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Kinetics of Hydrolysis of a New Peptide Substrate Containing *p*-Guanidino-L-phenylalanine by Trypsin and Thrombin

HIDEAKI TSUNEMATSU,^{*,a} KOICHI MIZUSAKI,^b YOSHIHIRO HATANAKA,^b
MASAO KAMAHORI^b and SATORU MAKISUMI^b

*Faculty of Pharmaceutical Sciences, Fukuoka University,^a Jonan-ku, Fukuoka 814-01, Japan
and Department of Chemistry, Faculty of Science, Kyushu University,^b
Higashi-ku, Fukuoka 812, Japan*

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A new peptide substrate containing *p*-guanidino-L-phenylalanine, *N*^α-benzoyl-L-phenylalanyl-L-prolyl-*p*-guanidino-L-phenylalanine *p*-nitroanilide (Bz-Phe-Pro-GPA-*p*NA), was synthesized, and the rates of hydrolyses of this substrate by bovine trypsin and thrombin were compared with those of the corresponding arginine peptide substrate (Bz-Phe-Pro-Arg-*p*NA). The specificity constants (k_{cat}/K_m) for the hydrolysis of GPA-peptide by the two enzymes were much smaller than those for Arg-peptide. Remarkably low k_{cat} values were found in the hydrolyses of GPA-peptide by the two enzymes compared with the values in those of Arg-peptide. The effect of the peptide chain elongation was observed in the hydrolysis of GPA-peptide by thrombin, while it was not in the case of trypsin, suggesting that the subsite of trypsin is very different from that of thrombin. GPA-peptide was ascertained to be a useful peptide substrate to study the subsite specificities of trypsin-like enzymes.

Keywords—*p*-guanidinophenylalanine; peptide substrate; trypsin; thrombin; hydrolysis kinetics

Introduction

We have recently reported that the amide and anilide substrates of *N*^α-substituted *p*-guanidino-L-phenylalanine (GPA), in which the alkyl methylenes in the arginyl residue are replaced by a benzene ring, are good substrates for bovine and porcine trypsins, and showed that GPA substrates are useful for the study of the specificity of trypsins as well as arginine (Arg) substrates.^{1,2)} Synthetic peptide substrates are a valuable tool for determining enzyme specificity. Tripeptide substrates containing Arg at the P₁ position have been widely used to study the secondary interaction between peptide substrates and the subsites of trypsin-like enzymes.

In the hope of obtaining better peptide substrates than Arg peptide ones for trypsin and thrombin, we synthesized a tripeptide substrate containing GPA at the P₁ position, *N*^α-benzoyl-L-phenylalanyl-L-prolyl-*p*-guanidino-L-phenylalanine *p*-nitroanilide (Bz-Phe-Pro-GPA-*p*NA) and compared the rates of the hydrolysis of this peptide substrate by bovine trypsin and thrombin with those of the corresponding Arg peptide substrate, *N*^α-benzoyl-L-phenylalanyl-L-prolyl-L-arginine *p*-nitroanilide (Bz-Phe-Pro-Arg-*p*NA). In this paper, we report the syntheses of Bz-Phe-Pro-GPA-*p*NA and Bz-Phe-Pro-Arg-*p*NA as well as the kinetics of the interactions of these peptide substrates with bovine trypsin and thrombin.

Experimental

N^α-Benzoyl-L-arginine *p*-nitroanilide hydrochloride (Bz-Arg-*p*NA · HCl) was purchased from Protein Research Foundation. *N*^α-Benzoyl-*p*-guanidino-L-phenylalanine *p*-nitroanilide hydrobromide (Bz-GPA-*p*NA · HBr) and

GPA-pNA·2HBr were prepared as described previously.^{1,2)} Bz-Phe-Pro-OH was synthesized by the usual method using dicyclohexylcarbodiimide. All other chemicals were of analytical or reagent grade. Optical rotations were measured on a Union Giken high-sensitivity polarimeter, type PM-71. All melting points are uncorrected. The purity of each compound was checked by thin layer chromatography (TLC) on silica-gel plates (Kiesel-gel 60 PF₂₅₄, Merck, Darmstadt) with *n*-BuOH-AcOH-H₂O (4:1:1, v/v), CHCl₃-MeOH (5:1, v/v) and CHCl₃-MeOH-ethyl acetate (4:3:1, v/v) as developing systems. The structures of the synthesized peptides were confirmed with the aid of fast atom bombardment-mass spectrometry (FAB-MS) using a JEOL JMS-DX 300/JMA 3500 Data Analysis System.

Bovine trypsin (lyophilized, salt-free; Lot 1081346) was obtained from Boehringer Mannheim-Yamanouchi Co. and was 57% active, as described previously.¹⁾ Bovine thrombin (lyophilized powder, 500 NIH units/vial, Lot SA 400) was purchased from Mochida Pharmaceutical Co., and the enzyme concentration of this enzyme was determined by using $E_{1\text{cm}}^{1\%}(280\text{nm}) = 19.5^3$ and a molecular weight of 36600.

Synthesis of Bz-Phe-Pro-GPA-pNA·HBr—Bz-Phe-Pro-OH (150 mg, 0.41 mmol) and GPA-pNA·2HBr (207 mg, 0.41 mmol) were suspended in 1 ml of dimethylformamide. Triethylamine (0.058 ml, 0.041 mmol) and 1-hydroxybenzotriazole (1-HOBt) (0.13 g, 0.82 mmol) were added to the mixture, which was kept at -5°C , then dicyclohexylcarbodiimide (84.7 mg, 0.41 mmol) was added. The reaction mixture was stirred at -5°C for 3 h and then for a further 20 h at room temperature. A drop of glacial acetic acid was added before filtering off the dicyclohexylurea. On the addition of diethyl ether to the filtrate, an oily material separated out. When it was treated with a saturated aqueous solution of sodium bicarbonate to remove unreacted 1-HOBt and water-soluble impurities, it changed to a powder, which was collected by filtration to give the crude product (244 mg). This was applied to a column (2.8 × 120 cm) of Sephadex LH-20, and the column was eluted with MeOH (flow rate, 18 ml/h; fraction volume, 5 ml). Fractions of eluate containing the desired material (as judged by paper chromatography with nitroprusside-ferricyanide reagent) were combined and concentrated to a small volume, and the product was crystallized by addition of ether. The crystalline product was washed with ether and dried over P₂O₅. Yield, 158 mg (50%), mp 149–150°C (dec.), $[\alpha]_D^{20} = -39.8^\circ$ ($c=1$, MeOH). *Anal.* Calcd for C₃₇H₃₈N₈O₆·HBr·H₂O: C, 56.27; H, 5.24; N, 14.19. Found: C, 56.55; H, 5.11; N, 14.44. The structure of this compound was ascertained by FAB-MS. m/z : 691 [M-Br]⁺.

Synthesis of Bz-Phe-Pro-Arg-pNA·HBr—This compound was prepared by the same procedure as described for Bz-Phe-Pro-GPA-pNA. Yield, 180 mg (55%), mp 140–141°C, $[\alpha]_D^{20} = -78.8^\circ$ ($c=1$, MeOH). *Anal.* Calcd for C₃₃H₄₀N₈O₆·HBr·H₂O: C, 53.39; H, 5.71; N, 15.10. Found: C, 53.60; H, 5.58; N, 15.31. FAB-MS m/z : 644 [M-Br]⁺.

Kinetic Studies—The rates of hydrolysis of the peptide substrates were followed in terms of the increase in absorbance at 410 nm at 25°C at pH 8.2⁴⁾ with a Shimadzu UV-200 spectrophotometer equipped with a thermostated cell compartment and U-125 MU recorder. The buffer solution used was 0.05 M Tris-HCl buffer containing 0.02 M CaCl₂ for bovine trypsin and the same buffer containing 0.3 M NaCl for bovine thrombin. The concentrations of trypsin in the assay mixture were 6.20×10^{-7} M for Bz-Phe-Pro-GPA-pNA and 6.33×10^{-9} M for Bz-Phe-Pro-Arg-pNA, and those of thrombin were 8.00×10^{-8} M for Bz-Phe-Pro-GPA-pNA and 3.69×10^{-9} M for Bz-Phe-Pro-Arg-pNA, respectively. The values of K_m and k_{cat} were calculated from a plot of $[E]_0/v$ vs. $1/[S]$ at 8–9 different substrate concentrations using the least-squares method.⁵⁾

Results and Discussion

Before determining the kinetic constants, the pH-activity relationship for the hydrolysis of the peptide anilide substrate of GPA by trypsin was examined. A plot of the initial velocity at a fixed substrate concentration (1×10^{-4} M) against pH gave a bell-shaped curve with a maximum at around 8.2; the pattern of the curve is quite similar to that for the tryptic hydrolysis of Arg peptide substrate. A similar relationship was obtained for the hydrolyses of the peptide substrates of GPA and Arg by thrombin. The actions of both trypsin and thrombin on the peptide substrates followed Michaelis-Menten kinetics in the range of substrate concentration below 2×10^{-4} M. The kinetic parameters for bovine trypsin and thrombin with Bz-Phe-Pro-GPA-pNA (GPA-peptide) and Bz-Phe-Pro-Arg-pNA (Arg-peptide) are presented in Table I together with those for Bz-GPA-pNA and Bz-Arg-pNA. Based on the second-order rate constant (k_{cat}/K_m), the value for GPA-peptide in the hydrolysis by trypsin is 224 times smaller than that for Arg-peptide. The K_m value for GPA-peptide is almost the same as that for Arg-peptide while the k_{cat} value is 172 times smaller than that for the latter. If K_m reflects the binding affinity of these substrates to trypsin, no significant difference appears to exist between the binding abilities of these two peptide substrates to the specificity site of this enzyme. The very small k_{cat} value for GPA-peptide may

TABLE I. Kinetic Parameters for the Hydrolysis of Bz-Phe-Pro-GPA-pNA and Bz-Phe-Pro-Arg-pNA by Bovine Trypsin and Thrombin at 25°C

Substrate		Trypsin	Thrombin
Bz-Phe-Pro-GPA-pNA	K_m ^{a)}	0.0635 ± 0.0048	0.0162 ± 0.0012
	k_{cat} ^{a)}	0.365 ± 0.021	0.438 ± 0.013
	k_{cat}/K_m ^{a)}	5.75 ± 0.29	27.0 ± 1.8
Bz-Phe-Pro-Arg-pNA	K_m	0.0488 ± 0.0033	0.0335 ± 0.0012
	k_{cat}	62.8 ± 3.5	18.1 ± 0.5
	k_{cat}/K_m	1287 ± 48	540 ± 10
Bz-GPA-pNA	K_m	0.0156 ± 0.004 ^{b)}	— ^{c)}
	k_{cat}	0.081 ± 0.001 ^{b)}	— ^{c)}
	k_{cat}/K_m	5.19 ± 0.14 ^{b)}	— ^{c)}
Bz-Arg-pNA	K_m	0.461 ± 0.016 ^{b)}	0.152 ± 0.009
	k_{cat}	1.67 ± 0.05 ^{b)}	0.130 ± 0.004
	k_{cat}/K_m	3.61 ± 0.14 ^{b)}	0.855 ± 0.043

a) K_m (mM); k_{cat} (s^{-1}); k_{cat}/K_m ($\times 10^3 \cdot M^{-1} \cdot s^{-1}$). b) Tsunematsu *et al.* (Reference 1). c) The rate of hydrolysis of this substrate by thrombin was very slow.

be due to the poor orientation of the scissile bond to the catalytic serine residue of trypsin. These are unexpected results, because Bz-GPA-pNA was hydrolyzed by trypsin as fast as Bz-Arg-pNA. GPA-peptide was hydrolyzed by trypsin as fast as Bz-GPA-pNA. The K_m value for GPA-peptide was about 5 times larger than that for Bz-GPA-pNA while the k_{cat} value was also 4 times larger than that for the latter. On the other hand, Arg-peptide was hydrolyzed about 360 times faster than Bz-Arg-pNA. The K_m value for Arg-peptide was about 10 times smaller than that for Bz-Arg-pNA while the k_{cat} value is 38 times larger than that for the latter. These results indicate that chain elongation affects the hydrolysis of Arg-peptide by trypsin, but not that of GPA-peptide. The reason for this was discussed in the previous paper.⁶⁾

GPA-peptide was hydrolyzed by thrombin at about one-twentieth of the rate of Arg-peptide. The K_m value for GPA-peptide was about half of that for Arg-peptide, while the k_{cat} value is about 40 times smaller than that for the latter. In the hydrolysis of the peptide substrates by thrombin, as well as in that by trypsin, no significant difference was observed between the binding abilities of these two peptide substrates to the specificity site of thrombin, and the orientation of the scissile bond of GPA-peptide to the catalytic serine residue was assumed to be inappropriate. GPA- and Arg-peptides were hydrolyzed by thrombin much faster than the corresponding amino acid substrates. These results suggest that chain elongation does affect the hydrolysis of these two peptide substrates by thrombin. It is likely that the subsite of trypsin is very different from that of thrombin.

We have recently found a new serine protease, which preferentially recognizes the *p*-guanidino-L-phenylalanyl residue, in ascitic plasma from Ehrlich tumor-bearing mice and referred to this enzyme as "*p*-guanidinophenylalanine specific enzyme" (GPASE).⁶⁾ GPA-peptide was ascertained to be a good substrate for this enzyme. Consequently, GPA-peptide should be a useful peptide substrate in the study of the subsite specificities of trypsin-like enzymes and for the assay of GPASE.

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