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Purification and Characterization of Four Proteases from a Clinical Isolate of *Serratia marcescens* kums 3958

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Four distinct proteases were purified to homogeneity from culture filtrates of *Serratia marcescens* kums 3958, a fresh isolate from a patient with a severe corneal ulcer. Purification was achieved by ammonium sulfate precipitation, DEAE-cellulose ion-exchange chromatography, and Sephadex gel filtration chromatography. The proteases were differentiated from each other by polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate and by immunodiffusion in agarose gels. The molecular weights of these purified proteases were estimated to be 56×10^3, 60×10^3, and 73×10^3 (hereafter designated 56K, 60K, and 73K proteases, respectively). The 73K protease was separated into 73Ka and 73Kb upon isoelectricfocusing. The isoelectric points of the 56K (major) and 60K, 73Ka, and 73Kb proteases (minors) were approximately 5.3, 4.4, 5.8, and 7.3, respectively. Both 56K and 60K enzymes were completely inactivated by EDTA at pH 5.0 and were reactivated by zinc ion; thus, they are metalloenzymes, whereas 73K (73Ka and 73Kb) enzymes appear to be thiol proteases. Carbohydrate, cysteine, and cystine were not detected in the 56K and 60K proteases. Amino acid compositions, partial amino acid sequence, and enzymological and immunological properties revealed that these four enzymes are distinct from each other.

Isolations of multiply drug-resistant *Serratia* sp. from various disease sites suggest that *Serratia marcescens* is more important than previously realized as a potential pathogen, especially to immunologically impaired patients or those undergoing steroid therapy. Many of these isolates do not form pigment and are resistant to various antibiotics, including cephalosporins. At the Kumamoto University Medical School Hospital, about 85% of the isolates from eye infections showed multiple drug resistance. Since 1974 we have observed many cases of corneal ulcers and one of panophthalmitis due to nonpigmented *S. marcescens*.

The mechanism of ulcer formation as related to the serratial infection is not fully understood. We envisaged a potential role of the protease produced by this organism because proteolytic destruction of corneal tissue may be a cause of the corneal lesion. A number of strains of *S. marcescens* have been reported to produce one to four proteases (8). Most of them were nonclinical isolates. One extensively studied serratial peptidase was produced by *Serratia piscatorum* isolated from a silkworm (14). Furthermore, most of the investigations were limited to the main (major) protease, and little is known about the minor proteases.

The present report concerns the isolation and characterization of the major and minor proteases and comparison of presently and previously known proteases.

MATERIALS AND METHODS

Bacteria and culture. The strain of *S. marcescens* kums 3958 used in this study was isolated from a patient with a severe corneal ulcer. The strain produced no pigment and the serotype was O-5.

Chemicals and inhibitors. Leupeptin and antipain were gifts from Matsushima and Umezawa of Tokyo. Phenylmethylsulfonyl fluoride was obtained from Calbiochem, Los Angeles, Calif. Fluorescein isothiocyanate I (FITC), EDTA, and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA) were from Dojindo Laboratories, Kumamoto, Japan. Dansyl chloride and diisopropyl fluorophosphate were from Sigma Chemical Co., St. Louis, Mo. Polyamide sheets and the silica gel thin layer were from Chen Ching Trading Co. Ltd., Taipei, Taiwan, and Wako Pure Chemical Co., Osaka, Japan, respectively. All other chemicals were from commercial sources.

Purification. (i) Culture and ammonium sulfate precipitation. A 0.5-ml sample of a stationary-phase culture of the organism was introduced into and cultured in 250 ml of tryptosoy broth (Eiken Chemical, Tokyo, Japan) in a 1-liter flask, with reciprocal shaking at 1.75 Hz and 30°C for 36 h. A 3-liter sample of the culture was centrifuged at $8,000 \times g$ for 30 min followed by filtration through a membrane filter (0.22 μm), both at 4°C. Ammonium sulfate was added slowly to the supernatant, with gentle stirring, to a final concentration of 90% saturation. After standing for 24 h at 4°C, the precipitates were collected by centrifugation at $8,000 \times g$ for 45 min, dissolved in about 500 ml of cooled distilled water, and dialyzed for 24 h with 15 liters of distilled water and finally twice with 15 liters of 10 mM Tris hydrochloride buffer (pH 8.3).

(ii) Column chromatography. The dialysate (630 ml) was applied to a column (4 by 38 cm) of DEAE-cellulose (DE52) equilibrated with 10 mM Tris hydrochloride buffer (pH 8.3) and eluted with 1 liter of 10 mM Tris hydrochloride buffer (pH 8.3) at a flow rate of 125 ml/h. A linear gradient consisting of 2 liters of 10 mM Tris hydrochloride buffer (pH 8.3) and 2 liters of the same buffer with 0.3 M NaCl was then used.

Eluate (5.0-ml fractions) was collected for the measurement of absorbance at 280 nm and enzyme activity. The active fractions were pooled, dialyzed against distilled water at 4°C, and lyophilized. The lyophilized preparations were applied to a column (4.0 by 68 cm) of Sephadex G-100 equilibrated and eluted with 10 mM sodium phosphate buffer (pH 6.8). The active fractions were pooled and applied to a column (2.2 by 30 cm) of DEAE-cellulose equilibrated with

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10 mM sodium phosphate buffer (pH 6.8) for rechromatography. The column was eluted with 600 ml of 10 mM phosphate buffer (pH 6.8) at a flow rate of 40 ml/h, after which a linear gradient consisting of 400 ml of 10 mM sodium phosphate buffer (pH 6.8) and 400 ml of the same buffer with 0.2 M NaCl was used. Fractions (3.9 ml) of the eluate were collected similarly and dialyzed with distilled water followed by lyophilization. The lyophilized powders were stored at −70°C until used.

Protease assay. Protease activity was measured based on the fluorescence polarization (FP) value by using FITC-labeled gelatin as a substrate (11). The final concentration of the substrate was about 100 nM of the fluorescein equivalent. Portions of the chromatography fractions were placed in cuvettes containing the substrate in 2.0 ml of 50 mM Tris hydrochloride buffer (pH 8.0), and FP values were measured automatically at 30°C by a fluorescence spectropolarimeter (Japan Immunoresearch Inc., Co., Takasaki, Japan; also sold by Meloy Laboratories in the United States). Since the FP value reflects the molecular volume, any decrease in FP value indicates decreased molecular weight due to proteolysis.

Determination of pH optimum. Protease preparations (56×10^3, 60×10^3, and 73×10^3 [hereafter designated 56K, 60K, and 73K, respectively]; 10, 10, and 2 μg in 10 μl, respectively) were added to 0.5 ml of the substrate solution in the following buffers: pH 2.0 to 3.0, 0.1 M KCl-HCl; pH 3.5 to 5.5, 0.1 M sodium acetate; pH 5.5 to 8.5, 0.1 M sodium phosphate, and 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–NaOH; pH 7.5 to 9.0, 0.1 M Tris hydrochloride; pH 9.0 to 11.5, 0.1 M sodium carbonate-sodium bicarbonate. After preparations were incubated at 30°C for 5 min, a 100-μl portion of the reaction mixture was placed in a cuvette containing 1.8 ml of 0.1 M Tris hydrochloride buffer (pH 8.0), and the FP value was measured at 30°C.

Heat and pH stability. To determine heat inactivation, both 56K and 60K protease preparations (40 μg/ml) were incubated at 37, 45, 50, 60, and 65°C for 60 min in 0.1 M Tris hydrochloride buffer (pH 8.0). A portion (20 μl) was then removed for the enzyme assay at pH 8.0. To test pH stability, the purified 56K and 60K enzymes were dissolved in various buffers as described above at 0.2 mg/ml and incubated for 24 h at 37°C. Enzyme activity was then determined as described above.

Effects of metal ions and protease inhibitors. These effects were tested at two different pH values. To test the effect at pH 8.0, purified proteases were added to 50 mM Tris hydrochloride buffer to a concentration of 1.5 μM (56K and 60K proteases) or 225 nM (73K protease) and the following substances were added to each solution to give a concentration of 8.3 mM: $MgSO_4$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $CoCl_2$, $Cd(CH_3COO)_2 \cdot 2H_2O$, $FeSO_4$, $HgCl_2$, and $ZnCl_2$ (metal ions); leupeptin, diisopropylfluorophosphate, and phenylmethylsulfonyl fluoride (serine protease inhibitors); mercaptoethanol, reduced glutathione, L-cysteine, dithiothreitol, antipain (papain inhibitor), iodoacetic acid, and *N*-ethylmaleimide (sulfhydryl reagents); EDTA and EGTA (chelating agents). These mixtures were incubated at 30°C for 20 min. Subsequently, 50 μl of each mixture was added to the assay mixture for FP as described. To test the effect at pH 5.0, the proteases purified above were added to 50 mM acetate buffer to a concentration of 340 nM (56K and 60K proteases) or 52 nM (73K protease). The same test chemicals described above were added to the reaction mixtures to give a final concentration of 1.92 mM. Mixtures were incubated at 30°C for 20 min, and 10 μl of FITC-labeled gelatin was added and incubated at 30°C for 5 min. Subsequently, 0.1 ml of each reaction mixture was added to 1.8 ml of 50 mM Tris hydrochloride buffer (pH 8.0) for measurement of the FP value.

Reactivation of the EDTA-inactivated proteases. Both of the metalloproteases (56K and 60K) at 350 nM in 0.5 ml of acetate buffer (pH 5.0) were inactivated with 2 mM EDTA by incubation at 30°C for 20 min. To this inactivated enzyme solution, the metal ions noted above were added to yield a final concentration of 2 mM. Each of these mixtures was further incubated at 30°C for 20 min, and their restored enzyme activities were measured.

Isoelectric focusing. Isoelectrofocusing in a sucrose density gradient was carried out at 4°C for approximately 48 h in a 35-ml column (1 by 40 cm) between pH 3.5 and 10 with a final potential at 750 V (19). The gradient was prepared with a low-density solution of 15.8 ml of water and 0.2 ml of 40% (wt/vol) ampholine (pH 3.5 to 10; LKB Instruments, Inc., Rockville, Md.) and a high-density solution of 15.4 ml of 50% (wt/vol) sucrose and 0.6 ml of 40% ampholine (pH 3.5 to 10). The pH of each 0.5-ml fraction was determined at 4°C, and then the fraction was assayed for protease activity.

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Davis (3), except that the sample solutions were applied directly in 60% glycerol. Gels (7.5% in acrylamide) were stained with 0.04% Coomassie brilliant blue and were destained with 7% acetic acid.

Molecular weight estimation by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. The purified proteases and various reference proteins were denatured by boiling for 2 min in 2% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol and were then subjected to gel electrophoresis as described above in the gel containing 0.1% SDS. The molecular weights of the samples were estimated by their relative mobility as described by Weber et al. (20).

Amino acid, sulfhydryl, and carbohydrate analyses. Amino acid analyses were performed by the method of Spackman et al. (17). Samples (ca. 0.1 to 0.3 mg) of the purified enzymes were hydrolyzed for 24, 48, and 72 h at 110°C with 0.2 ml of 6 M HCl in sealed Pyrex tubes in vacuo. The hydrolysates were dried in vacuo, and amino acid analyses were performed. The tryptophan content was estimated by *p*-dimethylaminobenzaldehyde (18) and by magnetic circular dichroism (2, 7), with free tryptophan and chicken lysozyme used as standards. The sulfhydryl content was determined with 5,5'-dithiobis(2-nitrobenzoic acid) (6). Carbohydrate content was determined by the phenol-sulfuric acid method (5).

Amino-terminal residue and amino acid sequence. We have adopted two different methods for determination of the amino-terminal residue: dansyl chloride (21) and FITC (12, 16). Dansylated and FITC-coupled proteases were subjected to hydrolysis in 6 M HCl at 110°C in vacuo or were treated with trifluoroacetic acid under a nitrogen atmosphere at 37°C, respectively. Both the dansyl and FITC derivatives of amino acids were identified on either polyamide or silica gel thin-layer sheets. The amino acid sequence of the amino terminal was determined by using the FITC method developed by Maeda (12) with some modifications (16).

Preparation of antisera and the Ouchterlony immunodiffusion method. Antibodies against the crude serratial protease preparation (ammonium sulfate precipitates) were raised in a rabbit with complete Freund adjuvant. Ouchterlony double immunodiffusion was performed in agarose in 30 mM Veronal buffer (pH 8.6). In addition to our purified serratial proteases, *S. piscatorum* peptidase and *Pseudomonas* protease were also tested for their possible common antigenicity.

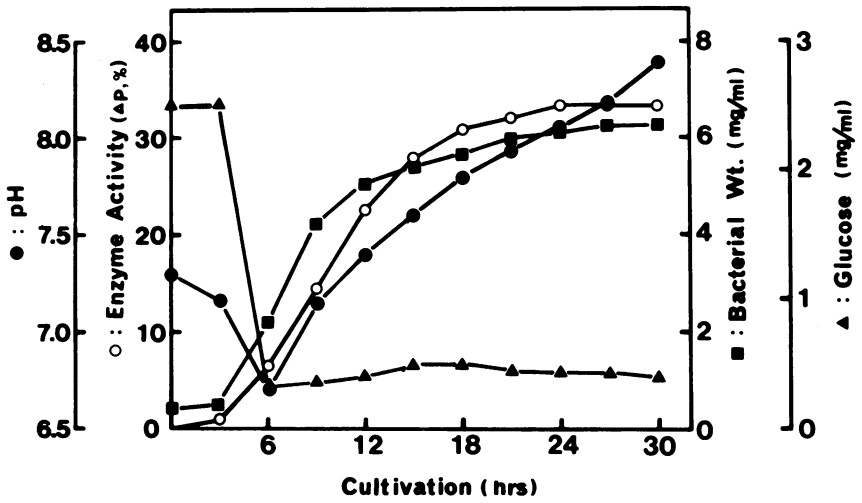

FIG. 1. Time course of culture of *S. marcescens* kums 3958 and production profile of protease activity. Enzyme activity (Δp) is based on the change of the FP value (see text).

RESULTS

Production of proteases and purification. The time course of culture and the protease activity are shown in Fig. 1. Culture filtrate was harvested at 36 h after inoculation. DEAE-cellulose chromatography (pH 8.3) of the ammonium sulfate precipitates yielded three peaks of proteolytic activity (Fig. 2). These active peaks were designated SM 73K protease, SM 60K protease (minor proteases), and SM 56K protease (major protease), according to their molecular weights. A pooled fraction of each protease was subjected to gel filtration on a Sephadex G-100 column. In all cases, enzyme activity was shown as a discrete single peak. Polyacrylamide disc gel electrophoresis of these protease preparations revealed a single band for the 56K protease and at least two bands for the 73K and 60K proteases. After a second DEAE-cellulose chromatography at pH 6.8, the 60K protease preparation revealed homogeneity on electrophoresis in 7.5% polyacrylamide gel (Fig. 3A), whereas 73K protease revealed two peaks on isoelectric focusing (73Ka and 73Kb) (Fig. 4). Molecular weights of purified enzyme preparations were estimated by SDS-polyacrylamide gel electrophoresis to be approximately 73K, 60K, and 56K (Fig. 3B).

From 2.7 liters of the culture fluid, approximately 0.7, 15, and 113 mg of the purified 73K, 60K, and 56K proteases, respectively, were obtained (Table 1). The specific activities of these enzymes were increased about 264-, 45-, and 32-fold compared with the original culture fluid.

Protease activity and pH. The 56K protease showed maximal activity at about pH 5, whereas the 60K and 73K proteases had relatively broad pH optima of pH 5 to 8 and pH 6 to 9, respectively.

Heat and pH stability. The 60K protease was more stable

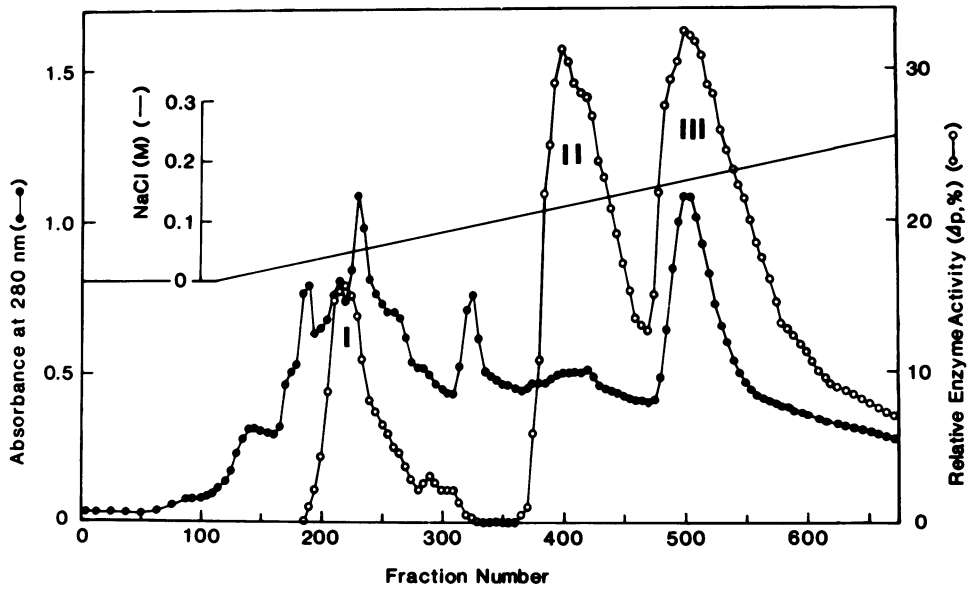

FIG. 2. DEAE-cellulose chromatography of serratial proteases (column size, 4.0 by 38 cm). See text and Table 1 for details. A 5-ml fraction was collected in a test tube.

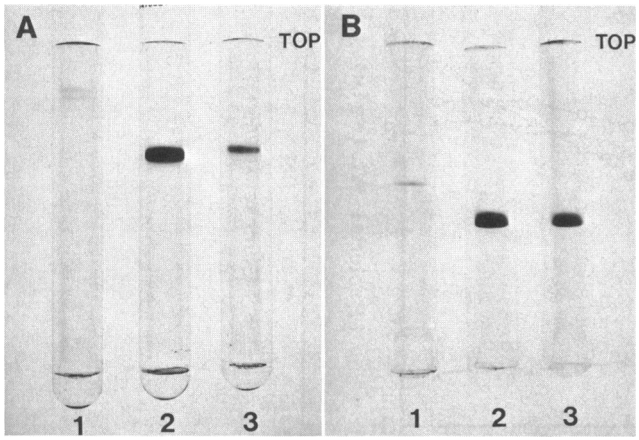

FIG. 3. Disc gel electrophoresis of *S. marcescens* proteases without (A) or with (B) SDS. (A) Test tube 1, 15 μg of 73K protease; test tube 2, 60 μg of 60K protease; test tube 3, 30 μg of 56K protease. (B) Test tube 1, 10 μg of 73K protease; test tube 2, 50 μg of 60K protease; test tube 3, 50 μg of 56K protease. Migration was from top to bottom.

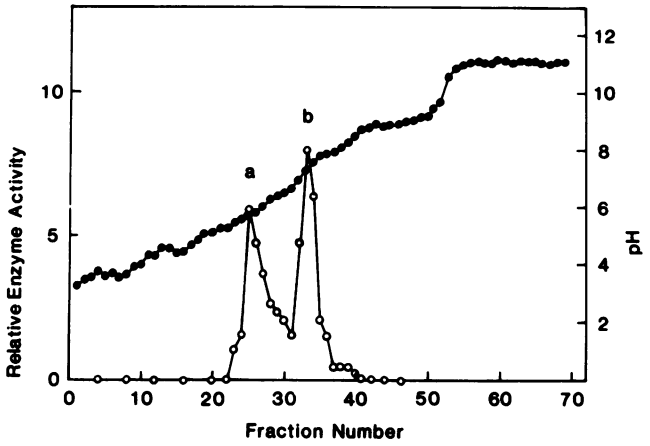

FIG. 4. Isoelectrofocusing of 73K protease of *S. marcescens* at pH 3.5 to 10 at 4°C. Fractions of 0.5 ml were collected. Peaks a and b correspond to 73Ka and 73Kb proteases, respectively. Symbols: ○, enzyme activity; ●, pH. The 56K and 60K proteases showed only a single peak in the same system.

than the 56K protease, although both enzymes were inactivated above 65°C at pH 8.0. The alkaline treatment also revealed that the 60K protease was more stable.

Electrophoresis and electrofocusing. Disc gel electrophoresis showed that all three protease preparations were homogeneous with SDS (Fig. 3B). Isoelectric focusing showed that these proteases had different isoelectric points (pI): 56K protease, 5.3; 60K protease, 4.4; 73Ka protease, 5.8; 73Kb protease, 7.3 (Fig. 4). These results agreed with the electrophoretic mobility in the polyacrylamide gel at pH 8.6 (Fig. 3A).

Effects of metal ions and protease inhibitors. The effects of various inhibitors on the proteolytic activities are shown in Table 2. Both 56K and 60K enzymes were inactivated almost completely at pH 5.0 but partially at pH 8.0 in 2 mM Na_2EDTA. They were not inhibited by EGTA. Among metal ions, only Hg^{2+} inactivated both proteases completely at both pH values. However, there was no indication of involvement of the sulfhydryl group because no effect was observed by iodoacetic acid and *N*-ethylmaleimide. Addition of Zn^{2+} to the EDTA-inactivated 56K and 60K proteases restored the original activities (Table 3). Calcium also activated the enzymes but to a lesser degree (Table 3). Fe^{2+} activated only the SM 56K protease.

The 73K protease was not affected by the chelating agents, but it was activated significantly by thiol agents; and *p*-chloromercuribenzoic acid inactivated this enzyme completely, whereas *N*-ethylmaleimide and iodoacetic acid did so a little. Among various metal ions, Hg^{2+} and Cu^{2+} inactivated 73K enzyme completely. These results indicated that both 56K and 60K proteases were metalloenzymes, whereas 73K protease was, perhaps, a thiol enzyme.

TABLE 1. Purification steps and recovery of *S. marcescens* proteases

Purification steps	Vol (ml)	Protein (mg)	Activity (FP; %)[a]	Sp act (FP; %/mg)[a]	Purification (fold)	Recovery (%)
I Culture supernatant	2,700	10,152[b]	4,389,700	432	1	100
II 90% saturated $(NH_4)_2SO_4$ precipitate	620	794	4,333,300	5,458	12.6	99
III DEAE (pH 8.3)						
73K (active P-I)[c]	273	66.6	133,500	2,005	4.6	3.1
60K (active P-II)[c]	483	76.3	630,000	8,257	19.1	14.4
56K (active P-III)[c]	716	188.5	1,626,800	8,630	20.0	37
IV Sephadex G-100						
73K	103	0.7	79,900	114,143	264.2	1.8
60K	147	24.1	625,500	25,954	60.1	14.3
56K	129	109.0	1,457,000	13,367	30.9	33.2
V Rechromatography from step IV, 60K, on DEAE (pH 6.8)						
60K	156	14.8	285,200	19,270	44.6	6.5
56K	91	4.4	87,600	19,909	46.1	2.0

[a] Proteolytic activity was measured with gelatin as a substrate by fluorescence polarimetry. See text for details.
[b] A part of this value might have been derived from the culture medium itself.
[c] These notations correspond to the peaks in Fig. 2.

Immunological identity. Using the antibody raised against the crude serratial protease preparation as described above, we compared six related bacterial proteases: 56K, 60K, and 73K (a and b) proteases; serratiopeptidase (*S. piscatorum* from the intestinal canal of a silkworm); and *Pseudomonas* protease. Common antigenicity was found between the 56K protease and serratiopeptidase, but the 56K, the 60K, the 73K, and the *Pseudomonas* proteases were different from each other (Fig. 5).

Amino acid analysis. The results of amino acid analyses of the SM 56K, 60K, 73Ka, and 73Kb proteases are shown in Table 4. Data show the presence of about 6 (56K), 2 (60K), and 6 (mixture of 73Ka and 73Kb) mol of tryptophan per mol, respectively. The amino acids of the 56K protease were similar to those of previously published serratial protease, which came from strains of nonclinical isolates, except for tryptophan and methionine content. However, those of the 60K, 73Ka, and 73Kb proteases differed greatly. No cysteine was found in the 56K or 60K enzyme as judged by the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (Table 4). On the other hand, both the 73Ka and 73Kb proteases contained large amounts of cysteine residues, respectively.

Amino-terminal amino acid residues and preliminary amino-terminal sequences. Amino-terminal residues of the SM 60K and SM 56K proteases were identified as glycine and arginine, respectively. Preliminary partial amino acid sequences of the 60K and 56K proteases were as follows: Gly-Gln-( )-Gly-Arg-Gly- and Arg-Gly-Asp-(Gly)-Ala-, respectively.

DISCUSSION

A number of previous reports have documented the isolation and characterization of serratial proteases (8, 11, 14). These organisms were isolated mainly from nonclinical sources including insects and plants. Furthermore, the characteristics of only the main protease were described, although multiple types of the enzymes were known to exist. In this report, we describe at least four different proteases of *S. marcescens* isolated from the lesion of a human corneal ulcer.

These proteases, designated SM 56K, SM 60K, and SM 73K protease, have different molecular weights, based on SDS disc gel electrophoresis (Fig. 3B). Only the SM 56K and SM 60K proteases had molecular weights similar to the previously reported serratial proteases; that is, the molecular weights of proteases from *S. marcescens* ATCC 25419 (4), strain BG (10), and *S. piscatorum* (14) were 51,900, 52,500 and 60,000, respectively. We found 73Ka and 73Kb were much larger than previously known serratial proteases. The pIs of the 60K, 56K, 73Ka, and 73Kb proteases were 4.4, 5.3, 5.8, and 7.3, respectively (Fig. 4).

The Ouchterlony immunodiffusion test showed that the 56K protease from *S. marcescens* kums 3958 was immuno-

TABLE 2. Effects of various enzyme inhibitors on the activities of *S. marcescens* proteases

Reagents[a]	Residual protease activity (%) for the following proteases:[b]		
	56K	60K	73K
None (control)	100	100	100
EDTA	0	0	85
EGTA	95	98	65
L-Cysteine	92	94	157
Glutathione (SH)	98	97	168
Dithiothreitol	71	85	161
2-Mercaptoethanol	99	94	121
Iodoacetic acid	92	100	31 (85)
N-Ethylmaleimide	99	100	72 (100)
p-Chloromercuribenzoic acid	—	—	0 (0)
Antipain	60	95	89
Diisopropylfluorophosphate	103	97	100
Phenylmethylsulfonyl fluoride	88	94	58
Leupeptin	90	102	77

[a] Enzyme inhibitors were used at a concentration of 1.92 mM.
[b] The assay procedure is as described in the text at pH 5.0. The result at pH 8.0 was essentially similar to that at pH 5.0. The I/E (mole/mole) ratio was 5,600 for both 56K and 60K and 37,000 for 73K. The values in parentheses were obtained after treatment at pH 8.0.

TABLE 3. Reactivation of the EDTA-inactivated 56K and 60K proteases by various divalent metal ions[a]

Metal ions	Restored activity (%) for the following proteases:	
	56K	60K
$ZnCl_2$	72	104
$CaCl_2$	10	23
$MgSO_4$	0	0
$MnCl_2$	5	0
$FeSO_4$	90	7
$CoCl_2$	3	0
$CuSO_4$	9	4
Controls		
Native enzyme + EDTA (2 mM)	0	0
Native enzyme alone	100	100

[a] To the EDTA-inactivated enzyme solution, metal ions at 2 mM were added and incubated in 50 mM sodium acetate buffer (pH 5.0) at 30°C for 20 min. Substrate was then added. The enzyme concentration was 0.35 μM in this assay.

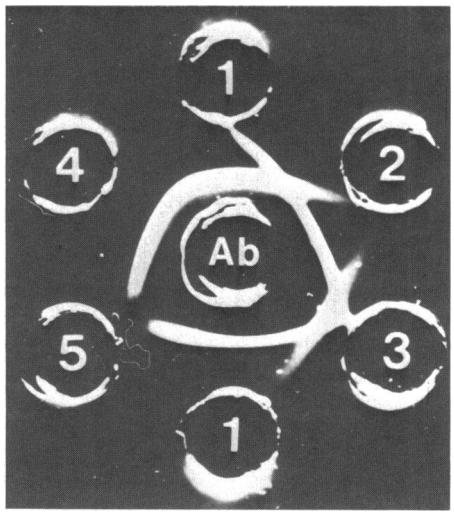

FIG. 5. Ouchterlony immunodiffusion in agarose gel. The center well (Ab) contained 20 μl of antiserum against *S. marcescens* crude protease. The outer wells contained 15 μg each of 56K protease (1); 60K protease (2); 73K protease (contains a and b) (3); *S. piscatorum* peptidase (4); *Pseudomonas* protease (5).

TABLE 4. Amino acid compositions of the 56K, 60K, 73K proteases from *S. marcescens* kums 3958 and previously isolated *Serratia* proteases[a]

Amino acids	56K protease				60K protease				73Ka protease[b]		73Kb protease[b]		Results from previous studies (reference no.)		
	No. of amino acids after the following hydrolysis times (h):			Nearest integers	No. of amino acids after the following hydrolysis times (h):			Nearest integers	24[c]	Nearest integers	24[c]	Nearest integers	4	10	14
	24	48	72		24	48	72								
Lys	18.8	20.4	19.6	20	22.0	22.1	18.9	21	(36.2)	(36)	(61.3)	(61)	15	14	20
His	13.6	13.6	13.5	14	13.5	18.0	12.6	15	11.9	12	18.5	19	10	10	14
Arg	10.9	11.3	11.0	11	14.5	18.6	13.4	16	15.7	16	13.8	14	9	8	10
Asp	85.6	83.5	85.7	86	78.9	96.8	104.7	93	80.0	80	71.7	72	76	68	90
Thr	34.2	31.8	31.6	37[d]	35.1	31.6	30.3	39[d]	42.0	42	34.4	34	36	28	35
Ser	35.0	30.6	28.4	39[d]	45.6	37.1	33.1	53[d]	76.8	77	89.4	89	38	31	48
Glu	47.4	46.5	47.9	47	50.0	42.5	45.5	46	78.0	78	88.4	88	42	36	48
Pro	12.8	13.7	12.6	13	—	16.5	22.5	20	(22.7)	(23)	(8.9)	(9)	11	9	11
Gly	71.7	70.5	72.0	71	70.4	67.1	67.8	68	76.0	76	85.5	86	59	53	66
Ala	58.1	58.0	58.0	58	63.3	66.4	64.1	65	71.7	72	71.4	71	47	43	52
Cys (1/2)	0	0	0	0	0	0	0	0	4.3	4	8.3	8	0	0	0
Val	34.2	33.0	33.5	34[e]	17.4	23.7	28.7	29[e]	47.2	47	38.0	38	27	23	27
Met	1.2	1.2	0.7	1	5.7	5.7	2.3	6	trace	trace	trace	trace	1	0	0
Ile	25.6	25.2	25.5	26[e]	21.8	24.1	25.2	25[e]	37.7	38	33.5	34	20	20	25
Leu	33.5	32.8	33.1	33[e]	36.4	37.6	36.5	37[e]	65.0	65	54.2	54	27	26	30
Tyr	20.6	19.5	19.8	20	—	26.9	19.0	23	22.4	22	18.8	19	21	15	20
Phe	34.8	33.8	34.5	34	29.4	30.2	28.2	29	26.1	26	18.2	18	31	26	32
Trp	7.0[f]	6.1[g]		7(6)			1.9[g]	2	(5.5)[g]	(6)	(5.5)[g]	(6)	8	7	10
Total no. of residues				551				587		(720)		(720)	478	417	529

[a] The estimated approximate molecular weights of 56K, 60K, 73Ka, and 73Kb proteases were 55,293, 59,385, 73,053, and 72,815, respectively. The latter two values are tentative and need reconfirmation. Molecular weights determined previously were 51,900 (4) and 60,000 (14) as determined by the sedimentation method and 52,500 (10) as determined by SDS-polyacrylamide disc gel electrophoresis.

[b] Note that data of the 73Ka and 73Kb proteases were the result of 24-h hydrolysis only. Trp. value for the 73Ka and 73Kb proteases were based on the mixture of the two. All values in the parentheses are tentative and need reconfirmation.

[c] Time (h) of acid hydrolysis in 6M HCl determined by HPLC and detected by *o*-phthalaldehyde.

[d] Intrapolated to zero hydrolysis time.

[e] Calculated from the 72-h hydrolysis samples.

[f] Determined by the diaminobenzaldehyde method (18).

[g] Determined by magnetic circular dichroism (2, 7).

logically identical to the peptidase from *S. piscatorum* indicating that different *Serratia* sp. may produce immunologically identical proteases (Fig. 5). The same test also showed that 56K, 60K, and 73K proteases were immunologically nonidentical (Fig. 5). In addition, *Pseudomonas* protease was different from *Serratia* proteases based on a similar experiment with antibodies prepared against a mixture of serratial proteases, as well as that against *Pseudomonas* protease. McQuade and Crewther (13) and Lyerly et al. (10) observed more than one component with the proteolytic activity having a pI of about 5 in the purified final preparation after isoelectric focusing in polyacrylamide gel. Although the pI of our 56K protease was very similar to those of previous reports (10, 13), the pI values of 60K and 73K proteases were different. The pH optima for proteolytic activity of serratial enzymes has been reported to be about pH 9, when casein, hemoglobin, and azocasein were used as substrates (1, 4, 10). We found that the 56K protease (major) possessed a pH optimum of about 5, with gelatin as the substrate (data not shown). The phosphate buffer appeared to inhibit its activity to an appreciable extent.

McQuade and Crewther (13) reported that the protease from pigmented *S. marcescens* showed optimal activity at pH 9 with casein or tendon as a substrate and an optimum at pH 5 with a synthetic substrate, Z-Gly-Pro-Gly-Gly-Pro-Ala (13). Our 60K protease showed maximal activity at pH 5.0 to 8.0, whereas 73K showed maximal activity between pH 6.0 and 9.0, which was different than that of the 56K protease. In spite of a much lower production of the 60K protease than the 56K protease, the former was more stable than the latter to heat and alkaline treatment.

Since both the 56K and 60K proteases were inactivated completely by EDTA at pH 5 and reactivated by Zn^{2+}, they may be classified as metalloproteases. However, they were affected only slightly by EGTA, which entraps Ca^{2+} more preferentially than EDTA. This result suggests that Ca^{2+} is not essential for the activity of the enzymes. Lyerly and Kreger (10) and Miyata et al. (15) reported that Zn^{2+} was essential for enzyme activity. On the other hand, Aiyappa and Harris (1) reported that Fe^{2+} was essential for proteolytic activity and was an essential part of the holoenzyme. Our results showed that either Fe^{2+} or Zn^{2+} could affect reactivation of the EDTA-inactivated 56K protease, whereas the 60K protease was affected by Fe^{2+} very little. The 73K proteases appeared to be thiol enzymes as judged by the inhibition by sulfhydryl reagents (Table 2). Thus, they are different from 56K or 60K proteases.

Both the 56K and 60K proteases contained a high percentage of acidic amino acids, but they contained neither cysteine nor cystine. Preliminary amino acid analyses of 73Ka and 73Kb proteases showed that they differed somewhat from each other, and only these proteases contained cystine residue (Table 4). When we compared our data with previous data on serratial proteases from other sources, the amino acid composition of only the 56K protease was similar to them, whereas that of the 60K protease was different from them in the content of methionine, tryptophan, and others. The data for the 73K proteases were completely different from all other previous ones with regard to the content of lysine, serine, cystine, leucine, tryptophan, and many others (Table 4) as well as enzymatic and antigenic properties. 56K and 60K enzymes did not have cystine residue or sugar, and it was suggested that they were monomers of a simple protein without disulfide bridges.

Another clinical strain of *S. marcescens* also showed similar production of multiple proteases (data not shown). The relative amount of these enzymes varied as the time of cultivation progressed. In any event, they were different from each other by various criteria, and a possibility of conversion of, for instance, 60K to 56K or 73Kb to 73Ka through a limited proteolysis or spontaneous deamidation appears to be unlikely due to antigenicity, amino acid composition, and enzymatic properties.

The role of these proteases in pathogenesis can be envisaged, particularly in ulcer formation. Our preliminary results and also recent work of Lyerly et al. (9) support such a mechanism of pathogenesis.

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