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PEPTIDE SUBSTRATES FOR CHYMOSIN (RENNIN)

KINETIC STUDIES WITH PEPTIDES OF DIFFERENT CHAIN LENGTH INCLUDING PARTS OF THE SEQUENCE 101-112 OF BOVINE κ-CASEIN

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Summary

Kinetic parameters have been determined for the reaction between milk-clotting chymosin (EC 3.4.23.4) and a series of peptides (or their methyl esters) including the amino acid sequence around the enzyme-sensitive Phe(105)-Met (106) bond of bovine κ-casein. In particular, the influence of the substrate's chain length on the kinetic parameters has been studied. Evidence is presented that in the model peptides studied the sequence -Ser-Phe-Met-Ala- with a further residue added to either end (in casu Leu(103) or Ile(108)) is necessary to induce any cleavage by the enzyme. When both the Leu(103) and Ile(108) residues form part of the peptide chain, a marked improvement of the substrate properties is observed. It is suggested that prolyl residues on either side of the sensitive peptide bond form additional sites for secondary enzyme-substrate interactions.

Introduction

Chymosin (rennin, EC 3.4.23.4) is a milk-clotting enzyme, the precursor of which is secreted by the fourth stomach of the calf [1]. During the last few decades it has been established that the process of milk clotting is initiated by the specific proteolysis of so-called κ-casein by the enzyme [2]. This initial action on κ-casein is restricted to the cleavage of the peptide bond 105-106 between phenylalanine and methionine [3—5]. To gain information how this specific cleavage is connected with the structure of the substrate, we have undertaken a systematic study of the substrate specificity of chymosin. For this purpose we have utilized a series of synthetic peptides of different chain length includ-

ing parts of the sequence

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-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-
 101    103     105 106    108    110    112
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around the chymosin-labile Phe-Met bond of bovine κ-casein.

In previous communications [6,7] some of us have reported on the substrate properties of a number of such peptides. Conclusions were based on high-voltage paper electrophoretic patterns after prolonged incubation of the peptides with the enzyme. The main features arising from this work were: First, a minimum chain length of five amino acid residues seems to be a prerequisite for cleavage by the enzyme. Second, the sequence Leu(103)-Ser(104) is of importance. When this sequence is reversed, a case studied by Hill [8,9], a sharp decrease in the rate of cleavage is observed. Third, further chain extension at either side of the sensitive bond enhances the capacity of the peptide to function as a substrate for chymosin.

Independently of this work, other groups have also studied the action of chymosin on parts of κ-casein containing the Phe-Met bond in question. Hill [8, 9] concluded from his experiments with short synthetic peptides as well as with photo-oxidized κ-casein [10] that the enzymic action is accelerated by serine and histidine side chains located close to the Phe-Met bond. Polzhofer [11] found a synthetic pentadecapeptide to be split rapidly and calculated a Michaelis constant for this reaction. Furthermore, he concluded that the His (102) residue has an important role, since the hexapeptide His-Leu-Ser-Phe-Met-Ala appeared to be split by chymosin whereas the pentapeptide Leu-Ser-Phe-Met-Ala was found to be resistant to enzymatic cleavage. Evidence for the importance of the hydroxyl group of Ser(104) was presented by Raymond et al. [12,13] working with peptide substrates slightly different from the parent primary structure.

In the present paper kinetic studies are reported in which special attention has been given to the influence of the chain length of the substrate on the kinetics of its reaction with chymosin. This approach permits an investigation of the effect of secondary enzyme-substrate interactions and may lead to some conclusions about the size of the enzyme's active centre [14].

Materials and Methods

Peptides and their derivatives were synthesized and characterized as described by Schattenkerk et al. [15]. The specificity of their cleavage by chymosin was checked by thin-layer chromatography, paper electrophoresis and by N-terminal group analysis using dansyl chloride [16]. Crude chymosin, isolated as an extract ("rennet") from stomachs of newborn calves, was supplied by the "Coöperatieve Stremsel- en Kleurselfabriek" (Leeuwarden, The Netherlands). From this extract chymosin was purified by DEAE-Sephadex chromatography as described by De Koning [3,4]. The milk-clotting activity of the purified enzyme amounted to $6.5 \cdot 10^6$ Soxhlet units [17,18]. As a reference the "Netherlands Standard for the determination of (calf-)rennet strength" furnished by the Government Dairy Station (Leiden, The Netherlands), was used.

Enzyme solution. The enzyme was dissolved to a final concentration of 0.418 M in 0.05 M sodium acetate buffer containing 1 M NaCl (pH 5.25) assuming a molecular weight of 30 000 [1,3,4]. Small portions of the enzyme solution, sufficient for a 1-day experiment, were kept frozen until needed in tightly closed 1 ml vials. With this procedure the proteolytic activity remained unaffected for many months.

Substrate solutions. Freeze-dried peptide (5—10 mg for one duplicate experiment) was brought into contact with a suitable volume of 0.05 M sodium acetate buffer (pH 4.7). After centrifugation of non-dissolved material *, the supernatant was utilized as a stock solution, the concentration of which was established as follows. Duplicate samples, to which known amounts of L-norleucine had been added as an internal standard, were made 6 M in HCl and heated at 110°C in small evacuated tubes for 22 h. The contents were then evaporated to dryness in vacuo. The residues were taken up with sodium citrate buffer (pH 2.2) and analyzed with the aid of an automatic amino acid analyzer (JEOL JLC-5AH). The concentration of peptide initially present was calculated by comparison with a standard mixture of amino acids analyzed in the same way.

Duplicate series of 5—8 different concentrations were prepared by diluting samples of the stock solution with 0.05 M sodium acetate (pH 4.7).

Kinetic measurements. Reactions were carried out at 30°C in a 0.05 M sodium acetate buffer, pH 4.7. The enzymatic cleavage was monitored by the automated ninhydrin assay described elsewhere [20]. Each experiment was started with equal volumes of substrate solution (800 μl) in standard size reaction tubes (5.5 × 0.6 cm). The time of mixing the enzyme with the substrate was kept as short as was necessary to ensure complete mixing (5 s). By doing so, we could minimize the effect of a gradually decreasing initial reaction rate which in preliminary experiments had been observed when the reaction mixture was continuously stirred. This effect, the magnitude of which also depended on the total volume stirred, is probably to be attributed to an inactivation of the enzyme at the air-water interface. The same phenomenon was observed when a substrate in which methionine-106 had been replaced by a different residue (e.g. leucine or norleucine), was used. This rules out air oxidation of the substrate's methionine side chain, resulting in decreasing substrate properties (cf. Results), as the main source of this effect.

Evaluation of kinetic parameters. The kinetic parameters, V and K_m, were calculated from the collected data of two independent experiments each carried out with 5—8 substrate concentrations. A BASIC-programmed Hewlett-Packard calculator, model 9830 A, was used routinely to determine slope and intercept from plots of c/v vs c [21] and $1/v$ vs $1/c$ [22]. The programme provides all necessary calculations including a weighted (v^{-4}) least-squares fit to a straight line with standard errors for the computed parameters [23] and the correction for hydrolytic cleavage discussed below.

Correction of parameters for the extent of initial hydrolysis.
For the process:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2,$$

* Addition of an organic solvent, which is sometimes used to enhance the solubility of substrates, has been avoided in this study as this may affect the kinetics of enzymic action [19].

(E = enzyme, S = substrate, and P = product) the rate equation can be written as:

$$v_0 = \frac{Vc_s^0}{K_m + c_s^0}, \tag{1}$$

where v_0 is the initial velocity; c_s^0 the initial substrate concentration; V the maximal velocity (= k_{cat} × total enzyme concentration) and K_m the Michaelis constant (= $(k_{cat} + k_{-1})/k_1$). When the substrate concentration changes to a considerable extent during the time course of measurement (t) the integrated rate equation should be used:

$$Vt = c_p^t + K_m \ln \frac{c_s^0}{c_s^0 - c_p^t} \tag{2}$$

where c_p^t stands for the concentration of product formed. After expanding the logarithm and rearrangement, one obtains

$$\frac{c_p^t}{t} + \frac{K_m\left\{\frac{1}{2}\left(\frac{c_p^t}{c_s^0}\right)^2 + \frac{1}{3}\left(\frac{c_p^t}{c_s^0}\right)^3 + \frac{1}{4}\left(\frac{c_p^t}{c_s^0}\right)^4 + \ldots\right\}}{t\left(\frac{c_s^0 + K_m}{c_s^0}\right)} = \frac{Vc_s^0}{K_m + c_s^0} \tag{3}$$

The ratio c_p^t/c_s^0 is a measure of the extent of hydrolysis. Its value can be established for each substrate concentration [20]. In Eq. 3 the second term on the left-hand side represents the correction of the apparent initial velocity c_p^t/t for substrate depletion. Since this term contains the parameter K_m, we have used an iterative procedure for the evaluation of correct Michaelis parameters. As a starting value of K_m the result from a linear plot with the apparent initial velocities was taken.

Results

Identical kinetic parameters, V and K_m, were obtained by analysing the c_s^0/v vs c_s^0 plot and the $1/v$ vs $1/c_s^0$ plot only after proper weighting for inversion [24].

In Table I the kinetic parameters k_{cat}, K_m and k_{cat}/K_m for a number of peptides are presented together with their standard errors. The experimental conditions are specified in columns 4 and 5. The maximal depletion of substrate during the time used for the determination of initial velocities, is given in column 6. In the last column the number of experimentally determined initial velocities over the concentration range given, is listed. As may be expected, the values of the separate parameters, k_{cat} and K_m, are more reliable when K_m is well within the range of substrate concentrations.

The pentapeptide ester Ser-Phe-Met-Ala-Ile-OMe (I), which previously [6] was found to be split to a reasonable extent during a 24-h incubation period with the enzyme, appeared to be a poor substrate in terms of k_{cat}/K_m as compared with the substrates V—XIII. The same can be said of other peptides with N-terminal serine and chains extended in the C-terminal direction (substrates

TABLE I

KINETIC PARAMETERS OF THE REACTION BETWEEN CHYMOSIN AND SYNTHETIC SUBSTRATES CONTAINING PARTS OF THE κ-CASEIN SEQUENCE

- Pro -	His -	Leu -	Ser -	Phe -	Met -	Ala -	Ile -	Pro -	Pro -	Lys -	Lys -
101	103	105	106	108	110	112					

All experiments were carried out at 30°C in 0.05 M sodium acetate buffer (pH 4.7).

No.	Substrate	Number of residues	Substrate concentration (mM)	Enzyme [a] concentration (nM)	Percentage of hydrolysis	k_{cat} [a] (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	n
I	Ser(104)⟷Ile(108)OMe	5	0.43—1.72	2960	0.8— 1.2	0.33 ± 0.10 [b]	8.5 ± 2.7 [b]	0.038 ± 0.002	10
II	Ser(104)⟷Pro(109)OMe	6	0.20—1.60	1490	0.9— 3.2	1.05 ± 0.45 [b]	9.2 ± 4.0 [b]	0.114 ± 0.007	10
III	Ser(104)⟷Pro(110)OMe	7	0.66—1.76	775	2.1— 3.3	1.57 ± 0.82 [b]	6.8 ± 3.6 [b]	0.231 ± 0.022	12
IV	Ser(104)⟷Lys(111)OH	8	0.30—1.20	1540	4.4— 5.4	0.75 ± 0.15 [b]	3.2 ± 0.6 [b]	0.239 ± 0.013	10
V	Leu(103)⟷Ile(108)OMe	6	0.10—0.80	66	8.6—14.6	18.3 ± 0.9	0.85 ± 0.05	21.6 ± 0.7	15
VI	Leu(103)⟷Pro(109)OMe	7	0.08—0.64	38	12.2—21.3	37.5 ± 1.7	0.71 ± 0.04	52.8 ± 1.6	16
			0.10—0.83	30	9.1—17.3	38.7 ± 1.4	0.67 ± 0.03	57.5 ± 1.7	13
VII	Leu(103)⟷Pro(110)OMe	8	0.09—0.76	19	7.9—18.3	43.3 ± 2.3	0.41 ± 0.03	105.1 ± 6.9	15
VIII	Leu(103)⟷Lys(111)OH	9	0.06—0.50	19	7.4—14.2	33.6 ± 1.2	0.43 ± 0.02	78.3 ± 2.3	13
IX	Leu(103)⟷Lys(112)OH [c]	10	0.11—0.45	43	15.9—24.9	31.4 ± 1.6	0.49 ± 0.03	63.7 ± 2.2	13
			0.06—0.45	29	10.6—18.0	29.0 ± 1.2	0.43 ± 0.02	66.9 ± 2.1	16
X	Leu(103)⟷Lys(112)OH [d]	10	0.05—0.37	29	10.9—19.2	25.3 ± 1.5	0.40 ± 0.03	63.0 ± 2.6	7 [f]
XI	Leu(103)⟷Lys(112)OH [e] [(106)Met(O)]	10	0.09—0.36	143	9.7—12.3	7.1 ± 0.4	0.84 ± 0.05	8.41 ± 0.16	6 [f]
XII	His(102)⟷Ile(108)OMe	7	0.09—0.65	52	8.8—17.5	16.0 ± 0.8	0.52 ± 0.03	30.8 ± 1.4	12
XIII	Pro(101)⟷Ile(108)OMe [c]	8	0.10—0.83	15	5.4—13.0	34.8 ± 0.5	0.37 ± 0.01	94.6 ± 2.1	15
			0.09—0.71	14	6.2—16.9	32.3 ± 0.5	0.31 ± 0.01	105.9 ± 2.6	16

[a] Calculated assuming a molecular weight of 30 000 for the enzyme.

[b] Values of the separate parameters, k_{cat} and K_m, are rather uncertain, since the v vs c_s^0 plot largely showed first-order kinetics.

[c] Results are given of two duplicate experiments done at a time interval of at least one month.

[d] After preliminary treatment with 2-mercaptoethanol.

[e] After preliminary oxidation of the methionyl residue to its sulphoxide according to the method described by Iselin [25].

[f] Data obtained from a single experiment.

II—IV). Addition of a leucyl residue to the N-terminal part of the peptides I—IV caused an increase of the k_{cat}/K_m parameter by more than two orders of magnitude (substrates V—VIII). Extension with a histidyl residue at the N-terminal side of the hexapeptide ester V hardly influenced the k_{cat}; the increase of the k_{cat}/K_m ratio was predominantly brought about by a change of K_m (XII). Further extension in N-terminal direction with a prolyl residue brought about an increase in k_{cat} and a decrease in K_m. The k_{cat} value (32—35 s^{-1}) found for this octapeptide ester XIII is about the same as that of the heptapeptide ester VI obtained by extension with only one (prolyl) residue at the C-terminal side of peptide V. Comparing the kinetic parameters of the substrates V and VI, one sees that for the longer peptide the K_m is somewhat lower while the k_{cat} is doubled. Addition of a second proline to this sequence, leading to the octapeptide ester Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-OMe (VII), resulted in a further decrease of K_m leaving k_{cat} almost unchanged. As soon as the lysine-111 had been joined to the sequence, the k_{cat} value decreased slightly while the K_m remained unaltered (VIII). However, no definite conclusions in terms of electrostatical effects can be drawn from this, since a free carboxylic end group was introduced simultaneously in the peptide chain in place of the methyl ester group, leaving the net peptide charge unaffected. In fact, an extra positive charge was added only by introduction of the second lysyl residue at position 112 (IX). This hardly altered the kinetic parameters as compared with those of the substrate VIII.

Treatment of the peptide IX with 2-mercaptoethanol to eliminate the possible effect of methionine oxidation did not influence the enzyme kinetics (X). Apparently methionine sulphoxide was not present in the peptide preparation in significant amount. This argument is corroborated by the fact that careful oxidation of the methionyl residue to the sulphoxide resulted in a striking fall of the substrate properties as expressed by a decreasing k_{cat} and an increasing K_m (XI).

Discussion

Considering the results listed in Table I, it appears that the leucyl residue at position 103 is of great importance for the rate of hydrolysis of the substrate, as revealed by a sharp increase in k_{cat} after the introduction of this residue. Two factors might be responsible for this effect. First, the extension of the peptide backbone at the "left-hand" side, which, in addition, may lead to a suitable location of the N-terminal charge with respect to a counterion in the enzyme. Second, the increase in hydrophobicity of the peptide by the addition of the leucyl side chain.

From earlier work [7] it follows that the isoleucine-108 also strongly influences the rate of hydrolysis, since the peptide ester Leu-Ser-Phe-Met-Ala-OMe (thus containing Leu(103) but missing Ile(108)) showed equally poor substrate properties as the peptide ester Ser-Phe-Met-Ala-Ile-OMe (I) listed in Table I. Further evidence for an important role of the residue in position 108 was presented by Raymond et al. [12,13] in comparing the peptides Leu-Ser-Phe(NO_2)-Nle-Ala-OMe (k_{cat}/K_m = 0.1 mM^{-1} s^{-1}) and Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe (k_{cat}/K_m = 12.7 mM^{-1} s^{-1}) as substrates for chymosin.

The tetrapeptide esters Leu-Ser-Phe-Met-OMe, Ser-Phe-Met-Ala-OMe and Phe-Met-Ala-Ile-OMe were all found to be completely resistant to cleavage by chymosin [6].

From the above it is apparent that a minimum chain length of five amino acid residues including the sequence -Ser-Phe-Met-Ala- is essential to bring about any cleavage of the Phe-Met bond. A large jump in the substrate quality is effected when both the Leu(103) and the Ile(108) form part of the peptide chain, as is reflected by the marked differences in k_{cat}/K_m between the substrate series I—IV and series V—VIII. The contribution to the rate of proteolysis of substrate groups at some distance from the bond to be split by the enzyme has also been reported for the hydrolysis of peptides by other proteases such as pepsin [26,27], and chymosin-like enzymes from *Mucor miehei* [28] or from *Mucor pusillus* [29].

As is evident from Table I, additional sites of secondary interaction can be located on amino acid side chains more distant from the labile Phe-Met bond. These substrate groups, however, contribute to a much lesser extent to the substrate quality of the peptide than do the leucine-103 and isoleucine-108 discussed above. The kinetic parameters of the substrates V—XIII (except the oxidized peptide XI) all fall within the same range: k_{cat} 16—43 s^{-1} and K_m 0.3—0.9 mM. The most suitable substrates found in the present study were the octapeptide esters VII and XIII ($k_{cat}/K_m \approx 100\ s^{-1}\ mM^{-1}$). These substrates contain, in addition to the important Leu(103) and Ile(108), one or two prolyl residues which evidently further add to the secondary enzyme-substrate interaction. The prolyl residues might also impart some stabilization to a preferential conformation of the substrate molecule.

A discussion about the role of the histidyl and lysyl side chains is hampered by the fact that all our experiments were carried out at the same pH and ionic strength. The pH of 4.7 has been chosen as being the optimum pH for the action of chymosin on small peptide substrates [12]. It cannot be ruled out that the substitution of an additional charged group in a peptide substrate will have a considerable effect on the pH optimum for enzymic cleavage. The conclusion that the introduction of the lysyl residues at the positions 111 and 112 would not greatly affect the substrate quality (cf. Table I) must therefore be considered with care. In addition to this, a second aspect has to be taken into account when considering the function of the side chain of histidine-102. One can conclude from Table I that under the conditions of our studies, coupling of a histidyl residue to the peptide ester V, leading to substrate XII, only influences the K_m of the reaction to a reasonable extent leaving the k_{cat} almost unchanged. This does not imply, however, that in a more extended molecule (e.g. in intact κ-casein) the positive charge of the histidine side chain will not have any effect on the k_{cat} of the enzymic cleavage. In the present case of small peptide esters the function of the protonated imidazole group may have been taken over completely or partly in the hexapeptide ester V by the positively charged N-terminal residue. Definite conclusions as to the function of the histidyl and lysyl side chains in the enzyme-substrate interactions have to await the results of further experimental work.

A marked influence on the kinetic parameters was observed when the methionyl residue of peptide IX had been oxidized to its sulphoxide (XI). This treat-

ment apparently affected the primary interactions between the enzyme and its substrate.

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