Kinetics of the action of chymosin (rennin) on a peptide bond of bovine α_{s1}-casein

Comparison of the behaviour of this substrate with that of β- and κ_o-caseins

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The action of chymosin on the Phe23–Phe24 bond of bovine α_{s1}-casein, in citrate buffer (pH 6 2) at 30°C, was followed by reversed-phase HPLC quantification of residual α_{s1}-casein or fragment 24–199 after different time periods and at different substrate concentrations This allowed determination of the Michaelian parameters for the reaction under study which were compared with those previously obtained for the action of chymosin on β- and κ_o-casein under identical reaction conditions The whole efficiency of the three reactions, as estimated by k_{cat}/K_m, was 1 8, 20 6 and 1405 0 for α_{s1}-, β- and κ_o-caseins, respectively The specificity of chymosin is discussed in the light of these results and of the known sequences of the 3 caseins

Casein *Chymosin* *Kinetic study* *Reversed-phase HPLC*

1. INTRODUCTION

Caseins are the natural substrates of chymosin (EC 3.4 23.4) and pepsin A (EC 3.4 23.1) in the stomach of suckling calves A number of studies have been devoted to the action of both enzymes on the main bovine caseins (α_{s1}, β and $\varkappa$) However, most were of a qualitative nature, mainly achieved in order to characterize the first reaction products visible on gel electrophoresis. We have recently carried out kinetic studies of the action of chymosin on β- and $\varkappa_o$-caseins [1,2]. Here, the work has been completed by the determination of the catalytic constants relative to the action of chymosin on α_{s1}-casein under identical conditions, thus giving comparable data on the action of chymosin on the 3 main caseins.

2. MATERIALS AND METHODS

Lichrosolv 2-propanol from Merck (Darmstadt, FRG) was used for HPLC. The ammonium acetate Normapur was from Prolabo (Paris), and SDS from Eastman-Kodak (Rochester, NY). All other chemicals were from Merck. Buffers and solvents for HPLC were filtered through 0.45 μm Millipore filters (Millipore Corp., Bedford, MA) and degassed under vacuum before use.

The proportion of active enzyme in the freeze-dried chymosin preparation obtained from Sigma (St Louis, MO) was determined as described by Martin et al [3]. The preparation was shown to be free of pepsin by using the immunological technique of Collin et al. [4].

α_{s1}-Casein B was prepared as described by Mercier et al [5] from the milk of a cow homozygous at the 4 casein loci. The concentration of the casein solutions was determined, after filtration on 0 45 μm filters, from the extinction coefficient $E^{1\,mg/ml}_{280nm} = 1\ 05$ given by Swaisgood [6]

α_{s1}-Casein was digested with chymosin at 30°C in 50 mM sodium citrate buffer, pH 6.2, and 0.1% NaN_3 (w/v). Aliquots were taken at intervals, and the reaction was stopped by adding a known volume of concentrated ammonia to bring the pH to 9–10 It was shown that the inhibition was im-

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mediate and irreversible. Volumes of 10–50 μl were injected on the HPLC column after filtration on 0.45 μm filters. It was checked that there was no adsorption of any protein material on the filter.

Amino acids were determined after acid hydrolysis (5.7 N HCl, 110°C, 24 h under vacuum) with an LC 5000 amino acid analyser (Biotronik, FRG) equipped with a single column (20 cm × 3.2 mm i.d.).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to O'Farrell [7] on 1.5 mm thick gels (stacking gel, 7.5% acrylamide; running gel, linear gradient from 12 to 15% acrylamide). Samples were in 0.12 M Tris (pH 6.8), 3.5 M urea, 10% (w/v) sucrose, 2% (w/v) SDS, 2% (w/v) 2-mercaptoethanol.

The HPLC equipment consisted of two 6000 A solvent delivery systems, a U6 K septumless injector, a microBondapak C18 column, a model 720 system controller (Waters, Milford, MA) and a variable wavelength CE 2012 UV monitor (Cecil, Cambridge, England). The chromatographic conditions were: column and solvent temperature, 40°C; flow rate, 2 ml/min; solvent A, 50 mM ammonium acetate, 10 mM SDS adjusted to pH 7.2 with concentrated ammonia; solvent B, 66% 2-propanol, 34% water (v/v), 40 mM ammonium acetate, 10 mM SDS, final concentrations, pH to 7.2 as above. The elution was obtained by using a linear gradient from 0 to 100% B in 30 min. The absorbance was recorded at 220 nm. The collected fractions were dried using a Speed Vac Concentrator (Savant, Hicksville, NY). Quantitative evaluation of peak areas was performed manually In a preliminary trial all fractions of interest were collected. Increasing amounts of each were injected In each case, excellent linearity was found when the areas were plotted against the injected amounts.

The direct linear plot of Eisentahl and Cornish-Bowden [8] was employed for the determination of kinetic parameters. M_r values of 35650 [9] and 23600 [6] were taken for chymosin and α_{s1}-casein, respectively.

3. RESULTS

The action of chymosin on α_{s1}-casein was followed by HPLC analysis of aliquots taken at intervals for 3 h. Three well resolved peaks, whose height varied with time, were observed during this reaction period (fig.1). For the trial shown in fig.1, each peak was collected and analysed. The unretained fraction (t_r = 2 min) corresponded only to NaN_3 and citrate as shown by amino acid analysis and injection of these components. Fraction C was easily identified from its amino acid composition and corresponded to peptide 1–23. As the other fractions contained larger peptides, the amino acid compositions were not accurate enough to identify them unambiguously On gel electrophoresis, fractions A and B migrated as α_{s1}-I (fragment 24–199) according to the nomenclature of Fox [10], and α_{s1}, respectively. That peak B was intact α_{s1}-casein was verified from the retention time by chromatographing the protein alone.

The quantitative evolution of components α_{s1}-I and α_{s1} during a 3 h period is shown in fig.2 for one of the 5 studied concentrations. The curves

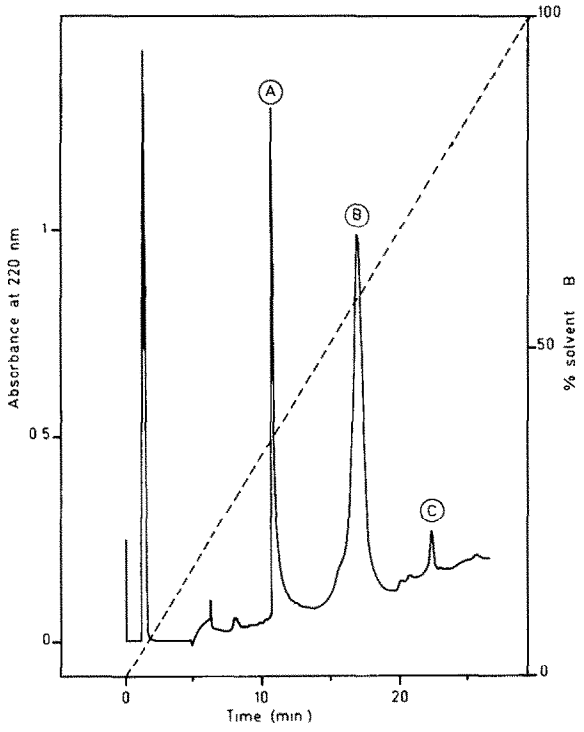

Fig.1 RP-HPLC pattern showing the molecular species that appear at the beginning of chymosin action on α_{s1}-casein Conditions for digestion and elution are described in the text A, α_{s1}-I, B, α_{s1}-casein, C, fragment 1–23. The non-retained fraction corresponds to NaN_3 and citrate (– – –) % solvent B

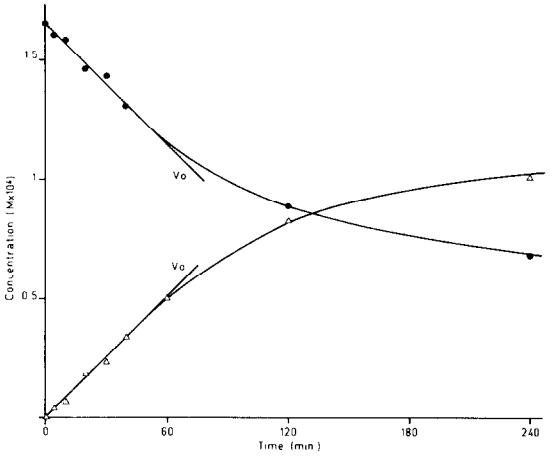

Fig 2 Curves representing the disappearance of α_{s1}-casein (●) and the appearance of α_{s1}-I fragment (△) as a function of time during the action of chymosin on α_{s1}-casein for one substrate concentration Determination of the initial rate of reaction v_o

of appearance of α_{s1}-I and disappearance of α_{s1} are symmetrical, confirming that only the Phe23–Phe24 bond was cleaved. Under these conditions the Michaelian parameters relative to splitting of the bond 23–24 could be determined. This was done by quantitative determination on HPLC of the disappearance of α_{s1}-casein after 5, 10, 15, 20 and 30 min reaction times, respectively, with an enzyme concentration of $2\,8 \times 10^{-5}$ mM. This gave the initial rate of reaction at each substrate concentration (0.66, 0.33, 0.17, 0.08 and 0 04 mM) Quite similar initial velocities were obtained by following the appearance of α_{s1}-I as a function of time. From the results thus obtained the following values were determined: k_{cat} = 0 7 s^{-1} and K_m = 0.37 mM.

4 DISCUSSION

Our aim was to compare the catalytic parameters relative to the early action of chymosin on the 3 main caseins, α_{s1}, β and $\varkappa$, under identical reaction conditions. Indeed, a number of qualitative studies have been performed showing that a few peptide bonds in each were especially chymosin sensitive. However, no quantitative data were available which could allow a clear comparison of the susceptibility of the 3 caseins to chymosin. We have recently obtained such data concerning the cleavage of the Leu192–Tyr193 and Phe105–Met106 bonds of β- and $\varkappa_o$-caseins, respectively [1,2] The reaction conditions were identical with those employed here At the substrate concentrations used the 3 proteins were in polymeric forms, micelles for β- and $\varkappa_o$-caseins, and unordered structures for α_{s1}-casein. The state of aggregation of the 3 proteins could be considered as invariant over the range of substrate concentrations employed [11]. Only for β-casein has it also been possible to determine the kinetic parameters on the monomeric form by working at low substrate concentration [1] In each case the reactions were quantitatively followed by HPLC. It was clearly shown that each reaction led to the cleavage of a single peptide bond in each casein The results are compared in table 1. This table also shows values obtained by Hill et al. [12] as far as

Table 1

Kinetic parameters relative to the early cleavage of α_{s1}-, β- and $\varkappa_o$-caseins[a] by chymosin (30°C, 50 mM Na citrate buffer, pH 6 2, I = 0 2)

	Association state	Bond cleaved	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	Ref
α_{s1}-Casein	aggregates	Phe 23–Phe 24	0 370	0 66	1.8	this work
	aggregates	Phe 23–Phe 24	0 450	3 80	8 4	[12][b]
$\varkappa_o$-Casein	micelles	Phe 105–Met 106	0 049	68 87	1402 0	[2]
β-Casein	micelles	Leu 192–Tyr 193	0 075	1 54	20 6	[1]
	monomers	Leu 192–Tyr 193	0.007	0 56	79 7	[1]

[a] The carbohydrate-free fraction of $\varkappa$-casein
[b] 0 1 M sodium acetate, pH 6 4, 30°C

the cleavage of the Phe23–Phe24 bond of α_{s1}-casein is concerned, using a rather tedious and inaccurate procedure (determination of the rate of appearance of peptide 1–23 by Sakaguchi reaction performed on trichloroacetic acid supernatants obtained after filtratiton on paper). The reaction conditions were somewhat different from ours (Na acetate, pH 6.4, 30°C).

Stopped-flow kinetic measurements have shown for porcine pepsin that the K_m approximates the dissociation constant of the enzyme-substrate complex [13]. We will assume that this also applies to chymosin, an enzyme closely related to the former, and that k_{cat} reflects the catalytic efficiency, even though the two parameters are indeed not completely independent, in particular because part of the enzyme-substrate binding energy can be used to lower the free energy of activation [13]. The affinity of the region of α_{s1}-casein surrounding bond 23–24 appears to be low and the overall substrate capacity, as expressed by k_{cat}/K_m, is moderate It is equal to that of peptide Leu-Gly-Phe-Nle-Ala-OMe, a synthetic peptide analogous to the sensitive region of κ-casein, for which the k_{cat} value is similar. As shown by Visser [14] a similar peptide, with only Ser instead of Gly at position P2 (using the representation of Schechter and Berger [15]) as in κ-casein, displays a much better substrate capacity. It can thus be inferred that, as postulated earlier, the Ser residue should be essential for efficient catalysis Since Gly, and Arg in α_{s1}-casein, seem to exert a similar adverse effect, the size and charge of the amino acid in this position do not seem to be involved.

Fig 3 shows the alignment of the sequences around the 3 chymosin-sensitive bonds of the caseins. It shows in addition the sequence 10–24 of κ-casein which displays a striking homology with that of α_{s1}-casein around bond 23–24. The cleavage of Phe17–Phe18 of κ-casein by chymosin has not been reported. As it is known that the active site of the aspartyl proteinases is located in a cleft lined with hydrophobic residues, it can be suggested that the low hydrophobicity and high number of charged groups in the sequence 10–24 of κ-casein prevent its binding to the enzyme.

Many investigations have been devoted to kinetic studies on peptides of various lengths which mimic κ-casein around the most sensitive bond 105–106. Besides demonstrating the role of

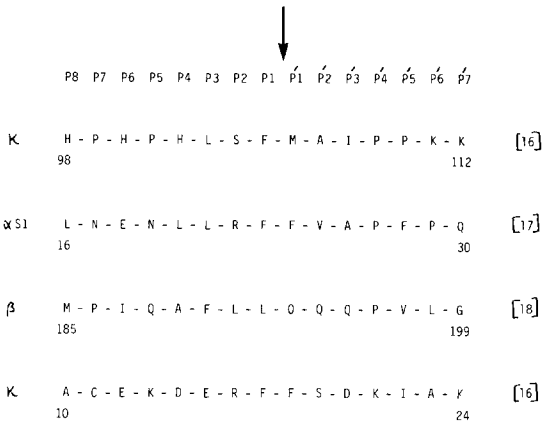

Fig.3 Alignment of the sequences around 3 chymosin-sensitive bonds of the caseins (Phe23–Phe24 for α_{s1}, Phe105–Met106 for κ and Leu192–Tyr193 for β) and 1 chymosin-non-sensitive bond (Phe17–Phe18 for κ)

Ser104, they also show that the smallest peptide with good substrate properties is the hexapeptide 103–108, that it is mainly the length of the peptide backbone which determines the improved substrate quality of this segment in comparison with shorter ones, and that hydrophobic or bulky residues at positions P1, P3 and P'1 are important for a good fit on the enzyme's active center and/or for catalytic efficiency [14]. Experiments on longer peptides, up to peptide 98–112, have indicated that lengthening both sides of the hexapeptide mentioned above increases considerably the proteolytic coefficient k_{cat}/K_m. It seems that this could be due to the occurrence of non-polar residues on the right side and to the positive cluster on the left side, acting as secondary binding sites. Finally, prediction of the secondary structure of κ-casein assigns an extended β-structure to segment 103–108, which is assumed to provide a good fit in the active center [19].

This study confirms some of these hypotheses and provides some new indications. However, further comments must first be added as far as the state of aggregation of β-casein in solution is concerned. While the chymosin-sensitive bonds in polymeric κ- and α_{s1}-caseins are likely quite accessible to the enzyme, this is not the case for β-casein as indicated by the kinetic parameters. Furthermore, previous reports have shown that the segment which contains the sensitive bond of β-

casein is implied in the aggregation of this molecule [20]. Therefore, it seems wise to compare the catalytic parameters of polymeric α_{s1}- and $\varkappa_o$-casein with those of monomeric β-casein (table 1). This protein appears to be a better substrate for chymosin than α_{s1}-casein. A comparison of the 3 protein substrates shows that each has similar hydrophobic residues at positions P3, P1 and P'1, and a proline at P'4. A role for the proline residues located each side of segment 98–112 of $\varkappa$-casein in the conformation of the substrate was postulated in [21] A proline residue is located at position P7 in $\varkappa$- and β-casein. The absence of such a residue in α_{s1}-casein, the occurrence of a negative charge in P6, might be responsible for its weak affinity for chymosin, while the absence of serine at P2, of a positive cluster on the left side, could explain the lower catalytic efficiency of chymosin for β-casein than for $\varkappa$-casein.

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