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STUDIES ON A NEW PROTEOLYTIC ENZYME FROM *ACHROMOBACTER LYTICUS* M497-1

II. SPECIFICITY AND INHIBITION STUDIES OF *ACHROMOBACTER* PROTEASE I *

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The unique specificity of *Achromobacter* protease I for lysine residue was investigated using synthetic and natural substrates, i.e., lysine derivatives, arginine derivatives, lysine vasopressin, substance P, ACTH and insulin. The enzyme cleaved only the -Lys-X- bonds in the above substrates. The binding affinity of alkylamines as determined by K_i was much stronger than that of the corresponding alkylguanidines.

Introduction

We have reported [1] that an affinity chromatography was effective in the purification of *Achromobacter* protease I on an AH-Sepharose 4B column chromatography. This phenomenon was interpreted as being based on the unique substrate specificity of this enzyme. This enzyme hydrolyzes the peptide bonds at the carboxyl side of the lysine residue in a similar manner to trypsin. However, on the contrary this enzyme attacks the Lys-Pro bond which is resistant to trypsin, and does not attack the arginyl bond which is easily hydrolyzed by trypsin. The conspicuous difference of this enzyme from trypsin in the proteolytic activity prompted us to compare the substrate specificities of this enzyme with those of trypsin in esterolytic activity. The aim, in this paper, is to investigate the substrate specificity and the competitive inhibition of the enzyme using various substrate analogues, such as alkylamines, alkylguanidines, lysine, ornithine and arginine.

* This is the third paper in a series. The previous papers are Ref. 1 and 11.

Materials and Methods

Materials. Lysine vasopressin (grade IV) and diisopropylfluorophosphate (iPr_2P-F)-treated carboxypeptidase B were purchased from Sigma Chemicals Co., U.S.A. *N*-Benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) and *N*-tosyl-L-lysine methyl ester (Tos-Lys-OMe) were from the Protein Research Foundation, Japan. *N*-Benzoyl-L-lysine methyl ester (Bz-Lys-OMe) and *N*-benzoyl-L-ornithine methyl ester (Bz-Orn-OMe) were from Vega-Fox Biochemicals, U.S.A. Adrenocorticotropic hormone (ACTH 1–24) and substance P were kindly offered by Dr. M. Yoneda, Microbiological Research Laboratories, Takeda Chemical Industries, Japan. Porcine insulin (zinc-free) was also kindly offered by Dr. H. Kimura, Central Research Laboratory, Nippon Soda Chemical Ltd., Japan.

Achromobacter protease I. The enzyme was purified according to the method described in the previous paper [1].

Amino acid composition. The amino acid analysis of peptides was carried out on a Hitachi Model KLB-3B autoanalyzer according to the method of Spackman et al. [2]. Cystine and cysteine were determined according to the method of Spencer and Wold [3].

Isolation of peptides. Isolation of peptides was performed according to the method of Katz et al. [4] using a Toyo Roshi filter paper No. 51. High voltage paper electrophoresis was carried out at pH 3.7 (pyridine/acetic acid/water, 1 : 10 : 289, v/v) with a voltage gradient of 50 V/cm. Paper chromatography was developed in butanol/pyridine/acetic acid/water (15 : 10 : 3 : 12, v/v) [5]. Peptides were detected by spraying 0.02 or 0.2% ninhydrin in acetone.

NH_2- and COOH-terminal amino acid analysis. The NH_2-terminal amino acid analysis of peptides was performed according to the method of Gray [6], and the COOH-terminal amino acid analysis according to the method of Amber [7].

Digestion of polypeptide substrates. (a) ACTH, lysine vasopressin and substance P. ACTH (3.4 mg), lysine vasopressin (2 mg) and substance P (1 mg) were digested under the following conditions. The weight ratio of substrate to the enzyme was 100 : 1 in a buffer of 50 mM triethylamine-acetate (pH 9.5) at 30°C for 24 h. The digest was lyophilized and separated by the method of Katz et al. [4]. Then amino acid composition and NH_2-terminal amino acid were determined. (b) Insulin. Insulin (10 mg) was digested in 50 mM Tris-HCl buffer (pH 9.2) using a similar procedure. After lyophilization, the products of digestion were separated by gel filtration on a column (1.3 X 60 cm) of Sephadex G-15 equilibrated with 0.4 M acetic acid. Separated products were subjected to the amino acid analysis and the COOH-terminal amino acid was determined.

Kinetic studies. The esterolytic activities against *N*-benzoylated and *N*-tosylated substrates were measured at 30°C by the method of Schwert and Takenaka [8] and Hummel [9], or the method of Masaki et al. [10]. The Michaelis constant (K_m) and V of the reaction were estimated by a Lineweaver-Burk plot. The k_{cat} values were calculated from the V values. The K_i values for competitive inhibitors were estimated by the method of Dixon from the intersection of thee lines determined in the presence of inhibitors. Activities against Bz-Lys-pNA and Tos-Lys-OMe were measured in 40 mM Tris-HCl buffer (pH 8.0).

Protein concentration. Protein concentration was determined using $E^{1\%}_{1cm} = 18.77$ at 280 nm.

Results

(A) Digestion of polypeptide substrates. (a) ACTH, lysine vasopressin and substance P: As shown in Table I, only the Lys^8-Gly^9NH_2 bond in lysine vasopressin was hydrolyzed. The Lys^3-Pro^4 bond was hydrolyzed in the substance P, and in the case of ACTH, the Lys^{11}-Pro^{12}, Lys^{15}-Lys^{16}, Lys^{16}-Arg^{17} and Lys^{21}-Val^{22} bonds were hydrolyzed.

(b) Insulin: The digestion mixture was fractionated by passage through Sephadex G-15 column equilibrated with 0.4 M acetic acid. The elution profile (Fig. 1) showed only two peaks. Amino acid analysis showed that the second peak B consisted of only alanine. The COOH-terminal amino acid of the lyophilizate of peak A was determined to be lysine, since carboxypeptidase B liberated 1.11 μmol lysine per 1.17 μmol peak A after 24 h incubation. This fact indicates that the enzyme split only the Lys^{29}-Ala^{30} bond in the B-chain of insulin.

(B) Hydrolysis of synthetic substrates. Table II gives the kinetic parameters for the hydrolysis of esters by the enzyme. Bovine trypsin was used for

TABLE I

HYDROLYSIS OF PEPTIDES BY PROTEASE I

Tuftsin (H-Thr-Lys-Pro-Arg-OH) was not hydrolyzed by this enzyme.

Peptide
Lysine vasopressin *
H-Cys-Tyr-Phe-Gln-Asn(5)-Cys-Pro-Lys↓GlyNH$_2$
Substance P **
H-Arg-Pro-Lys↓Pro-Gln(5)-Gln-Phe-Phe-Gly-Leu-MetNH$_2$
ACTH$^{1-24}$ **
H-Ser-Tyr-Ser-Met-Glu(5)-His-Phe-Arg-Trp-Gly(10)-Lys↓Pro-Val-Gly-Lys(15)↓Lys↓Arg-Arg-Pro-Val(20)-Lys↓Val-Tyr-Pro-OH

* Amino acid analysis was done after separation of the digestion products by high voltage paper electrophoresis.

** NH_2-terminal amino acid and amino acid analysis were done after separation of the digestion products by the method of Katz et al. [4]. In the case of ACTH, three peptides were detected on the peptide map. The NH_2-terminal amino acids of each peptides were determined to be Ser, Pro, Lys and Arg. From the direct NH_2-terminal amino acid analysis of the digestion mixture, Val was newly detected in addition to other four amino acids, indicating a ninhydrin insensitive oligopeptide (-Val-Tyr-Pro-OH) was contained in the digestion mixture.

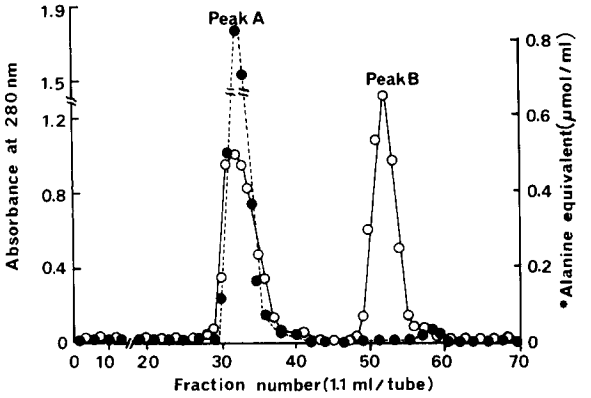

Fig. 1. Gel filtration of the digest of insulin with protease I on a Sephadex G-15 column. ○———○, The alanine equivalent was determined by the ninhydrin method; ●———●, absorbance at 280 nm.

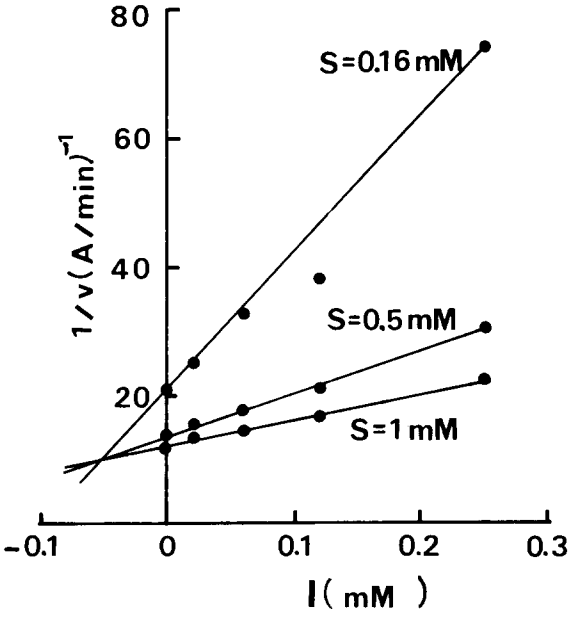

Fig. 2. Dixon plot for inhibition of the esterolytic activity by butylamine. Enzyme activity was assayed in the the presence or absence of butylamine. The reactions were carried out at 30°C for 5 min. Velocity (V) was expressed as the net increase in absorbance at 247 nm/min. The reaction mixture contained various concentrations of Tos-Lys-OMe and 0.26 μg enzyme in 40 mM Tris-HCl buffer (pH 8.0), in a total volume of 3.0 ml.

comparison. The k_{cat}/K_m (app) value for the hydrolysis of Tos-Lys-OMe was 40 000-times higher than that of Tos-Arg-OMe. The value for hydrolysis of Bz-Lys-OMe was 6 700-times higher than that of Bz-Arg-OEt. The enzyme hydrolyzed Bz-Orn-OMe more slowly than Bz-Lys-OMe but faster than Bz-Arg-OEt.

(C) Inhibition of the enzyme by various analogues of substrates. As shown in Fig. 2, the inhibition of the enzyme by butylamine was typically competitive as evidenced by the intersection of lines above the I-axis. K_i values for various analogues of substrates of the enzyme were compared with those of bovine trypsin (Table III). It is evident that alkylamines inhibit the enzyme 10^1–10^2-times more strongly than the corresponding alkylguanidines. K_i values of

TABLE II

KINETIC PARAMETERS FOR THE HYDROLYSIS OF ESTERS BY PROTEASE I AND BOVINE TRYPSIN

The activity with each substrate was measured in 40 mM Tris-HCl buffer (pH 8.0) at 30°C. The kinetic parameters were obtained using a plot of 1/v vs. 1/s. For the calculation of k_{cat}, the molecular weight of protease I was assumed to be 30 000.

Substrate	Protease I			Bovine trypsin		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Bz-Arg-OEt	20.0	7.41	0.37 *	0.0066	17.9	2700 *
Bz-Lys-OMe	0.091	225.3	2480	0.017	17.0	1000 **
Bz-Orn-OMe	1.0	10.4	10.4			
Tos-Arg-OMe	35.7	5.04	0.14 *	0.015	65.2	4350 *
Tos-Lys-OMe	0.1	570.0	5700 *	0.19	98.4	520 *

* From Ref. 11.
** From Ref. 12.

TABLE III

COMPARISON OF THE COMPETITIVE INHIBITION OF PROTEASE I AND TRYSIN BY VARIOUS ANALOGUES OF SUBSTRATES

The activity with each substrate was measured at 30°C in 40 mM Tris-HCl buffer (pH 8.0) as in the previous paper [1]. K_i values were calculated from a Dixon plot of 1/v vs. [I] in the presence of different concentrations of substrate.

Inhibitor	K_i (mM)			
	Protease I		Bovine trypsin	
	Tos-Lys-OMe	Bz-Lys-pNA	Tos-Lys-OMe	Bz-Lys-pNA
Arginine	>100.0	n.d.	75.0	n.d
Citrulline	>100.0	n.d.	>100.0	n.d.
Lysine	10.0	n.d.	>100.0	n.d.
Ornithine	22.5	n.d.	>100.0	n.d.
Methylamine	4.6	2.7	>100.0	>100.0
Ethylamine	1.0	0.7	60.0	64.0
Propylamine	0.25	0.32	9.0	2.65
Butylamine	0.05	0.03	2.5	3.5
Amylamine	0.03	0.04	4.5	n.d.
Hexylamine	0.35	n.d.	n.d.	n.d.
Methylguanidine	25.0	20.0	7.65	3.5
Ethylguanidine	22.0	n.d.	1.40	n.d.
Butylguanidine	21.0	16.0	0.41	1.44

n.d. Not determined.

alkylamines decreased as the carbon chain length increased, from the K_i value of 4.6 mM for methylamine to 0.03 mM for amylamine.

Both L-lysine and L-ornithine were weak inhibitors. On the other hand, L-arginine or L-citrulline, when tested up to 100 mM, caused no change in the rate of the hydrolysis of Tos-Lys-OMe.

Discussion

In our study, it was clearly shown that *Achromobacter* protease I has a greatly different substrate specificity from that of bovine trypsin. The differences of this enzyme from bovine trypsin in substrate specificities are summarized as follows. (a) This enzyme hydrolyzes only the lysyl substrate, while trypsin hydrolyzes both lysyl and arginyl substrates. (b) This enzyme hydrolyzes the Lys-Pro bonds in ACTH and substance P, while trypsin does not hydrolyzes them at all [13]. (c) The same substrate specificity was observed both for the esterolytic and amidolytic reaction of this enzyme, except for the very weak esterolytic activity found for Bz-Arg-OEt and Tos-Arg-OMe (Table II and Ref. 11).

As is well established, trypsin has a pocket containing at its bottom the negatively charged carboxyl group of Asp^{177} which is able to bind arginine and lysine residues in peptidic substrates [14]. If we assume a similar substrate binding pocket in this enzyme, the following interpretation of the data in our study may be obtained. (a) Butylamine (K_i 0.05 mM) was a stronger inhibitor than butylguanidine (K_i 21 mM) (Table III). This seems to indicate that the guanidine group is too bulky to fit correctly into such a pocket. (b) K_i values of this enzyme decreased as the number of methylene groups of the tested alkylamines increased, but did not depend at all on the number of the methylene group of alkylguanidines (Table III). This implies that the nature of the assumed pocket in the enzyme is different from that of trypsin.

The above results showed that *Achromobacter* protease I could be a valuable tool not only for studies of protein sequence but also for studies of peptide bond synthesis. In fact, Morihara et al. [15,16] used this enzyme for the replacement of Ala-

B30 with threonine in porcine insulin for the semi-synthesis of human insulin.

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