Caseinolytic Specificity of Cardosin, an Aspartic Protease from the Cardoon *Cynara cardunculus* L.: Action on Bovine α_s- and β-Casein and Comparison with Chymosin

I. Queiroz Macedo,*,† Carlos J. Faro,‡ and Euclides M. Pires‡

Department of Chemistry, University of Aveiro, 3810 Aveiro, Portugal, and Department of Biochemistry, University of Coimbra, 3049 Coimbra Codex, Portugal

The action of cardosin on bovine α_s- and β-casein at 30 °C in 50 mM citrate buffer (pH 6.2) was studied. Peptides were isolated by reversed-phase HPLC on C_{18} columns and identified from their amino acid composition and *N*-terminal amino acid sequence. The relative susceptibility of peptide bonds cleaved was Phe_{23}-Phe_{24} > Trp_{164}-Tyr_{165} > Tyr_{166}-Val_{167} > Tyr_{165}-Tyr_{166} > Phe_{153}-Tyr_{154} > Phe_{145}-Tyr_{146} ≈ Leu_{149}-Phe_{150} ≈ Leu_{156}-Asp_{157} ≈ Ala_{163}-Trp_{164} for α_{s1}-casein and Leu_{192}-Tyr_{193} > Leu_{191}-Leu_{192} ≈ Leu_{165}-Ser_{166} > Phe_{190}-Leu_{191} ≥ Ala_{189}-Phe_{190} ≈ Leu_{127}-Thr_{128} for β-casein. In α_{s2}-casein, cardosin cleaved the bonds Phe_{88}-Tyr_{89} and Tyr_{95}-Leu_{96}. The enzyme shows a clear preference for bonds between hydrophobic, bulky amino acids, cleaving four consecutive peptide bonds in extremely bulky, hydrophobic regions of both α_{s1}-CN (Ala_{163}-Val_{167}) and β-CN (Ala_{189}-Tyr_{193}), which was less attacked by chymosin in various experimental conditions. The active site cleft of cardosin accommodates sequences as bulky as Trp-Tyr-Tyr in different subsites (S_1 to S'_2, S_2 to S'_1, and probably S_3 to S_1). Several bitter peptides were identified in the digests.

Keywords: *α_s-Casein; β-casein; cardosin; vegetal rennet; specificity; proteolysis; bitter peptides*

INTRODUCTION

Cynara cardunculus L. is a cardoon that grows wild in various regions of Portugal and other Mediterranean countries, as well as in Argentina. Dried flowers of this cardoon have been used since ancient times for the production of high quality sheep-milk cheese. The clotting enzyme, cardosin, is an aspartic protease that was isolated from these flowers, purified, and partly characterized (Heimgartner et al., 1990; Faro et al., 1992). The flowers of *Cynara cardunculus* L. are not used for the production of cheese of cow milk. Studies performed with extracts of other cardoon species on bovine caseins indicate some differences with respect to specificity; preliminary results point to the production of cheeses of better texture and flavor. The investigation of the caseinolytic specificity of the clotting enzymes present in the various cardoon species is thus important and of great help for the understanding of these differences in cheese quality and may lead to a more extended application of milk-clotting proteases extracted from cardoon.

Studies performed in our laboratory on the specificity of cardosin toward isolated bovine κ-casein (κ-CN) showed that, like other milk-clotting enzymes (namely, chymosin), it only cleaves the Phe_{105}-Met_{106} bond, the proteolytic coefficient being of the same order of magnitude (Macedo et al., 1993). In the present work, the action of cardosin on isolated bovine α_s- and β-CN was studied under the experimental hydrolysis conditions used by other investigators for chymosin studies (Carles and Ribadeau-Dumas, 1984 and 1985).

Cheeses of cow milk prepared with cardosin extracted from *Cynara cardunculus* L. tend to taste bitter and to present texture defects (Sá and Barbosa, 1972). The proteolytic action on α_s- and β-CN is known to affect the yield, texture, and flavor of the cheese. The effects on the yield and texture are due to the formation of soluble peptides that are lost to the whey and to the exposure of new (or loss of) protein−protein interaction sites, thus affecting syneresis. The effect on the flavor is essentially due to the formation of bitter peptides. Although the experiments were not performed in a cheese-like environment, a relationship between the caseinolytic specificity of cardosin and its cheese making performance is suggested.

MATERIALS AND METHODS

Materials. α_s-Casein (α_{s1}-CN with a small proportion of α_{s2}-CN), β-CN, and phenyl isothiocyanate (PITC) were from Sigma Chemical Company (St. Louis, MO). All reagents were of analytical grade.

Enzyme Preparation. The protease was extracted and purified as described by Faro et al. (1992). One gram of styles, obtained from dried flowers of *Cynara cardunculus* L., was macerated in 10 mL of a 100 mM sodium citrate/citric acid (pH 3.0) solution. After centrifugation at 12000*g* for 5 min, the resultant supernatant was applied to a Sephadex G-100 column (2.5 × 75 cm), previously equilibrated with 50 mM NH_4HCO_3. The sample was eluted at room temperature with the equilibrium solution and collected in 10-mL fractions. The active enzyme-containing fractions were pooled and lyophilized.

***β*-Casein Purification.** β-Casein was purified by anion-exchange chromatography (Mono Q HR 5/5 column), with FPLC equipment (Pharmacia Fine Chemicals), according to the method of Guillou et al. (1987) as slightly modified by Macedo (1993). The changes involved column temperature, eluent flow, and NaCl gradient. The β-CN sample was dissolved in 5 mM Tris-HCl (pH 8.0), 4.5 M urea, and 8×10^{-4} dithiothreitol. Elution was performed at room temperature, at a flow rate of 0.75 mL/min, with a 5 mM Tris-HCl (pH 8.0), 4.5 M urea, 6.4×10^{-5} M dithiothreitol solution, and a linear gradient of NaCl from 0.15 to 0.32 M between 5 and 40 mL, respectively. Solutions were prepared with Milli Q water, and

* Author to whom correspondence should be addressed [fax (351) 34-25143; e-mail IQM@dq.ua.pt].
† Department of Chemistry.
‡ Department of Biochemistry.

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the eluents were deaerated with helium. The fractions were dialyzed against 3 × 1 L of distilled/deionized water at 4 °C with gentle stirring and lyophilized. The purity was checked by reversed-phase (rp)-HPLC with the conditions used for peptide separation.

Enzymatic Hydrolysis of α_s- and β-Casein. Casein hydrolysis was carried out at 30 °C in 50 mM sodium citrate/citric acid buffer (pH 6.2) and 0.1% (w/v) NaN_3. After "blank aliquots" were taken, the enzyme was added to the casein solution (enzyme/substrate mass ratio of 1/1000), and the reaction was allowed to proceed. At selected times, aliquots were taken, and the reaction was quenched either by raising the pH to 9−10 with ammonia or by keeping the mixture in liquid nitrogen.

Peptide Separation. Separation of peptides obtained upon casein digestion was achieved by rp-HPLC (Vydac TP, C_{18}, 5-μm, 0.46 × 25-cm column). The elution was carried out with linear gradients of acetonitrile in 0.1% trifluoroacetic acid. After a 2-min isocratic run, a gradient from 0 to 28% (0 to 30% for β-CN) acetonitrile was developed in 8 min, followed by a 28−65% (30−77% for β-CN) gradient over 25 min. The elution rate was 1.0 mL min^{-1}, and the column temperature was 38 °C (21 °C for β-CN). The peptides were detected at 220 nm, pooled over several runs, and concentrated for subsequent analysis. The time course chromatograms of α_s-CN and β-CN degradation are presented in Figures 1 and 2, respectively.

Amino Acid Analysis. The peptides collected by HPLC were dried and hydrolyzed under reduced pressure with 6 M HCl containing 0.01% phenol at 110 °C for 18 h. After removal of HCl, the amino acids were derivatized with PITC by the method of Heinrikson and Meredith (1984). The phenylthiocarbamoyl amino acid derivatives were separated by rp-HPLC as described previously (Macedo et al. 1993).

***N*-Terminal Sequence Determination.** The peptides collected by HPLC were concentrated in a Speed-vacuum concentrator (Hetovac VR-1) and partially sequenced on an automatic gas−liquid protein sequencer (Applied Biosystems 473A).

RESULTS AND DISCUSSION

β-Casein Purification. The purified β-CN, although homogeneous in SDS-PAGE, shows two peaks in rp-HPLC, eluting 2% acetonitrile apart from each other. The two fractions have identical amino acid composition, and rechromatography of each peak leads to the same doublet, suggesting two different forms of aggregation or different conformations. Prolonged (>15 min) interactions between proteins and the rp-HPLC stationary phase or acetonitrile may induce conformational changes (Dorsey et al., 1990). Furthermore, in organic solvent gradient elution, conformations with less affinity for the stationary phase may be desorbed, and the molecule readsorbed in a higher binding affinity orientation (Geng and Regnier, 1984). The hypothesis of two different conformations of β-CN can thus not be ruled out.

Peptide Identification. Amino acid analyses were made in triplicate and all but those of fractions C and K from α_s-CN and F from β-CN proved reproducible. Indeed, the amino acid analysis of these three fractions, although reproducible within each pool, varied significantly between pools, despite repeated experiments. This variation raised the hypothesis of coelution of peptides, which obviously are present in different amounts in the various pools. The peptide sequence results confirm this hypothesis. Sequences obtained for fraction C (D and Y, A and P, Y and E, P and L, S, G, A, W) allow the identification of two peptides, namely, α_{s1} Asp_{157}-Trp_{164} and α_{s1} Tyr_{146}-Leu_{149}. The results obtained for fraction K (F and K, V, and N, A and T, P and M, F and E, P and H, E, and V) show that it is a mixture of two peptide chains, with the sequences determined corresponding to the fragments α_{s1} Phe_{24}-Glu_{30} and α_{s2} Lys_1-Val_7. The identification of the complete chains was then deduced from the timecourse chromatographic profile of the reaction mixture. Fraction F from β-CN is a two-peptide mixture consisting of peptide Leu_{191}-Val_{209} and of an apparently big peptide having Arg_1 as the *N*-terminal.

The various fragments (Table 1), as well as the corresponding cleavage sites were identified by matching the results of amino acid analysis, *N*-terminal sequencing, and timecourse profile chromatograms with the known amino acid sequences of α_{s1}-CN (Mercier et al., 1971; Nagao et al., 1984; Stewart et al., 1984), α_{s2}-CN (Brignon et al., 1977) and β-CN (Ribadeau-Dumas et al., 1972; Carles et al., 1988).

The results obtained in this study also give additional information on some controversial segment sequences of α_{s1}- and β-CN. Indeed, the primary structure of α_{s1}-CN was determined by Edman degradation (Mercier et al., 1971) and later confirmed by the study of the corresponding cDNA (Nagao et al., 1984; Stewart et al., 1984) with the exception of residue 30, which appears to be Glu instead of Gln. In the present study, sequencing of the first eight amino acids of peptide k_2 (α_{s1}-CN Phe_{24}-Trp_{199}) by Edman degradation confirmed the results obtained for residue 30 by cDNA sequencing. β-Casein was completely sequenced by Edman degradation in 1972 by Ribadeau-Dumas et al. and in 1988 by Carles et al. Four differences were observed; namely, Gln_{117} → Glu; Pro_{137} → Leu; Leu_{138} → Pro; Glu_{175} → Gln; and Gln_{195} → Glu. This new sequence is in agreement with one of the sequences deduced from that of β-CN cDNA (Bayev et al., 1987), but not with the other one (Jimenez-Flores et al., 1987). The partial sequencing of peptides C, A, and E made it possible to confirm the identity of four of these "controversy" residues: the 10^{th} and 11^{th} residues of peptide C are Leu and Pro, respectively (Leu_{137}, Pro_{138}); the 10^{th} residue of peptide A is Gln (Gln_{175}); and the 3^{rd} residue of peptide E is Glu (Glu_{195}). These results agree with those of Carles et al. (1988) and Bayev et al. (1987).

Action on α_{s1}-Casein. For comparative purposes, casein hydrolysis was performed under the experimental conditions chosen by Carles and Ribadeau-Dumas (1985) for the study of chymosin action on α_{s1}-CN, the enzyme/substrate ratio lying near to the maximum used by these authors. Under these conditions, chymosin only cleaved the Phe_{23}-Phe_{24} bond of α_{s1}-CN, whereas cardosin cleaved nine bonds over the same period of time (with no additional bonds being cleaved in the following 3 h). Four sets of peptides were observed, which started to show at 0.5 min [G, K_2], 20 min [H, I, J], 30 min [E, F], and 60 min [A, B, C, D] after the enzyme addition (Figure 1).

The time course HPLC profile of the α_s-CN digestion mixture (Figure 1 and intermediate reaction times, not shown) suggests the following pathway of proteolysis. The primary cleavage site of cardosin is Phe_{23}-Phe_{24}, with the formation of Arg_1-Phe_{23} and the complementary polypeptide, Phe_{24}-Trp_{199}. The next bonds cleaved are Trp_{164}-Tyr_{165}, Tyr_{166}-Val_{167}, Tyr_{165}-Tyr_{166} and Phe_{153}-Tyr_{154}, in this order, producing the peptides Tyr_{165}-Trp_{199}, Val_{167}-Trp_{199}, Tyr_{166}-Trp_{199}, Tyr_{154}-Trp_{164}, and Tyr_{154}-Tyr_{165}. The last four bonds to be cleaved are Phe_{145}-Tyr_{146}, Leu_{149}-Phe_{150}, Leu_{156}-Asp_{157}, and Ala_{163}-Trp_{164}, leading to the formation of the fourth set of peptides just mentioned.

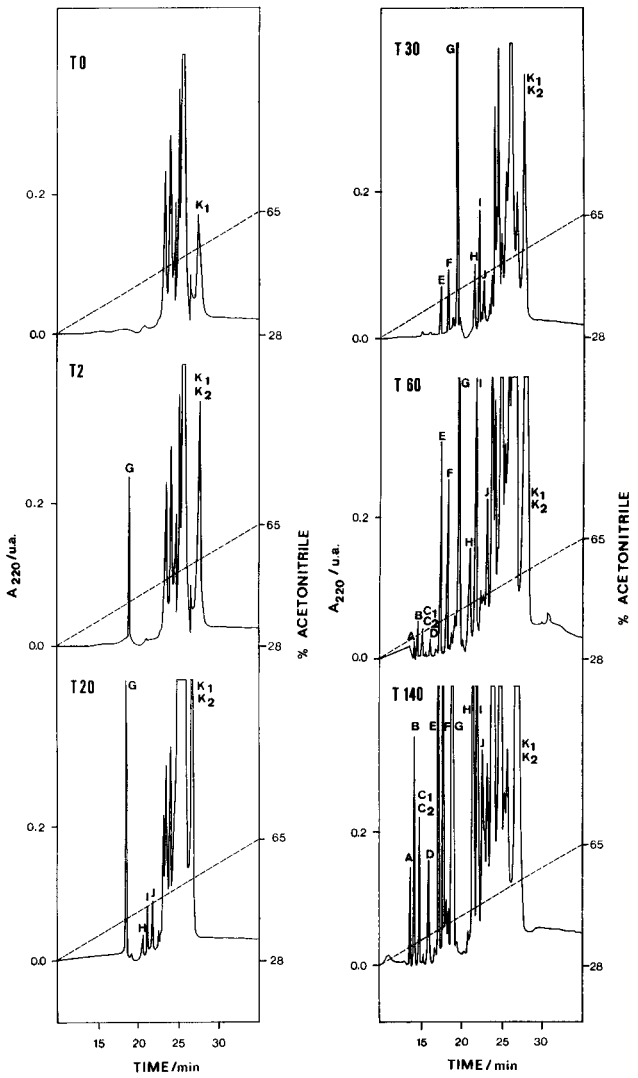

Figure 1. Reversed-phase HPLC pattern of the products obtained following 2−140-min incubation of α_s-CN with cardosin [30 °C, pH 6.2, 50 mM sodium citrate/citric acid buffer, 0.1% (w/v) NaN_3]. The column was a C_{18}, Vydac TP (0.46 × 25 cm), the elution was made with a linear acetonitrile gradient in 0.1% trifluoroacetic acid (dotted line), the flow rate was 1.0 mL/min, and the column temperature was 38 °C. Key: (T_0) prior to enzyme addition; (T_2 to T_{140}) reaction times of 2−140 min; (u.a.) arbitrary units.

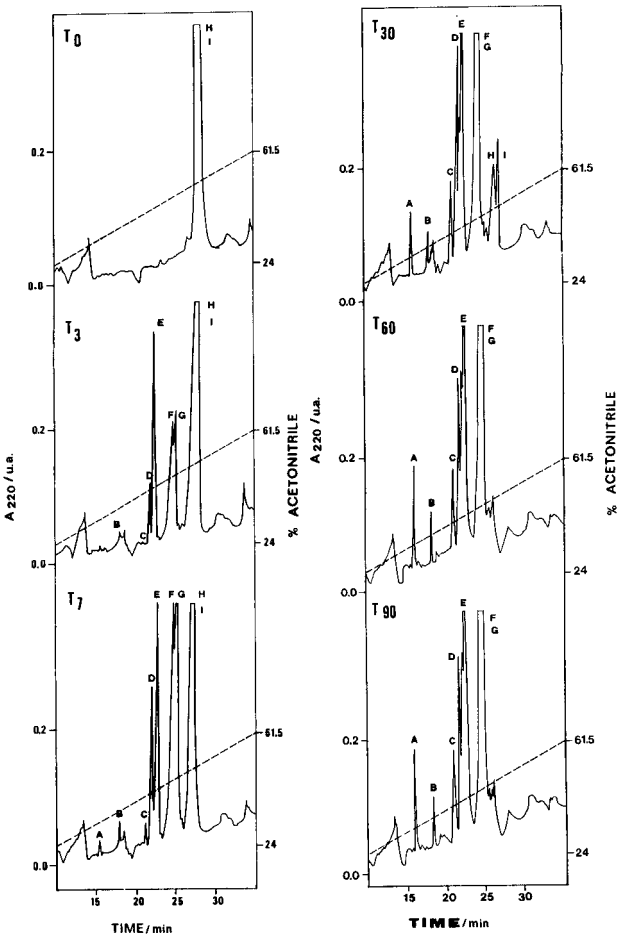

Figure 2. Reversed-phase HPLC pattern of the products obtained following 3−90-min incubation of β-CN with cardosin [30 °C, pH 6.2, 50 mM sodium citrate/citric acid buffer, 0.1% (w/v) NaN_3]. The column was a C_{18}, Vydac TP (0.46 × 25 cm), the elution was made with a linear acetonitrile gradient in 0.1% TFA (dotted line), the flow rate was 1.0 mL/min, and the column temperature was 21 °C. (T_0) prior to enzyme addition; (T_3 to T_{90}) reaction times of 3−90 min; (u.a.) arbitrary units.

McSweeney et al. (1993) identified, at pH 6.5 and 5% NaCl, seven cleavage sites of chymosin on α_{s1}-CN. Five of these are susceptible to cardosin in the conditions of our study, as shown in Figure 3; cardosin did not cleave the two bonds that contain a prolyl residue at the C-terminal side. It is worth noting that three of the four bonds that are cleaved by cardosin (this study) and not by chymosin (McSweeney et al., 1993) belong to an extremely hydrophobic and bulky region of α_{s1}-CN; that is, Ala_{163}-Trp-Tyr-Tyr-Val_{167}. This segment remained unattacked by chymosin in mediums of various ionic strengths, pH, and urea concentrations (Carles and Ribadeau-Dumas, 1985; Mulvihill and Fox, 1977, 1979, and 1980), and, in conditions where only the Phe_{23}-Phe_{24} bond was cleaved by this enzyme (Carles and Ribadeau-Dumas, 1985), cardosin cleaved all peptide bonds. In the study of McSweeney et al., only the bond Trp_{164}-Tyr_{165} was cleaved in this region. The cleavage by chymosin of the bonds Ala_{163}-Trp_{164} and Tyr_{165}-Tyr_{166} has not been reported so far, and the hydrolysis of Tyr_{165}-Val_{167} was only reported in studies with calf rennet (Pélissier et al., 1974). The active-site cleft of cardosin is able to accommodate sequences as bulky as Trp-Tyr-Tyr in different subsites; namely, S_1 to S'_2, S_2 to S'_1, and S_3 to S_1. Tryptophan (Trp), the bulkiest amino acid, fits into S_1 as well as into S_2, S'_1, and probably S_3. Eight of the nine bonds cleaved in α_{s1}-CN include a phenylalanine, a tyrosine, or a tryptophan residue in at least one of the primary binding sites, each appearing in either position. The remaining bond, Leu_{156}-Asp, although still including a relatively hydrophobic residue, is hydrolyzed to a lesser extent.

The hydrolysis by chymosin of the bond Phe_{145}-Tyr_{146} has not been reported. Of the 15 cleavage sites identified by Pélissier et al. (1974) in their studies with calf rennet, only four (Phe_{23}-Phe_{24}, Leu_{156}-Asp_{157}, Trp_{164}-Tyr_{165}, and Tyr_{166}-Val_{167}) were found to be susceptible to cardosin in the conditions of this study. Mulvihill and Fox (1977, 1979, 1980) identified several potential chymosin cleavage sites, but only two of these are susceptible to cardosin under the conditions of our study (Phe_{23}-Phe_{24} and Leu_{149}-Phe_{150}). Despite the differences in hydrolysis conditions and the lack of data concerning relative bond cleavage rates, it is reasonable to suggest that cardosin shows a stronger preference for bulky, hydrophobic sequences and for bonds between hydrophobic residues than chymosin.

Cardosin : **Phe_{23}-Phe_{24} > Trp_{164}-Tyr_{165}** > Tyr_{166}-Val_{167} > Tyr_{165}-Tyr_{166} > **Phe_{153}-Tyr_{154}** >

Phe_{145}-Tyr_{146} ≈ **Leu_{149}-Phe_{150} ≈ Leu_{156}-Asp_{157}** ≈ Ala_{163}-Trp_{164} (present work [1])

Chymosin : Phe_{23}-Phe_{24} (Carles and Ribadeau-Dumas 1984 [1])

Chymosin : **Phe_{23}-Phe_{24} > Trp_{164}-Tyr_{165} > Leu_{156}-Asp_{157} > Phe_{153}-Tyr_{154}** >

Tyr_{159}-Pro_{160} > Phe_{28}-Pro_{29} > **Leu_{149}-Phe_{150}** (McSeeney et al. 1993 [2])

Figure 3. Peptide bonds of α_{s1}-CN hydrolyzed by cardosin and chymosin. The bonds in boldface are those cleaved in both studies. Key: (1) hydrolysis conditions: 30 °C, 50 mM sodium citrate/citric acid buffer, pH 6.2, 0.1% (w/v) NaN_3, 90 min; (2) hydrolysis conditions: 30 °C, 100 mM sodium phosphate buffer, pH 6.5, 5% (w/v) NaCl, 0.05% (w/v) NaN_3, 12 or 24 h.

Table 1. Peptides Formed from the Action of Cardosin on α_s-CN and β-CN

HPLC peak[a]	α_s-CN fragment	HPLC peak[b]	β-CN fragment
A	Tyr_{154}-Ala_{163}	A	Ser_{166}-Phe_{190}
B	Tyr_{89}-Tyr_{95} α_{s2}-CN[c]	B	Ser_{166}-Ala_{189}
C1	Asp_{157}-Trp_{164}	C	Thr_{128}-Leu_{165}
C2	Tyr_{146}-Leu_{149}	D	Ser_{166}-Leu_{191}
D	Asp_{157}-Tyr_{165}	E	Tyr_{193}-Val_{209}
E	Tyr_{154}-Trp_{164}	F1	Arg_1-Leu_{191}-Val_{209}
F	Tyr_{154}-Tyr_{165}	F2	Arg_1-Leu_{192}
G	Arg_1-Phe_{23}	G	Arg_1-Leu_{192}
H	Tyr_{166}-Trp_{199}	H	β-CN
I	Val_{167}-Trp_{199}	I	β-CN
J	Tyr_{165}-Trp_{199}		
K1	α_{s2}-CN		
K2	Phe_{24}-Trp_{199}		

[a] See Figure 1. [b] See Figure 2. [c] B is a fragment from α_{s2}-CN; all other fractions are α_{s1}-CN fragments; digestion conditions: 30 °C, 50 mM sodium citrate buffer (pH 6.2), 0.1% (w/v) NaN_3.

Some bonds that would at first inspection be susceptible to hydrolysis upon cardosin action, namely, Ile_{71}-Val, Tyr_{91}-Leu, Tyr_{94}-Leu, and Leu_{98}-Leu, were not cleaved. Ile_{71}-Val is situated in a highly negatively charged cluster, rich in phosphoserine and glutamic acid. The other three bonds, although somewhat exposed as judged by the three-dimensional structure proposed for α_{s1}-CN (Kumosinski et al., 1991), are also situated in a rather acidic region (25% Glu+Asp in the Glu_{84}-Leu_{99} segment). According to Payens and Visser (1981), the active site of chymosin has a nucleus of negative charge. If we assume that the same is true for cardosin, the electrostatic repulsion would account for the non-susceptibility of these bonds to this enzyme, preventing the active site from approaching the regions of the substrate molecule just referenced.

Action on β-Casein. Under the conditions used by Carles and Ribadeau-Dumas (1984), only the bonds Ala_{189}-Phe_{190} and Leu_{192}-Tyr_{193} of β-CN are cleaved upon chymosin action. In the present study, carried out under the same conditions, cardosin cleaves six bonds over the same period of time, leading to the formation of eight peptides (Figure 2). Leu_{192}-Tyr_{193} is the easiest hydrolyzed bond, also the most susceptible to attack by chymosin and other milk-clotting enzymes (Pélissier et al., 1974; Creamer, 1976; Visser and Slangen, 1977; Carles and Ribadeau-Dumas, 1984). Indeed, in the molecular model of β-CN predicted by Kumosinski et al. (1993), this region is exposed on the (monomer) surface and accessible to enzyme action. The relative susceptibility of the peptide bonds cleaved by cardosin under the conditions of this study was Leu_{192}-Tyr_{193} > Leu_{191}-Leu_{192} ≈ Leu_{165}-Ser_{166} > Phe_{190}-Leu_{191} ≥ Ala_{189}-Phe_{190} ≈ Leu_{127}-Thr_{128}.

Chymosin cleaves two peptide bonds in the sequence Ala_{189}-Phe-Leu-Leu-Tyr_{193} of β-CN, whereas cardosin cleaves all four bonds under the same conditions. Even under harder conditions, [i.e., E/S 10 times higher and 5 h-incubation time (Visser and Slangen, 1977), or E/S 10 times higher, 116-h incubation time, and pH 5.5 and 3.5 (Guillou et al., 1991)], chymosin was unable to cleave the bonds Phe_{190}-Leu_{191} and Leu_{191}-Leu_{192}. The conjunct analysis of several studies on the specificity of chymosin suggests that neither the primary nor the secondary specificity of each residue alone (from P_3 to P'_3) is responsible for the non-hydrolysis of these two bonds. We suggest that bulky sequences are the main cause for this non-hydrolysis. Residues Phe_{190}, Leu_{191}, Leu_{192}, and Tyr_{193} of β-CN interact respectively with subsites S'_1 to S'_4, S_1 to S'_3, S_2 to S'_2, and S_3 to S'_1 of the cardoon protease.

Outside this segment, cardosin cleaved bonds Leu_{165}-Ser_{166} and Leu_{127}-Thr_{128}, which are also cleaved by chymosin, although mostly under more extreme conditions (Visser and Slangen, 1977; Pélissier et al., 1974; Visser, 1981 and references therein; Guillou et al., 1991). Creamer (1976) showed that bonds Ala_{189}-Phe_{190}, Ser_{164}-Leu_{165} or Ser_{166}-Gln_{167}, and Leu_{139}-Leu_{140} are the bonds cleaved at pH 6.25. In the study of Guillou et al. (1991) on the hydrolysis of β-CN by chymosin [37 °C, pH 5.5, 116 h, E/S = 1/100 (w/w)], nine peptide bonds were split. Only three of these bonds were also split by cardosin in our study (Leu_{165}-Ser_{166}, Ala_{189}-Phe_{190}, and Leu_{192}-Tyr_{193}); the cleavage at Leu_{127}-Thr_{128} was observed by these authors at pH 3.5. In the study of Yvon and Pélissier (1987), who analyzed the peptides leaving the stomach of calves fed diets of skim milk and casein solution, three bonds of β-CN were cleaved (namely, Leu_{192}-Tyr_{193}, Leu_{127}-Thr_{128}, and Leu_{139}-Leu_{140}). We would expect calf rennet to cleave more bonds than chymosin alone; nevertheless, this is not evident in these results. Differences in reaction conditions may be the cause for this discrepancy and make it difficult to compare the results in the literature. Nonetheless, the cardoon protease seems to cleave more bonds than chymosin in the bulky, hydrophobic segment Ala_{189}-Phe-Leu-Leu-Tyr_{193} of β-CN.

Analogy between the Activity on Fragments α_{s1}-CN Ala_{163}-Val_{167} and β-CN Ala_{189}-Tyr_{193}. An analogy can be established between the proteolytic activity of cardosin in the rather bulky hydrophobic sequences of α_{s1}-CN (Ala_{163}-Trp-Tyr-Tyr-Val_{167}) and β-CN (Ala_{189}-Phe-Leu-Leu-Tyr_{193}), which contain the four bulkiest amino acids tryptophan, tyrosine, phenylalanine, and leucine (Figure 4). In both cases, the enzyme cleaved all peptide bonds, exhibiting its affinity for bulky, hydrophobic environments, and the capacity of its active site to accommodate this type of sequences. Chymosin, despite its specificity for peptide bonds between hydro-

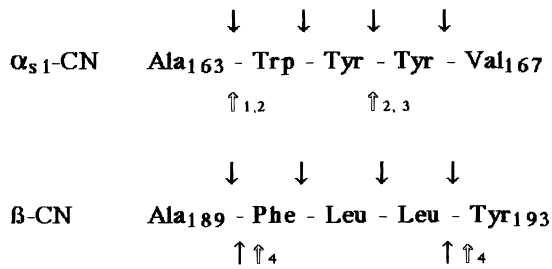

Figure 4. Bonds of α_{s1}-CN and β-CN cleaved by cardosin and chymosin in two bulky, hydrophobic sequences. Key: (↓) bonds cleaved by cardosin (this work); (↑) bonds cleaved by chymosin on α_{s1}-CN or β-CN under conditions identical to those used in this work (Carles and Ribadeau-Dumas, 1984 (β-CN) and 1985 (α_{s1}-CN)); ($\uparrow_1$) bonds cleaved by chymosin on α_{s1}-CN (McSweeney et al., 1993); ($\uparrow_2$) bonds cleaved by calf rennet on α_{s1}-CN or β-CN (Pélissier et al., 1974); ($\uparrow_3$) bond cleaved by calf gastric enzymes *in vivo* on α_{s1}-CN or β-CN (Yvon and Pélissier, 1987); ($\uparrow_4$) bonds cleaved by chymosin on β-CN (Guillou et al., 1991).

phobic amino acids, does not seem to react as easily in the presence of very bulky sequences.

Action on α_{s2}-Casein. Although our aim was to study the proteolysis of α_{s1}-CN, the presence of α_{s2}-CN in the substrate solution allowed the detection of a peptide originating from this protein. In α_{s2}-CN, cardosin catalyses the hydrolysis of two peptide bonds between very hydrophobic amino acids (Phe_{88}-Tyr_{89} and Tyr_{95}-Leu_{96}), leading to a rather lipophilic heptapeptide, Tyr_{89}-Tyr_{95}. This peptide is well resolved from the peptide mixture by rp-HPLC, but elutes somewhat earlier than would be expected on the basis of its hydrophobicity and molecular weight.

According to the extensive review of Grappin et al. (1985), α_{s2}-CN was considered to be totally resistant to the action of chymosin. Very recently, a study on the proteolysis of α_{s2}-CN by chymosin (McSweeney et al., 1994) indicated that the primary site of chymosin action appears to be Phe_{88}-Tyr_{89}. The bond Tyr_{95}-Leu_{96} was also cleaved, but the peptide Tyr_{89}-Tyr_{95} was not identified in the digestion mixture. Chymosin cleavage sites were restricted to the hydrophobic regions of the molecule.

Cheese Quality. Milk-clotting enzymes affect cheese quality through their action on κ-, α_s-, and β-CN. Manufacturing and ripening conditions influence the protein degradation pattern in a maturing cheese, the total amount of bitter peptides in the cheese being the net result of their formation and breakdown by rennet, endogenous milk proteases, and starter enzymes. The threshold values for bitter taste perception as well as eventual synergetic or antagonistic effects are also important in determining whether or not a cheese is rated bitter. It is thus clear that the degradation studies in solution are only a first approximation of the behavior of the milk-clotting enzyme in cheese and that great care is needed in interpreting the relationship between caseinolytic specificity in solution and cheese quality. On the basis of the observed differences in caseinolytic behavior of cardosin and chymosin in solution and in the quality of cheeses prepared with these enzymes, some working suggestions can be made.

Ney (1979) established a relationship between the average hydrophobicity (Q) of a peptide, as measured by the hydrophobicity of amino acid side chains determined by Tanford (1962), and bitterness; that is, peptides with Q values >1400 cal mol^{-1} $residue^{-1}$ and molecular weights up to 6000 Da (bigger molecules are likely too large to interact with the taste receptors) taste bitter, and no bitterness occurs when Q is <1300 cal mol^{-1} $residue^{-1}$. As judged by the Q principle, all the α_s-CN peptides and four of the β-CN peptides formed by cardosin action are bitter. The former are more lipophilic (Table 2), and some of them have not been found in chymosin digests. However, there is evidence (Visser et al., 1983a) that because of their association properties, bitter peptides originating from β-CN and especially the peptides of the C-terminal region are much less degraded in cheese than the α_s- or κ-CN peptides. The work of Visser et al. (1983a and b) and Creamer (1976) support the idea that cleavage near residue 190 is of significance in cheese. It thus seems reasonable to suggest that the apparent high activity of cardosin on the segment Ala_{189}-Phe-Leu-Leu-Tyr_{193} may contribute to the bitter taste of cheese at the end of the maturation period. At first sight we would expect small peptides to be lost to the whey or to be easily broken down, but their high hydrophobicity suggests that they tend to be retained in the protein and fat curd and to associate, especially those that form later. Indeed, a strong association of short, hydrophobic peptides has been observed (Visser et al., 1983a, 1976 and references therein; Visser and Slangen, 1977). In view of the similar specificity (same site of attack, and identical catalytic coefficients) of chymosin and cardosin against κ-CN (Macedo et al., 1993), it seems unlikely that the poor performance of cardosin in the production of cow milk-cheese be due to its action on κ-CN.

Some or all the 20 C-terminal amino acids of β-CN are involved in the association of this protein with α_s- and β-CN molecules, and the polypeptide 1−189 has no ability to associate (Berry and Creamer, 1975). A reduction of these protein−protein interaction sites may inhibit curd syneresis and hence have adverse effects on the rheological properties of the curd (Noel et al., 1987). These results suggest a relationship between the cleavage by cardosin of the four consecutive bonds in segment Ala_{189}-Tyr_{193} of β-CN and texture defects.

Table 2. *Q* Values of Peptides Formed from the Action of Cardosin on α_{s1}-, α_{s2}-, and β-Casein

parent casein	fragment	Q,[a] cal mol^{-1} $residue^{-1}$	time of formation[b]
α_{s1}-CN	146−149	2110	4
α_{s2}-CN	89−95	1755	4
α_{s1}-CN	1−23	1745	1
α_{s1}-CN	154−165	1700	3
α_{s1}-CN	157−165	1672	4
α_{s1}-CN	154−164	1582	3
α_{s1}-CN	165−199	1581	2
α_{s1}-CN	166−199	1536	2
β-CN	190−209	1534	
α_{s1}-CN	157−164	1504	4
α_{s1}-CN	167−199	1489	2
β-CN	128−165	1486	2
β-CN	191−209	1469	2
α_{s1}-CN	154−163	1424	4
β-CN	192−209	1410	
β-CN	193−209	1342	1
β-CN	166−190	908	3
β-CN	166−189	835	3
β-CN	166−191	966	1

[a] Q value, calculated according to Ney (1979), on the basis of the hydrophobicity of the amino acid side chains, as determined by Tanford (1962). [b] 1 to 4 indicate early to late appearance.

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