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Specificity and Kinetics of the Milk-Clotting Enzyme from Cardoon (*Cynara cardunculus* L.) toward Bovine κ-Casein

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The action of *Cynara cardunculus* L. protease on whole bovine κ-casein, over a 3-h period at pH 6.4, was investigated. Rp-HPLC of the 3% trichloroacetic acid (TCA)-soluble fraction of the κ-casein digestion mixture showed three peptide peaks, which were identified by amino acid analysis and N-terminal analysis as the 106–169 fragment [caseinomacropeptide (CMP)]. Upon selective precipitation with 12% TCA, one glycosylated and two nonglycosylated forms of CMP were distinguished. Analysis of the whole digestion mixture showed no additional peptides. The kinetics of hydrolysis of the Phe105–Met106 bond was studied by spectrofluorometry, using fluorescein isothiocyanate-labeled κ-casein (FTC-κ-casein). The values obtained for k_{cat}, k_m, and k_{cat}/k_m were 1.04 s^{-1}, 0.16 μM, and 6.5 $\mu M^{-1} s^{-1}$, respectively. The proteolytic coefficient is of the same order of magnitude as those obtained for other milk-clotting enzymes, but the k_m is significantly lower, which reflects the higher affinity of *Cynara* protease to κ-casein.

INTRODUCTION

The coagulation of milk is the basic step in the manufacture of all cheeses. Milk-clotting enzymes suitable for cheese-making must have a high, specific cleavage capacity towards κ-casein at the pH of milk. Calf rennet, which contains chymosin as the main enzyme component, has been the most widely used milk-clotting enzyme preparation, but, in the past few years, increasingly high prices have led to a systematic investigation of suitable substitutes, from either animal, bacterial, or plant origin. From this last class, ficin, from the fig tree, papain, from papaya, and bromelain, from pineapple, have been tried, but they all proved to be unsuitable, since they produce extremely bitter cheeses. To our knowledge, there is only one exception to this general unacceptability of plant clotting enzymes—the protease present in the flowers of the cardoon (*Cynara cardunculus* L.), which is used traditionally in Portugal in the manufacture of the highly appreciated sheep-milk Serra cheese. This aspartic protease has been recently isolated, purified, and partly characterized by Heimgartner et al. (1990) and Faro (1991).

Twenty years ago, Sá and Barbosa (1972) studied the total protein breakdown and the rheological behavior of curds prepared with this enzyme and different types of milk. In their study they concluded that, despite its high proteolytic activity, cardoon extract could satisfactorily replace rennet in the manufacture of soft cheeses; for other cheeses, however, lower yields were observed. These authors suggested that cleavage sites of bovine κ-casein by chymosin and the *cardoon protease* might be different.

To get more information on the action of the cardoon protease on caseins, the cleavage of whole bovine κ-casein by the protease was investigated in this study. The kinetic parameters k_{cat} and k_m for the cleavage of the Phe105–Met106 bond were determined and compared to those published for other milk-clotting enzymes.

MATERIALS AND METHODS

Materials. κ-Casein, phenyl isothiocyanate (PITC), and fluorescein isothiocyanate (FITC) were from Sigma Chemical Co. All reagents were of analytical grade.

Enzyme Extraction and Purification. The protease was extracted and purified as described by Faro (1991). One gram of styles, obtained from dried flowers of *C. cardunculus* L., was macerated in 10 mL of a 100 mM sodium citrate, 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

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in 10-mL fractions. The active-enzyme-containing fractions were pooled and lyophyilized.

Protein Assays. κ-Casein was determined according to the Lowry–Peterson method (Peterson 1977), using κ-casein as standard. Cardoon protease concentration was determined according to the method of Katzenellenbogen and Dobryszycka (1959), using bovine serum albumin as standard.

Specificity Studies. Digestions of κ-casein were carried out at 30 °C in 50 mM sodium phosphate buffer/0.1% (w/v) NaN_3, pH 6.4. After "blank aliquots" where taken, enzyme was added to κ-casein solution (enzyme/substrate mass ratio of 1/1000) and the reaction allowed to proceed. At selected times (up to 3 h), aliquots were taken and the reaction stopped with 3% or 12% TCA (final concentrations). After 30 min at room temperature, the precipitate was removed by centrifugation at 12000*g* for 15 min. The supernatant was filtered through a 0.22-μm pore size filter (Millipore) and analyzed by rp-HPLC.

Peptide Separation. The separation of peptides obtained upon casein digestion was achieved by rp-HPLC, on a Vydac C_{18}, 0.46 × 25 cm column. Elution was carried out with linear gradients of acetonitrile in 0.1% TFA; after a 2-min isocratic run, a gradient of 0–24% acetonitrile was developed in 8 min, followed by a 24–56% gradient over 20 min. The elution rate was 1.0 mL min^{-1} and the column temperature 38 °C. Eluants were prepared with Milli Q water and kept under a flow of helium. Peptides were detected at 220 nm, collected, and pooled for subsequent analysis.

Amino Acid Analysis. The peptides collected in HPLC were dried down and hydrolyzed under vacuum 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 (PTC) amino acid derivatives were injected onto a Spherisorb ODS2 (250 × 4 mm) and eluted, at a flow rate of 1.5 mL min^{-1}, with a CH_3CN gradient in sodium phosphate buffer, pH 6.5; a 2-min isocratic run was performed, followed by linear gradients from 0 to 22.5% over 22.5 min and from 22.5 to 70% over 2 min. Eluants were filtered through a 0.22-μm pore size filter (Millipore) before use and degassed with a flow of helium. The column was kept at 38 °C.

N-Terminal Analysis. N-Terminal amino acid identification was carried out by thin-layer chromatography, on 5 × 5 cm polyamide layer sheets, of the dansylamino acid derivatives (Bruton and Hartley, 1970).

Kinetic Studies. The method for determination of initial rates of caseinomacropeptide (CMP) production was adapted from the assay method for proteolytic enzymes developed by Twining (1984). CMP concentrations were determined by the fluorescence intensity of the 3% TCA-soluble fraction of enzyme digests of the fluorescein thiocarbamoyl derivative of κ-casein (FTC-κ-casein). Fluorescence intensities were converted to concentrations by means of a calibration curve, made up for each set of analysis. The FTC-κ-casein solution was diluted with assay buffer to appropriate concentrations (2.2–25 μM). Individual reaction tubes were prepared, containing 10 μL of this solution, 5 μL of enzyme solution, and 30 μL of assay buffer. After selected times (2, 3, 4, 5, 7, 10 min) the reaction was stopped by addition of 67 μL of 5% TCA and vigorous vortex mixing. The tubes were allowed to sit overnight at 4 °C. The 3% TCA-insoluble protein was sedimented by centrifugation at 12000*g* for 5 min and the supernatant filtered through a 0.22-μm pore size filter (Millipore); and 60-μL aliquots were diluted to 3.000 mL with 500 mM Tris buffer, pH 8.5. Fluorescence intensities were then read at 525 nm, using an excitation wavelength of 490 nm. Blanks, with no enzyme, were treated the same way. The kinetic parameters k_m and k_{cat}, were calculated from Lineweaver–Burk plots.

Preparation of FTC-κ-Casein. κ-Casein was labeled with FITC by a modification of the method proposed by Twining (1984). The protein was dissolved in 50 mM phosphate buffer, pH 9.5, containing 0.1% NaN_3 and allowed to react with a 2.5-fold molar excess of FITC for 1 h, in the dark, at room temperature, with gentle stirring. The pH was then lowered to 6.4 and excess FITC removed by gel filtration on Sephadex G-25; 500 μL aliquots were applied to 60 × 15 mm G-25 columns and eluted by centrifugation at 3000 rpm (Sigma 302k centrifuge) for 2 min. The substrate was stored in 500 μL aliquots at −20 °C. The FTC content of labeled κ-casein was determined by measuring the absorbance at 490 nm of a pH 8.6 solution (Twining, 1984).

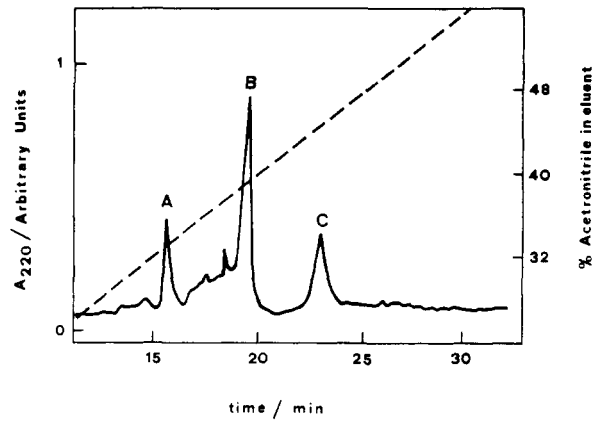

Figure 1. Chromatogram of the 3% TCA-soluble fraction of κ-casein digest with cardoon protease. See text for conditions of HPLC.

Table I. Amino Acid Composition (Moles of Amino Acid per Mole of Peptide) of the Peptides Formed upon Reaction of κ-Casein with Cardoon Protease

amino acid	CMP B-A sequence derived	A	B	C
Asx	4–5	3	4	3
Glx	10	8	9	8
Ser	6	6	6	4
Gly	1	2	1	1
Thr	11–12	10	9	9
Ala	6–5	6	6	6
Pro	8	9	10	8
His	0	0	0	0
Arg	0	0	0	0
Tyr	0	0	0	0
Val	6	6	7	6
Met	1	nd[a]	nd	1
Cys	0	0	0	0
Ile	7–6	5	8	7
Leu	1	2	1	1
Phe	0	0	0	0
Lys	3	nd	3	3
N-terminal	Met	Met	Met	Met

[a] nd, not determined, owing to artifact peaks.

RESULTS AND DISCUSSION

Analysis of κ-Casein Digests. *3% TCA-Soluble Fraction.* The action of the cardoon protease on bovine κ-casein was investigated by analysis of the peptides present in the supernatant upon precipitation with 3% (w/v) TCA. According to Vreeman et al. (1986), 3% (w/v) is the optimum TCA concentration for following the caseinomacropeptide formation from whole κ-casein digests, i.e., the concentration at which maximal protein precipitation and minimal macropeptide precipitation occur. The HPLC chromatograms of the 3% TCA-soluble fraction of κ-casein digest (Figure 1) showed three peptide-containing peaks (A, B, and C), all of which were identified as CMP by amino acid analysis and N-terminal analysis (Table I).

12% TCA-Soluble Fraction. It has been reported (Leonil and Mollé, 1990) that 12% TCA leads to precipitation of carbohydrate-free CMP, keeping carbohydrate-containing forms in solution. Therefore, to distinguish between glycosylated and nonglycosylated CMP, 12% TCA-soluble fractions of the digestion mixture were also analyzed. Two of the peaks (B and C) correspond to nonglycosylated CMP, probably coming from the two

genetic variants of κ-casein, while the other one is likely glycosylated CMP.

Whole Digest Analysis. Protein precipitation with 3% TCA prior to HPLC analysis was routinely used to prevent κ- and *para*-κ-caseins from entering the HPLC column. However, to check on the formation of non-3%-TCA-soluble fragments, aliquots to which no TCA was added were also analyzed by HPLC. In this case the reaction was stopped, either by raising the pH to 9–10 (with NH_4OH) or by keeping the aliquots in liquid nitrogen until analysis. The same results were obtained in both cases: no additional peptides were observed. To prevent the unavailability of *para*-κ-casein by precipitation during renneting and, consequently, unaccessibility of "susceptible bonds", eventually present in this part of the κ-casein molecule, assays at 0 °C were also performed. Again, no bonds other than the Phe105–Met106 were cleaved.

Comparison with Other Milk-Clotting Enzymes. *Specificity.* In several countries, the use of calf rennet substitutes for cheese-making, such as porcine pepsin A, porcine pepsin C, *Mucor miehei* protease, and *Endothia parasitica* protease, became general practice. Specificity studies of these enzymes toward κ-casein (Drohse and Foltmann, 1989), carried out at the same experimental conditions we used, although over a 30-min period rather than 3 h, showed that only the Phe105–Met106 bond was cleaved by all but *E. parasitica*, which cleaved only the preceding bond, i.e., Ser104–Phe105; this difference in cleavage site seemed not to affect clotting. Yvon and Pélissier (1987) analyzed the peptides leaving the stomach of calves fed diets of skim milk and casein solution and came to the conclusion that only CMP was released from κ-casein within 6 h (after ingestion). Besides the case mentioned above, there is no report of a cleavage site other than Phe105–Met106 when κ-casein is incubated with a milk-clotting enzyme under the conditions of milk clotting. So, as far as the κ-casein cleavage site is concerned, *C. cardunculus* L. protease behaves similarly to other milk-clotting enzymes. This behavior seems to be determined by both the specificity of the enzymes and the three-dimensional molecular structure of κ-casein. Predictive models suggest that residues lying between Pro101 and Pro109 are either in a β-sheet or in an α-helical conformation. In either case, the Pro–Pro and Pro–X–Pro turns would present this segment on the surface (Kumosinski et al., 1991) and make it readily accessible to enzymic attack. This, together with the specificity of the cardoon enzyme toward hydrophobic residues (Faro et al., 1992), would explain the high susceptibility of the Phe105–Met106 bond. Studies on the action of the cardoon protease towards α_s-casein, which are in progress in our laboratory, would suggest that other bonds of κ-casein might be cleaved (Phe17–Phe18, Tyr30–Val31, Tyr42–Tyr43, and Tyr60–Tyr61). However, residues 20–68 represent an exceptionally hydrophobic area, with almost no charge, organized in nonstranded β-sheet conformations. These β-sheets make ideal sites for sheet by sheet interactions with other κ-casein molecules (and with hydrophobic sites of α_s- and β-caseins). This could be a reason why bonds Tyr30–Val31, Tyr42–Tyr43, and Tyr60–Tyr61 of κ-casein are not hydrolyzed. The Phe17–Phe18 bond, although somewhat exposed, is not cleaved, likely because of the high charge density in the sorrounding area: 11 ionizable residues between Glu 1 and Lys 24. The nonsusceptibility of these bonds would then be attributable either to secondary specificity hindrances, e.g., electrostatic repulsion between charged groups of the substrate and the enzyme (Phe17–Phe18), or to nonaccessibility to the enzyme, due to conformation (all others).

Table II. Kinetic Parameters of Cardoon Protease, Chymosin, and Pepsin A Action on Whole κ-Casein, Whole κ-Casein B, and κ-Casein B Fractions

substrate[a]	pH	k_{cat} (s^{-1})	k_m (μM)	k_{cat}/k_m ($\mu M^{-1}\ s^{-1}$)	ref
K-CN	6.4	1.04	0.16	6.5	*a*
K-CN B	6.6	93.3	31.8	2.9	*b*
K-CN B-7	6.6	24.9	3.5	7.1	*b*
K-CN B-1	6.6	43.4	8.9	4.9	*b*
K-CN B	6.2	68.7	4.9	1.4	*c*
K-CN B	6.2	45.6	1.9	2.4	*d*

[a] Present paper, cardoon protease. [b] Vreeman et al. (1986), chymosin. [c] Carles and Martin (1985), chymosin. [d] Carles and Martin (1985), pepsin A. [e] For the nomenclature of κ-casein components, the guidelines of the American Dairy Science Association Committee (Eigel et al., 1984) are followed: K-CN, whole κ-casein; K-CN B, whole κ-casein B; K-CN B-7, κ-casein B fraction containing one P and six NeuAc groups; K-CN B-1, κ-casein B fraction containing one P.

Kinetics. HPLC has been widely used in kinetic studies of κ-casein hydrolysis. However, in our hands, quantitative HPLC analysis was not reproducible, probably owing to unwanted retention of peptide material onto the HPLC column. Moreover, in our separation system, different forms of CMP elute separately. The kinetic studies were then based on the fluorescence intensity of the 3% TCA-soluble fraction of enzyme digests of FTC-κ-casein. [It is known, for sure, that this fraction contains no protein other than CMP (see Analysis of κ-Casein Digests) and that CMP contains residues that bind FITC.] The efficiency of our method to eliminate unreacted reagent from the FTC-κ-casein solution was checked by measuring the [FTC]/[protein] ratio in the various steps. The method was found to be reproducible, simple, and fast; a constant ratio (0.39 ± 0.01) was reached from the third eluate on, i.e., each 500-μL aliquot should be passed through three G-25 columns. The fluorescence method proved to be linear in the concentation range used. Preliminary experiments showed that, in our experimental conditions, hydrolysis times up to 10 min were suitable for initial rate determinations.

The kinetic parameters k_m, k_{cat}, and k_{cat}/k_m of *C. cardunculus* L. protease action on isolated bovine κ-casein are shown in Table II. For comparison, the results obtained by other authors for chymosin and pepsin A are also presented. Although there are slight differences in substrate (as to amino acid composition and glycosylation level) and pH between our experiments and the others, it seems not very likely that the amino acid replacements that occur in the genetic variant A (136 Ile/Thr and 148 Ala/Asp) influence significatively the kinetics of the enzymatic reaction, since they are situated far from the cleavage site, as judged from the secondary structure presented by Kumosinski et al. (1991). The discrepancy of opinions concerning the influence of carbohydrate content on the kinetics of CMP release upon κ-casein enzymic hydrolysis led Vreeman et al. (1986) to carry out an extensive study on the importance of this effect. They concluded that NeuAc content plays a minor role (cf. data for K-CN B-1 and K-CN B-7 in Table II). This is in agreement with the studies of Dalgleish (1986), who also observed no effect of the extent of glycosylation of κ-casein on the rate of its breakdown during renneting. Finally, the pH in our experiments is in the range of those used by the other authors. Thus, on the basis of what was said above, it seems fair to compare the data presented in Table II.

From the data shown, it is seen that the proteolytic

coefficients k_{cat}/k_m for the various enzymes differ only to a small extent. However, the k_m value found for the cardoon protease is remarkably lower than those obtained for the other two enzymes, thus reflecting a substantially higher affinity of this enzyme for the substrate.

ABBREVIATIONS USED

CMP, caseinomacropeptide, 106–169 fragment of κ-casein; FITC, fluorescein isothiocyanate; FTC, fluorescein thiocarbamoyl derivative; K-CN, whole κ-casein; K-CN B, whole κ-casein B; K-CN B-1, κ-casein B fraction containing one phosphate group; K-CN B-7, κ-casein B fraction containing one phosphate group and six NeuAc groups; NeuAc, *N*-acetylneuraminic acid; TCA, trichloroacetic acid.

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