Purification and Some Properties of *Achromobacter* Protease Ia from *Achromobacter lyticus* M497-1

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Received June 26, 1986

A protease with strict specificity to lysyl peptide bonds like that of *Achromobacter* protease I was purified from a crude enzyme powder obtained from a culture filtrate of *Achromobacter lyticus* M497-1 and characterized. The purified enzyme had the following differences from protease I. The enzyme had an isoelectric point of 5.3, lower than the value of 6.9 for protease I. The amino acid composition of the enzyme had higher proportions of His, Glu, and Gly and lower proportions of Arg and Thr than protease I. The enzyme was unstable (30% residual activity) in the presence of 7 M urea (pH 8.0, 30°C, 20 min); protease I was resistant to the same conditions (80% residual activity). The k_{cat}/K_m values for the hydrolysis of Tos-Lys-OMe and Lys-pNA by the enzyme were lower than those of protease I.

We have purified *Achromobacter* protease I (EC 3.4.21.50) from *Achromobacter lyticus* M497-1 in five steps, including affinity chromatography on AH-Sepharose 4B and isoelectric focusing.^{1~3)} With isoelectric focusing, we isolated a second lysine-specific endopeptidase that differs from protease I. This paper describes the purification and some properties of the enzyme and compares them with those of protease I.

MATERIALS AND METHODS

Materials. *N*-Benzoyl-DL-lysine-*p*-nitroanilide (Bz-Lys-pNA) and *N*-benzoyl-L-ornithine methyl ester (Bz-Orn-OMe) were purchased from Serva. *N*-Benzoyl-L-arginine-*p*-nitroanilide (Bz-Arg-pNA), L-arginine-*p*-nitroanilide (Arg-pNA), *N*-tosyl-L-arginine methyl ester (Tos-Arg-OMe), and *N*-tosyl-L-lysine methyl ester (Tos-Lys-OMe) were from the Protein Research Foundation. L-Lysine-*p*-nitroanilide (Lys-pNA) was obtained from E. Merck. *N*-Tosyl-L-arginine chloromethyl ketone (Tos-ArgCH₂Cl) was kindly supplied by Dr. Kazuyuki Morihara, Shionogi Research Laboratory. Acetone powder fractionated with 70% acetone from a culture filtrate was kindly offered to us by Dr. Masahiko Yoneda, Takeda Chemical Industries.

Assay of enzyme activity. Amidolytic activity was assayed at pH 8.8 with Bz-Lys-pNA as substrate.⁴⁾

Esterolytic activity was assayed at pH 8.0 with Tos-Lys-OMe as substrate.⁵⁾

Assay of protein. Protein concentration was estimated at different stages of the purification procedure by measurement of the absorbance at 280 nm. In the purified enzyme preparations, the protein concentration was estimated by method of Lowry *et al.*⁶⁾ with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was done at pH 9.5.⁷⁾

Estimation of molecular weight. The molecular weight was estimated by Andrew's method⁸⁾ with a column of Sephadex G-75 (2.0 × 81 cm).

Isoelectric focusing. Electrofocusing was done on a column (about 140 ml) containing 0.5% carrier ampholyte with pH ranges from 3.5~10 and 4~6 (LKB) at 600 V for 48 hr at 4°C.

Amino acid composition. The purified enzyme was hydrolyzed with 6 N HCl in a sealed evacuated glass tube at 110°C for 24 hr. The hydrolysate was analyzed on a Hitachi model-635 high-pressure liquid chromatograph with a column of Lichrosorb PR-18 resin by the *o*-phthalaldehyde method of Benson and Hare.⁹⁾

Purification of the enzyme. All steps were done in a cold room at 4°C.

Step 1. CM-cellulose treatment. The acetone powder

(10 g) was dissolved in 10 mM Tris-HCl buffer, pH 8.0, (buffer A) and centrifuged. The supernatant was mixed with CM-cellulose (wet weight, about 200 g) equilibrated with buffer A. The I and Ia enzymes were not adsorbed under these conditions, whereas a bacteriolytic enzyme was tightly bound. The fractions (protease I + Ia) were collected and concentrated by an Amicon UM-10 ultrafiltration membrane.

Step 2. DEAE-cellulose treatment. The concentrated enzyme solution (500 ml) was mixed with DEAE-cellulose (wet weight, about 400 g) equilibrated with buffer A. The mixture was passed through a glass filter (26G-3), and the filtrate was concentrated. This solution was then dialyzed against 2 mM Tris-HCl buffer, pH 8.0 (buffer B), and concentrated again. Most of the colored material was removed by this treatment.

Step 3. AH-Sepharose 4B chromatography. The concentrated solution (250 ml) from step 2 was put on an AH-Sepharose 4B column (4 × 21 cm) equilibrated with buffer B. The charged column was washed with buffer B and then proteases I and Ia were eluted with a 0 ~ 1.0 M NaCl linear gradient in buffer B. The fractions (Nos. 170 ~ 220) that were active against Bz-Lys-pNA were collected, dialyzed against buffer B, and concentrated. Proteases I and Ia behaved similarly during this step.

Step 4. First isoelectric focusing. The sample from step 3 was processed by isoelectric focusing (carrier ampholyte, pH 3.5 ~ 10). Activity against Bz-Lys-pNA was found in both the first (protease Ia; pI 5.3) and the second (protease I; pI 6.9) peaks (Fig. 1). The first active fraction (Nos. 42 ~ 51) was collected, dialyzed against buffer B, and concentrated.

Step 5. Second isoelectric focusing. The concentrated enzyme (13 ml) from step 4 was again processed by isoelectric focusing (carrier ampholyte, pH 4 ~ 6). The active fractions (Nos. 65 ~ 75) were dialyzed against buffer B, concentrated, put on a Sephadex G-50 column equili-

brated with buffer B to remove the carrier ampholyte,¹⁰⁾ and stored at -20°C.

RESULTS

I. Purification

The results of the purification are summarized in Table I. The final yield of the amidolytic activity was 7.0%, with 40-fold purification. The purified enzyme gave a single band on polyacrylamide gel electrophoresis.

FIG. 1. Isoelectric Focusing of Protease I and Ia.

Ampholine, pH 3.5 ~ 10; total volume of column, 140 ml; separation time, 48 hr; current, 600 V; temperature, 4°C; flow volume, 1.6 ml; —●—, absorbance at 280 nm; ---×---, activity against Bz-Lys-pNA; —△—, pH.

TABLE I. SUMMARY OF PURIFICATION OF PROTEASE Ia FROM *Achromobacter lyticus* M497-1

Step	Total protein ($A_{280\text{ nm}}$)	Total activity (U) ^a	Specific activity (U/ $A_{280\text{ nm}}$)	Recovery (%)
Acetone powder (10 g)	3,870	216.0	0.056	100
CM-cellulose treatment	3,380	216.0	0.064	100
DEAE-cellulose treatment	600	216.0	0.36	100
AH-Sepharose 4B chromatography	65.0	144.0	2.22	66.7
1st isoelectric focusing (pH 3.5 ~ 10)	14.6 (43.0) ^b	18.9 (110.9) ^b	1.29 (2.58) ^b	8.7 (51.4) ^b
2nd isoelectric focusing (pH 4 ~ 6) (pH 5 ~ 8)	7.2 (41.6) ^b	15.1 (108.2) ^b	2.10 (2.60) ^b	7.0 (50.1) ^b

^a Units of Bz-Lys-pNA-hydrolyzing activity.

^b *Achromobacter* protease I.

TABLE II. AMINO ACID COMPOSITION OF *Achromobacter* PROTEASES I AND Ia

Amino acid	Protease Ia ^a	Protease I ^b
	Number of residues	Number of residues
Lysine	9	6
Histidine	15	6
Arginine	6	13
Aspartic acid	30	33
Threonine	12	26
Serine	36	35
Glutamic acid	32	17
Proline	n.d.	15
Glycine	51	39
Alanine	39	31
Half-cystine	n.d.	6
Valine	18	15
Methionine	3	4
Isoleucine	6	10
Leucine	9	14
Tyrosine	6	9
Phenylalanine	6	8
Tryptophan	n.d.	7
Total	>278	294

^a The amino acid composition of the enzyme was found by the *o*-phthalaldehyde method of Benson and Hare.⁸⁾ The values are expressed as the number of residues/molecule with a molecular weight of 30,000 assumed.

^b From ref. 2. n.d., not determined.

II. Properties of the purified enzyme

Molecular weight. The molecular weight of the enzyme as estimated by gel filtration on Sephadex G-75 was 30,000.

Amino acid composition. The amino acid composition of the enzyme, summarized in Table II, had several differences from that of protease I. The proportions of histidine, glutamic acid, and glycine were greater than in protease I, whereas arginine and threonine were present at lower levels.

Optimum pH and pH stability. The optimum pH for the esterolytic activity of the enzyme was 7.8~8.2, which was the same as that of protease I,²⁾ and the amidolytic activity had an optimum pH of 8.8. This value differed from that of protease I, which had a broad optimum pH of 9.0~9.5.

The enzyme solution was incubated at 4°C

FIG. 2. Effects of Urea and SDS on Protease I and Ia.

Protease I (0.22 μ g, —○—) and protease Ia (0.27 μ g, —×—) were incubated (40 mM Tris-HCl buffer, pH 8.0, 30°C, 20 min) with various concentrations of denaturants, and the residual enzyme activities were assayed with Tos-Lys-OMe as the substrate.

for 24 hr at various pH (3.0~12) and the remaining amidolytic activity was measured at pH 8.8. The enzyme was stable within the pH range of 5.0~11.0.

Thermal stability. The enzyme was incubated in 0.1 M Tris-HCl buffer (pH 8.8) at various temperatures (20~60°C) for 20 min and the remaining amidolytic activity was measured at the same pH. The enzyme was stable up to 40°C, and completely lost its activity when incubated above 60°C for 20 min. The thermal stability curve was very similar to that of protease I.²⁾

Effect of various metal ions and inhibitors. The effect of various reagents on the esterolytic activity was examined. The esterolytic activity was neither inhibited nor stimulated by Ca^{2+} , Mn^{2+} , Mg^{2+} , Ni^{2+} , or Co^{2+} at 1 mM or 0.1 mM, but was inhibited by Zn^{2+} at 1 mM. The enzyme activity was inhibited by Tos-LysCH₂Cl (0.1 mM), iPr₂P-F (1 mM or 20 mM), and PhCH₂SO₂F (2 mM), but was affected little or not at all by thiol reagents (0.1 or 1 mM), metal-chelating reagents (1 mM), reducing reagents (1 mM), Tos-ArgCH₂Cl (0.1 mM), or Tos-PheCH₂Cl (1 mM). Thus, these inhibitory effects in proteases I and Ia were similar.

Effects of protein denaturants. The effects of urea and SDS on the esterolytic activity of protease Ia were examined. After incubation of the enzyme with 7 M urea or 0.1% SDS, the enzyme retained 30% and 100% of its activity, respectively; protease I retained 80% and 100% of its activity, respectively (Fig. 2).

Substrate specificity. Table III summarizes

TABLE III. KINETIC CONSTANTS FOR HYDROLYSIS OF ESTERS AND ANILIDES
 BY *Achromobacter* PROTEASES I AND I_a

The enzyme activity with each substrate was measured at 30°C and at a given pH by (A) the spectrophotometric method of Schwert and Takenaka⁵⁾ and (B) the colorimetric method of Tuppy *et al.*⁴⁾ as described elsewhere.¹⁾

The kinetic parameters were obtained with a plot of $1/v$ vs. $1/s$. For the calculation of k_{cat} , the molecular weight of protease I_a was assumed to be 30,000.

Substrate	Assay method	Protease I _a			Assay method	Protease I ^a		
		<i>K_m</i> (mM)	<i>k_{cat}/K_m</i> (mM ⁻¹ s ⁻¹)	pH		<i>K_m</i> (mM)	<i>k_{cat}/K_m</i> (mM ⁻¹ s ⁻¹)	pH
Tos-Arg-OMe	A	2.5	0.17	8.0	A	35.7	0.14	8.0
Tos-Lys-OMe	A	0.083	2570	8.0	A	0.1	5700	8.0
Bz-Orn-OMe	A	0.63	4.22	8.0	A	1.0	10.4	8.0
Bz-Arg-pNA	B	Not hydrolyzed		8.8	B	Not hydrolyzed		9.5
Bz-Lys-pNA	B	0.09	11.1	8.8	B	0.07	12.3	9.5
Arg-pNA	B	Not hydrolyzed		9.0	B	Not hydrolyzed		9.2
Lys-pNA	B	0.11	0.20	9.0	B	0.08	1.75	9.2

^a From refs. 1 and 3.

some of the kinetic constants for hydrolysis of esters and anilides by protease I_a. The effective substrate was Tos-Lys-OMe; the specificity constant (k_{cat}/K_m) for Tos-Lys-OMe was 15,000 times that of Tos-Arg-OMe. The hydrolytic rate for Bz-Orn-OMe was lower than that of its lysine derivative, although Ac-Tyr-OEt and Bz-Tyr-OEt were not hydrolyzed by this enzyme. The enzyme did not hydrolyze Bz-Arg-pNA and Arg-pNA at all. Of the lysine derivatives, Bz-Lys-pNA was hydrolyzed more readily than Lys-pNA. A considerable difference was thus observed between proteases I and I_a in their k_{cat}/K_m values for Tos-Lys-OMe, Bz-Orn-OMe, and Lys-pNA; these values for the hydrolysis of Tos-Lys-OMe and Bz-Orn-OMe by protease I_a were only about half those of protease I, and the k_{cat}/K_m values for the hydrolysis of Lys-pNA by protease I_a was one-ninth that of protease I. These differences were mainly due to their different k_{cat} values.

To identify the cleavage site in protein hydrolysis by the enzyme, a reaction mixture containing 5.5 mg (960 nmol) of insulin and 5.5 μg (0.18 nmol) of protease I_a in 2 ml of 50 mM Tris-HCl buffer, pH 9.0, was incubated at 30°C for 24 hr. The digestion mixture was fractionated by passage through a Sephadex

FIG. 3. Gel Filtration of a Digest of Insulin with Protease I_a on a Sephadex G-15 Column.

Column, 1.3 × 60 cm; solvent, 0.5 M acetic acid; —○—, alanine equivalent assayed by the ninhydrin method; —●—, absorbance at 280 nm.

G-15 column equilibrated with 0.5 M acetic acid. The elution profile (Fig. 3) had only two peaks. Amino acid analysis showed that peak II consisted of alanine only (570 nmol). We concluded that protease I_a cleaves the Lys (29)–Ala (30) bond in the B-chain of porcine insulin.

DISCUSSION

Protease I_a isolated by the method shown in Table I was purified to homogeneity by polyacrylamide gel electrophoresis and isoelectric focusing. This enzyme is the third proteolytic

enzyme found to have strict specificity for lysyl peptide bonds. *Achromobacter* protease I²⁾ (EC 3.4.21.50) and endoproteinase Lys-C^{11,12)} with a similar specificity have been isolated from *Achromobacter lyticus* M497-1 and *Lysobacter enzymogens* ssp DSM 1895, respectively, but the enzymatic properties of the latter enzyme have not been reported in detail.

Protease I hydrolyzes Lys-pNA as well as Bz-Lys-p-NA,¹⁾ and further evidence for its exopeptidase activity is that incubation of Lys-Lys with protease I for 48 hr at 37°C resulted in the formation of Lys only (unpublished data). Such exopeptidase activity was also observed for protease Ia, although its specific activity (U/mg) was only 13% that of protease I. On the other hand, endoproteinase Lys-C does not hydrolyze Lys-pNA at all¹²⁾

Like protease I²⁾ and endoproteinase Lys-C,¹²⁾ this enzyme could be classified as a serine protease. However, like protease I, the enzyme was strongly inhibited by Tos-LysCH₂Cl, but little by Tos-ArgCH₂Cl.

The enzyme was similar to protease I in its enzymatic properties, including molecular weight, optimum pH, pH stability, and absence of a requirement for metal ions. However, the enzyme was quite different from protease I in its amino acid composition and isoelectric point, the former enzyme being an acidic protein with a pI 5.3 and the latter a neutral protein with a pI of 6.9. The lower isoelectric point of the enzyme might be due to the higher proportion of acidic amino acid forms than in protease I.

The similarity in molecular weight and the difference in amino acid composition of the

two *Achromobacter* proteases show that protease Ia cannot have been derived from protease I by autolysis. Also, the enzyme was more unstable than protease I in the presence of 7 M urea.

From the above results, we concluded that protease Ia is an enzyme specific for lysyl peptide bonds and distinguishable from protease I.

Acknowledgments. The authors thank Drs. Yoshichika Takamura of Ibaraki University and Masahiko Yoneda of Takeda Chemical Industries (Japan) for their kind support. The authors are also indebted to Dr. Kazuyuki Morihara of Kyoto Research Laboratories, Toho Pharmaceutical Industries (Japan) for kindly supplying Tos-ArgCH₂Cl.

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