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

I. PURIFICATION AND SOME ENZYMATIC PROPERTIES *

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Achromobacter lyticus **M497-1 produces three kinds of alkaline proteases (protease I, II and III) in culture medium along with the bacteriolytic enzyme (Masaki, T., Nakamura, K., Isono, M. and Soejima, M. (1978) Agric. Biol. Chem. 42, 1443–1445). Among these three proteases, *Achromobacter* protease I (EC 3.4.21.-) shows strict splitting for lysine residues at the carboxyl side of the splitting point. This enzyme was purified through a sequence of benzalkonium chloride treatment, acetone fractionation, CM-cellulose and DEAE-cellulose treatment, chromatography on AH-Sepharose 4B and isoelectric focusing method. This form was shown to be homogeneous by polyacrylamide gel electrophoresis and ultracentrifugation analysis. The physicochemical properties of the enzyme were: M_r 30500; partial specific volume ($\bar{v}$), 0.717 ml/g; intrinsic viscosity (η), 0.0385 dl/g; isoelectric point (p*I*) 6.9; and $E^{1\%}_{1cm}$ at 280 nm, 18.77. The enzyme was composed of 294 residues of amino acid per molecule, with glycine as NH_2-terminal and lysine as COOH-terminal amino acids. The optimum pH values with casein, Bz-Lys-pNA and Tos-Lys-OMe were 8.5–10.7, 9.0–9.5 and 7.8–8.2, respectively. The enzyme was inhibited by iPr_2P-F, $PhCH_2SO_2F$ and Tos-LysCH_2Cl but not by Tos-ArgCH_2Cl, EDTA, *o*-phenanthroline and PCMB.**

Introduction

In the previous paper [1], we reported that *Achromobacter* protease I isolated from the culture filtrate was a new type of protease which has strict specificity for splitting the carboxyl side of lysine residues in the synthetic substrates (e.g., Bz-Lys-pNA, Bz-Lys-NH_2 and Tos-Lys-OMe) and glucagon.

This paper describes the purification procedure, and some physicochemical and enzymatic properties of this enzyme.

* This is the second paper in a series. The previous paper is Ref. 1.

Materials and Methods

Materials. *N*-Benzoyl-DL-lysine-*p*-nitroanilide (Bz-Lys-pNA) and *N*-tosyl-L-lysine-chloromethyl ketone (Tos-LysCH_2Cl) were purchased from E. Merck, F.R.G. *N*-Tosyl-L-lysine methyl ester (Tos-Lys-OMe) was from Protein Research Foundation, Japan. *N*-Tosyl-L-arginine chloromethyl ketone (Tos-ArgCH_2Cl) [2] was kindly supplied by Dr. K. Morihara, Shionogi Research Laboratory, Japan. *N*-Tosyl-L-phenylalanine chloromethyl ketone (Tos-PheCH_2Cl) was from Nakarai Chemicals, Ltd., Japan. Diisopropylfluorophosphate (iPr_2P-F) and phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) were from Sigma Co., U.S.A.

Microorganism and culture conditions. Achromo-

bacter lyticus M497-1 [3] was kindly supplied from Microbiological Research Laboratories (Central Research Division, Takeda Chemical Industries, Japan). The microorganism was innoculated into 400 ml nutrient broth (1% sucrose/1% polypeptone/0.5% milk casein/0.01% KH_2PO_4/0.01% K_2HPO_4/0.01% $MgSO_4 \cdot 7H_2O$ in distilled water (pH 7.2)). After cultivation at 28°C for 100 h on a shaker (135 rev./min), the cells were removed by centrifugation, and the supernatant was used as the starting material for enzyme purification.

Enzyme assays. Proteolytic activity was determined at 40°C with 0.7% denatured casein in 50 mM Tris-HCl buffer (pH 9.0) [4]. 1 unit of proteolytic activity was defined as the amount of the enzyme that liberated trichloroacetic acid-soluble hydrolyzate corresponding to 1 μmol tyrosine/min. Amidolytic activity was determined at 405 nm in 170 mM 2-amino-2-methyl-1,3-propanediol buffer (pH 9.0) with 0.25 mM Bz-Lys-pNA [5]. Esterolytic activity was determined at 247 nm in 40 mM Tris-HCl buffer (pH 8.0) with 0.33 mM Tos-Lys-OMe as substrate [6]. 1 unit of amidolytic and esterolytic activity was defined as the amount of the enzyme that hydrolyzed 1 μmol of respective substrates in 1 min at 30°C.

Inhibition studies. The enzyme solutions were preincubated in 40 mM Tris-HCl buffer (pH 8.0) at 30°C for 30 min with various inhibitors, and residual enzyme activities were assayed.

Protein concentration. Protein concentrations at each stage of purification were determined spectrophotometrically at 280 nm assuming the specific extinction coefficient to be $E^{1\%}_{1cm} = 18.77$.

Analytical electrophoresis. Polyacrylamide gel electrophoresis was carried out at pH 4.3 [7].

Molecular weight estimation. The molecular weight of the enzyme was estimated chromatographically [8], using a 2.2 × 98 cm column of Sephadex G-100 in 50 mM Tris-HCl buffer (pH 7.5)/0.1 M NaCl, and electrophoretically [9] with cytochrome *c* (M_r 12400), α-chymotrypsinogen A (25000), egg-albumin (45000) and bovine serum albumin (67000) as standards.

Isoelectric focusing. Electrofocusing was carried out on a column (about 140 ml) containing 0.5% carrier ampholyte with pH ranges from 3.5–10 and from 5–8 (LKB) at 600 V, for 48 h at 4°C.

Ultracentrifugal analysis. The sedimentation coefficient in 50 mM Tris-HCl buffer (pH 8.0) was determined with a Spinco Model E ultracentrifuge at 20°C and 56960 rev./min.

Viscosity measurement. Viscosity was measured with an Ostwald viscometer at 20°C.

Partial specific volume. Partial specific volume was determined by the method of Schachman [10], and by that of Cohn and Edsall [11] based on the amino acid composition.

Amino acid analysis. The purified enzyme was hydrolyzed with 6 N HCl in a sealed, evacuated glass tube at 110°C for 24, 48, 72 and 96 h. The hydrolyzate was analyzed using a Hitachi Model KLB-3B amino acid analyzer. The contents of cystine and cysteine, tryptophan and amide ammonia were determined by the methods of Spencer and Wold [12], Liu and Chang [13] and Chibnall et al. [14], respectively.

NH_2- and COOH-terminal amino acid analysis. NH_2-terminal amino acid was determined qualitatively by the dansyl chloride procedure [15] and quantitatively by the phenylisothiocyanate procedure [16]. The COOH-terminal amino acid was determined by the method of Amber [17] using iPr_2P-F-treated carboxypeptidase B (Sigma).

Purification of the enzyme

Unless otherwise indicated, all steps were carried out in a cold room at 4°C.

Step 1. Benzalkonium chloride treatment. To the culture broth fluid (20.6 l), benzalkonium chloride was added slowly with stirring to give a concentration of 0.04%. After standing overnight at 4°C, the mixture was centrifuged and the supernatant was obtained.

Step 2. Acetone fractionation. To the supernatant (20 l) from Step 1, 3 vol. acetone (precooled to −15°C) were added gradually with stirring. The suspension was centrifuged after standing at −15°C for 12 h. The precipitate was washed twice with cold acetone and dried in vacuo (acetone powder).

Step 3. CM-cellulose treatment. The acetone powder (14 g) was dissolved in 200 ml 10 mM Tris-HCl buffer, pH 8.0 (buffer A) and centrifuged. The supernatant was desalted on Sephadex G-15 and applied to a CM-cellulose column (6 × 46 cm) equilibrated with buffer A. The I and II forms of the alkaline enzymes were not adsorbed under these,

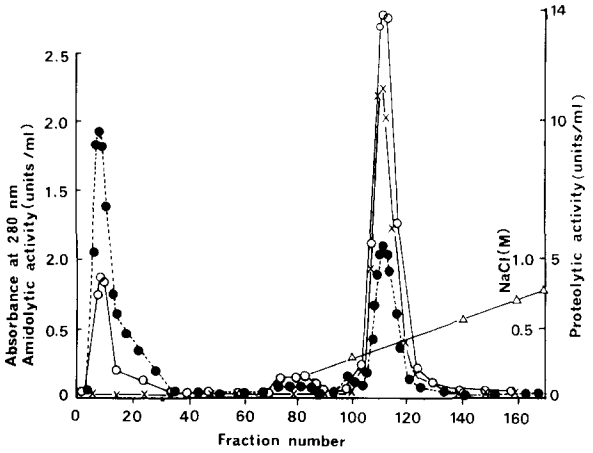

Fig. 1. AH-Sepharose 4B chromatography of protease I from Step 4. Flow volume, 5.1 ml; Flow rate, 20 ml/h. ●------●, absorbance at 280 nm; ○———○, activity against casein; X———X, activity against Bz-Lys-pNA; △———△, NaCl concentration.

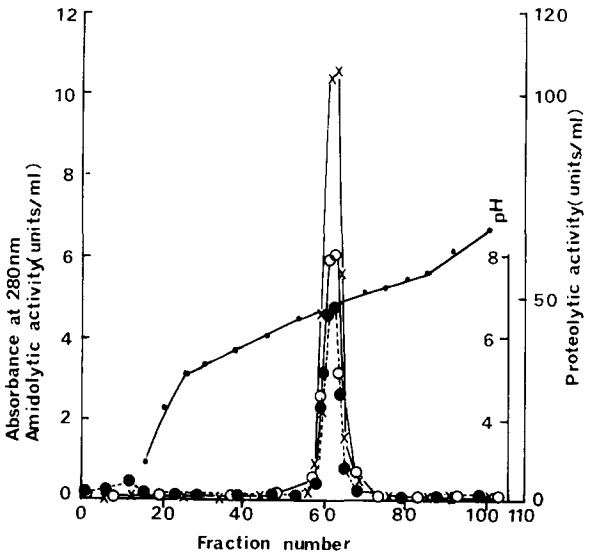

Fig. 2. Refocusing of protease I from step 6. Ampholyte, 0.5% (pH 5–8); Total volume of column, approx. 140 ml; Separate time, 48 h; Voltage, 600 V; Temperature, 4°C. Flow volume, 1.6 ml; ●------●, absorbance at 280 nm; ○———○, activity against casein; X———X, activity against Bz-Lys-pNA; ●———●, pH.

whereas a bacteriolytic enzyme and protease III were tightly bound. The active fractions (protease I and II) were collected and concentrated by Amicon UM-10 ultrafiltration membrane.

Step 4. DEAE-cellulose treatment. The concentrated enzyme solution (1–1) was applied to a DEAE-cellulose column (6 X 35 cm) equilibrated with buffer A. The initial effluent (800 ml) was discarded and the following effluent (3 l) was collected and concentrated. The enzyme solution was dialyzed against 2 mM Tris-HCl buffer, pH 8.0 (buffer B) and reconcentrated. Most of the coloured material was removed by this treatment.

Step 5. AH-Sepharose 4B chromatography. The concentrated enzyme solution (35 ml) from Step 4 was applied to an AH-Sepharose 4B column (1.9 X 20 cm) equilibrated with buffer B. The charged column was washed with buffer B (300 ml) to remove protease II. Then protease I was eluted with a 0–0.8 M NaCl linear gradient in buffer B. The fractions (Nos. 105–120) which were active against casein and Bz-Lys-pNA were collected, dialyzed against buffer B, and then concentrated (Fig. 1).

Step 6. First isoelectric focusing. The sample from Step 5 was processed by the isoelectric focusing (carrier ampholyte, pH 3.5–10), and the active fraction was collected, dialyzed against buffer B and then concentrated.

Step 7. Second isoelectric focusing. A portion (18 ml) of the concentrated sample from Step 6 was again subjected to isoelectric focusing (carrier ampholye, pH 5–8). The small amount of impurity was completely removed after this second isoelectric focusing. As shown in Fig. 2, the isoelectric point of the enzyme was 6.9. The active fraction was dialyzed against buffer B, concentrated, then applied to a Sephadex G-50 column equilibrated with buffer B in order to remove carrier ampholyte [18], and stored at −15°C. The purified enzyme was stable for over 1 year under these conditions.

Results

Purification. The results of purification are summarized in Table I. Final yields of caseinolytic activity and amidolytic activity were 4.1 and 29%, with 100- and 700-fold purification, respectively.

Homogeneity. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis in the absence and presence of SDS, as shown in Fig. 3A and B. Ultracentrifugation analysis of the enzyme also showed a single symmetrical peak, with a sedi-

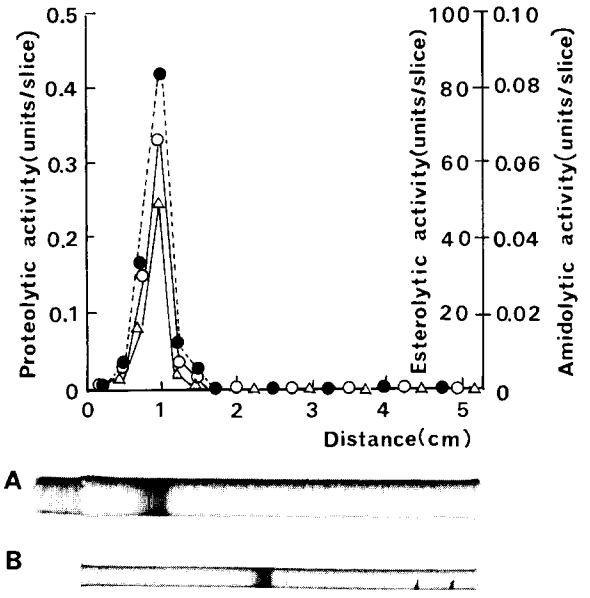

Fig. 3. Polyacrylamide gel electrophoresis of the purified protease I. (A) Electrophoresis at pH 4.3 in a 7.5% gel. A duplicate gel was cut into 0.25 cm sections and the enzyme activity of their extracts was assayed. Applied sample, 67 μg of the purified enzyme; ●-------●, activity against casein; ○———○, activity against Bz-Lys-pNA; △———△, activity against Tos-Lys-OMe. (B) Electrophoresis in the presence of sodium dodecyl sulfate at pH 7.0 in a 10% gel. Applied sample, 50 μg purified enzyme.

mentation coefficient ($s^{\circ}_{20,w}$) of 3.01 S.

Molecular weight. The molecular weight of the enzyme was estimated to be 27 000 by gel filtration on Sephadex G-100, and 30 000 by SDS-polyacrylamide gel electrophoresis. The intrinsic viscosity (η) and partial specific volume ($\bar{v}$) were 0.0385 dl/g and 0.717 ml/g, respectively. From these data, the molecular weight of the enzyme was calculated to be 31 900 according to the Scheraga and Mandelkern equation [19].

Amino acid composition. The amino acid composition of the enzyme is given in Table II. The values are expressed as the number of residues/molecule assuming a molecular weight of 30 000. Six half-cystine residues/molecule were found, but no free sulfhydryl group was detected by Ellman's reaction. These results indicate that the enzyme consists of 294 residues of amino acid per molecule and contains three intramolecular disulphide bridges.

NH_2- and COOH-terminal amino acids. The NH_2-terminal amino acid of the enzyme was glycine, and the COOH-terminal amino acid was lysine. Quantitative determination of the NH_2-terminal glycine showed the value of 0.8 mol/mol protein.

Optimum pH and pH stability. The optimum pH values for amidolytic, esterolytic and caseinolytic activities of the enzyme were 9.0–9.5, 7.8–8.2 and 8.5–10.7, respectively (Fig. 4).

TABLE I

SUMMARY OF PURIFICATION OF PROTEASE I FROM *ACHROMOBACTER LYTICUS* M497-1

Step	Volume (ml)	Total protein (A_{280nm})	Total activity (U) [a]	Specific activity		Recovery (%)
				U/280 nm [a]	U/280 nm [b]	
Culture broth	20 600	245 000	869	0.0036	0.15	100
Benzalkonium chloride treatment	20 000	216 000	843	0.003	0.13	97
Acetone fractionation	500 (14g) [c]	3 180	608	0.16	5.93	70
Sephadex G-15	2 000	3 760	598	0.16	5.93	69
CM-cellulose treatment	1 000	2 170	571	0.26	4.25	66
DEAE-cellulose treatment	105	493	387	0.79	5.88	45
AH-Sepharose 4B chromatography	59	135	359	2.66	14.1	41
1st isoelectric focusing (pH 3.5–10)	53	114	294	2.58	14.0	34
2nd isoelectric focusing (pH 5–8)	26	98.4	254	2.58	14.59	29

[a] Unit of Bz-Lys-pNA hydrolyzing activity.
[b] Unit of casein hydrolyzing activity.
[c] Acetone powder.

TABLE II

AMINO ACID COMPOSITION OF PROTEASE I

Molecular weight calculated from the total integral number of residues is 30 500.

Amino acid	Residues in 30 000	Residues to nearest integer
Lysine [a]	5.57	6
Histidine [a]	5.99	6
Arginine [a]	12.80	13
Asparatic acid [a]	32.55	33
Threonine [a]	25.80 [e]	26
Serine [a]	34.76 [e]	35
Glutamic acid [a]	16.54	17
Proline [a]	15.25	15
Glycine [a]	38.69	39
Alanine [a]	30.76	31
Half-cystine [b]	6.49 [f]	6
Valine [c]	14.88	15
Methionine [c]	3.48	4
Isoleucine [d]	9.60	10
Leucine [a]	14.16	14
Tyrosine [a]	8.84 [e]	9
Phenylalanine [a]	7.50	8
Tryptophan [a]	6.72 [e,g]	7
Amide-NH_3	15.0 [e]	
Total		294

[a] Average of two hydrolysates at 24, 48, 72 and 96 h.
[b] Average of two hydrolysates at 21 h.
[c] Average of two hydrolysates at 48, 72 and 96 h.
[d] Average of two hydrolysates at 72 and 96 h.
[e] Values extrapolated to zero time of hydrolysis.
[f] Determined as cysteic acid after dimethylsulfoxide oxidation [12].
[g] Determined after hydrolysis with *p*-toluenesulfonic acid [13].

The enzyme solution was incubated at various pH values (pH 3.1–12) at 4°C for 24 h and the remaining amidolytic activity was measured at pH 9.0. The enzyme was stable in a pH range of 4.0–11.0.

Optimum temperature and thermal stability. The amidolytic, esterolytic and caseinolytic activities of the enzyme were measured at various temperatures (30–70°C) for 10 min. These three activities showed the same optimum temperature of 50°C.

The enzyme was incubated in 0.1 M Tris-HCl buffer (pH 9.0) at various temperatures for 30 min and the remaining amidolytic activity was measured at the same pH. The enzyme was stable up to 45°C, and completely lost the activity above 60°C for 30 min.

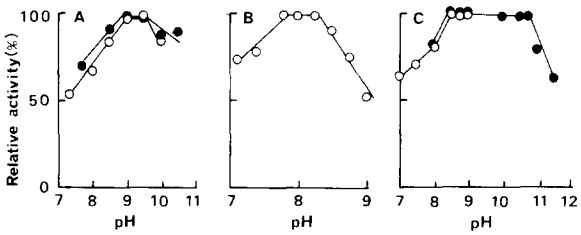

Fig. 4. Effect of pH on the rate of hydrolysis of Bz-Lys-pNA (A), Tos-Lys-OMe (B) and casein (C) by protease I. The activity at each pH was expressed as a percentage of the maximum activity. (A) The reaction mixture contained 0.25 mM Bz-Lys-pNA and 2.04 μg enzyme in a final volume of 3.0 ml. The buffers used were 100 mM Tris-HCl (○———○, pH 7.3–10.0) or 100 mM diol-HCl (●———●, pH 7.7–10.5). (B) The reaction mixture contained 0.33 mM Tos-Lys-OMe and 0.255 μg enzyme in a final volume of 3.0 ml. The buffer used was 40 mM Tris-HCl (○———○, pH 7.1–9.0). (C) The reaction mixture contained 0.7% casein and 0.82 μg of the enzyme in a final volume of 1.2 ml. The buffers used were 60 mM Tris-HCl (○———○, pH 7.0–9.0) or 60 mM borate (●———●, pH 8.0–11.5).

Effects of various metal ions and inhibitors. The effects of various metal ions and inhibitors on the amidolytic and esterolytic activities were examined. Monovalent cations such as Li^+, Na^+, K^+, Rb^+, Cs^+ and NH_4^+, and divalent cation, Ba^{2+}, were competitive inhibitors of the enzyme in order of increasing effectiveness (Table III).

The amidolytic and esterolytic activities were neither inhibited nor stimulated by Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Sr^{2+}, Cd^{2+} and Be^{2+} at 1 mM, or Hg^{2+} at

TABLE III

CONSTANTS FOR THE INHIBITION OF PROTEASE I BY ALKALI METAL IONS AND Ba^{2+}

K_i values were calculated from a Dixon plot of $1/v$ vs. [I] in the presence of different concentrations of subtrate.

Inhibitor	K_i (mM)	
	Bz-Lys-pNA	Tos-Lys-OMe
LiCl	24.0	90.0
NaCl	15.0	86.0
KCl	5.2	45.0
RbCl	3.3	45.0
CsCl	5.5	40.0
NH_4Cl	2.5	28.0
$BaCl_2$	1.6	3.4

TABLE IV

EFFECT OF VARIOUS REAGENTS ON THE HYDROLYSIS OF Bz-Lys-pNA AND Tos-Lys-OMe BY PROTEASE I

0.1 ml enzyme solution was preincubated with 0.1 ml of the reagents in the 80 mM Tris-HCl buffer (pH 8.0) for 30 min, at 30°C. The amounts of the enzyme used for the assay of amidolytic and esterolytic activities were 2.05 μg and 0.255 μg, respectively. Results are expressed as a percentage of the activity in the absence of added reagents.

Reagent	Concentration (mM)	Remaining activity (%)	
		Bz-Lys-pNA	Tos-Lys-OMe
None		100.0	100.0
$ZnCl_2$	1.0	59.8	15.0
EDTA	1.0	103.1	93.5
iPr_2P-F	1.0	39.6	50.8
	10.0	0	0
$PhCH_2SO_2F$	5.0	10.0	0
Tos-$ArgCH_2Cl$	0.1	82.5	88.1
Tos-$LysCH_2Cl$	0.1	0	0
Tos-$PheCH_2Cl$	1.0	98.2	n.d.

n.d. Not determined.

0.1 mM, but clearly inhibited by Zn^{2+} at 1 mM (Table IV). Both activities were inhibited by Tos-$LysCH_2Cl$, iPr_2P-F and $PhCH_2SO_2F$, while little or not at all by thiol reagents, metal-chelating reagents, reducing reagents, Tos-$PheCH_2Cl$ and Tos-$ArgCH_2Cl$ at concentrations of 1 or 0.1 mM (Table IV).

Discussion

Achromobacter protease I prepared according to the procedure in Table I has a high degree of purity as indicated by isoelectric focusing, polyacrylamide gel electrophoresis and ultracentrifugation. Among the seven steps in the purification procedure, DEAE-cellulose treatment (Step 4) and AH-Sepharose 4B chromatography (Step 5) were important. The treatment of the enzyme solution with DEAE-cellulose was effective in removing the viscous substance (coloured impurity) in the solution. AH-Sepharose 4B chromatography was the most effective step, by which the purity of the enzyme was elevated to 700-fold. The main role of this step was to separate protease I from co-existing protease II. This specific separation of the two proteases seems to be based on the principle of affinity chromatography: aminohexyl group of the Sepharose 4B resembles the lysine residue which is strictly specific for the enzyme. The enzyme was inhibited by iPr_2P-F and $PhCH_2SO_2F$, so the enzyme could be classified as a trypsin-like serine protease similar to bovine trypsin (EC 3.4.21.4) and *Streptomyces* protease (EC 3.4.21.-) [20]. Tos-$ArgCH_2Cl$, a specific inhibitor of trypsin [2], had little effect, whereas Tos-$LysCH_2Cl$ had a strongly inhibitory effect on this enzyme. These results reflect the strict substrate specificity of this enzyme for the lysine residue.

The molecular weight (30 500) and isoelectric point (p*I* 6.9) of this enzyme were slightly larger and lower than those of other trypsin-like proteases (bovine trypsin, M_r 24 000, p*I* 10.9; *Streptomyces griseus* protease, M_r 19 000, p*I* 9.0; *Streptomyces fradiae* protease, M_r 16 500, p*I* 9.0) [20,21]. SDS-polyacrylamide gel electrophoresis, NH_2-terminal amino acid and amino acid composition studies indicate that this enzyme consists of a single peptide chain of 294 amino acid residues similar to the other trypsin-like proteases listed above. Bovine trypsin is unstable under alkaline conditions, whereas this enzyme is stable at a wide pH range of pH 4.0–11.0. Unlike bovine trypsin, this enzyme was inhibited by monovalent metal ions, Li^+, Na^+, K^+, Rb^+, Cs^+ and NH_4^+. Some divalent metal ions, Zn^{2+} and Ba^{2+} were also inhibitory, but the others such as Ca^{2+}, Mn^{2+} and Co^{2+} were not. In contrast with this, bovine trypsin is reported by Green and Neurath [22] to be activated by some divalent metal ions such as Ca^{2+}, Mn^{2+}, Co^{2+} and to lesser extent by Ba^{2+}.

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