Serratia Protease

Part II. Substrate Specificity of the Enzyme

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The substrate specificity of *Serratia* protease was determined using various synthetic substrates. The enzyme did not participate in the hydrolysis of di- and tri-peptides except benzoyl-glycylleucinamide which was split at a limited rate into hippuric acid and leucinamide. The enzyme action on larger peptides was also studied. The enzyme cleaved the gly-leu bond in eledoisin related peptide and the gly-phe bond in bradykinin. The enzyme split oxidized insulin B-chain at twelve different peptide bonds.

The previous paper¹⁾ has elucidated the purification procedure of protease from the culture broth of *Serratia* sp. E-15, and also characterized physicochemical, chemical and general enzymatic properties of the purified enzyme. In the present paper, is described the substrate specificity of the enzyme comparing with those of other bacterial enzymes.

METHODS

Enzyme. The purified enzyme used in this study was prepared from the culture broth of *Serratia* sp. E-15²⁾ according to the method described in the previous paper.¹⁾ The purified enzyme was confirmed to be homogeneous by ultracentrifugation and electrophoresis. Enzyme concentration was determined spectrophotometrically as previously described.¹⁾

Assay of proteolytic activity. Proteolytic activity was determined according to the method described in the previous paper.¹⁾

Assay of peptidase, esterase and amidase activities. About 1 mg of peptide was dissolved in 1.0 ml of

0.02 M Tris-HCl buffer, pH 9.0, containing 0.1 mg of the enzyme, and incubated at 25°C for 1 day. The cleavage of the peptide bond was measured by the ninhydrin method of Yemm and Cocking.³⁾ The cleavage of the peptide was also checked by thin-layer chromatography with silica gel plate using a solvent system of *n*-butanol, acetic acid and water (3:1:1, v/v). Esterase activity⁴⁾ was assayed by titrating released carboxy groups with 0.1 N NaOH using a Radiometer model TTT1 autotitrator. Amidase activity was assayed according to Conway's method⁵⁾ after an enzyme-substrate mixture was incubated at pH 7.5 and at 37°C for 30 min.

Analysis of hydrolyzate of eledoisin related peptide and bradykinin. About 5 mg of the peptide was dissolved in 2.0 ml of 0.02 M ammonium carbonate buffer, pH 8.5, containing 0.05 mg of the enzyme. The mixture was incubated at 37°C for 5 hr. The hydrolyzate of the peptide was subjected to paper chromatography using a solvent system of *n*-butanol, pyridine, acetic acid and water (15:10:3:12, v/v). The amino acid composition of the respective peptides isolated from

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the paper-chromatogram was further analyzed by two dimensional thin-layer chromatography⁶⁾ using solvent systems of chloroform, methanol and 17% ammonium hydroxide (2:2:1, v/v) followed by phenol and water (75:25, w/w), after hydrolysis in 6N HCl at 110°C for 24 hr. The N-terminal amino acid of the peptide was determined by Sanger's DNP-method.⁷⁾

Determination of substrate specificity on oxidized insulin B-chain. The B-chain was prepared from crystalline whale insulin according to the method of Griffin *et al.*⁸⁾

a) *Hydrolysis.* To a solution of about 100 mg of the B-chain in 5.0 ml of water was added 1.0 mg of the enzyme, then pH of the solution was adjusted to pH 9.0 by the addition of $(\text{NH}_4)_2\text{CO}_3$. After 24 hr incubation at 37°C, the solution was chilled to 4°C and the reaction was stopped by the addition of 2 ml of cold 10% NH_4OH . After lyophilization the remaining $(\text{NH}_4)_2\text{CO}_3$ was removed in a vacuum desiccator over P_2O_5 and NaOH.

b) *Column chromatography of the hydrolyzate.*⁹⁾ To a Dowex 50×2 column (0.9×105 cm), equilibrated with 0.2M pyridine-acetate buffer, pH 3.1, at 38°C, was applied 95 mg of the hydrolyzate. After the column was washed with the same buffer, a logarithmic gradient elution was performed with 500 ml of 0.2M pyridine-acetate buffer, pH 3.1, in a mixing flask, and 2.0M pyridine-acetate buffer, pH 5.0, in a reservoir. Finally the elution was conducted with 8.5M pyridine-acetate buffer, pH 5.6, at 40°C. The elution was carried out at a flow rate of 16 ml per hr, and the every 1.6 ml fraction was collected. Peptides in the eluate were determined by the ninhydrin method.³⁾ Each peptide fraction was dried in a vacuum desiccator. The isolated peptides were subjected to paper chromatography of the previously described method. The peptides further fractionated by the paper chromatography were extracted with water and lyophilized.

c) *Amino acid analysis of peptides.* The peptides obtained by column or paper chromatography were hydrolyzed in 6N HCl for 24 hr at 110°C. The amino acid composition in the hydrolyzate was determined with a Hitachi amino acid analyzer KLA-3B according to the method of Moore and Stein.¹⁰⁾

d) *Identification of N-terminal amino acid of peptides.* The DNP-method of Sanger⁷⁾ was used. The identification of DNP-amino acids was performed by two dimensional paper chromatography according to the method of Koch and Weidel.¹¹⁾

MATERIALS

Benzoylglutylleucinamide was purchased from Mann Res. Inc. The other peptides were obtained from Protein Res. Inst. of Osaka University, casein (Hammarsten, milk) and egg albumin from Merck Co., hemoglobin (crystalline, bovine) from Sigma Co., lysozyme (crystalline, egg) from Worthington Biochem. Corp., insulin (crystalline, whale) from Shimizu Pharm. Ltd., γ -globulin (bovine fraction II) from Tokyo Kasei Kogyo Ltd. and gelatin from Difco Co., respectively.

Silica gel plates used were TLC-plates No. 5721/0025 of Merck Co. for two dimensional developments and Eastman Chromagram sheet No. 6061 of Eastman Kodak Co. for one dimensional development.

RESULTS

Hydrolysis of proteins

The hydrolysis rates toward various native and urea-denatured proteins were measured according to the standard assay method except that the substrate was substituted. The results are shown in Table I. Casein was most rapidly hydrolyzed and the denaturation of casein caused no change in the susceptibility to the enzyme. The other native proteins were hydrolyzed at a slower rate than casein, but the susceptibility of these proteins was

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TABLE I. RELATIVE HYDROLYSIS RATE TOWARD VARIOUS PROTEINS

Substrate	Native substrate	Urea denatured substrate ^{a)}
	(Relative rate)	
Casein	100(%)	100(%)
Hemoglobin	28	60
Egg albumin	11	26
Lysozyme	6	90
Insulin	5	25
γ -Globulin	3	80

^{a1} The protein was denatured with 7M urea in 1N NaOH at 37°C for 1 hr. Urea concentration in the assay solution was 1 M.

TABLE II. MAXIMUM HYDROLYSIS DEGREE OF VARIOUS PROTEINS

Protein	Maximum hydrolysis degree	
	TCA-soluble	Peptide bond cleaved
Casein	95(%)	10(%)
Egg albumin	31	8
Hemoglobin	39	9
Insulin	100	9
Gelatin	—	13

The reaction mixture containing 10 mg of the substrate and 0.1~1.0 mg of the enzyme in 10 ml of 0.05 M Tris-HCl buffer, pH 9.0, was incubated at 37°C for 30 hr. At intervals, an aliquot was taken to estimate hydrolysis degree of the respective protein. To 2.0 ml of the aliquot, 3.0 ml of 5.0% TCA was added. The precipitate was removed by filtering with Toyo-roshi No. 5c and the absorbancy of the filtrate at 280 m μ was measured. The aliquot was also used for the ninhydrin determination.³¹

greatly increased by the denaturation. In Table II are summarized the maximum hydrolysis degrees of various proteins catalyzed by the enzyme after a long time incubation. Percent of the hydrolysis was calculated by comparing the absorbancy at 280 m μ of TCA-soluble materials of the hydrolyzate with that of the native protein, and the cleavage of the peptide bond by comparing the ninhydrin color value of the hydrolyzate with that of

the acid hydrolyzate of the protein with 6N HCl for 24 hr, respectively. The hydrolyzates of casein and insulin by the enzyme were completely TCA-soluble, while those of egg albumin and bovine hemoglobin were precipitated with TCA. On the other hand, the hydrolysis degrees of various proteins based on the peptide bond cleavage approximated to a value of 10%.

Hydrolysis of peptides

The substrate specificity of the enzyme on various peptides amides and esters was quali-

TABLE III. SUBSTRATE SPECIFICITY OF THE ENZYME ON VARIOUS PEPTIDES, AMIDES AND ESTERS

Substrate	Degree of hydrolysis
Gly-Pro	—
Gly-DL-Phe	—
Gly-DL-Ala	—
Gly-Leu	—
Leu-Gly	—
DL-Ala-DL-Val	—
DL-Ala-DL-Phe	±
DL-Leu-Gly-DL-Phe	±
Leu-Gly-Gly	±
Bz-Gly-Leu-NH ₂	+
Di-Bz-Cys	—
Bz-DL-Val	—
Cbz-Glu-Phe	—
Cbz-Gly-Pro-Leu-Gly-Pro	±
Cbz-Gly-Phe	—
Cbz-Phe-NH ₂	—
Cbz-Gly-Phe-NH ₂	—
Leu-NH ₂	—
N-Ac-Met	—
N-Ac-Val	—
BAEE	—
Ac-Tyr-EE	—
Gly-EE	—
Tosyl-Arg-EE	—

About 1 mg of peptide was dissolved in 1.0 ml of 0.02 M Tris-HCl buffer, pH 9.0, containing 0.1 mg of the enzyme. The mixture was incubated at 25°C for 1 day. The detection of the hydrolysis by the enzyme was described in Methods. The both assays of esterase and amidase activity were also described in Methods. Bz: benzoyl, Cbz: carbobenzoxy, BAEE: benzoyl-arginine-ethylester, EE: ethylester. —: not hydrolyzed, ±: slightly hydrolyzed, +: detectably hydrolyzed.

TABLE IV. AMINO ACID COMPOSITION AND N-TERMINAL AMINO ACID OF PEPTIDES SEPARATED BY COLUMN CHROMATOGRAPHY

Peptide fraction	Amino acid composition	N-terminal amino acid	Most probable sequence in insulin B-chain
1* a	His, Glu, Leu, CySO_3H	Glu	4~7
b	Gly, Val, Leu, CySO_3H	Leu	17~20
2	Gly, Ser	—	8~9
3	Glu, Ala, Val, Leu	Leu	11~14
4	Arg, Glu, Gly, Val, Leu, CySO_3H	Leu	17~22
5	Asp, Val, Phe	Phe	1~3
6	Arg, Glu, Gly, Val, Leu_2 , Tyr, CySO_3H	Leu	15~22
7	Ala, Leu, Tyr	—	14~16
8	Leu, Tyr	Leu	15~16
9* a	His, Ser, Gly	Gly	8~10
b	Gly, Phe	Gly	23~24
10	not detected		
11	Lys, Thr, Pro, Ala	—	27~30
12* a	His, Glu, Ala, Val, Leu_2 , Tyr	His	10~16
b	Arg, Glu, Gly	Glu	21~23
13	Arg, Glu, Gly, Phe_2 , Tyr	Glu	21~26
14* a	Arg, Glu, Gly, Phe_2 , Tyr	Glu	21~26
b	Gly, Tyr, Phe_2	Gly	23~26

* Peptide fraction was further separated by paper chromatography.

FIG. 3. Cleavage Sites of Oxidized Insulin B-Chain by Proteases.

The arrows indicate major sites of peptide bonds cleaved by the enzyme and the dashed arrows indicate the bond cleaved less readily.

subtilis neutral protease¹³⁾ and *Pseudomonas aeruginosa* alkaline protease,¹⁴⁾ both known as metalloenzymes, are also indicated there.

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Although peptide bonds involving amino groups of leucine and phenylalanine were cleaved in the hydrolyses of eledoisin related peptide and bradykinin by the enzyme, the result from the hydrolysis of the B-chain showed that the peptide bonds susceptible to the enzyme were not restricted to these specific

sites in the hydrolysis of larger molecular peptides.

DISCUSSION

Of smaller peptides tested, only benzoyl-glycylleucinamide was hydrolyzed detectably by the enzyme. This specificity is characteristic of neutral proteases from microorganisms such as *Bacillus subtilis*¹⁵⁾ and *Pseudomonas aeruginosa*,¹⁶⁾ although the hydrolysis rate of benzoylglycylleucinamide by *Serratia* sp. E-15 protease was about 1/5000 fold as less as that by *Bacillus subtilis* neutral protease.¹⁵⁾

Toward larger peptides such as eledoisin related peptide and bradykinin, the enzyme split the specific peptide bonds of gly-hydrophobic amino acid, whereas, toward oxidized insulin B-chain, the enzyme attacked not only the peptide bonds of gly-hydrophobic amino acid but also other bonds. With respect to the action of the enzyme on the B-chain, *Serratia* sp. E-15 protease resembles *Pseudomonas aeruginosa* alkaline protease¹⁴⁾ rather than *Bacillus subtilis* enzyme.¹³⁾

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