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PEPTIDE SUBSTRATES FOR CHYMOSIN (RENNIN)

KINETIC STUDIES WITH BOVINE κ-CASEIN-(103—108)-HEXAPEPTIDE ANALOGUES

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Summary

Kinetic parameters have been determined for the reaction between chymosin (EC 3.4.23.4) and synthetic peptide analogues of the sequence Leu-Ser-Phe-Met-Ala-Ile around the chymosin-sensitive Phe(105)-Met(106) bond of bovine κ-casein. From the present and earlier results it is concluded that a minimum length of the molecular backbone with three amino acid units on both sides of the scissile bond is required to make the peptide a good substrate for the enzyme. In addition, hydrophobic side chains in the positions 103 and 108, and particularly the hydroxyl group of Ser-104 contribute to the effectiveness of the enzyme-substrate interactions. The substrate properties are markedly influenced by changes in the steric and/or polar character of the amino acid side chains in the positions 105 and 106.

Introduction

In a previous paper [1] we described the kinetics of the action of the milk-clotting enzyme chymosin (or rennin) (EC 3.4.23.4) on peptide substrates of different chain lengths including parts of the sequence:

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

around the enzyme-sensitive Phe(105)-Met(106) bond of bovine κ-casein. It was found that both the pentapeptide esters Leu-Ser-Phe-Met-Ala-OMe and Ser-Phe-Met-Ala-Ile-OMe are poor substrates ($k_{cat}/K_m < 0.1\ s^{-1} \cdot mM^{-1}$) as compared with the hexapeptide ester Leu-Ser-Phe-Met-Ala-Ile-OMe (k_{cat}/K_m 22

Abbreviations used: Cha: β(cyclohexyl)-L-alanyl; Phe(NO_2): *p*-nitro-L-phenylalanyl.

$s^{-1} \cdot mM^{-1}$). The substrate properties of the hexapeptide His-Leu-Ser-Phe-Met-Ala-OMe ($k_{cat}/K_m \approx 0.15\ s^{-1} \cdot mM^{-1}$, unpublished result) and of hexa- to octapeptides having Ser-104 at the N-terminus ($k_{cat}/K_m \leqslant 0.2\ s^{-1} \cdot mM^{-1}$, cf. ref. 1) are of the same order as those of the above pentapeptides. Moreover, di-, tri- and tetrapeptide esters containing the natural 105-106 sequence of κ-casein, and also the heptapeptide with Phe(105)-Met(106)- at the N-terminus, are completely resistant to cleavage by chymosin [2]. From these data it follows that the presence of at least three residues on both sides of the scissile bond is necessary to obtain good substrate properties. This condition, emphasizing the importance of "secondary" interactions, is reminiscent of that obtained from studies on other proteinases [3—9].

In the present study kinetic experiments were carried out with bovine κ-casein-(103—108)-hexapeptide analogues. This allowed us to establish the contribution to the enzymic action of some individual amino acid side chains located in the region around the chymosin-sensitive bond of κ-casein.

Materials and Methods

The synthesis of the peptide ester Leu-Ser-Phe-Met-Ala-Ile-OMe was described previously [10]. The peptides Leu-Ser-Phe-Met(O)-Ala-Ile-OMe and Leu-Ser-Cha-Nle-Ala-Ile-OMe were obtained by modification of Met and Phe in the corresponding peptides, by limited oxidation (11) and by prolonged catalytic reduction under pressure with H_2 and excess of Pd/C *, respectively. The hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe and the octapeptide Pro-His-Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe were gifts from M.N. Raymond and E. Bricas (Université Paris-Sud, Orsay, France). A commercial sample of the last-mentioned hexapeptide ("Cyclo Chemical") was provided to us by P. Martin (INRA, Jouy-en-Josas, France). Details of the synthesis of the other peptide-substrates will be published elsewhere (Schattenkerk, C., to be published).

The check on the specificity of enzymic cleavage, the preparation of enzyme and substrate solutions, the kinetic measurements, and the evaluation of kinetic parameters were carried out as described previously [1]. A correction was made for the extent of initial hydrolysis [1] since for the various peptide substrates studied different ranges of substrate depletion (not exceeding 20 %) were found within the period of measurement.

Results and Discussion

During the initial rate measurement the enzymic cleavage of each substrate studied was restricted to the peptide bond between the residues in the positions

* Actually, reduction of the aromatic nucleus of Phe under these conditions had been observed earlier [12] as a side reaction during the reductive splitting of protective groups from synthetic peptide derivatives. In the present case about 60% conversion to the cyclohexyl analogue could be obtained. In a "blank" experiment with corresponding concentrations of enzyme and non-modified peptide alone, it was found that complete splitting of the latter under these conditions had taken place within 2 min of incubation. As this is before the time at which actual measurement of the absorbance increase is started [13], the initial rate measurement of the modified peptide, which is a relatively poor substrate, was not influenced by the presence of the remaining non-modified one.

105 and 106. For a few peptides, in particular substrate X in Table I, some cleavage between the residues 104 and 105 could be observed, but only after extended incubation with increased amounts of enzyme.

In Table I the kinetic parameters k_{cat}, K_m and k_{cat}/K_m are presented for a number of κ-casein-(103—108)-hexapeptide analogues.

Replacement of Met-106 in the parent sequence (I) by the isosteric Nle (IV) leads to a threefold improvement of the substrate properties (as expressed by the proteolytic ratio k_{cat}/K_m) mainly as a consequence of a lower K_m value. This may be due to an enhanced binding capacity (i.e. if K_m approaches K_s, the enzyme-substrate dissociation constant). On the other hand, substitution of Leu for Met-106 (III) lowers the substrate quality despite the somewhat lower K_m obtained. Apparently, the introduction in position 106 of a residue with a branched side chain retards the action of chymosin. This might explain why the proteolytic action of chymosin on human κ-casein, which contains a Phe-Ile instead of a Phe-Met unit in the same position [18], is much slower than that on bovine κ-casein under identical conditions (unpublished result). The different substrate behaviour may also be caused by a difference in the carbohydrate content of both proteins [19]. Oxidation of Met-106 to the corresponding sulphoxide (II) also decreases the substrate quality which confirms our earlier observation with a synthetic decapeptide [1]. This decreased susceptibility to enzymatic attack can be ascribed to the altered steric and/or polar properties of the side chain in position 106. The result may be related to those of other investigators who have found that the action of chymosin on casein or skim milk is much reduced or even blocked by peroxide treatment [20] or photooxidation [21—23]. It is known that these reactions can oxidize the methionine side chain, although other residues are susceptible as well [24,25]. In bovine κ-casein the oxidation of Met-106 may be favoured by the fact that this residue is readily accessible to specific attack [23].

Modification of Phe-105 is also reflected by a change of the kinetic parameters of the enzymic reaction. The unfavourable effect of a non-aromatic/non-planar substituent in this position is evident from the k_{cat}/K_m ratio obtained with the cyclohexylalanine-containing analogue (V). Para-substitution of the phenyl nucleus by a nitro group (VI), which increases the polarity but does not affect the planarity of the ring system in the side chain, also decreases the substrate quality although to a much lesser extent than in the case of the Phe → Cha replacement.

The exchange of Ile-108 for Leu (VII) has no influence on the substrate properties. Earlier results [1] left the question unanswered whether the sharp decrease in substrate properties if the hexapeptide Leu-Ser-Phe-Met-Ala-Ile-OMe is shortened by one residue at either end, is to be ascribed to the absence of the hydrophobic Leu-103 or Ile-108 side chain, or to the decreased length of the molecular backbone. It is now clear, however, that both these factors are of influence with a principal contribution of the backbone length. This is corroborated by the finding that removal of one of the hydrophobic side chains through replacement by Gly (cf. IV and VIII or IX respectively) reduces the proteolytic ratio to about 10% of its original value while shortening of the peptide chain by one residue altogether reduces it to 0.2—0.3% [1].

The importance of the Ser-104 side chain is demonstrated by the fact that

TABLE I

Kinetic parameters of the reaction between chymosin and κ-casein 103—108 analogues.
All experiments were carried out at 30°C in a 0.05 M sodium acetate buffer (pH 4.7); kinetic constants are given together with standard errors as defined by Cleland [14].

No.	Substrate	Initial substrate concentration (mM)	Total * enzyme concentration (nM)	k_{cat} * (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	n **
I	Leu-Ser-Phe-Met-Ala-Ile-OMe	0.10—0.80	66	18.3 ± 0.9	0.85 ± 0.05	21.6 ± 0.7	15
II	Leu-Ser-Phe-Met(O)-Ala-Ile-OMe	0.10—0.78	262	7.0 ± 1.3 ***	3.5 ± 0.7 ***	2.0 ± 0.1	16
III	Leu-Ser-Phe-Leu-Ala-Ile-OMe	0.10—0.82	119	4.6 ± 0.2	0.53 ± 0.03	8.7 ± 0.4	13
IV	Leu-Ser-Phe-Nle-Ala-Ile-OMe ‡	0.08—0.71	31	24.7 ± 1.1	0.39 ± 0.03	63.2 ± 3.2	13
		0.11—0.83	30	25.0 ± 0.5	0.33 ± 0.02	75.2 ± 2.4	13
V	Leu-Ser-Cha-Nle-Ala-Ile-OMe	0.09—0.38	537	4.5 ± 2.6 ***	3.1 ± 1.9 ***	1.4 ± 0.1	10
VI	Leu-Ser-Phe(NO_2)-Nle-Ala-Ile-OMe	0.08—0.64	72	7.6 ± 0.5	0.32 ± 0.04	23.9 ± 2.1	15
VII	Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe ‡	0.10—0.79	60	8.7 ± 0.3	0.35 ± 0.02	25.2 ± 1.1	15
		0.08—0.53	72	15.8 ± 1.4	0.68 ± 0.07	23.3 ± 1.1	13
		0.07—0.34	72	11.6 ± 0.6	0.48 ± 0.03	24.0 ± 0.9	13 ‡‡
VIII	Leu-Ser-Phe-Nle-Ala-Gly-OMe	0.10—1.37	208	9.9 ± 1.0	1.4 ± 0.2	7.2 ± 0.5	12
IX	Gly-Ser-Phe-Nle-Ala-Ile-OMe	0.10—1.66	149	18.3 ± 2.0	1.7 ± 0.2	10.9 ± 0.8	12
X	Leu-Gly-Phe-Nle-Ala-Ile-OMe	0.21—1.72	879	1.8 ± 0.2	1.0 ± 0.1	1.8 ± 0.2	15

* Calculated assuming a molecular weight of 30 000 for the enzyme [15—17].
** Number of experimentally determined initial velocities over the concentration range given in the third column.
*** Values of the separate parameters, k_{cat} and K_m, are rather uncertain since K_m lies far beyond the concentration range used.
‡ Data presented from several duplicate experiments done with different batches of the synthetic substrate.
‡‡ Data obtained with a commercial product (Cyclo Chemical).

replacement of this residue by Gly (X) reduces the proteolytic ratio by more than 95%. Ser-104 is not involved directly in the cleavage of the susceptible peptide bond since the replacement does not completely abolish the substrate activity. The possibility exists that the hydroxyl group of the serine side chain participates in a specific hydrogen bond which in turn favours the interaction between the substrate and the enzyme. The finding [2,26] that the sequence Leu(103)-Ser(104)- makes the substrate a far better one than does the sequence Ser(103)-Leu(104)- is in line with the above. Our observation is in good agreement with earlier results of Raymond et al. [27,28] who found the substrate properties of a pentapeptide ester strongly diminished after replacement of Ser-104 by Ala.

It is worth mentioning that for the substrates VI and VII, both containing a *p*-nitrophenyl group in position 105, strong inhibition became obvious from the $1/v$ vs. $1/s$ plot at substrate concentrations exceeding 1 mM under the experimental conditions. This was not the case with the corresponding hexapeptide ester without the para-substituted Phe-105 (IV). Several other peptides (cf. Table I) were also studied at higher concentrations (up to 1.7 mM). The results did not give any indication of an inhibitory effect in the absence of a Phe(NO_2) group in position 105. In this respect we have also compared the octapeptide esters Pro-His-Leu-Ser-Phe-Met-Ala-Ile-OMe [1] and Pro-His-Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe [28]. Whereas for the former peptide ($k_{cat} \approx 35\ s^{-1}$; $K_m \approx 0.35$ mM; $k_{cat}/K_m \approx 100\ s^{-1} \cdot mM^{-1}$) no inhibition was found in the concentration range studied (0.1—0.8 mM), the latter substrate (k_{cat} 15—16 s^{-1}; K_m 0.16—0.18 mM; k_{cat}/K_m 90—93 $s^{-1} \cdot mM^{-1}$ as measured in our laboratory) showed strongly increasing inhibition at concentrations above 0.25 mM under identical experimental conditions. Since the substrate concentration at which inhibition becomes observable is shifted towards a higher level when the enzyme concentration is enhanced (Raymond, M.N., personal communication), substrate or product inhibition may be involved. The latter possibility was further investigated. To this end, increasing amounts of the potentially inhibiting product Leu-Ser-Phe(NO_2)-OH (and also of its methyl ester) were added to Leu-Ser-Phe-Nle-Ala-Ile-OMe. The kinetics of splitting by chymosin were not influenced, however, which rules out product inhibition.

Summarizing the results of our studies, we come to the following conclusions:

1. For peptide esters containing the sequence around the labile Phe(105)-Met(106) bond of κ-casein a minimum length of the molecular backbone is required to make them good substrates for chymosin. There exists a high preference for the presence of at least three residues on both sides of the scissile bond [1]. In this respect the substrate specificity of chymosin resembles that of several other proteinases (cf. for example ref. 8).

2. Sites of enzyme-substrate interaction are formed by hydrophobic side chains located at the positions 103 and 108.

3. The hydroxyl group of serine-104 is strongly involved, possibly via hydrogen bond formation, in the interacting enzyme-substrate system.

4. As reported before [1], prolyl residues more remote from the chymosin-sensitive bond are also of some influence on the stabilization of the enzyme-substrate complex.

5. The steric or polar properties of the side chains adjacent to the labile peptide bond are of crucial importance for a good fit on the enzyme's active centre.

A better understanding of the above findings can undoubtedly be obtained when the tertiary structure of the enzyme, and particularly the geometry of the active site herein, have been elucidated.

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