SPECIFICITY OF PEPSIN: SIZE AND PROPERTY OF THE ACTIVE SITE

T.OKA and K.MORIHARA
Shionogi Research Laboratory, Shionogi and Co., Ltd., Fukushima-ku, Osaka, Japan

Received 7 September 1970

1. Introduction

The method of measuring the size of an enzyme's active site by presenting it with substrates large enough to show up interactions with further-most parts of the site was first applied to papain by Schechter and Berger [1]. The active site there was found to cover 25 Å accomodating at least seven amino acid residues of a peptide substrate. The method was also applied to other proteolytic enzymes such as carboxypeptidase A [2], various neutral proteases from bacterial origin [3], and subtilisin BPN′ and α-chymotrypsin [4], and the results indicated that these enzymes possess a considerably large active site corresponding to five or more amino acid residues of a peptide substrate.

The present study was thus undertaken to investigate the size and property of the active site in pepsin by applying the above method: various oligopeptides Z-A-Phe↓Leu-Ala, Z-A-Gly-Phe↓Tyr, Z-Phe↓Leu-B and Z-Phe↓Leu-Ala-B (A or B = glycine and D- and L-alanine) were used as substrates. In view of the specificity of pepsin, these peptides should be split at the peptide bond shown by the arrow, and we could therefore examine the effects of different kinds of amino acid residue two or three amino acid residues distant on either side of the cleavage site, and thus determine whether pepsin has a large active site corresponding to these amino acid residues.

2. Materials and methods

Worthington pepsin (three times crystallized, salt-free) was used without further purification. The peptides Z-Phe-Leu, Z-Phe-Leu-NH_2, and Z-Phe-Tyr were obtained from the Institute for Protein Research at Osaka University. Z-A-Phe-Leu-Ala (A = glycine, and D- and L-alanine), Z-Phe-Leu-B (B = glycine, D- and L-alanine, and L-leucine) and Z-Phe-Leu-Ala-B (B = D- and L-alanine), were synthesized according to the method described in the previous paper [5]. Z-Gly-Phe-Tyr and Z-A-Gly-Phe-Tyr (A = glycine, and D- and L-alanine) were synthesized in this laboratory by usual procedure, as will be described in an another paper. Z-free peptides were also prepared as usual. Except when specified the constituent amino acids were all of the L-configuration.

The hydrolysis of peptide substrates was determined as follows: A reaction mixture (5 ml) containing 0.5 mM substrate, 2.5 mg of pepsin, 0.05 M citric acid, and 20% dimethylformamide (for low solubility of most of these peptides presented here), final pH 2.2, was incubated at 40°. At various intervals, 1 ml of the reaction mixture was withdrawn and put into a test tube containing 1 ml of 0.5 M citrate (pH 5), 0.6 ml of 5% ninhydrin solution (in methyl cellosolve) and 2.5 ml of 0.2 mM KCN solution (in methyl cellosolve), which had previously been cooled in ice-water. The extent of hydrolysis was measured by the usual ninhydrin method. The color yield by the ninhydrin method of the compounds such as Leu-Gly, Leu-Ala, Leu-D-Ala, Leu-Ala-Ala, and Leu-Ala-D-Ala was described in the previous paper [5]. Rate of hydrolysis was also determined as above, where the enzyme concentration was suitably adjusted in the reaction mixture.

The sites of action of the enzyme were determined by paper chromatography of the hydrolysates, or by the usual DNP-method.

North-Holland Publishing Company – Amsterdam

3. Results and discussion

Table 1 indicates the peptidase activity against Z-A-Phe-Leu-Ala, Z-A-Gly-Phe-Tyr and others, where the positions of amino acid residues (P) in each peptide are numbered at P_1, P_2, etc., respectively, for those toward the NH_2-end from the cleavage site, and as P'_1, P'_2, etc., respectively, for those toward the COOH-end. It indicates that pepsin shows stringent stereo- and sidechain-specificities at P_2 and P_3. The presence of a charged α-amino group at P_2 or P_3 also affects the hydrolysis markedly as does such a group at P_1. On the other hand, the rate of hydrolysis of peptide substrates is dramatically increased by elongation of the peptide chain towards the *N*-terminus, to P_2 and P_3, as seen in the correlation between Z-Phe↑Leu-Ala and Z-Gly-Phe↑Leu-Ala or Z-Phe↑Tyr and Z-Gly-Phe↑Tyr (P_1 to P_2), or between Z-Gly-Phe-Tyr and Z-Gly-Gly-Phe↑Tyr (P_2 to P_3)

Table 2 indicates the hydrolysis of Z-Phe-Leu-B and Z-Phe-Leu-Ala-B. It shows that pepsin shows some stereospecificity at P'_2 but little at P'_3. The study of sidechains-specificity at P'_2 using Z-Phe-Leu-B as substrates was difficult, because the peptide bond of Leu-B also became susceptible depending upon the nature of B, as seen in Z-Phe-Leu-Leu. The increased ninhydrin color value by hydrolysis of Z-Phe-Leu-Leu under the conditions described in table 2, however, was not higher than that of Z-Phe↑Leu↑Gly. The rate of hydrolysis of Z-Phe-Leu-Ala described in table 2 must be somewaht higher than the true one, because the calculation was made ignoring the hydrolysis at Leu-Ala. These results would indicate that the sidechains-specificity at P'_2 is small.

In contrast to the small stereo- or sidechain-specificity at P'_2 and P'_3, a remarkable increase of hydrolysis is observed by elongation of the peptide chain towards the *C*-terminus, to P'_2 or P'_3, as seen in the correlations among Z-Phe↑Leu, Z-Phe↑Leu-Ala and Z-Phe-Leu-Ala-Ala. The enzymatic hydrolyses of the present study were carried out at pH 2.2, thus making the terminal carboxyl group of peptide substrates unionizable. The small rate of hydrolysis in Z-Phe↑Leu-NH_2 is not inconsistent with these results. Therefore, the increased

Table 1
Effects of neighboring amino acid residues at *N*-terminal side of the cleavage site in peptide substrates.

Peptide $P_4-P_3-P_2-P_1\downarrow P'_1-P'_2$	Rate of hydrolysis (nmoles/min/mg enzyme)	% of hydrolysis (hr)		
		0.5	3	22
Z–Phe–Leu–Ala*	0.4	< 5	16	49
H–Phe–Leu–Ala				< 5
Z–Gly–Phe–Leu–Ala*	3.3	12	53	80
Z–Ala–Phe–Leu–Ala*	34	74	100	100
H–Ala–Phe–Leu–Ala				< 5
Z–D-Ala–Phe–Leu–Ala*	0.2	5	18	53
Z–Phe–Tyr			5	10
Z–Gly–Phe–Tyr	0.6	< 5	17	50
Z–Gly–Gly–Phe–Tyr**	22	32	74	80
Z–Ala–Gly–Phe–Tyr**	182	72	73	76
H–Ala–Gly–Phe–Tyr				< 5
Z–D-Ala–Gly–Phe–Tyr	0.5		6	31

Reaction mixture: [S] = 0.5 mM, [E] = 0.5 mg/ml, 0.05 M citric acid, 20% dimethylformamide, final pH 2.2. Reaction temperature, 40°. Rate of hydrolysis was determined by varying the enzyme concentration in the reaction mixture to measure the initial velocity, and the other conditions were as above. The positions of amino acid residues (P) in each peptide are numbered as P_1, P_2, etc., respectively, for those toward the NH_2-end from the cleavage site, and as P'_1, P'_2, etc., respectively, for those toward the COOH-end. The arrow shows the bond split.

* The other peptide bond (Leu-Ala) was also susceptible, but the hydrolysis was much less rapid than that of the main cleavage site (Phe-Leu) and so neglected for the calculation.

** The hydrolysis did not exceed over 80%, which might indicate that the peptide synthesized in our laboratory was partly racemized.

Table 2
Effects of neighboring amino acid residues at C-terminal side of the cleavage site in peptide substrates. The conditions were described in table 1.

Peptide $P_2-P_1\downarrow P'_1-P'_2-P'_3$	Rate of hydrolysis (nmoles/min/mg enzyme)	% of hydrolysis (hr)		
		0.5	3	22
Z–Phe–Leu				< 5
Z–Phe–Leu–NH_2				< 5
Z–Phe–Leu–Gly		< 5	8	18
Z–Phe–Leu–Ala	0.4	< 5	16	49
Z–Phe–Leu–D-Ala				< 5
Z–Phe–Leu–Ala–Ala	26.5	53	100	100
Z–Phe–Leu–Ala–D-Ala	13.5	30	82	97

susceptibility of peptides by elongation of the peptide chain towards the C-terminus cannot be related to blocking of the α-carboxyl group present at P'_1 or P'_2.

The present study indicated that pepsin has an active site large enough to correspond to at least six amino acid residues P_1–P_3 and P'_1–P'_3 in peptide substrates. The size of the active site in pepsin is comparable with those in the other proteolytic enzymes so far investigated [1–4]. With regards to the properties of this large active site, pepsin seemed to resemble subtilisin BPN′ [6] rather than the other proteolytic enzymes; i.e. (1) the enzyme shows a stringent stereo- or sidechain-specificity against amino acid residues, such as P_2 and P_3, present at the N-terminal side from the cleavage site in peptide substrates, while it shows little specificity for those at the C-terminal side, P'_2 and P'_3; (2) the enzymatic reaction is remarkably promoted when the peptide chain of the substrate is elongated from P_1 to P_2 or P_3 at the N-terminal side from the cleavage site; a similar promoting effect was also observed on elongation to the C-terminus, a situation not observed with subtilisin BPN′.

Observation (2) has already been reported by Fruton and his coworkers [7, 8]; they reported that the k_{cat} values of pepsin for substrates of the type A-Phe-Phe-B or A-Phe$(NO)_2$)-Phe-B were remarkably increased when the A group was changed from Z to Z-Gly-Gly and the B group was unchanged, or when the B group was changed from OMe to Ala-Ala-OMe or Ala-Phe-OMe and the A group was unchanged, respectively, while the K_m values were little changed. The rate enhancement was attributed to secondary enzyme-substrate interactions involving groups in the A or B portion of the substrate. Our present study coincides with this view. Nevertheless, the catalytic role of the large active site in pepsin still remains to be resolved.

It is well-known that pepsin shows a broad specificity against a large molecular peptide such as oxidized insulin B chain [9], while the enzyme shows a considerably strict specificity against dipeptide substrates [10]. The difference might partly be ascribed to whether or not the whole active site of the enzyme participates in the hydrolytic reaction.

References

[1] I.Schechter and A.Berger, Biochem. Biophys. Res. Commun. 27 (1967) 157.
[2] N.Abramowitz, I.Schechter and A.Berger, Biochem. Biophys. Res. Commun. 29 (1967) 862.
[3] K.Morihara and T.Oka, Biochem. Biophys. Res. Commun. 30 (1968) 625.
[4] K.Morihara, T.Oka and H.Tsuzuki, Biochem. Biophys. Res. Commun. 35 (1969) 210.
[5] K.Morihara, T.Oka and H.Tsuzuki, Arch. Biochem. Biophys. 135 (1969) 311.
[6] K.Morihara, T.Oka and H.Tsuzuki, Arch. Biochem. Biophys. 138 (1970) 515.
[7] G.P.Sachdev and J.S.Fruton, Biochemistry 8 (1969) 4231.
[8] K.Medzihradszky, I.M.Voynick, H.Medzihradszky-Schweiger and J.S.Fruton, Biochemistry 9 (1970) 1154.
[9] F.Sanger and H.Tuppy, Biochem. J. 49 (1951) 481.
[10] G.E.Trout and J.S.Fruton, Biochemistry 8 (1969) 4183.