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Purification and Characterization of an Extracellular Metalloprotease from *Pseudomonas fluorescens*[1]

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An extracellular metalloprotease was purified from the culture supernatant of *Pseudomonas fluorescens* strain KT1 to apparent homogeneity and shown to consist of a single polypeptide chain (M_r 46,000-47,000). The enzyme was strongly inhibited by chelating agents such as EDTA and *o*-phenanthroline, and activated by certain detergents. Among the peptidyl 4-methylcoumaryl-7-amide (MCA) substrates examined, *t*-butyloxycarbonyl-Arg-Val-Arg-Arg-MCA was the best one. With this substrate, the enzyme exhibited a pH optimum of around pH 5.5 in the absence of Co^{2+} ions, whereas it showed two different pH optima (at pHs around 5.5 and 8-9) in the presence of Co^{2+} ions due to remarkable activation by Co^{2+} ions in the alkaline pH range. On the other hand, a single broad pH optimum of around 6 to 8 was obtained with some peptides in both the presence and absence of Co^{2+} ions, and no activation by Co^{2+} was observed. The enzyme showed trypsin-like specificity, preferentially cleaving certain arginyl peptide bonds, and hydrolyzed the basic protein, histone, most rapidly among various proteins examined. Partial amino acid sequence analysis revealed that the enzyme is highly homologous with proteases of the serralysin family, a group of zinc metalloproteases.

Key words: Co^{2+} ion activation, metalloprotease, partial amino acid sequence, *Pseudomonas fluorescens*, sequence homology.

Pseudomonas sp. is ubiquitously distributed in water and soil, and frequently isolated from clinical and environmental specimens. This species produces several proteases and often causes serious problems as a pathogen and/or as a source of food deterioration. Among these proteases, the alkaline metalloprotease from *Pseudomonas aeruginosa* has been most extensively studied (*1*), including determination of its primary (*2, 3*), and tertiary (*4*) structures. Furthermore, the primary structures of the related metalloproteases from *Serratia marcescens* (*5*) and *Erwinia chrysanthemi* (*6, 7*), and the tertiary structure of the *Serratia* enzyme (*8*) have also been reported. These proteases have similar structures and thus are classified as members of the serralysin family, a family of zinc metalloproteases (*9*) different from the thermolysin family. *Pseudomonas fluorescens* also secretes several proteases which may differ among its biotypes (*10*). Because this species is known as the major source of food deterioration, studies on these proteases were performed mainly on their heat stability and production control, their molecular and enzymatic characterization having not been performed in detail (*10, 11*). Furthermore, no information is available about their primary structures. Therefore, it seems interesting to elucidate their properties in detail and to compare them with those of proteases from related species.

As the first step toward this end, we isolated in this study an extracellular metalloprotease from a wild strain of *P. fluorescens*, and investigated its molecular and enzymatic properties, including its partial amino acid sequence. The results indicated that this protease has trypsin-like specificity, preferentially cleaving certain arginyl peptide bonds, and is closely related in structure with the alkaline protease from *P. aeruginosa* and related proteases of the serralysin family.

EXPERIMENTAL PROCEDURES

Materials—Peptide 4-methylcoumaryl-7-amide (MCA) substrates, L-*trans*-epoxysuccinyl-leucylamide-(4-guanido)-

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Abbreviations: BAM-12P, bovine adrenal medulla dodecapeptide; Boc, *t*-butyloxycarbonyl; BPTI, bovine pancreatic trypsin inhibitor; DFP, diisopropylfluorophosphate; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanido)butane; MCA, 4-methylcoumaryl-7-amide; MES, 2-(morpholino)ethane sulfonate; MOPS, 3-(morpholino)propane sulfonate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; TLCK, N^{α}-tosyl-L-lysine chloromethyl ketone; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone.

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butane (E-64), leupeptin, bestatin, chymostatin, bovine adrenal medulla dodecapeptide (BAM-12P), dynorphin A, neurotensin, and α-neoendorphin were purchased from Peptide Institute. Proalbumin(1-10) peptide was synthesized in our laboratory. DEAE-cellulose (DE-52) was purchased from Whatman, and chelating Sepharose 6B, Sephacryl S-200, and a Mono Q (HR5/5) column were from Pharmacia Biotechnology. A BCA protein assay kit was obtained from Pierce. Fluorescamine, histone, bovine serum albumin, ovalbumin, myoglobin, γ-immunoglobulin, pancreatic trypsin inhibitor (Kunitz) (BPTI), soybean trypsin inhibitor (SBTI), diisopropylfluorophosphate (DFP), N^{α}-tosyl-L-phenylalanine chloromethyl ketone (TLCK), N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethanesulfonyl fluoride (PMSF), and *o*-phenanthroline were purchased from Sigma. Lubrol PX, sodium deoxycholate, histone (calf thymus), and EDTA were from Nacalai Tesque, and casein (Hammasten) from Merck. A mixture of molecular weight marker proteins for SDS-polyacrylamide gel electrophoresis (PAGE) was obtained from BioRad. Other reagents used were of the highest grade available.

Bacterial Strain—When a rat liver homogenate prepared in 50 mM triethanolamine-HCl buffer, pH 8.0, containing 0.25 M sucrose was kept standing at room temperature for a few days, a bacterium was found to grow in the homogenate and to produce an extracellular protease hydrolyzing Boc-Arg-Val-Arg-Arg-MCA. This bacterial strain, named KT1, was isolated and identified by the conventional microbiological method.

Determination of taxonomic characteristics: Motility was observed under a light microscope using cells in the logarithmic growth phase grown on nutrient agar. Catalase activity was determined by bubbling in a 33% hydrogen peroxide solution. Oxidase activity was determined by the oxidation of 1% tetramethyl-*p*-phenylenediamine on filter paper. For the determination of other physiological and biochemical characteristics, API (Appareilis et Procedes' Identification; La Balme les Grottes, Montalieu Vercieu, France) 20E was used.

Cellular fatty acid analysis: Cells harvested after culturing for 24 h in nutrient broth were freeze-dried, and then 50 mg of the dried cells was suspended in 10% HCl in methanol and heated at 100°C for 3 h. Fatty acid methyl esters extracted with *n*-hexane were separated by TLC using a solvent system of *n*-hexane/diethyl ether (1 : 1, v/v). Nonpolar, 2-hydroxy (2-OH) and 3-hydroxy (3-OH) fatty acids, visualized by spraying 0.02% dichlorofluorescein in ethanol, were extracted with diethyl ether and analyzed by GC.

DNA base composition analysis: DNA was isolated by the method of Saito and Miura (*12*). The G+C content of DNA was determined by reversed-phase HPLC (*13*) after nuclease P_1 and alkaline phosphatase treatment.

Analysis of isoprenoid quinones: Isoprenoid quinones were extracted twice with chloroform/methanol (2 : 1, v/v) for 4 h, and purified by TLC using *n*-hexane/diethyl ether (85 : 15, v/v) as the solvent, and then analyzed by HPLC.

Enzyme Assay—Enzyme activity toward MCA substrates was determined as described by Tamanoue *et al.* (*14*) with a slight modification. For routine assaying, *t*-butyloxycarbonyl (Boc)-Arg-Val-Arg-Arg-MCA was used exclusively unless otherwise specified. The assay was carried out at 37°C for 30 min in 200 μl of a reaction mixture comprising 0.1 M Tris-HCl buffer (pH 8.0), 0.1 mM MCA substrate, 1 mM $CoCl_2$, and the enzyme. When necessary, 0.1 M 2-(morpholino)ethane sulfonate (MES) buffer (pH 5.5) was used in the absence of Co^{2+} ions. The reaction was stopped by the addition of 1 ml of 0.1 M sodium acetate buffer (pH 4.3) containing 0.1 M monochloroacetic acid. The amount of 7-amino-4-methylcoumarin released was measured with a Hitachi spectrofluorometer at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. One unit of activity was defined as the amount of enzyme releasing 1 pmol of 7-amino-4-methylcoumarin per min.

Kinetic parameters (k_{cat} and K_m values) for the hydrolysis of peptide MCA substrates were determined according to Lineweaver-Burk (*15*) under the standard conditions described above except that the pH, Co^{2+} ion concentration and/or substrates were varied.

Enzyme Purification—All purification procedures were performed at 4°C in the presence of 0.02% NaN_3. *Pseudomonas fluorescens* KT1 was grown in LB broth containing 1 mM $CaCl_2$ at 30°C for 2 days. The culture fluid was obtained by centrifuging the whole culture ($5,000\times g$, 20 min). The supernatant was dialyzed against 10 mM *N*-methyldiethanolamine, pH 9.0 (buffer A), for 8 h and then overnight with one change of buffer A. The dialyzed fraction was centrifuged ($5,000\times g$, 30 min) and then applied to a DE-52 column (3×29 cm) equilibrated with buffer A at a flow rate of 30 ml/h. The column was washed with 2 volumes of buffer A, followed by elution with a linear gradient of NaCl (0-0.25 M) in a total volume of 1,000 ml, 9.0-ml fractions being collected. The active fractions (Nos. 41-49) were pooled and applied to a chelating Sepharose 6B column (1.8×5 cm) equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer B). The column was washed with 0.5 M NaCl, followed by elution with 0.2 M glycine at a flow rate of 30 ml/h, 3.0-ml fractions being collected. The active fractions (Nos. 34-37) were pooled and concentrated to 2 ml by ultrafiltration. An aliquot (1 ml) of the enzyme solution was applied to a Sephacryl S-200 column (1×145 cm) equilibrated with buffer B, and elution was carried out at a flow rate of 6 ml/h, 1.5-ml fractions being collected. The active fractions (Nos. 54-57) were pooled and dialyzed against 0.02 M triethanolamine-HCl buffer, pH 8.0. One-third of the sample was applied to a Mono Q (HR5/5) column equilibrated with 0.02 M triethanolamine-HCl buffer, pH 8.0. Elution was performed with a gradient of NaCl (0 to 0.5 M) in the same buffer (15 ml) at a flow rate of 0.5 ml/min, 0.3-ml fractions being collected. The active fractions were pooled.

Protein Determination—Protein was determined by measuring the absorbance at 280 nm of the sample solution or by the method of Smith *et al.* (*16*) using the BCA reagent with bovine albumin as a standard.

Electrophoresis—SDS-PAGE (12% acrylamide gel) was performed according to Laemmli (*17*), and the gel was stained with Coomassie Brilliant Blue. Electrophoresis under non-reducing conditions was performed at pH 9.5 and 4°C using 4.5% acrylamide gel containing 1 mM $CoCl_2$ and 0.02 mM $ZnCl_2$. The gel was sliced into 1.5-mm pieces, and each piece was immersed in 100 μl of distilled water before freezing and thawing for extraction of the enzyme.

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The supernatant of the gel-containing solution, which had been vortexed and centrifuged, was used for the determination of protease activity at pH 5.5 and 8.0.

Determination of the Molecular Mass—The molecular mass was determined by SDS-PAGE using molecular weight markers (Bio-Rad). It was also determined using a calibrated column of Sephacryl S-200.

Hydrolysis of Peptides—Each peptide (1 nmol) was hydrolyzed with 15 units of enzyme in 50 μl of 0.04 M Tris-HCl buffer, pH 8.0 (or other buffers of different pHs when necessary) at 37°C for 30 min. The digest was immediately analyzed by HPLC on a TSKgel ODS-120T column (0.46×25 cm) in a Hitachi 655A-11 HPLC apparatus. Elution was performed with a gradient of acetonitrile (0 to 50% in 60 min) in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min and monitored at 215 nm. The isolated peptides were hydrolyzed with 6 N HCl at 110°C for 24 h in evacuated sealed tubes and then submitted to amino acid analysis with an Applied Biosystems derivatizer/analyzer (420A/130A-920A).

Hydrolysis of Proteins—Each protein solution (1%) in 0.2 M sodium borate buffer, pH 8.0, was incubated with the enzyme at 37°C for 30 min. The extent of hydrolysis was analyzed with fluorescamine as described previously (*4*).

Thermal Stability Measurement—The enzyme (24 units) was incubated at 43°C in 50 μl of 0.1 M Tris-HCl buffer, pH 8.0, or in 0.1 M MES buffer, pH 5.5, and at appropriate intervals aliquots were removed for assaying with Boc-Arg-Val-Arg-Arg-MCA as a substrate.

Determination of the NH_2-Terminal and Internal Sequences—The NH_2-terminal sequence and internal sequences were analyzed according to Iwamatsu and Yoshida-Kubomura (*18, 19*). The purified enzyme was electroblotted onto a polyvinylidene difluoride membrane after separation by SDS-PAGE. For determination of the NH_2-terminal sequence, the enzyme on the membrane, that had been reduced and *S*-carboxymethylated, was directly submitted to amino acid sequencing with a Shimadzu RSQ-1 gas phase sequencer. On the other hand, for analysis of the internal sequences, the reduced and *S*-carboxymethylated enzyme on the membrane was further treated with *Achromobacter* protease I (lysylendopeptidase). The released proteolytic fragments were separated by HPLC on a μ-Bondasphere 5 μ C8-300 A column (2.2×150 cm; Waters) (data not shown) and then submitted to amino acid sequencing.

TABLE I. **Taxonomic characteristics of strain KT1.**

Characteristic	Strain KT1	*Pseudomonas aeruginosa* IAM 1514^T
Gram stain	Negative	Negative
Motility	Motile	Motile
Isoprenoid quinone	Q-9	Q-9
Cellular fatty acids[a]:		
non-polar	16:0, 17:0	16:0, 16:1
2-hydroxy	2-OH 12:0	2-OH 12:0
3-hydroxy	3-OH 10:0	3-OH 10:0
G+C content of DNA (mol%)	60.4	67.2
Formation of:		
oxidase	+[b]	+
catalase	+	+
L-arginine dihydrolase	+	+
L-lysine decarboxylase	−	−
L-ornithine decarboxylase	−	−
L-tryptophan deaminase	−	−
urease	−	−
indole	−	−
Hydrolysis of:		
o-nitrophenol	−	−
Utilization of citrate	+	+
H_2S formation	−	−
Voges-Proskauer reaction	−	+
Liquefaction of gelatin	+	+
Acid formation from:		
glucose	+	+
mannitol	−	−
inositol	−	−
sorbitol	−	−
L-rhamnose	+w	−
saccharose	−	−
melibiose	+	+w
amygdalin	−	−
L-arabinose	+	+
Nitrate reduction	−	−
Growth at: 16:0,		
4°C	+	−
41°C	−	+

^{T}, type strain. [a]16:0, hexadecanoic acid; 16:1, hexadecenoic acid; 17:0, heptadecanoic acid; 2-OH 12:0, 2-hydroxy dodecanoic acid; 3-OH 10:0, 3-hydroxy decanoic acid; 3-OH 12:0, 3-hydroxy dodecanoic acid. [b]+, positive; −, negative; +w, weakly positive.

RESULTS AND DISCUSSION

Identification of Pseudomonas Strain KT1—Table I shows the taxonomic characteristics of strain KT1. Based on the morphological, biochemical, and chemotaxonomic characteristics thus determined, the isolate, KT1, was identified as *Pseudomonas fluorescens* according to Bergey's Manual of Systematic Bacteriology (*20*).

Purification of a Protease from P. fluorescens—The results of purification of a protease from the culture supernatant of *P. fluorescens* strain KT1 are shown in Table II. On DE-52 column chromatography the protease activity was eluted as a single peak at about 0.12 M NaCl. Upon subsequent chromatography on chelating Sepharose 6B with Zn^{2+} as a ligand, the activity was eluted at 0.2 M glycine. The enzyme was further purified by chromatography on a Sephacryl S-200 column, followed by chromatography on a Mono Q column. The protein and activity peaks coincided well with each other. Through these procedures, the enzyme was purified about 700-fold in a 6% yield (Table II), and only a single protein band was detected on SDS-PAGE (Fig. 1a). The molecular mass of the purified protease was estimated to be approximately 47,000 Da by SDS-PAGE under both reducing and non-reducing condi-

TABLE II. **Purification of the protease from the culture supernatant of *Pseudomonas fluorescens* KT1.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Culture supernatant	1,440	823,500	572	1	100
DE-52	24.8	569,000	22,900	40	69
Chelating Sepharose	1.0	154,000	154,000	270	19
Sephacryl S-200	0.20	57,900	289,000	499	7.0
Mono Q	0.12	47,800	398,000	696	5.8

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tions, indicating that the enzyme is composed of a single polypeptide chain. It was also estimated by gel filtration on a column of Sephacryl S-200 to be around 46,000 Da (data not shown).

Enzymatic Properties of the Purified Protease—The activity at pH 8.0 toward Boc-Arg-Val-Arg-Arg-MCA was found to be significantly affected by Co^{2+} ions as shown in Fig. 2a. In the absence of Co^{2+} ions, the enzyme exhibited a pH optimum of pH 5.5. On the other hand, the activity at around pH 8.0 was increased markedly at above 0.1 mM Co^{2+}ions and maximally an about 6-fold increase in activity was observed at around 1 mM Co^{2+} ions, whereas the activity at around pH 5.5 was not affected so much by 1 mM Co^{2+} ions. Thus, in the presence of 1 mM Co^{2+} ions, pH optima were observed at both pH 8.0 and 5.5 (Fig. 2b). Such activation did not occur with Ca^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} ions.

Figure 1b shows the native PAGE profile of the enzyme, the activity being measured at pH 8.0 in the presence of 1 mM Co^{2+} ions and at pH 5.5 in the absence of Co^{2+} ions. The electrophoretic mobility/activity profiles at the two pH values coincided well with each other. The two activities also overlapped upon chromatography on a Superose 12 column, and showed essentially the same thermal stability profile; both activities were lost in parallel and completely at 43°C in 30 min (data not shown). Therefore, we conclude that the activities at both pH 8.0 and 5.5 are due to the same enzyme. The relatively low thermal stability of the enzyme is interesting considering the fact that *P. fluorescens* KT1 cannot grow at 41°C but can grow at 4°C, whereas *P. aeruginosa* can grow at 41°C but not at 4°C.

The protease activity toward various MCA substrates was determined at pH 8.0 in the presence or absence of 1 mM Co^{2+} ions and at pH 5.5 in the absence of Co^{2+} ions, similar activity profiles being obtained (Table III). Among the MCA substrates examined, Boc-Arg-Val-Arg-Arg-MCA was the best one. Boc-Val-Leu-Lys-MCA, a sensitive substrate for plasmin, was also hydrolyzed well. Although the substrate specificity of the protease was not so clear in this analysis, the basic residue, arginine and lysine, at the P_1 and P_2 sites seemed to be favorable for cleavage [designation according to Schechter and Berger (*22*)]. Since Boc-Arg-Val-Arg-Arg-MCA was hydrolyzed most rapidly, the arginine residue at the P_4 site may also contribute to the rate of hydrolysis by the enzyme. Previously, the substrate specificity of the metalloprotease of *Serratia marcescens*, a member of the serralysin family, toward some peptide MCA-substrates was examined (*21*). The results were partially similar to those obtained in the present study; however, the *Serratia* protease was reported to hydrolyze Boc-Phe-Ser-Arg-MCA and Boc-Val-Pro-Arg-MCA fairly well, which were scarcely hydrolyzed by the present enzyme. The k_{cat} and K_m values toward Boc-Arg-Val-Arg-Arg-MCA and Boc-Val-Leu-Lys-MCA were estimated at pH 8.0 and/or 5.5 (Table IV). At pH 8.0 in the presence of 1 mM Co^{2+} ions, the k_{cat} values were increased

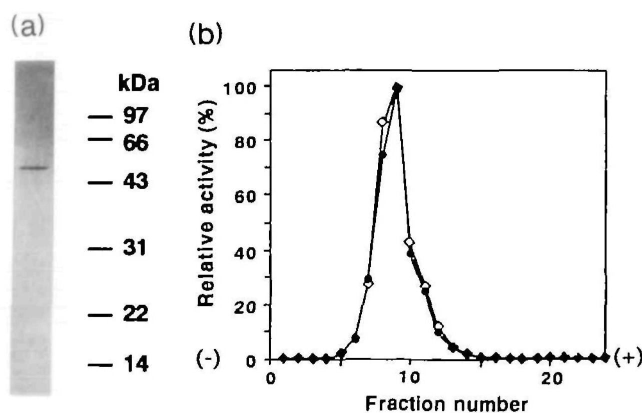

Fig. 1. **Polyacrylamide gel electrophoreses (PAGE) of the purified enzyme.** (a) SDS-PAGE. Slab gel electrophoresis was performed by the method of Laemmli (*17*), and the gel was stained with Coomassie Brilliant Blue. (b) Native PAGE. Electrophoresis (4.5% gel) was performed at pH 9.5. The gel was sliced into 24 pieces, and the enzyme was extracted by repeated freezing and thawing, and then assayed. The activity at pH 8.0 was assayed in 0.1 M Tris-HCl buffer with 1 mM Co^{2+} ions (●), and that at pH 5.5 in 0.1 M MES-NaOH buffer without Co^{2+} ions (◇).

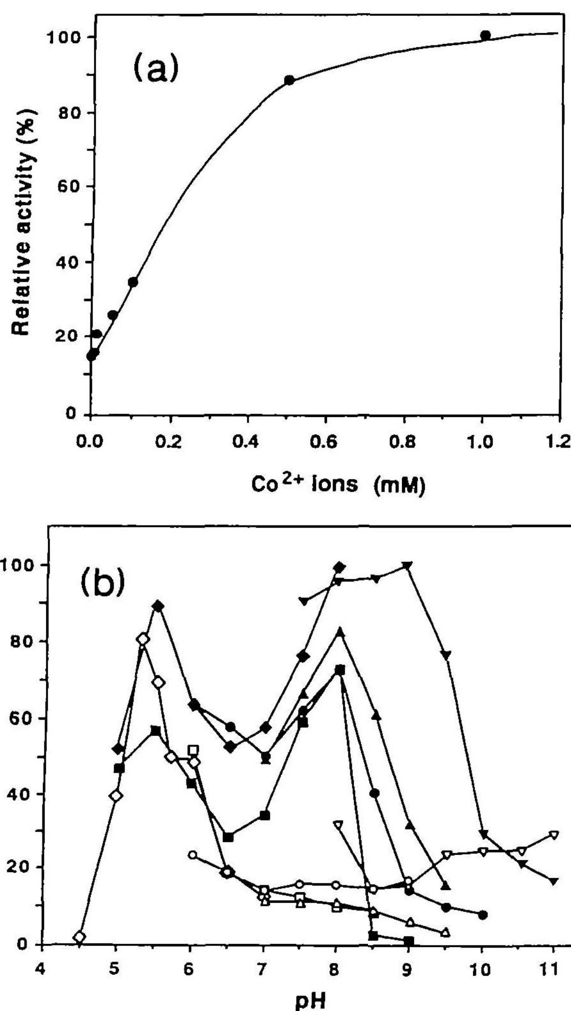

Fig. 2. **Effects of the Co^{2+} ion concentration on the activity at pH 8.0 (a) and pH/activity profiles (b).** The activity was determined in 0.1 M buffer with Boc-Arg-Val-Arg-Arg-MCA as a substrate. (a) Activity was determined at various concentrations of Co^{2+} ions. At 10 mM Co^{2+} ions, the activity was 82% of that at 1 mM Co^{2+} ions (data not shown). (b) The activity was determined in the presence and absence of 1 mM Co^{2+} ions at various pHs. ●, Tris-HCl+Co^{2+}; ○, Tris-HCl; ▲, triethanolamine-HCl +Co^{2+}; △, triethanolamine-HCl; ◆, MES-NaOH+Co^{2+}; ◇, MES-NaOH; ■, MOPS-NaOH+Co^{2+}; □, MOPS-NaOH; ▼, borate-NaOH+Co^{2+}; ▽, borate-NaOH.

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several fold as compared with those obtained in the absence of Co^{2+} ions, whereas the K_m values changed to a lesser extent. Therefore, the activation by Co^{2+} ions at pH 8.0 is largely due to the increase in the k_{cat} value. On the other hand, at pH 5.5, these values did not change significantly in the presence or absence of Co^{2+} ions. In this connection, it is interesting to note that Morihara and Tsuzuki (*23*) previously reported marked activation by Co^{2+} ions at pH 7.0 of *P. aeruginosa* alkaline metalloprotease as to benzyl-

TABLE III. **Activity toward peptide MCA substrates.**

Substrate	Relative activity (%)		
	pH 8.0		pH 5.5
	$+Co^{2+}$ (1 mM)	$-Co^{2+}$	$-Co^{2+}$
Boc-Arg-Val-Arg-Arg-MCA	100	100	100
Boc-Val-Leu-Lys-MCA	44	39	28
Boc-Gln-Arg-Arg-MCA	35	49	19
Boc-Gly-Lys-Arg-MCA	25	24	13
Boc-Gly-Arg-Arg-MCA	21	21	7.2
Boc-Gln-Ala-Arg-MCA	14	17	nd
Boc-Leu-Ser-Thr-Arg-MCA	3.7	5.7	1.1
Boc-Leu-Lys-Arg-MCA	3.3	2.6	2.0
Boc-Glu-Lys-Lys-MCA	2.9	0	1.2
Boc-Leu-Arg-Arg-MCA	2.5	1.3	1.7
Boc-Ala-Gly-Pro-Arg-MCA	1.3	nd	1.3
Boc-Phe-Ser-Arg-MCA	0.8	0.8	0.2
Boc-Leu-Leu-Val-Tyr-MCA	0.7	nd	0
Boc-Gln-Gly-Arg-MCA	0.6	nd	0.3
Boc-Ile-Glu-Gly-Arg-MCA	0.4	nd	0.5
Boc-Leu-Thr-Arg-MCA	0.2	1.0	0
Boc-Val-Pro-Arg-MCA	0.2	nd	0.1
Boc-Leu-Gly-Arg-MCA	0	nd	0.7
Suc-Gly-Pro-MCA	0	nd	1.0
Suc-Ala-Ala-Pro-Phe-MCA	0	nd	0.3

nd, not determined; suc, succinyl.

TABLE IV. **Kinetic parameters for the hydrolysis of peptide MCA substrates.**

Substrate	pH	Co^{2+} (1 mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
Boc-Arg-Val-Arg-Arg-MCA	8.0	+	0.53	0.035	15.1
Boc Arg-Val-Arg-Arg-MCA	8.0	−	0.064	0.015	4.2
Boc-Arg-Val-Arg-Arg-MCA	5.5	+	0.39	0.023	16.9
Boc-Arg-Val-Arg-Arg-MCA	5.5	−	0.46	0.023	20.1
Boc-Val-Leu-Lys-MCA	8.0	+	0.11	0.023	4.6
Boc-Val-Leu-Lys-MCA	8.0	−	0.046	0.016	2.9

DYNORPHIN Tyr-Gly-Gly-Phe-Leu-Arg-Arg-▼-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln

BAM-12P Tyr-Gly-Gly-Phe-Met-Arg-Arg-▼-Val-Gly-Arg-Pro-Glu

NEUROTENSIN <Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-▼-Arg-Pro-Tyr-Ile-Leu

PROALBUMIN (1-10) PEPTIDE Arg-Gly-Val-Phe-Arg-Arg-▼-Asp-Ala-His-Lys

α-NEOENDORPHIN Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys (not cleaved)

Fig. 3. **The sites of cleavage of some peptides by the enzyme.** Each peptide was hydrolyzed with the enzyme at pH 8.0 in both the presence and absence of 1 mM Co^{2+} ions. The digest was analyzed as described under "MATERIALS AND METHODS". Arrowheads indicate the sites of cleavage. <Glu, pyroglutamic acid residue.

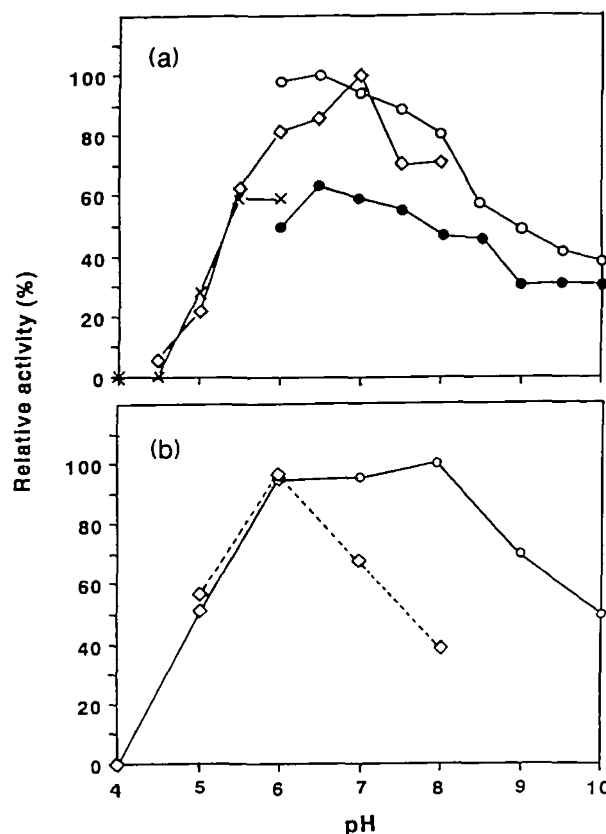

Fig. 4. **pH-rate profiles of the hydrolysis of peptides by the enzyme.** (a) Dynorphin A, (b) BAM-12P (——), and proalbumin (1-10) peptide (---). 0.04 M buffers were used: ●, Tris-HCl+0.1 mM Co^{2+} ions; ○, Tris-HCl; ◇, MES-NaOH; ×, sodium acetate. The maximal activity was taken as 100%.

TABLE V. **Effects of protease inhibitors on the activity.** The enzyme (15 ng) was preincubated with each inhibitor at the given concentration in 0.5 ml of 0.1 M Tris-HCl buffer, pH 8.0, or 0.1 M MES-NaOH buffer, pH 5.5, at room temperature for 1 h, and then the activity toward Boc-Arg-Val-Arg-Arg-MCA as a substrate was measured at the same pH.

Inhibitor	Concentration (mM)	Relative activity (%)		
		pH 8.0		pH 5.5
		$+Co^{2+}$ (1 mM)	$-Co^{2+}$	$-Co^{2+}$
None		100	100	100
EDTA	10	0.5	0	0.1
o-Phenanthroline	10	0.8	9	9.6
Phosphoramidone	0.1	100	74	74
DFP	1.0	88	109	91
PMSF	1.0	83	84	86
E-64	0.1	74	98	84
N-Ethylmaleimide	0.1	86	nd	nd
TLCK	0.1	nd	101	nd
TPCK	0.1	nd	82	nd
Chymostatin	0.1	nd	82	nd
Leupeptin	0.1	90	93	nd
Elastatinal	0.1	nd	89	nd
Bestatin	0.1	nd	107	nd
BPTI	0.01	nd	100	95
SBTI	0.01	nd	100	91

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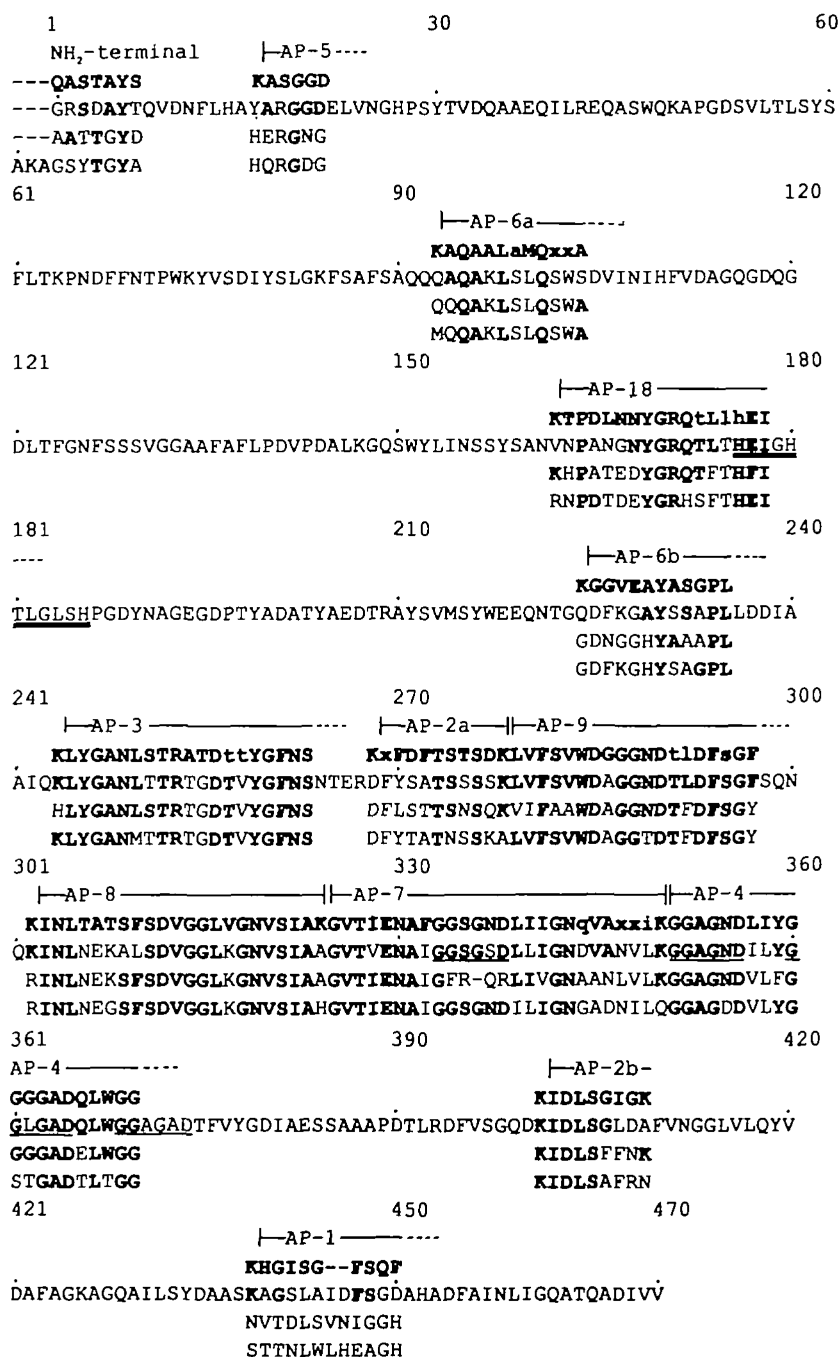

Fig. 5. **Comparison of the partial amino acid sequence of the protease with the sequences of some serralysin proteases.** The sequences are aligned as four lines using the one letter notation, the residues in the present enzyme and the residues identical with them being shown in bold face. First line: the partial sequences of the present protease; second line: the complete sequence of *Pseudomonas aeruginosa* alkaline protease (*2, 3*); third line: *Serratia marcescens* protease (*5*); and fourth line: *Erwinia chrysanthemi* protease B (*6, 7*). The peptides derived from the present enzyme are numbered in the order of elution on HPLC (data not shown) except for AP-18, which was obtained under different HPLC conditions. AP stands for *Achromobacter* lysylendopeptidase. Small letters indicate residues not fully identified. Dashes indicate gaps introduced to maximize the homology. Each peptide was partially sequenced from the NH_2-terminus except for AP-8, AP-7, and AP-2b, which were sequenced completely. Each peptide was assumed to be preceded by a lysine residue from the specificity of lysylendopeptidase. For the *Pseudomonas aeruginosa* protease, the zinc binding motif and calcium binding sites are indicated by double and single underlines, respectively. For the *Serratia* and *Erwinia* proteases, only the sequences corresponding to the partial sequences of the present enzyme are shown.

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oxycarbonyl-Ala-Phe-Gly-Ala as a substrate; however, they found that the activation was mainly due to a decrease in the K_m value rather than an increase in the k_{cat} value.

Figure 3 shows the cleavage sites of some peptides at pH 8.0. The same results were obtained in both the presence and absence of 1 mM Co^{2+} ions. Although the peptides used contained several sites which are thought to be potentially susceptible to the enzyme, only the Arg-Ile bond in dynorphin, the Arg-Val bond in BAM-12P, the Arg-Arg bond in neurotensin, and the Arg-Asp bond in proalbumin(1-10) peptide were cleaved specifically, whereas no cleavage of α-neoendorphin occurred under the conditions used. These results seem to indicate that the enzyme preferentially hydrolyzes certain Arg-X bonds. It may be noteworthy that the cleavage occurred at the sites of paired arginine residues. These results are largely consistent with those obtained with the MCA substrates.

The pH-rate profiles of the enzyme toward dynorphin A, BAM-12P and proalbumin(1-10) peptide are shown in Fig. 4. Contrary to the results obtained with the peptide MCA substrates (Fig. 2b), the enzyme showed a broad pH/rate profile with a single pH optimum at around pH 6-8 in both the presence and absence of Co^{2+} ions. No Co^{2+} ion activation was observed and the activity was even reduced in the presence of 1 mM Co^{2+} ions, as shown for dynorphin A (Fig. 4a). Taken together, therefore, the apparently anomalous

pH-rate profile and Co^{2+} ion activation observed with Boc-Arg-Val-Arg-Arg-MCA may be a specific phenomenon due to the use of these small synthetic substrates.

Among the various proteins examined as substrates, the enzyme hydrolyzed histone most rapidly at pH 8.0 in the presence or absence of Co^{2+} ions. The relative rates of hydrolysis obtained under the conditions used were in the following order: histone (100), myoglobin (39), bovine serum albumin (11), casein (8.9), ovalbumin (1.2), and γ-immunoglobulin (0). The high activity toward histone is consistent wth the trypsin-like specificity of the enzyme found with peptide substrates.

The inhibition profile of the protease with various protease inhibitors was examined at pH 8.0 in the presence or absence of Co^{2+} ions and at pH 5.5 in the absence of Co^{2+} ions, and the results were essentially the same, as shown in Table V. The protease was strongly inhibited by typical metal chelators such as EDTA and *o*-phenanthroline, which indicates that it is a metalloprotease. The activity lost with these metal chelators was restored partially (about 60% for each) on the addition of 1 mM Zn^{2+} or Co^{2+} ions after removal of the chelator. Various inhibitors for serine and cysteine proteases failed to inhibit the enzyme. On the other hand, the protease was found to be markedly activated by detergents such as lubrol and sodium deoxycholate in both the presence and absence of Co^{2+} ions; nearly three-fold activation was observed at detergent concentrations of 10^{-3} to 1 mM (data not shown). The reason for this activation is not certain.

NH_2-Terminal and Internal Amino Acid Sequences—The NH_2-terminal part of the protease and its internal sequences determined from the various fragments obtained on lysylendopeptidase digestion are compared with the sequences of three serralysin family proteases: *P. aeruginosa* alkaline protease (*2, 3*), *S. marcescens* protease (*5*), and *E. chrysanthemi* protease (protease B) (*6*) in Fig. 5. Peptide AP-18 appears to include the first three residues of the zinc binding motif (His-Glu-X-X-His-X-X-Gly-X-X-His) (*4, 9, 24*) of serralysin proteases. Three repeats of a typical calcium binding motif (Gly-Gly-X-Gly-X-Asp) were found in the proteolytic fragments of the present enzyme. Overall identities of the partial sequences (total 193 residues) thus determined were 65, 55, and 53% with the corresponding sequences of the metalloproteases from *P. aeruginosa*, *S. marcescens*, and *E. crysanthemi*, respectively. Therefore, the present enzyme is suggested to belong to this protease family, although the complete amino acid sequence and the zinc content of the present enzyme remain to be determined. Based on these data, an attempt to clone the gene for the present enzyme is underway.

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