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Comparison of the Hydrolysis of Bovine κ-Casein by Camel and Bovine Chymosin: A Kinetic and Specificity Study

Kirsten Kastberg Møller,*[,†,‡] Fergal P. Rattray,[‡] Jens Christian Sørensen,[†] and Ylva Ardö[†]

[†]Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

[‡]Chr. Hansen A/S, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark

Supporting Information

ABSTRACT: Bovine chymosin constitutes a traditional ingredient for enzymatic milk coagulation in cheese making, providing a strong clotting capacity and low general proteolytic activity. Recently, these properties were surpassed by camel chymosin, but the mechanistic difference behind their action is not yet clear. We used capillary electrophoresis and reversed-phase liquid chromatography-mass spectrometry to compare the first site of hydrolysis of camel and bovine chymosin on bovine κ-casein (CN) and to determine the kinetic parameters of this reaction (pH 6.5; 32 °C). The enzymes showed identical specificities, cleaving the *Phe105−Met106* bond of κ-CN to produce *para*-κ-CN and caseinomacropeptide. Initial formation rates of both products validated Michaelis−Menten modeling of the kinetic properties of both enzymes. Camel chymosin bound κ-CN with ∼30% lower affinity (K_M) and exhibited a 60% higher turnover rate (k_{cat}), resulting in ∼15% higher catalytic efficiency (k_{cat}/K_M) as compared to bovine chymosin. A local, less dense negatively charged cluster on the surface of camel chymosin may weaken electrostatic binding to the *His−Pro* cluster of κ-CN to simultaneously impart reduced substrate affinity and accelerated enzyme−substrate dissociation as compared to bovine chymosin.

KEYWORDS: *camel chymosin, bovine chymosin, κ-casein, Michaelis−Menten kinetics, enzyme specificity*

INTRODUCTION

The transformation of liquid milk into a gel is a key step of the cheese-making process. Milk coagulation is typically achieved by enzymatic hydrolysis of κ-casein (CN) at the surface of CN micelles. κ-CN covers the predominantly hydrophobic core of the micelles by its polar, net negatively charged C-terminal, thus preventing micellar aggregation by steric hindrance and charge repulsion. Milk-clotting enzymes specifically remove this part of the protein by cleavage at the *Phe105−Met106* bond in bovine κ-CN, which leaves the CN micelles deprived of colloidal properties, thus leading to aggregation. While the hydrophobic *para*-κ-CN (*Glu1−Phe105*) remains in the cheese curd, the hydrophilic caseinomacropeptide (CMP) (*Met106−Val169*) dissolves in the whey (all κ-CN residues are typed in italics).[1]

Chymosin (EC 3.4.23.4) of bovine origin (*Bos taurus*) represents the principal milk-clotting enzyme used in cheese making.[2] It belongs to the aspartic proteinase family and is as such structured mainly in β-sheets that form the N- (Gly1−Tyr175) and C-terminal (Tyr176−Ile323) barrel domains. These domains are divided by a deep substrate binding cleft, at the base of which the two catalytic aspartates (Asp34 and Asp216) reside.[3] Bovine chymosin (pI ≈ 4.7) has a net negative charge at the pH of milk (∼6.6), just like its natural substrate κ-CN (pI ≈ 5.6).[1]

The ratio between milk-clotting activity (C) expressed in International Milk-Clotting Units (IMCU) and general proteolytic activity (P) represents a useful measure of the combined activity and specificity of the coagulant and, hence, of its performance during cheese making.[4] Generally, enzymes combining a high C with a low P are desirable to minimize the coagulation time and loss of CN-derived peptides to the cheese whey. In this respect, bovine chymosin is superior to most other aspartic proteinases in use for bovine milk coagulation.[5] Porcine chymosin, for example, has been examined in detail, as it has a higher C/P ratio than bovine chymosin.[6] However, because of a low C, it is not an economic alternative to bovine chymosin and, hence, not commercially available.

On the basis of available structural data, numerous kinetic studies have sought to unveil the mechanism behind the highly efficient and specific action of bovine chymosin using as substrate various whole κ-CN fractions,[7,8] peptides of various sizes corresponding in amino acid sequence to the extended chymosin-sensitive region of κ-CN,[9−11] or peptide mutants.[12] The finding that *His98−Lys112* corresponding to the peptide joining the N- and C-terminal domains of κ-CN provides kinetic parameters (K_M and k_{cat}) similar to those for whole κ-CN represents an important observation of previous work.[11] Visser et al.[12] proposed a model for the enzyme−substrate (ES) complex in which *Leu103−Ile108* is accommodated within the active-site cleft of the enzyme and kept in position through hydrophobic and hydrogen binding to the enzyme. Enzymatic preference to the *Phe105−Met106* bond was further enhanced via electrostatic binding of positively charged residues delineating the extended chymosin-sensitive region (*His98−His102* and *Lys111*), whereas *Pro99,101,109,110* residues acted as steric stabilizers of a particular constrained substrate conformation in the ES complex.[12] Advances in molecular modeling supported that the substrate binds to bovine

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chymosin in an extended conformation with charged residues on either side of the scissile bond playing an important role in stabilizing the binding pose.[13] Moreover, Gustchina et al.[9] pointed to a pivotal role of the electrostatic binding of the *His*−*Pro* cluster (*His98*−*His102*) in acting as an allosteric activator of bovine chymosin that involves a conformational change of Tyr77 located within the flap entrance to the active-site cleft and, hence, conversion from the self-inhibited to the active form of the enzyme.

Recently, chymosin of camel origin (*Camelus dromedarius*) produced in *Aspergillus niger* demonstrated an impressive 7-fold higher C/P ratio (∼70% higher C and only ∼25% of P) as compared to bovine chymosin in bovine milk.[14] Thus, although κ-CN is generally assumed to possess superior substrate properties to chymosin of the same species, camel chymosin interestingly clots bovine and camel milk equally well, whereas bovine chymosin fails to coagulate camel milk.[15] As a consequence of the improved C/P ratio of camel chymosin, similar curd strengths and renneting times were obtained with almost 30% less IMCU in cheddar cheese trials, while the extent of primary proteolysis was significantly lower in cheeses made with camel as compared to bovine chymosin.[16]

With the aim to unravel the mechanism behind this remarkably specific action of camel chymosin on bovine κ-CN exceeding even that of bovine chymosin, different comparative investigations have been initiated. Commercially available camel chymosin has been broadly characterized.[14] Unrestrained molecular dynamics simulations of the enzymes complexed with peptides corresponding to the extended chymosin-sensitive region of bovine κ-CN (*Arg97*−*Lys112*) and camel κ-CN (*Arg89*−*Ile104*) were evaluated,[17] and the 3D enzyme structures determined to high resolutions.[18] The present work aimed to contribute to this compilation of experimental evidence by investigating for the first time the proteolytic specificity of camel chymosin on bovine κ-CN and comparing the kinetic parameters for the action of camel and bovine chymosin on whole κ-CN purified from bovine milk. The enzymatic reactions were followed at pH 6.5 and 32 °C, and the N- and C-terminal products of κ-CN were analyzed. This experimental setup enabled the application of Michaelis−Menten kinetics under conditions of high relevance to cheese-making.

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), unless otherwise stated in the text.

Enzymes. Fermentation-produced camel chymosin (∼100% purity, CHY-MAX M, 1011 IMCU/mL) and fermentation-produced bovine chymosin [var. B, ∼100% purity, CHY-MAX Extra, 585 IMCU/mL (Chr. Hansen A/S, Hørsholm, Denmark)]. The activity of both enzyme preparations was determined according to ISO11815:-IDF157[19] immediately prior to use.

Preparation of Whole CN. Whole CN was prepared as described by Hollar et al.[20] Fresh, raw milk collected from the morning milking of a cow (Holstein-Friesian) heterozygous at the κ-CN locus (A/B) from the Research Centre Foulum herd (Aarhus University, Denmark) was skimmed by centrifugation (1400*g*, 45 min, 4 °C). The skim milk was filtrated twice through a double layer of Whatman GF/A glass fiber filter paper (Whatman, Clifton, NJ), then warmed to 30 °C, and adjusted to pH 4.6 by slow addition of 1 M HCl with constant stirring. The precipitated acid CN was collected by filtration through Whatman 113 V filter paper and washed twice by resuspension in water to remove lactose and milk salts. The washed acid CN was resuspended in water and slowly solubilized by addition of 1 M NaOH to produce a sodium caseinate solution of pH 6.7. This solution was frozen, lyophilized, and stored at −20 °C. The purity of the sodium caseinate was evaluated by capillary electrophoresis (CE) according to the method described below.

Purification of κ-CN. κ-CN was isolated by anion-exchange chromatography on a column (ϕ 5 cm × 30 cm) packed with DEAE Sepharose Fast Flow resin (GE Healthcare, Bio-Sciences, Hillerød, Denmark). The purification protocol was based on the method of Creamer[21] with minor modifications. Sodium caseinate (2.5 g) was dissolved in 100 mL of buffer (10 mM imidazole buffer, pH 7.0, containing 4.5 M urea and 0.1% β-mercaptoethanol), dialyzed overnight (MWCO = 12400 Da, gentle stirring, 4 °C) against 2 L of the same buffer, and filtered (0.45 μm). After equilibration of the column, the sample was loaded and eluted in aforementioned buffer with a linear NaCl gradient (0−0.36 M) at a flow rate of 1 mL/min over a total volume of 4.5 L. Fractions of 15.8 mL were collected, and their absorbance was measured (280 nm). The resulting chromatogram was evaluated by comparison to previous analytical anion-exchange separations of whole CN components,[22] and fractions containing κ-CN were pooled and dialyzed overnight against 20 L of water (MWCO = 12400 Da, gentle stirring, 4 °C). Following three exchanges of dialysis water (∼5 × 10^5 fold dilution of solutes), the κ-CN was lyophilized, and the purity of the κ-CN preparation was assessed by CE according to the method described below.

Enzymatic Hydrolysis of κ-CN. κ-CN was dissolved in 0.1 M sodium citrate, pH 6.5, and samples (1 mL) in stoppered tubes were placed in a water bath at 32 °C and allowed to temperature equilibrate for 15 min. For both enzymes, seven substrate concentrations were used (final concentrations from 0.016 to 1.0 mM), while the enzyme concentration of the reaction mixtures was constant at 1 nM. Thus, initial κ-CN/chymosin molar ratios ranged from 1.6 × 10^4 to 1.0 × 10^6, which were chosen to be well within the substrate excess for Michaelis−Menten kinetics to apply. Molar substrate concentrations were estimated based on nonglycosylated κ-CN A and B (1*P*) (M_W = 19022 Da). Molar enzyme concentrations were estimate based on M_W = 40000 and 35600 Da and specific milk-clotting activities of 462 and 223 IMCU/mg for camel and bovine chymosin, respectively.[4,14,23] The reaction was initiated by adding 40 μL of freshly diluted chymosin. The tubes were mixed by whirling, and the reactions were allowed to proceed for 25 min. Aliquots [30 μL for CE and 170 μL for reversed-phase (RP) HPLC] of the hydrolysates were removed at 5, 10, 15, and 25 min and prepared for CE and RP-HPLC analysis. Samples at time zero (t_0), to which no enzyme was added, were prepared for each substrate concentration.

CE. The degradation of κ-CN and formation of large CN fragments were measured by CE according to the method of Recio and Olieman[24] with some modifications. Hydrolysate aliquots (30 μL) were immediately mixed with 70 μL of sample buffer to stop the enzymatic reactions. The sample buffer (pH 8.6 ± 0.1) was composed of 167 mM Tris, 67 mM EDTA, 42 mM 3-morpholino-propane-sulphonic acid, 17 mM DL-DTT, and 1.6 mM Tyr-Ala (internal standard) dissolved in a 10 M urea solution containing 0.83 mg/mL methyl hydroxypropyl cellulose. After incubation for 1 h at room temperature, 2 μL of DL-lactic acid [18.0% (v/v)] was added (final pH 7.0). A control experiment in which chymosin was added to κ-CN solutions mixed with sample buffer, followed by prolonged incubation at room temperature (24 h after addition of lactic acid), validated the efficiency of the buffer system in stopping the reactions. Samples were injected (vacuum, 5 kPa, 7 s, vial content = 50 μL) from the anodic end of a PVA-coated fused silica capillary (i.d. = 50 μm, effective length = 56.0 cm, Agilent Technologies ApS, Hørsholm, Denmark) run at 45 °C in a HP ^{3D}CE system interfaced with ChemStation software, Rev. A.06.01 (Hewlett-Packard International Sarl, Allerød, Denmark). The run buffer (pH 3.0 ± 0.1) was composed of 20 mM sodium citrate, 190 mM citric acid, and 6 M urea containing 0.50 mg/mL methyl hydroxypropyl cellulose and was filtered before use (0.45 μm). Electrophoreses was carried out by applying a voltage of 25 kV for 40 min, and detection was at 214 nm. The capillary was rinsed with water (3 min) and run buffer (5 min) between each sample. Assignment of peaks was based on previous reports.[25]

RP-HPLC Coupled to Mass Spectrometry (MS). The formation of pH 4.6-soluble peptides was analyzed by LC-MS using a 1290 Infinity LC/quadrupole time-of-flight (TOF) MS equipped with a thermal focusing ESI source (Agilent Technologies ApS). Hydrolysate aliquots (170 μL) were withdrawn and immediately heat-treated (85 °C, 10 min) to inactivate the enzyme. The samples were cooled to room temperature and adjusted to a final pH of 4.6 by adding 10.5 μL of acetic acid [33.3% (v/v)], holding the samples for 10 min (room temperature), and then adding 10.5 μL of sodium acetate (3.33 M), as adapted from McGann et al.[26] The samples were centrifuged (16000*g*, 10 min), and the supernatants were filtered (0.20 μm) before injection (20 μL) onto the column (Zorbax 300 SB-C18; 2.1 mm × 150 mm, 5 μm). Binary gradient elution at 40 °C was applied by mixing 0.1% (v/v) TFA in water (eluent A) with 0.055% (v/v) TFA in acetonitrile:-water [80:20 (v/v)] (eluent B) according to a slightly modified version of the method described by Tolkach and Kulozik.[27] The gradient was generated by increasing the concentration of eluent B from 25 to 45% over 15 min and then from 45 to 51% over 10 min, after which the column was purged with 100% B for 3 min and re-equilibrated at 25% B for 16 min. The flow rate was 0.25 mL/min, and detection was at 214 nm. Each MS scan in the total ion chromatogram was an average of 5904 transients, producing a scan every second. MS spectra were created by averaging the scans across each peak. All data were corrected by reference masses m/z 121.051 and m/z 922.010. The MS conditions were as follows: positive ion mode; drying gas flow and temperature, 8 L/min and 300 °C; pressure of nebulizer, 35 psi; capillary, fragmentor, and skimmer voltages, 3500, 175, and 65 V, respectively; and scan range, MS: m/z 200−3000. Data acquisition and analysis were accomplished using MassHunter Workstation software, Ver. B.03.01. Identification of peptides by mass was supported by recognizing patterns of precursor ions with different numbers of protons $[M + H]^{n+}$.

Determination of Kinetic Parameters. Kinetic parameters were calculated based on initial reaction velocities of N- and C-terminal product formation. CE data of κ-CN depletion were used to convert peak areas of the hydrolysis products to molar concentrations. Integrated areas of CE peaks were divided by the migration time to obtain normalized values for comparison across the entire experiment. Subsequently, normalized areas of CN components were standardized to that of a Tyr-Ala dipeptide (internal standard) to correct for variation in injection volumes. Linear regressions of normalized, standardized peak areas of total κ-CN vs reaction time were calculated (R^2 = 0.73−0.97). Calibrated κ-CN peak areas were converted to molar concentration using a standard curve prepared from the series of t_0 samples of known molarity analyzed by CE (R^2 = 0.999). At pseudomonosubstrate reaction conditions, the molar difference, Δs, between the initial substrate concentration, s_0, and the substrate concentration at a given reaction time, s_X, equals the momentary concentration of the products, p_X:

$$\Delta s = s_0 - s_X = p_X \quad (1)$$

Hence, molar product concentrations were estimated from eq 1 and plotted against peak areas of N- (R^2 = 0.95−1.00) and C-terminal (R^2 = 0.94−1.00) products. Initial reaction velocities (v_0) of N- and C-terminal product formation were derived from linear regressions of the adjusted molar product concentrations versus reaction time. Enzyme saturation curves (v_0 vs s_0) were constructed, and the Michaelis−Menten constant, K_M, and the turnover number, k_{cat}, were calculated by Michaelis−Menten data fitting in GraphPad Prism, Ver. 5.04 (GraphPad Software, Inc., La Jolla, CA).

RESULTS AND DISCUSSION

Substrate Characterization. The purity of the bovine κ-CN preparation used as substrate in this study was assessed by CE (Figure 1). κ-CN was isolated from milk containing both genetic variants A and B, which had identical electrophoretic mobilities at pH 3.0. Additionally, three smaller peaks migrated longer (34, 36, and 40 min) than the main κ-CN peak (32 min), which represented glycosylated variants of κ-CN as supported by LC-MS analysis (Table 1) and earlier research.[28]

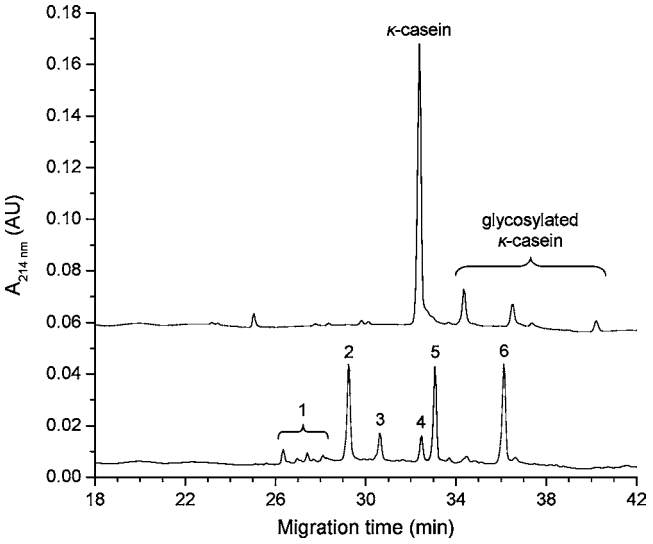

Figure 1. CE of purified κ-CN (10 mg/mL) (top) and sodium caseinate (10 mg/mL) (bottom). Peaks: 1, α_{S2}-CNs (n*P*); 2, α_{S1}-CN (8*P*); 3, α_{S1}-CN (9*P*); 4, κ-CN; 5, β-CN^{B}; and 6, β-CN^{A2}.

Table 1. LC-MS Results of pH 4.6-Soluble Peptides Produced from the Hydrolysis of κ-CN by Camel (C) and Bovine (B) Chymosin

	molecular weight (M_r)			
peak[a]	observed (C)	observed (B)	calculated[b]	suggested peptide
1	7702.71	7702.68	7703.04	CMP^{B}(1*P*)-1*E*[c]
	8651.03	8650.05	8650.62	CMP^{B}(1*P*)-2*E*
2	7733.67	7733.67	7735.00	CMP^{A}(1*P*)-1*E*
	8651.02	8650.05	8650.62	CMP^{B}(1*P*)-2*E*
3	6787.34	6786.33	6787.41	CMP^{A}(1*P*)
4	7702.71	7702.71	7703.04	CMP^{B}(1*P*)-1*E*
5	7702.69	7703.69	7703.04	CMP^{B}(1*P*)-1*E*
6	6754.37	6754.37	6755.45	CMP^{B}(1*P*)

[a]Peak numbers refer to Figure 2B. [b]www.expasy.ch/tools/pi_tool.html (average resolution). [c]*E*: Tetrasaccharide with chemical structure Neu5Acα(2−3)Galβ(1−3)[Neu5Acα(2−6)]GalNAc$_{OH}$ and M_r = 965.8 Da.[31]

A minor, unidentified peak (25 min) was also evident from the CE profile of purified κ-CN. Our substrate therefore comprised of a heterogeneous pool of κ-CN components with an estimated total purity of 95.5% of which 15−20% represented glycosylated variants (Figure 1). This level of glycosylation compares to a general ∼50% dominance of the nonglycosylated κ-CN component[29] and, hence, probably resulted from a partial separation of various glycosylated components during purification. The κ-CN preparation was considered highly suitable for the comparative kinetic analysis of its first hydrolysis by camel and bovine chymosin.

Enzyme Specificity. CE analysis revealed the appearance of a single peak at the migration time of *para*-κ-CN in the hydrolysates produced by camel and bovine chymosin and, hence, reflected a highly specific activity of both enzymes on κ-CN (Figure 2A). Likewise, RP-HPLC profiles revealed similar product compositions of the pH 4.6-fractionated hydrolysates indicating not only identical specificities but also relative

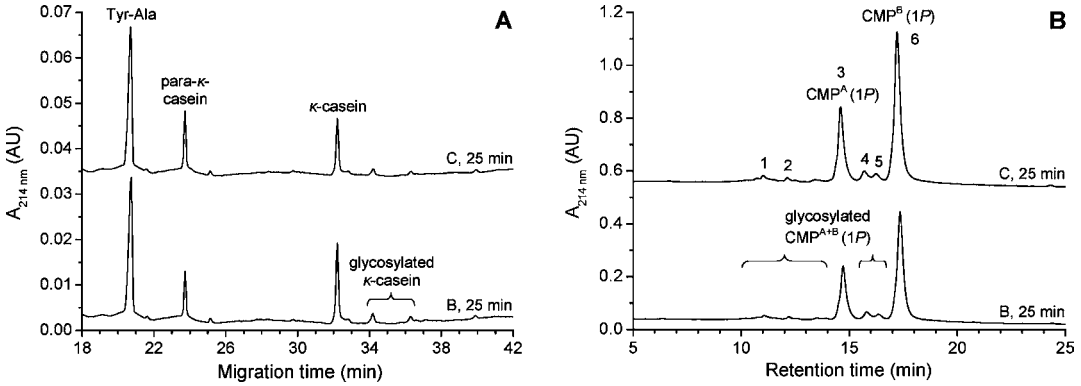

Figure 2. CE of *para*-κ-CN (A) and RP HPLC of CMPs (B) in the 25 min hydrolysates of κ-CN produced by camel (C) and bovine (B) chymosin. Initial substrate concentration, $[\kappa\text{-CN}]_0$, was 0.063 and 0.50 mM in A and B, respectively. (A) Tyr-Ala was used as internal standard. (B) Numbered peaks were identified by MS as specified in Table 1.

Table 2. Kinetic Parameters and Estimates of Specific Activity for the Hydrolysis of Bovine κ-CN by Camel and Bovine Chymosin

	K_M (mM)		k_{cat} (s^{-1})		k_{cat}/K_M (mM^{-1} s^{-1})		specific activity (μmol/min/mg)		
chymosin		mean		mean		mean		mean	product measured
camel	0.28 ± 0.05	0.29 ± 0.03	144 ± 10	146 ± 6	522 ± 102	509 ± 57	216	219	*para*-κ-CN
	0.30 ± 0.03		148 ± 6		496 ± 52		222		total CMP
bovine	0.19 ± 0.03	0.21 ± 0.02	86 ± 5	91 ± 3	462 ± 75	438 ± 47	145	153	*para*-κ-CN
	0.23 ± 0.03		95 ± 5		414 ± 58		161		total CMP

preferences to the various κ-CN components by the two enzymes (Figure 2B). Extracted mass spectra confirmed the former by assigning all significant HPLC peaks produced by both enzymes to various CMP components (Table 1). Thus, these results clearly demonstrated the exclusive preference for camel and bovine chymosin to cleavage of the *Phe105−Met106* bond of κ-CN. Chymosins of other mammalian species such as human, pig, rabbit, sheep, goat, and horse are also characterized by having a high degree of specificity toward this particular κ-CN bond.[30] In accordance with the initial substrate composition, pH 4.6-soluble profiles were strongly dominated by monophosphorylated, nonglycosylated CMP^A and CMP^B (peaks 3 and 6), which eluted subsequent to their glycosylated counterparts, as expected (peaks 1, 2, 4, and 5) (Figure 2B).[27] We identified only carbohydrate structure *E* [Neu5Acα(2−3)Galβ(1−3)[Neu5Acα(2−6)]$GalNAc_{OH}$] among the well-established post-translational glycosylations of κ-CN, the prevalence of which has been previously reported.[31] This tetrasaccharide was attached mainly at one position in the amino acid sequence, although traces of CMP components holding two *E* moieties were also observed for both enzymes (Table 1).

Kinetic Parameters. The initial formation rates of CMP and *para*-κ-CN as measured by RP-HPLC and CE, respectively, were used for kinetic calculations of camel and bovine chymosin-catalyzed cleavage of *Phe105−Met106*. Comparable values of both K_M and k_{cat} resulting from CMP and *para*-κ-CN analysis were obtained; hence, the means are used for further discussion. Thus, Michaelis−Menten constants (K_M) were 0.29 and 0.21 mM, and turnover numbers (k_{cat}) were 146 and 91 s^{-1} for the camel and bovine chymosin-catalyzed reaction, respectively (Table 2). Furthermore, mean specific enzyme activities of 219 and 153 μmol/min/mg could be estimated for the camel and bovine chymosin preparations, respectively, using assay conditions of pH 6.5, 32 °C, and I = 0.57 M and whole bovine κ-CN.

While a k_{cat} of 91 s^{-1} compared relatively well to previous references on bovine chymosin using purified bovine κ-CN in solution (2−100 s^{-1}), the K_M of 0.21 mM was about 2−8 times higher than the majority of earlier findings (0.03−0.10 mM).[7,8,32−34] However, multiple variable experimental conditions such as the temperature, pH, and ionic strength not to mention substrate and enzyme properties, complicate the comparison across different studies considerably as reflected by the broad ranges encountered of both parameters in the literature. The present research employed a relatively high ionic strength (I = 0.57 M), highly purified enzyme and substrate preparations, and s_0 concentrations predominantly above the cmc of κ-CN (~0.025 mM at comparable ionic strength).[35] In addition, data fitting to the nonlinear Michaelis−Menten equation is considered a more accurate approach than traditional linear transformations applied earlier.[8,32] Despite an obvious inadequacy of pinpointing single explanations, the ionic strength used in this study was a factor of 5−10 higher than what has been suggested for optimal electrostatic interaction during chymosin−κ-CN complex formation.[36] The ionic strength affects such interactions due to two opposing shielding effects: while reducing the overall repulsive energy barrier between net negatively charged enzyme and substrate, at the same time, a high ionic strength would diminish local electrostatic attractions of well-recognized importance to the rapid and firm ES complex formation.[12,36] Consequently, it is conceivable that the ionic strength caused a decrease in the rate of ES complex formation and, hence, a higher K_M. Furthermore, a probable micellelike polymerization of the reduced κ-CN molecules may be anticipated to restrict their accessibility to enter optimally into short-range attractive interactions with chymosin.[37] Also, κ-CN polymers (M_W ~ 600000 Da[35]) diffuse much slower than monomeric κ-CN; both effects of which

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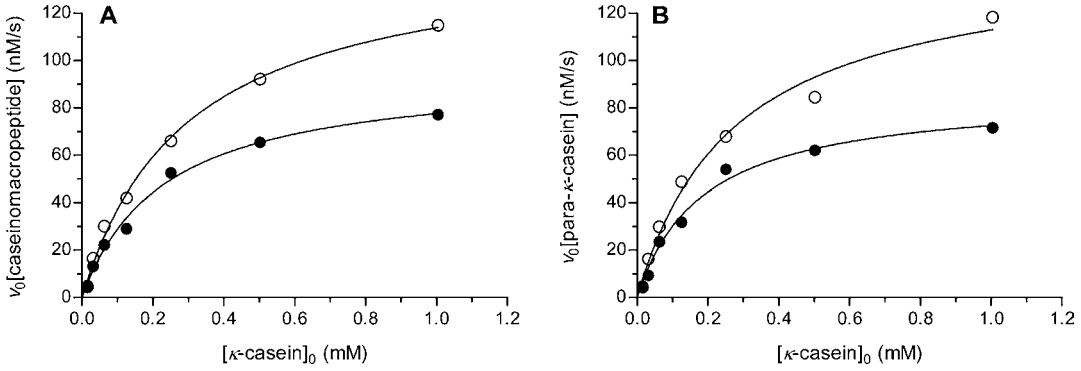

Figure 3. Reaction velocity, v_0, as a function of initial substrate concentration, $[\kappa\text{-CN}]_0$, for the formation of total CMP (A) and *para*-κ-CN (B) during hydrolysis of κ-CN by camel (○) and bovine (●) chymosin. Molar κ-CN concentrations were estimated based on the assumption of 95.5% substrate purity and using M_W = 19022 Da for nonglycosylated $\kappa\text{-CN}^{A/B}(1P)$.

again tend to decrease the affinity and rate of ES complex formation.[38] Such effects are indeed noticed in the difference between K_M values derived from systems of whole CN micelles (0.1−0.5 mM)[39] as compared to κ-CN in solution.[7,8,32−34] However, no break in the properties was observed on the enzyme saturation curves in Figure 3, as was neither the case in previous applications of Michaelis−Menten kinetics above the cmc of κ-CN.[7,8] These results may be rationalized in view of the existence of a highly dynamic equilibrium between monomer and micellar κ-CN, which represents a typical behavior of amphiphilic molecules above their cmc.

The most remarkable difference between the saturation curves of camel and bovine chymosin was found in the level of maximum reaction velocity (v_{max}), suggesting an estimated 60% higher turnover rate (k_{cat}) for camel chymosin as compared to the bovine enzyme (Figure 3 and Table 2). On the other hand, half saturation of bovine chymosin was achieved with only ∼70% of the κ-CN concentration required for camel chymosin, resulting in an estimated 15% higher catalytic efficiency (k_{cat}/K_M) of camel chymosin. Because of their coevolution, the preferential binding of bovine κ-CN by its native chymosin seems not surprising. While these results strongly support the superior milk-clotting capacity of camel relative to bovine chymosin previously demonstrated,[14,16] they contradict accompanied kinetic differences reported by Kappeler et al.[14] Their finding of a smaller Michaelis−Menten constant and a lower turnover number of camel as compared to bovine chymosin may, however, be explained by the fact that they used a κ-CN fragment (*His98*−*Ile108*) incorporating *Phe*(NO_2) at position *105*, which increases the polarity of *Phe105* and, hence, may disturb the hydrophobic interactions inside the enzyme's active-site cleft. Furthermore, kinetic properties as derived from the use of an undecapeptide versus whole κ-CN do not allow direct comparison. Surprisingly, a recent comparative study found that recombinant goat chymosin exhibited superior catalytic efficiency to that of buffalo, bovine, or camel chymosin, the latter of which was the least efficient catalyst.[40] However, it must be stated that the values of K_M and k_{cat} reported in the study were of a completely different magnitude to what is reported in our work or earlier studies.[7,8,32−34]

Can we, however, explain the kinetic differences observed in the present study based on structural differences between camel and bovine chymosin? They both consist of 323 amino acid residues in the mature form and share high sequence identity (85%) (www.ncbi.nlm.nih.gov) resulting not surprisingly in similar overall folds and identical specificities on κ-CN (Figure 2A,B). Still, the sequence dissimilarity existing between them was recently revealed to impart distinct 3D structural differences,[18] and likewise, dynamic computer simulations of contact positions to the chymosin-sensitive region of κ-CN provided clues of enzyme residues of probable importance to their catalytic efficiency.[13,17]

Much importance as to the specificity of bovine chymosin was placed upon electrostatic interactions between the positively charged *His98*−*His102* stretch of κ-CN and negatively charged C-terminal regions of the enzyme (at the pH of cheese making) in previous research.[3,12,41] Specifically, Safro et al.[41] proposed that on binding, this *His*−*Pro* cluster poses in direct proximity to Gln240−Cys250, located on the enzyme surface near the entrance to the active-site cleft. Thus, because of a distinct density of acidic residues within the Gln240−Cys250 stretch of the enzyme, it seems perfectly suited for efficient electrostatic fixation of the substrate. In this respect, Jensen et al.[18] emphasized the important contribution from Glu245, Asp247, and additionally from Asp249 and Asp251, which, interestingly, are substituted by uncharged residues Asn249 and Gly251 in camel chymosin. On the other hand, Palmer et al.[13] observed a stable interaction between Glu245 and Lys221 of bovine chymosin that effectively screened the Glu245−*His102* attraction. A Lys221Val substitution in camel chymosin would therefore enable stronger electrostatic forces between Glu245 and *His102* and, hence, reduce the net effect of aforementioned local surface charge differences. Still, the negative patch of the camel enzyme could be less well suited to pair up with the positively charged *His*−*Pro* cluster, which may play a role not only in a weaker affinity for the substrate (K_M) but also, and perhaps more importantly, by allowing a faster dissociation of the ES complex following cleavage (k_{cat}). The fact that a proportionally greater fraction of chymosin added during cheese making is retained in the curd supports the significance of molecular interactions between bovine chymosin and the *para*-κ-CN part of κ-CN.[4] The interaction between the *His*−*Pro* cluster of κ-CN and the C-terminal domain of both camel and bovine chymosin was confirmed recently.[17]

Although local charge differences may impart local electrostatic bonding, overall, κ-CN (and the CN micelle in milk) would be expected to repel the enzymes following their net negative charges at cheese-making pH. Therefore, the observation of an additional positive charge in camel chymosin (Gln56His substitution) within a distinctly positively charged stretch on the enzyme surface (Lys48−Lys62), previously

suggested to aid in the initial substrate recognition of bovine chymosin,[3] could add further to the superior catalytic efficiency of camel chymosin.[18]

Finally, we call attention to the paradox that the K_M and k_{cat} values obtained for camel chymosin point in opposing directions relative to those of bovine chymosin when considering the k_{cat}/K_M criterion as a measure of catalytic efficiency. The limit of the ultimate k_{cat}/K_M ratio is set by the rate of ES complex formation,[42] which was lower for camel chymosin due to reduced substrate affinity that in turn accelerated the rate of ES dissociation and provided an overall higher catalytic efficiency. Similarly, earlier research reported a positive relationship between the strength of bovine chymosin−κ-CN binding and the carbohydrate content of κ-CN, while, at the same time, the turnover number decreased drastically, resulting in the highest catalytic efficiency at an intermediate level of glycosylation.[8] This trade-off would require careful consideration in the attempt to engineer milk-clotting enzymes with improved catalytic properties.

■ ASSOCIATED CONTENT

Supporting Information

For the camel and bovine chymosin hydrolysates, averaged mass spectra of RP-HPLC peaks representing monophosphorylated, nonglycosylated CMP^A, and CMP^B (Figure 4) and plots of *para*-κ-CN and total CMP formation vs reaction time for each initial substrate concentration (Figure 5). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +45 30 13 06 11. Fax: +45 45 74 88 88. E-mail: dkkkm@chr-hansen.com.

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Notes

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■ ABBREVIATIONS USED

C, milk-clotting activity; CE, capillary electrophoresis; CMP, caseinomacropeptide; CN, casein; ES, enzyme−substrate; IMCU, international milk-clotting units; MS, mass spectrometry; P, general proteolytic activity; RP, reversed-phase

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