EFFECT OF SECONDARY INTERACTION ON THE ENZYMATIC ACTIVITY OF SUBTILISIN BPN′: COMPARISON WITH α-CHYMOTRYPSIN, TRYPSIN, AND ELASTASE

Kazuyuki MORIHARA and Tatsushi OKA
Shionogi Research Laboratory, Shionogi and Co., Ltd., Fukushima-ku, Osaka, 553 Japan

Received 29 March 1973

1. Introduction

It is well-known that the enzymatic activity of certain proteinases is affected by amino acid residues distant from the catalytic point in a peptide substrate. The effect may be called "secondary interaction", as has been proposed by Fruton [1]. In γ-chymotrypsin and subtilisin BPN′, the subsites which have been shown by X-ray studies to be involved in the binding of tripeptide chloromethyl ketone, Ser(214)–Trp(215)–Gly(216) of the backbone chain of the former enzyme [2] and Ser(125)–Leu(126)–Gly(127) in the latter [3], may possibly be related with their secondary interaction. This may be assumed to be true for the other serine proteinases, trypsin [4] and elastase.

The effect of secondary interaction on the enzymatic activity of subtilisin BPN′ has previously been determined [5, 6] using $Z–X–(Gly)_m–Tyr\downarrow NH_2$ or $Z–X–(Gly)_m–Leu\downarrow NH_2$ (X = various amino acid residues; m = 0, 1, and 2; the arrows show the bonds split) as substrates. The result indicated that hydrolysis is markedly increased by an increase in the peptide chain length from the catalytic point to the N-terminus in the substrate, where the specific amino acid residues L-tyrosine or L-leucine occupied the position at the carbonyl-side of the splitting point. A question then arises whether the effect of secondary interaction on the hydrolysis of peptide substrates is the same irrespective of the kind of amino acid residue at the carbonyl-side of the splitting point.

The activity of the four serine proteinases, subtilisin BPN′, chymotrypsin, trypsin, and elastase is due to an identical catalytic apparatus ("charged relay system" with Asp–His–Ser); and though their specificities are considerably different from each other, it is considered that they possess similar binding subsites as mentioned above. Another question then arises whether these four enzymes have similar secondary interaction effects on the hydrolysis of peptide substrates.

To clarify the above questions, a kinetic study was carried out with the four serine proteinases using $Ac–(Ala)_m–Phe–OMe$, $Z–(Ala)_m–Lys–OMe$, and $Ac–(Ala)_m–Ala–OMe$ (m = 0, 1, and 2) (designated as series A, B, and C) as substrates. Since these enzymes showed esterase activity on all of the peptides in the respective series, we could examine the effect of elongation of the peptide chain on the N-terminal side from the cleavage point with each enzyme. The results, as shown in this paper, indicate that the effect of secondary interaction on hydrolysis of subtilisin BPN′ is more marked the less specific the amino acid residue occupying the position at the carbonyl-side of the splitting point in the substrates. A comparative study of the four serine enzymes indicates that the effect of secondary interaction is smaller with enzymes which show more stringent specificity, and vice versa.

2. Materials and methods

Subtilisin BPN′ (Nagarse, crystals) was supplied by Nagase Co., Ltd., Osaka. Crystalline bovine α-chymotrypsin (3 times recrystallized) and crystalline bovine trypsin (twice recrystallized) were obtained from Worthington Biochemical Corporation, New Jersey. Crystalline pancreatic elastase was obtained from Mann Research Laboratory, New York. These enzyme preparations were used without further purification. The

North-Holland Publishisng Company – Amsterdam

ester substrates, Ac–$(Ala)_m$–Phe–OMe, Z–$(Ala)_m$–Lys–OMe, Ac–$(Ala)_m$–Ala–OMe (m = 0, 1, and 2) were synthesized in the usual manner in this laboratory; details of the syntheses will be reported in the near future.

Esterase activity was determined with the aid of a Radiometer type TTT1 pH-stat equipped with a syringe burette, a type SBR2c recorder, and a thermostatically controlled reaction vessel (30°C). The reaction was carried out in 0.1 M KCl at pH 7.5, with 0.05 N NaOH as titrant. In all cases, the data were accorded with Michaelis–Menten kinetics over the range of substrate concentration (0.02–20 mM) employed; 5–8 runs were performed for each determination of K_m and k_{cat} (V_m per molar equivalent of enzyme); the enzyme concentration was chosen to give reliable data for the initial rate of hydrolysis. In the calculation k_{cat}, the molecular weights of subtilisin BPN', α-chymotrypsin, trypsin, and elastase were assumed to be 27,600 [7], 25,000 [8], 24,000 [9], and 25,000 [10], respectively.

3. Results and discussion

The results are summarized in table 1. For convenience, the amino acid residues in the peptide substrates are numbered P_1, P_2, etc. to the N-terminus from the cleavage point. It is seen that the rate of hydrolysis by subtilisin BPN' is greatly increased by elongation of the peptide chain with L-alanine from P_1 to P_2 and from P_2 to P_3 in all the peptide series tested: Elongation of the peptide chain from P_1 to P_3 results in a one hundred-fold decrease in the K_m value

Table 1
Kinetics for hydrolysis of various ester substrates by subtilisin BPN', α-chymotrypsin, trypsin, and elastase.

(A) Ac–$(Ala)_m$–Phe–OMe (↑)

Substrate	Subtilisin BPN'		α-Chymotrypsin	
	K_m (mM)	k_{cat}/K_m ($sec^{-1} \cdot mM^{-1}$)	K_m (mM)	k_{cat}/K_m ($sec^{-1} \cdot mM^{-1}$)
Ac–Phe–OMe	28	3.6 (1)	0.57	55 (1)
Ac–Ala–Phe–OMe	1.1	230 (64)	0.05	330 (6)
Ac–$(Ala)_2$–Phe–OMe	0.32	930 (260)	0.03	2000 (36)

(B) Z–$(Ala)_m$–Lys–OMe (↑)

Substrate	Subtilisin BPN'		Trypsin	
	K_m (mM)	k_{cat}/K_m ($sec^{-1} \cdot mM^{-1}$)	K_m (mM)	k_{cat}/K_m ($sec^{-1} \cdot mM^{-1}$)
Z–Lys–OMe	20	2.3 (1)	0.2	470 (1)
Z–Ala–Lys–OMe	0.58	440 (190)	0.04	1700 (3.6)
Z–$(Ala)_2$–Lys–OMe	0.16	2800 (1200)	0.07	1400 (3)

(C) Ac–$(Ala)_m$–Ala–OMe (↑)

Substrate	Subtilisin BPN'		Elastase	
	K_m (mM)	k_{cat}/K_m ($sec^{-1} \cdot mM^{-1}$)	K_m (mM)	k_{cat}/K_m ($sec^{-1} \cdot mM^{-1}$)
Ac–Ala–OMe	36	0.25 (1)	76	0.11 (1)
Ac–$(Ala)_2$–OMe	5.8	53 (210)	9.2	0.48 (4.4)
Ac–$(Ala)_3$–OMe	0.45	570[a] (2300)	0.28	60[b] (550)

[a] 700 (K_m = 1.7 mM) [11]. [b] 170 (K_m = 0.43 mM) [12].

The methods were described in Methods. The arrows show bonds split. Relative activities are shown in parentheses.

independently of the kind of amino acid residue at P_1. The effect of elongation on the k_{cat} value, however, varies considerably depending upon the kind of amino acid residue at P_1; the increase in k_{cat} is about three-fold when P_1 is occupied by L-phenylalanine (A), about ten-fold when P_1 is L-lysine (B), and about thirty-fold when it is L-alanine (C).

The K_m and k_{cat} (sec^{-1}) values for hydrolysis of Ac–Lys–OMe by subtilisin BPN′, determined under the same experimental conditions as described in table 1, were 90 mM and 47, respectively, indicating that the proteolytic coefficient is about-seventh that of Ac–Phe–OMe. Therefore, the specificity of subtilisin BPN′ against these amino acid residues decreases in the order: L-phenylalanine > L-lysine > L-alanine. Accordingly, it can be said that the more enzyme-specific the amino acid residue at P_1, the less is the increase in k_{cat} value on elongation of the peptide chain; whereas the corresponding decrease in K_m value is independent of the kind of amino acid residue at P_1.

With α-chymotrypsin, elongation of the peptide chain from P_1 to P_2 or to P_3 in the peptide series (A) causes a considerable increase in hydrolysis, mainly relating to the change in K_m value. Similar results have previously been observed by Segal [13]. However, the increase in hydrolysis is considerably smaller than that observed with subtilisin BPN′ for the same peptide series. With series (B), the rate of hydrolysis by trypsin is promoted only two or three-fold by elongation of the peptide chain from P_1 to P_2 or P_3, mainly owing to the decrease in K_m value. On the other hand, the rate of hydrolysis by elastase in series (C) is markedly increased on elongation to P_3, similarly as in hydrolysis by subtilisin BPN′. The high esterase activity against Ac–$(Ala)_3$–OMe of either subtilisin or elastase has previously been observed by Gertler [11] or Gertler and Hofmann [12].

The present study would therefore indicate that the effect of secondary interaction on hydrolysis is less with enzymes which show more stringent specificity, the action of the enzymes being affected in the following decreasing order: subtilisin BPN′, elastase > α-chymotrypsin > trypsin. The effect of secondary interaction on hydrolysis by subtilisin BPN′ is more marked the less specific the amino acid residue occupying the P_1 position in the substrate. It might therefore be said that the effect of secondary interaction on hydrolysis by serine proteinases is greater or lesser, depending on whether the side-chain of the residue at P_1 in a peptide substrate is fixed loosely or tightly into the "tosyl hole" or "hydrophobic pocket" [14] in each of the enzyme molecules.

References

[1] J.S. Fruton, Adv. Enzymol. 22 (1970) 401.
[2] D.M. Segal, J.C. Powers, G.H. Cohen, D.R. Davies and P.E. Wilcox, Biochemistry 10 (1971) 3728.
[3] J.D. Robertus, R.A. Alden, J.J. Birktoft, J. Kraut, J.C. Powers and P.E. Wilcox, Biochemistry 11 (1972) 2439.
[4] D.M. Blow, C.S. Wright, D. Kukla, A. Rühlmann, W. Steigemann and R. Huber, J. Mol. Biol. 69 (1972) 137.
[5] K. Morihara, T. Oka and H. Tsuzuki, Biochem. Biophys. Res. Commun. 35 (1969) 210.
[6] K. Morihara, T. Oka and H. Tsuzuki, Arch. Biochem. Biophys. 138 (1970) 515.
[7] H. Matsubara, C.B. Kasper, D.M. Brown and E.L. Smith, J. Biol. Chem. 240 (1965) 1125.
[8] P.E. Wilcox, J. Kraut, R.D. Wade and H. Neurath, Biochim. Biophys. Acta 24 (1957) 72.
[9] K.A. Walsh, in: Methods in Enzymology, Vol. XIX, eds. G.E. Perlmann and L. Lorand (Academic Press, New York, 1970) p. 41.
[10] U.J. Lewis, D.E. Williams and N.J. Brink, J. Biol. Chem. 222 (1956) 705.
[11] A. Gertler, European J. Biochem. 23 (1971) 36.
[12] A. Gertler and T. Hofmann, Can. J. Biochem. 48 (1970) 384.
[13] D.M. Segal, Biochemistry 11 (1972) 349.
[14] T.A. Steitz, R. Henderson and D.M. Blow, J. Mol. Biol. 46 (1969) 337.