Caseinolytic Specificity of Cardosin, an Aspartic Protease from the Cardoon Cynara cardunculus L.: Action on Bovine $\alpha_s$- and $\beta$-Casein and Comparison with Chymosin

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

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

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 flowers of Cynara cardunculus L. is a cardoon that grows wild in various regionsofPortugalandotherMediterranean 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 action of cardosin on bovine $\alpha_s$- and $\beta$-casein at 30 °C in 50 mM citrate buffer (pH 6.2) was studied. Peptides were isolated by reversed-phase HPLC on C18 columns and identified from their amino acid composition and N-terminal amino acid sequence. The relative susceptibility of peptide bonds cleaved was Phe105-Met106 > Tyr165-Val167 > Tyr165-Tyr166 > Phe145-Tyr146 > Phe127-Thr128 for $\alpha_s$-casein and Leu192-Tyr193 > Leu193-Leu192 > Leu165-Ser166 > Phe190-Leu191 > Ala189-Phe190 > Leu127-Thr128 for $\beta$-casein. In $\alpha_s$-casein, cardosin cleaved the bonds Phe145-Tyr146 and Tyr165-Leu166. 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 $\alpha_s$-CN (Ala163-Val167) and $\beta$-CN (Ala136-Tyr193), 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 (S1 to S5).

Studies performed in our laboratory on isolated bovine k-casein (k-CN) showed that, like other milk-digesting enzymes (namely, chymosin), it only cleaves the Phe105-Met106 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 $\alpha_s$- and $\beta$-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 $\alpha_s$- and $\beta$-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. $\alpha_s$-Casein ($\alpha_s$-CN) with a small proportion of $\alpha_s$-CN, $\beta$-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 12000g for 5 min, the resultant supernatant was applied to a Sephadex G-100 column (2.5 × 75 cm), previously equilibrated with 50 mM NH4HCO3. 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.

$\beta$-Casein Purification. $\beta$-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 $\beta$-CN sample was dissolved in 5 mM Tris-HCl (pH 8.0), 4.5 M urea, and 8 × 10−4 M 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 × 10−4 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
the eluents were deaerated with helium. The fractions were diazylated against 3 x 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 αs1 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) NaN3. After “blank aliquots” were taken, the enzyme was added to the casein solution (enzymesubstrate 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, C18, 5-μm, 0.46 x 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 28–68% (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 αs-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 readsored 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 αs-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 reproducible HPLC profiles. 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, 2 Asp157-Trp164 and 1 Tyr165-Leu169. The results obtained for fraction K (K and K, V, and N, A and T, P and M, F and E, P and H, E, V) show that it is a mixture of two peptide chains, with the sequences determined corresponding to the fragments 3 Phe23-Glu30 and αs-Lys1-Val7. 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 Leu193-Val209 and of an apparently big peptide having Arg, 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 chromatograms with the known amino acid sequences of αs-CN (Mercier et al., 1971; Nagao et al., 1984; Stewart et al., 1984, αs-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 αs1- and β-CN. Indeed, the primary structure of αs1-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 k2, (αs1-CN Phe24-Trp199) 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, Glu117, Pro127, Leu137, Pro138, Leu138, Pro, Glu145, Gln, and Gln195, and 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 10th and 11th residues of peptide C are Leu and Pro, respectively (Leu137, Pro138); the 10th residue of peptide A is Gln (Gln117); and the 3rd residue of peptide E is Gln (Gln195). These results agree with those of Carles et al. (1988) and Bayev et al. (1987).

**Action on αs1-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 αs1-CN, the enzymesubstrate ratio lying near to the maximum used by these authors. Under these conditions, chymosin only cleaved the Phe23-Phe24 bond of αs1-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, K3], 20 min [H, L1 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 αs1-CN digestion mixture (Figure 1 and intermediate reaction times, not shown) suggests the following pathway of proteolysis. The primary cleavage site of cardosin is Phe23-Phe24, with the formation of Arg-Phe23 and the complementary polypeptide Phe24-Trp199. The next bonds cleaved are Trp165-Tyr166, Tyr166-Val167, Tyr167-Val168, and Phe153-Trp154, in this order, producing the peptides Tyr165-Trp199, Val167-Val168, Tyr166-Val168, and Tyr154-Trp156. The last four bonds to be cleaved are Phe145-Tyr146, Leu146-Phe147, Leu147-Phe148, and Ala163-Trp164, leading to the formation of the fourth set of peptides just mentioned.
McSweeney et al. (1993) identified, at pH 6.5 and 5% NaCl, seven cleavage sites of chymosin on \( \alpha_{\text{s1}} \)-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 \( \alpha_{\text{s1}} \)-CN; that is, Ala\textsubscript{163}-Trp-Tyr-Tyr-Val\textsubscript{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\textsubscript{145}-Tyr\textsubscript{146} 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\textsubscript{165}-Tyr\textsubscript{165} was cleaved in this region. The cleavage by chymosin of the bonds Ala\textsubscript{163}-Trp\textsubscript{164} and Tyr\textsubscript{165}-Tyr\textsubscript{166} has not been reported so far, and the hydrolysis of Tyr\textsubscript{165}-Val\textsubscript{167} was only reported in studies with calf rennet (Péllissier 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\textsubscript{1} to S\textsubscript{2}, S\textsubscript{2} to S\textsubscript{1}, and S\textsubscript{3} to S\textsubscript{1}. Tryptophan (Trp), the bulkiest amino acid, fits into S\textsubscript{1} as well as into S\textsubscript{2}, S\textsubscript{1}, and probably S\textsubscript{3}. Eight of the nine bonds cleaved in \( \alpha_{\text{s1}} \)-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\textsubscript{156}-Asp, although still including a relatively hydrophobic residue, is hydrolyzed to a lesser extent.

The hydrolysis by chymosin of the bond Phe\textsubscript{145}-Tyr\textsubscript{146} has not been reported. Of the 15 cleavage sites identified by Péllissier et al. (1974) in their studies with calf rennet, only four (Phe\textsubscript{23}-Phe\textsubscript{24}, Leu\textsubscript{156}-Asp\textsubscript{157}, Trp\textsubscript{164}-Tyr\textsubscript{165}, and Tyr\textsubscript{166}-Val\textsubscript{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\textsubscript{23}-Phe\textsubscript{24} and Leu\textsubscript{149}-Phe\textsubscript{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: Phε2-3-Phε2 > Trp164-Tyr165 > Tyr165-Val166 > Tyr165-Tyr166 > Phe153-Tyr154 > Phε145-Tyr146 = Leu49-Phe50 = Leu56-Asp57 = Ala163-Trp164 (present work 1)

Chymosin: Phε23-Phe24 (Carles and Ribadeau-Dumas 1984 1)

Chymosin: Phε2-3-Phε2 > Trp164-Tyr165 > Leu56-Asp57 > Phe153-Tyr154 > Tyr159-Pro160 > Phe28-Pro29 > Leu49-Phe50 (McSeeney et al. 1993 2)

Figure 3. Peptide bonds of α-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) NaN3, 90 min; (2) hydrolysis conditions: 30 °C, 100 mM sodium phosphate buffer, pH 6.5, 0.05% (w/v) NaN3, 12 or 24 h.

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

<table>
<thead>
<tr>
<th>HPLC peak a</th>
<th>α-CN fragment</th>
<th>HPLC peak a</th>
<th>β-CN fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Tyr154-Ala163</td>
<td>A</td>
<td>Ser160-Phe190</td>
</tr>
<tr>
<td>B</td>
<td>Tyr154-Val165</td>
<td>B</td>
<td>Ser160-Ala192</td>
</tr>
<tr>
<td>C1</td>
<td>Asp157-Trp164</td>
<td>C</td>
<td>Thr128-Leu195</td>
</tr>
<tr>
<td>C2</td>
<td>Tyr164-Leu165</td>
<td>D</td>
<td>Ser166-Leu191</td>
</tr>
<tr>
<td>D</td>
<td>Asp157-Tyr165</td>
<td>E</td>
<td>Tyr193-Val199</td>
</tr>
<tr>
<td>E</td>
<td>Tyr154-Trp164</td>
<td>F1</td>
<td>Arg1-</td>
</tr>
<tr>
<td>F</td>
<td>Tyr154-Tyr165</td>
<td>F2</td>
<td>Leu191-Val199</td>
</tr>
<tr>
<td>G</td>
<td>Arg-Phe192</td>
<td>G</td>
<td>Arg-Leu192</td>
</tr>
<tr>
<td>H</td>
<td>Tyr166-Trp199</td>
<td>H</td>
<td>α-CN</td>
</tr>
<tr>
<td>I</td>
<td>Val167-Val199</td>
<td>I</td>
<td>β-CN</td>
</tr>
<tr>
<td>J</td>
<td>Tyr165-Trp199</td>
<td>J</td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>Ala2-CN</td>
<td>K1</td>
<td></td>
</tr>
<tr>
<td>K2</td>
<td>Phe24-Phe29</td>
<td>K2</td>
<td></td>
</tr>
</tbody>
</table>

a See Figure 1. b See Figure 2. c B is a fragment from α2-CN; all other fractions are α1-CN fragments; digestion conditions: 30 °C, 50 mM sodium citrate buffer (pH 6.2), 0.1% (w/v) NaCl.

Some bonds that would at first inspection be susceptible to hydrolysis upon cardosin action, namely, Ile71-Val, Tyr91-Leu, Tyr93-Leu, and Leu92-Leu, were not cleaved. Ile71-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 α1-CN (Kumosinski et al., 1991), are also situated in a rather acidic region (25% Glu + Asp in the Gin44-Leu99 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 Ala189-Phe190 and Leu192-Tyr193 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). Leu192-Tyr193 is the easiest hydrolyzed bond, also the most susceptible to attack by chymosin and other milk-clotting enzymes (Pélessier et al., 1974; Creamer, 1976; Visser and Slagen, 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 Leu192-Tyr193 > Leu191-Leu192 ≈ Leu165-Ser166 > Phe290-Leu191 ≥ Ala189-Phe190 ≈ Leu127-Thr128.

Chymosin cleaves two peptide bonds in the sequence Ala189-Phe-Leu-Leu-Tyr193 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 Slagen, 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 Phe190-Leu191 and Leu191-Leu192. 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 to P') is responsible for the non-hydrolysis of these two bonds. We suggest that bulky sequences are the main cause for this non-hydrolysis. Residues Phe190, Leu191, Leu192, and Tyr193 of β-CN interact respectively with subsites S1 to S4, S5 to S5, S2 to S2, and S3 to S1 of the cardoon protease.

Outside this segment, cardosin cleaved bonds Leu165-Ser166 and Leu127-Thr128, which are also cleaved by chymosin, although mostly under more extreme conditions (Visser and Slagen, 1977; Pélessier et al., 1974; Visser, 1981 and references therein; Guillou et al., 1991). Creamer (1976) showed that bonds Ala189-Phe190, Ser164-Leu165 or Ser166-Gln167, and Leu139-Leu140 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 (Leu165-Ser166, Ala189-Phe190, and Leu192-Tyr193); the cleavage at Leu127-Thr128 was observed by these authors at pH 3.5. In the study of Yvon and Pélessier (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, Leu192-Tyr193, Leu127-Thr128, and Leu139-Leu140). 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 Ala189-Phe-Leu-Leu-Tyr193 of β-CN.

Analogies between the Activity on Fragments α2-CN Ala189-Val190 and β-CN Ala189-Tyr193. An analogy can be established between the proteolytic activity of cardosin in the rather bulky hydrophobic sequences of α2-CN (Ala189-Trp-Tyr-Val190) and β-CN (Ala189-Phe-Leu-Leu-Tyr193), 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-
enzymes, some working suggestions can be made. Action and in the quality of cheeses prepared with these enzymes.

It is thus clear that the degradation

threshold values for bitter taste perception as well as the total amount of bitter peptides in the cheese being the time of

imported in determining whether or not a cheese is

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-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 αs- 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 Alα₁CN-Phe-Leu-Leu-Tyr₁₉₃ 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 Slagen, 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 αs- 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 Alα₁CN-Phe-Leu-Leu-Tyr₁₉₃ and texture defects.

**LITERATURE CITED**


Carles, C.; Ribadeau-Dumas, B. Kinetics of the action of chymosin (rennin) on a peptide bond of \( \alpha_s \)–casein. Comparison of the behavior of this substrate with that of \( \alpha_s \) and \( \kappa \)-caseins. FEBS Lett. 1985, 185, 282–286.


Mulvihill, D. M.; Fox, P. F. Proteolytic specificity of chymosin on bovine \( \alpha_s \)-casein. J. Dairy Res. 1979, 46, 641–651.


Stewart, A.; Willis, I.; Mckinlay, A. Nucleotide sequences of bovine \( \alpha_s \)- and \( \kappa \)-casein cDNAs. Nucleic Acid Res. 1984, 12, 3895–3907.


Received for review December 7, 1994. Revised manuscript received August 31, 1995. Accepted September 27, 1995.* This work was supported by INIC and J NICT.