.2)[f] (comparison within enzyme)						0	2	0	0	1	2

[a]Peaks are numbered according to ascending retention interval (RI) (Figure 5). [b]Amino acids given in single capital letter symbols. Phosphorylated serine is indicated by lower case "s". [c]Parentheses indicate that the MS signal(s) of the peptide did not dominate the MS spectra. [d]Peaks 48 and 49 coeluted in BC hydrolysate, pH 6.5, and CC hydrolysate, pH 5.2, 0% NaCl. [e]MS signal dominant up to 6 h. [f]Corresponding peptides of different genetic variants (A2 and B) are counted as one.

toward Leu192−Tyr193 (Table 4). This difference compared well to that of β-CN breakdown estimated by CE (~15%; Figure 3B).

Another two early peptides were identified in quantifiable amounts in the CC hydrolysate, namely, f166−192 and f191−209, suggesting that Leu165−Ser166 and Phe190−Leu191 with cleavage rates of around 5% of that of Leu192−Tyr193 comprised the next most CC susceptible positions in β-CN (Table 4). In comparison, the bonds of the next greatest preference to BC included Leu165−Ser166, Gln167−Ser168, and Ala189−Phe190, which were also hydrolyzed at about 5% of the rate of Leu192−Tyr193. Although an early dominance of f190−192 over f166/168−189 in peak 9 (Figure 5B) was striking as evaluated by MS (not shown), hydrolysis of f1−192 at these three sites seemed to proceed in relatively close parallel at the early stage. Moreover, whereas the accumulation of f166/168−192 leveled off (Figure 6A, inset), peak 9 continued to increase at a linear rate, suggesting the further hydrolysis of f166/168−192 at Ala189−Phe190 and/or enhanced preference to Leu165−Ser166 and Gln167−Ser168 subsequent to Ala189−Phe190 cleavage. Less frequently, BC also hydrolyzed intact β-CN at Ala189−Phe190, liberating f190−209, which was, however, rapidly converted, presumably to f193−209 as shown previously.[16] Finally, f1−192 was hydrolyzed to a limited extent by both chymosins somewhat further along its C-terminal end, namely, at Leu163−Ser164 and Trp143−Met144 and, in the case of BC, also at Ser142−Trp143. In contrast, the hydrophilic N-terminal region of β-CN was cleaved sparsely at Ser57−Leu58 only, in the hydrolysate of both enzymes. Apart from this bond, all enzyme activity was thus directed toward a definite stretch of about 50 residues located at the hydrophobic C-terminal one-third of β-CN. As with α_{S1}-CN, CC was overall significantly less active than BC toward β-CN (Figure 3B).

The specificity of BC on β-CN agreed with earlier studies in which comparable experimental conditions were used,[11,38] except that we did not observe any cleavage at Leu139−Leu140−Gln141, but rather at Ser142−Trp143−Met144 (Table 3). The additional cleavage site identified at Ser57−Leu58 in this study was previously recognized at conditions of higher acidity.[13]

Overall Comparison of CC and BC. This paragraph serves to draw parallels in the mode of action of the two enzymes across the two different casein substrates. First, both CC and BC showed higher activity toward α_{S1}- as compared to β-CN. This preference was previously recognized for BC in both solution[14] and cheese[37] and may be explained in view of a more open conformation of α_{S1}-CN and a more amphiphilic (self-associative) nature of β-CN, favoring higher enzyme accessibility to α_{S1}-CN.[39] Second, CC and BC both showed strong

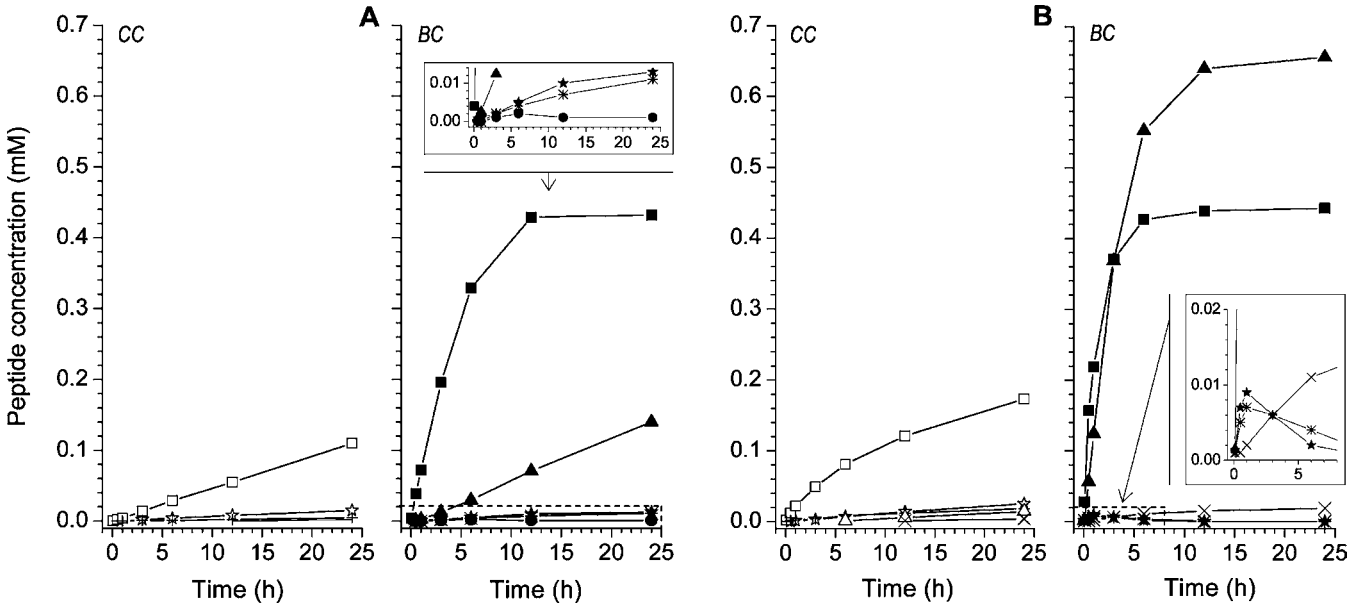

Figure 6. Peptide kinetics over 24 h of hydrolysis of β-casein by camel chymosin (CC) (open symbols) and bovine chymosin (BC) (solid symbols) as estimated from RP-HPLC analysis. (A) pH 6.5, 0% NaCl; (B) pH 5.2, 0% NaCl. (△, ▲) f190−192, f166−189, f168−189 (9); (×) f164−189 (11); (☆, ★) f166−192 (46); (∗) f168−192 (47); (□, ■) f193−209 (49); (+) f191−209 (53); (●) f190−209 (62). Numbers in parentheses after peptides in preceding details refer to Figure 5.

Table 4. Initial Rate for the Formation of Early Peptides during the Hydrolysis of β-Casein by Camel Chymosin (CC) and Bovine Chymosin (BC)

				initial rate of peptide formation[d] (nM/s)							
				pH 6.5		pH 5.2					
				0% NaCl		0% NaCl		2% NaCl		5% NaCl	
peak[a]	β-casein peptide	cleavage sites involved (P_1−P'_1)[b]	secondary structure[c]	CC	BC	CC	BC	CC	BC	CC	BC
11	164−189	*Leu_{163}*−Ser_{164}; *Ala_{189}*−*Phe_{190}*	r; β	0	0.044	0.077	0.63	0.051	0.41	0.037	0.30
46	166−192	*Leu_{165}*−Ser_{166}; *Leu_{192}*−*Tyr_{193}*	r; β	0.063	0.23	0.33	4.3	0.10	1.4	0.048	0.077
47	168−192	Gln_{167}−Ser_{168}; *Leu_{192}*−*Tyr_{193}*	r; β	0	0.18	0	3.3	0	1.1	0	0.040
9	190−192 +	*Ala_{189}*−*Phe_{190}*; *Leu_{192}*−*Tyr_{193}*	β; β	0	2.7	0.20	37.9	0.16	22.3	0.15	12.0
	166−189 +	*Leu_{165}*−Ser_{166}; *Ala_{189}*−*Phe_{190}*	r; β								
	168−189	Gln_{167}−Ser_{168}; *Ala_{189}*−*Phe_{190}*	r; β								
62	190−209	*Ala_{189}*−*Phe_{190}*	β	0	0.11						
53	191−209	*Phe_{190}*−*Leu_{191}*	β	0.057	0	0.36	0	0.17	0	0.092	0
49	193−209	*Leu_{192}*−*Tyr_{193}*	β	1.3	17.9	5.9	85.0	1.3	24.7	0.41	5.2

[a]Peak number refers to Figure 5. [b]Hydrophobic amino acids are indicated in italics. [c]Secondary structural assignments are based on Kumosinski et al.[48] β, β-sheet; r, reverse turn/random coil. [d]All data derive from LC-MS/MS analysis.

preferential cleavage of one particular bond in both caseins, a feature resembling the mode of action to their principal substrate, κ-CN. On the basis of this behavior, the affinity of BC was previously confined to peptide bonds located between residues of which at least one was highly hydrophobic and which were both in β-sheet conformation and surrounded by random coil or reverse turn structure.[32] As a result of identical sites of first hydrolysis of α_{S1}- and β-CN by the two enzymes, these criteria applied to the affinity of CC, too, and manifested as a general characteristic of bonds involved in the formation of early peptides by either enzyme (Tables 2 and 4). Moreover, such bonds tended to be located within pronounced hydrophobic stretches of the caseins.[39] In line with the findings of this study, proteolytic comparison of chymosins originating from bovine and other species previously revealed mainly quantitative differences, and the enzymes were suggested to be very closely related.[26]

Hydrolysis of β-CN at pH 5.2 and Variable NaCl Concentration. In the following sections we will extend and relate the proteolytic behavior revealed for CC and BC toward β-CN at pH 6.5 to the corresponding systems at pH 5.2 and containing 0, 2, or 5% (w/v) NaCl. α_{S1}-CN was not studied at pH 5.2 due to its high net negative charge and high phosphate content and, hence, poor solubility at low pH.

Effect of pH. Many of the peptides produced at pH 5.2 (0% NaCl) were also identified at pH 6.5, but the rates of formation were markedly higher, and several additional peptides were produced at pH 5.2 irrespective of the type of chymosin. Leu192−Tyr193 remained the highly preferred first site of hydrolysis by both enzymes at pH 5.2 (Table 4). Interestingly, whereas f193−209 was still resistant to BC hydrolysis, a small amount of f193−204 was identified in the CC hydrolysate (Table 3) that contributed to a weak plateauing in the kinetics of f193−209 accumulation (Figure 6B). Alternatively, enzymatic self-

inhibition may explain such kinetics.[19] Hence, Leu192−Tyr193 cleavage by both enzymes proceeded at a 5-fold higher initial rate at the lower pH (Table 4). The resistance of f1−192 to further conversion by CC as compared to BC became very apparent at pH 5.2. Whereas the two bonds of the next greatest susceptibility to CC cleavage (Leu165−Ser166 and Phe190−Leu191) were hydrolyzed at about 5% of the rate of Leu192−Tyr193 independent of pH, the corresponding relative hydrolysis rates of the three bonds of the next greatest susceptibility to BC cleavage (Leu165−Ser166, Gln167−Ser168, and Ala189−Phe190) increased from about 5 to 18% upon the pH decrease (Table 4). The higher activity of chymosin and other aspartic proteinases at acidic pH is well-recognized and relates to the enzyme rather than the substrate or environmental conditions.[11,14,40] Besides, due to net negative enzyme and substrate charges (p*I* ∼5), the pH decrease diminishes the overall electrostatic repulsive energy barrier between them, thus promoting initial recognition.[21,31]

Apart from accelerating the production of early peptides, a range of additional major and minor peptides were identified at the lower pH (Figure 5). The complexity of the CC hydrolysate increased more than that of BC and, consequently, the difference in terms of total number of peptides produced after 24 h was smaller at pH 5.2 (Table 3). New peptides arose from both new cleavage sites and higher activity to sites already observed at pH 6.5. In particular, several bonds at the acidic and polar N-terminal end of *β*-CN (residues 1−60) became susceptible to cleavage by both enzymes and produced a range of relatively large phosphopeptides. This effect has been reported previously for BC[41] and can be explained in view of a particular reduction in the repulsive energy barrier between the enzyme and this region of the substrate upon the pH decrease. The location of labile N-terminal bonds, however, depended on the enzyme (Table 3). Furthermore, some new bonds adjacent to previously recognized cleavage sites were hydrolyzed by CC (Gln188−Ala189, Ala189−Phe190, and Leu191−Leu192) and BC (Leu140−Gln141) at the lower pH. Interestingly, a few bonds located in the hydrophobic interior of the protein became sufficiently exposed at pH 5.2 to constitute a minor cleavage site of CC and a major one of BC, namely, Met93−Gly94, and a minor and major cleavage site of BC, namely, Glu121−Ser122 and Leu127−Thr128, respectively. These cleavage sites of BC at acidic pH have been reported previously.[13] The greater depth and complexity of acidic BC hydrolysis was also evident from comparison of the CE profiles (Figure 2).

Finally, some peptides observed in the 24 h hydrolysate produced by BC at pH 6.5 were not found in the lower pH counterpart (f143/144/164/166/168−192 and f190−209). These peptides were all labile intermediates and illustrate as such the central observation that the vast majority of proteolytic differences revealed across enzyme and pH level were predetermined by quantitative effects. The particularly low affinity of CC to Ala189−Phe190 cleavage is suggested to prevent much of the activity revealed by BC further along the C-terminal end of f1−192.

Effect of NaCl. Variation of the NaCl concentration at pH 5.2 elucidated several interesting effects common to both CC and BC as well as a number of differences between them. Both enzymes showed preferential activity to Leu192−Tyr193 across all NaCl concentrations (Table 4). Their inhibition to this hydrolysis was by far the most significant quantitative effect of NaCl, as reflected by a dramatic decrease in initial rate of f193−209 formation to around 6−7% at 5% NaCl. A similar effect of NaCl has been reported in a number of previous studies conducted on BC in model solution[42] and cheese[43] and was concurrently ascribed to restricted accessibility due to extensive hydrophobic self-association of the substrate rather than to the enzyme itself. An increase in the *β*-CN−*β*-CN binding response has indeed been measured in the presence of NaCl.[44] Also, the next most CC and BC susceptible bonds were the same at all three NaCl levels. Again, significant gradual NaCl inhibition was observed in the initial hydrolysis rates, but the degree of inhibition was much less severe than that of Leu192−Tyr193. In fact, first hydrolysis seemed to become rate limiting to second BC attack further along the truncated C-terminal at 5% NaCl (Table 4). This behavior can be explained by the aforementioned micellar association and, hence, inaccessibility of monomeric *β*-CN, which, once converted to f1−192, is readily accessible to further hydrolysis due to an almost complete loss of ability to self-associate and a highly open tertiary structure.[11,45]

No major quantitative impact of NaCl was revealed beyond that involving the release of early peptides during CC hydrolysis, whereas peptides involving cleavage of interiorly located bonds Met93−Gly94 (peaks 60, 63), Glu121−Ser122 (peaks 5, 6, 13), and Leu127−Thr128 (peak 64) accumulated at significantly NaCl-dependent rates in the BC hydrolysate (Figure 5). Although late eluting peaks were complex and poorly resolved, Leu127−Thr128 cleavage appeared strongly inhibited by NaCl, whereas the opposite tendency was observed for Glu121−Ser122. Met93−Gly94 is suggested to be optimally hydrolyzed by BC at a low level of NaCl (∼2%). In contrast, BC activity toward the distinctly hydrophilic N-terminal end of the molecule (residues 1−60) was always stimulated by increasing NaCl concentration, as revealed by the accumulation of an increasing number and/or quantity of different phosphopeptides (Figure 5B; Table 3). These findings may reflect an increased substrate hydration and, hence, exposure to enzymatic attack in the presence of NaCl and/or a reduction in electrostatic enzyme−substrate repulsion, which manifested similarly in the action of both enzymes as the pH was reduced. In contrast, the N-terminal activity of CC was not further stimulated by NaCl (Figure 5A), which may thus also have induced some conformational changes in either enzyme.

In line with the enzymatic behavior revealed hitherto, many peptides appeared and accumulated gradually as a function of NaCl concentration, indicating that NaCl did not alter enzyme specificity. As a result, hardly any peptides produced by either enzyme were unique to a specific level of NaCl (Table 3). Rather, NaCl restricted and promoted access of the enzymes to distinctly hydrophobic and hydrophilic regions of *β*-CN, respectively, whereas hydrolysis within regions of intermediate properties (residues 90−130)[39] showed mixed NaCl dependencies. This mode of impact manifested to an extent that depended on the enzymes' own conformational response to NaCl. Consequently, Leu192−Tyr193 cleavage by CC proceeded at only 5−8% of the initial rate of BC independent of NaCl concentration (and pH), whereas the quantitative impact of NaCl at regions not obviously influenced by this C-terminal truncation was almost fully reserved to BC. The rate of initial *β*-CN breakdown correlated inversely with NaCl concentration because it was governed by Leu192−Tyr193 cleavage (Figure 3B), whereas the pronounced hydrophobicity of *β*-CN overall eventually caused a net loss of activity but, in the case of BC, only as the NaCl concentration reached 5% (Figure 5B).

Most of the additional and early activity observed at pH 5.2 and variable NaCl concentration supported a high degree of

similarity between the modes of action of the two chymosins and that the differences revealed were mainly of quantitative origin. Still, within the degree of hydrolysis spanned by the present experimental field, the uniqueness of several cleavage sites could be proposed for either enzyme.

Cheese Quality. CC was previously shown to produce harder and less bitter cheese as compared to BC.[20,46] These effects may in part reflect directly on the present findings that α_{S1}-$CN_{23/24}$ and β-$CN_{192/193}$ were hydrolyzed by CC at only 36 and 7% of the rates of BC, respectively, as estimated at equivalent IMCU concentrations. Apart from the bitter-tasting f193−209 of β-CN, we suggest hydrophobic peptides such as β-CN(f168−189) and particularly β-CN(f190−192) could constitute potential sources of bitterness in cheese made with BC that may be practically absent with CC. Likewise, extensive BC activity toward β-$CN_{93/94}$ and β-$CN_{127/128}$ at pH 5.2 may be of importance for bitterness development in cheese and could probably be circumvented by using CC. The proteolytic behavior of CC during ripening of Cheddar cheese containing 2 and 5% (w/w) salt-in-moisture is currently being compared to that of BC.

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ABBREVIATIONS USED

BC, bovine chymosin; *C*, milk-clotting activity; CC, camel chymosin; CE, capillary electrophoresis; CN, casein; f, fragment; IMCU, international milk-clotting unit; *P*, general proteolytic activity; RP, reversed phase.

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