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Protease Production by *Pseudomonas aeruginosa* Isolates from Patients with Cystic Fibrosis

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The temporal appearance of extracellular proteases produced by *Pseudomonas aeruginosa* was analyzed by pH 9 and pH 4 polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-PAGE. Ammonium sulfate precipitates of culture supernatants from various stages of growth revealed a time-dependent increase in number and amount of proteolytically active proteins. One mucoid *P. aeruginosa* clinical isolate and its derived nonmucoid variant, as well as two other nonmucoid variant *P. aeruginosa* strains (all from cystic fibrosis patients), showed similar production of five differently migrating proteases (P1 to P5, numbered according to increasing net negative charge) in pH 9 PAGE and one protease in pH 4 PAGE. P2, P3, and P5 increased to maximum concentrations at 24 to 48 h, decreasing thereafter, whereas P4 continued increasing even at 83 h, and P1 fluctuated. P3 was identified as an elastase. P2 was possibly composed of polypeptide chains bridged by disulfide bonds, since without reduction it migrated in sodium dodecyl sulfate-PAGE as a single protein, and with reduction it migrated as three protein bands. Two-dimensional PAGE revealed multiple molecular weight species within protease-positive bands in pH 9 gel strips. Isoelectric focusing gave a pattern of protein separation that correlated with two-dimensional PAGE analysis. Thus, greater heterogeneity of active proteases than previously reported has been demonstrated in all *P. aeruginosa* clinical isolates studied by sensitive two-dimensional PAGE analysis.

Pseudomonas aeruginosa is a major pathogen in patients with cystic fibrosis (10, 12). A large proportion of clinical isolates are positive for protease (4) and elastase (2, 20) production. These enzymes have been implicated in the pathogenicity of this microorganism (3, 7, 8, 14) and have been studied in detail (6, 9, 16, 17, 19, 25). However, differences in the time sequence of appearance of the various proteases in the external medium during bacterial growth have not been studied. Such differences could easily be missed in the measurement of total protease activity. We report here for the first time on the appearance of *P. aeruginosa* proteases in the extracellular medium throughout culture growth, using the sensitive pH 9 polyacrylamide gel electrophoresis (PAGE) method of Jensen et al. (5) and expanding the analysis to include pH 4 PAGE and sodium dodecyl sulfate (SDS)-PAGE. In addition, we developed a two-dimensional PAGE system which allows identification of proteases in pH 9 PAGE with corresponding proteins in SDS-PAGE.

P. aeruginosa isolates from cystic fibrosis patients frequently produce a polyanionic polysaccharide extracellular matrix (11). This charged material may bind other exocellular products; therefore, we selected nonmucoid variants of the original mucoid *P. aeruginosa* isolates. One original mucoid isolate was also studied for comparison.

MATERIALS AND METHODS

Purified elastase from *P. aeruginosa* was a gift from K. Morihara, Shionogi & Co., Ltd., Osaka, Japan. The following materials were obtained from the indicated commercial firms: Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.); Biolyte electrofocusing gel, low-molecular-weight markers, and SDS (Bio-Rad Laboratories, Richmond, Calif.); trypsin (Boehringer Mannheim, Indianapolis, Ind.); pH isolytes, pH 2 to 10 (Brinkmann Instruments Inc., Westbury, N.Y.); acrylamide (enzyme grade), bisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine (Eastman Kodak Co., Rochester, N.Y.); Seakem LE agarose (FMC Corp., Rockland, Maine); and azocasein, Coomassie brilliant blue R, and elastin powder (Sigma Chemical Co., St. Louis, Mo.).

Bacteria and growth conditions. *P. aeruginosa* SC1 and SC2 were obtained from sputum samples from cystic fibrosis patients of D. Schidlow, St. Christopher's Hospital for Children, Philadelphia, Pa., and

strain HC1 was obtained from D. Holsclaw, Hahnemann Medical College, Philadelphia, Pa.

Sputum samples were streaked on blood and MacConkey agar plates. Colorless (i.e., non-lactose-fermenting) mucoid colonies were isolated from MacConkey plates. Oxidase-positive colonies were screened with a two-tube selective growth procedure (N/F system; Flow Laboratories, Roslyn, N.Y.) to determine whether the isolates were *P. aeruginosa*. Positive identification was based on the ability of the organisms to (i) grow and produce pyocyanin when incubated at 42°C, (ii) oxidize but not ferment glucose, (iii) elaborate N_2 gas, and (iv) produce fluorescent pigment when incubated at 37°C.

Nonmucoid, protease-positive variants were isolated from each mucoid strain by serial passage in standing broth cultures and selection of a rough colony on solid growth medium (23, 24). Nonmucoid variants of strains SC1, SC2, and HC1 are designated SC1v, SC2v, and HC1v, respectively.

Trypticase soy broth in flasks with a liquid to overall volume ratio of 1:5 was inoculated with a mid-logarithmic culture to an optical density of 0.002. (Spectronic 20, Bausch & Lomb, Inc., Rochester, N.Y.). This was incubated at 37°C on a shaker at 200 rpm.

Preparation of crude protease precipitates. All procedures were carried out at 4°C. Culture supernatant fluids were obtained by centrifugation (6,000 × *g* for 20 min) and filtration (0.45-μm pore size). A 25-ml amount from each time of culture growth was precipitated with 60% ammonium sulfate. Two hours after the final addition of ammonium sulfate, the precipitate was collected by centrifugation (46,000 × *g* for 15 min). The pellet was resuspended in 1 ml of 0.02 M Tris-hydrochloride (pH 7.5) and dialyzed against the same buffer. The dialyzed material was stored at −20°C. This was the crude protease precipitate used for further study.

Assays. Protein concentrations were determined by the method of Lowry et al. (13). Protease activity was measured by the method of Fisher and Allen (1). Azocasein substrate (0.5 ml of 5 mg/ml) was added to enzyme sample (0.5 ml of appropriate dilution into 0.1 M Tris-hydrochloride [pH 7.6], 0.007 M $CaCl_2$) and incubated at 37°C for 20 min. Trichloroacetic acid (3.5 ml of 5%) was added, and nonhydrolyzed substrate was removed by centrifugation (1,400 × *g* for 15 min). One volume of supernatant was neutralized with 1 volume of NaOH (0.5 M). The absorbance at 420 nm was read on a Stasse III automated spectrophotometer (Gilford Instruments, Oberlin, Ohio). Trypsin standards gave a linear response from 0.5 to 2.0 μg/ml. Enzymatic activity of unknown samples is expressed as equivalent units of trypsin activity.

PAGE. A high pH, discontinuous buffer system in 7.5% separating and 3% stacking polyacrylamide slab gels (15) was used for analysis of negatively charged pseudomonal proteases at pH 9. Samples were thawed just before use, mixed with an equal volume of sample buffer (1 ml of glycerol, 1.25 ml of 0.5 M Tris-hydrochloride [pH 6.7], 0.1 ml of 0.2% bromophenol blue, and 2.65 ml of water), and 50 μl was loaded in duplicate. Current (20 mA) was applied until the tracking dye had entered the separating gel. The current was then increased to 40 mA until the dye was 0.5 to 1 cm from the bottom edge of the 12-cm gel. Electrophoresis was performed at 15°C. One-half of the gel was stained for protein in Coomassie brilliant blue R (0.688 g in 125 ml of methanol, 125 ml of water, 25 ml of glacial acetic acid) for 1 h and then destained overnight (75 ml of glacial acetic acid, 50 ml of methanol per liter of water). The other half of the gel was laid atop 0.25% azocasein in 1% agarose gel–0.01 M Tris-hydrochloride (pH 7.5)–0.001 M $CaCl_2$. This was incubated at 37°C for 1 h. The polyacrylamide gel was removed, and unhydrolyzed azocasein was precipitated with cold 5% trichloroacetic acid. The agarose gel was photographed with darkfield illumination.

A low pH, discontinuous buffer system in 7.5% separating and 3% stacking polyacrylamide slab gels (15) was used for analysis of positively charged pseudomonal proteases at pH 4. One volume of thawed sample was mixed with one volume of sample buffer (1 ml of glycerol, 1 ml of stacking buffer, 3 ml of water), and 50 μl was loaded in duplicate. Electrophoresis was carried out similarly to that for high pH PAGE, except that the electrode polarity was reversed. One-half of the gel was stained for protein as described above. The other half was washed twice in 0.5 M Tris-hydrochloride (pH 8.0) (20 min each wash) and then laid atop the azocasein agarose gel as for high pH PAGE detection of protease-active bands.

Detection of all proteins present and estimation of molecular weights was made by SDS-PAGE (15). A 12.5% separating gel and a 5% stacking gel concentration provided the best separation of the proteins in the crude protease precipitates. Samples were diluted with SDS sample buffer (0.1 M Tris-hydrochloride [pH 6.8], 2% SDS, 1% dithiothreitol, 20% glycerol, and 0.004% bromophenol blue) and heated for 2 min before loading onto the gel. Electrophoresis was carried out at the same current settings as those for high and low pH gels. Staining and destaining of the gel for protein detection was done as described above. Bio-Rad low-molecular-weight protein standards were used for molecular weight determination.

Two-dimensional PAGE was achieved by high pH electrophoresis of the sample in the first dimension followed by SDS-PAGE. The high pH gel was cut into sample strips after electrophoresis. These were incubated for 15 min in 5 ml of sample buffer (0.5 ml of 0.5 M Tris-hydrochloride [pH 6.8], 0.5 ml of 10% SDS, 0.25 ml of 10% dithiothreitol, 0.05 ml of 0.02% bromophenol blue, 3.75 ml of water) which had been heated to boiling. This high pH gel strip was then laid lengthwise on top of the usual 12.5% acrylamide-SDS separating slab gel. A stacking gel was not necessary. Electrophoresis and staining of protein were done as described above.

Densitometer scans of photographic negatives of azocasein-agarose gels were made on a Zeineh soft laser densitometer (Biomed Instruments, Inc., Chicago, Ill.). Areas for each peak of protease activity were measured with a planimeter and expressed as percent of maximum total area.

Isoelectric Focusing. A crude protease precipitate (4 ml of 1.9 mg of protein per ml) from a 24-h culture supernatant was focused in a preparative flatbed LKB Multiphor apparatus with 1.6% pHisolytes (pH 2 to 10) and 5% glycerol in Biolyte electrofocusing gel (5 g/100 ml). The sample was focused for 16h initially at 20 mA and 300 V and finally at 4 mA and 1,000 V (maximum voltage setting). The gel bed was fractionated, and the fractions were eluted from the gel with 3 ml of water.

pH values were read at 4°C. Fractions were assayed for protease activity and, on the basis of these results, pooled and concentrated 10-fold by Amicon ultrafiltration. The concentrates were dialyzed against 0.9% NaCl and stored at −20°C before analysis by SDS-PAGE.

RESULTS

pH 9 PAGE. Crude protease preparations from various times of culture growth were electrophoresed on discontinuous pH 9 polyacrylamide slab gels and subsequently analyzed for protein and protease activity as represented in Fig. 1. Samples from mid-logarithmic and beginning stationary phases of culture were examined, but proteins and protease activity were generally not observed on the gels until 4 h after the beginning of stationary phase.

As seen in Fig. 1, the number of proteolytically active proteins increased with time to five, designated P1 to P5 in order of increasing net negative charge. The mucoid isolate (SC1), its paired nonmucoid variant, and the two other nonmucoid variants (from SC2 and HC1 isolates) all produced these five proteolytically active proteins (Table 1). The relative migrations for each individual protease were the same, indicating similar net charges on the molecules.

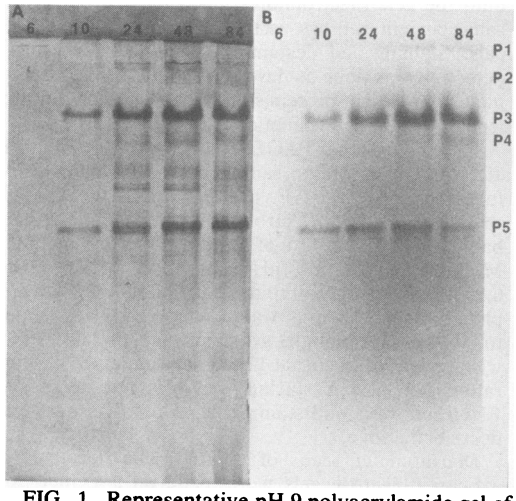

FIG. 1. Representative pH 9 polyacrylamide gel of SC2v culture supernatant samples from various times (in hours) after inoculation. (A) One-half of the gel stained for proteins. (B) Azocasein-agarose gel overlaid with duplicate half of polyacrylamide gel for detection of protease activities (designated P1 to P5). Protein hydrolysis appeared as dark bands by dark-field illumination. Equivalent volumes of culture supernatant material were loaded on the gel. Protein concentrations were as follows: 6 h, 33 μg; 10 h, 22 μg; 24 h, 39 μg; 48 h, 61 μg; and 84 h, 67 μg. The anode was at the bottom. A time-dependent increase in the number and amount of protease activities was evident.

TABLE 1. Protease activities in pH 9 PAGE in culture growth of four *P. aeruginosa* strains[a]

Protease	*P. aeruginosa* strain	% Protease activity			
		4 PS	24 h	48 h	84 h
P1	SC1	9.4	16	9	6
	Sc1v	6	7	12	9.6
	HC1v	12	8.2	9	5
	SC2v	13	9	14	22
P2	SC1		11	5	
	SC1v	6.3	7.7	6	8.4
	HC1v	7	13	10	6
	SC2v	2	3.7	5	3.6
P3	SC1	4.6	70	51	24
	SC1v	18	59	63	51
	HC1v	29	73	72	54
	SC2v	15	36	57	49
P4	SC1		1.6	2.6	
	SC1v		2.3	7	10.4
	HC1v		2	4	8
	SC2v			4	9
P5	SC1		2	2	
	CS1v		13.5	12.5	7
	HC1v		3.4	3	
	SC2v	12	16	20	12

[a] Protease activity in pH 9 gels detected by azocasein-agarose overlay was quantitated from densitometer scans as described in the text. The area for each protease peak is expressed as a percent of the maximum total area. For example, if the maximum activity (total area under protease peaks) in a culture occurred at 24 h, then the area for protease P1 at 84 h in the same culture is a percent of the 24-h total. The length of culture time is given in hours after inoculation, with the exception of 4 h post-stationary phase initiation (4 PS). This sample time varied for each strain, ranging from 6.5 to 9.7 h after inoculation.

There were few proteins that did not show protease activity.

Although the relative amounts of each protease activity varied for the different isolates, some general trends were noted (Table 1). Proteases P2, P3, and P5 increased to maxima at 24 to 48 h after inoculation, dropping off at 83 to 85 h. However, P4 continued to increase, even into this last time interval. P1 was more variable: SC1 mucoid isolate and nonmucoid variant P1 levels demonstrated maxima at 24 to 48 h, but SC2 and HC1 nonmucoid variants P1 levels fluctuated randomly.

Only P3 hydrolyzed elastin when this substrate was substituted for azocasein in the agarose gel. In addition, purified *P. aeruginosa* elastase migrated identically to P3 in high pH PAGE, with a faint, minor band corresponding to P4.

pH 4 PAGE. To ascertain that the full range of proteases were observed, including those that

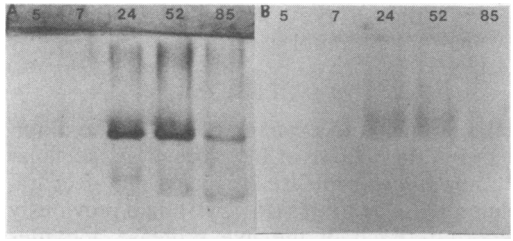

FIG. 2. Representative pH 4 polyacrylamide gel of SC1v culture supernatant samples from various times (in hours) after inoculation. (A) One-half of gel stained for proteins. (B) Azocasein-agarose gel overlaid with duplicate half for detection of protease activities. Equivalent volumes of culture supernatant material were loaded on the gel. Protein concentrations were as follows: 5 h, 57 μg; 7 h, 53 μg; 24 h, 88 μg; 52 h, 114 μg; and 85 h, 149 μg. The cathode was at the bottom. One diffuse band, sometimes resolving into a doublet, was observed.

might have a pI of >9, a similar analysis was performed on the same samples electrophoresed into pH 4 polyacrylamide gels (Fig. 2). A single diffuse band of proteolytic activity, possibly a doublet, was observed in all three nonmucoid variants and the one original mucoid isolate. This protease activity was maximal at 24 to 48 h. Since this may have been one of the slowest migrating proteases on high pH PAGE, the P1 and P2 regions from a sample on a high pH gel were excised and subsequently electrophoresed into a low pH gel. P2 was identified as migrating the same as the single protease observed. Neither protein nor protease activity from the P1 region was found, indicating that the migration at pH 9 was not perhaps according to charge. Otherwise, P1 should have been observed in pH 4 PAGE migrating cathodally to P2.

Purified *P. aeruginosa* elastase did not migrate into low pH gels.

SDS-PAGE. The same samples were also examined on SDS-PAGE, as shown in Fig. 3. Similar patterns of the main protein bands were observed for the three nonmucoid variants and the one mucoid isolate. Generally, more protein bands appeared than on both pH 4 and 9 PAGE. Also, with time, higher-molecular-weight proteins tended to decrease, whereas lower-molecular-weight proteins tended to increase. Despite an equivalent amount of protein loaded, only a few faint bands were observed in the 4-h (beginning stationary phase) time sample.

Purified *P. aeruginosa* elastase produced one major band on SDS-PAGE migrating identically to the major protein band in the culture supernatant at approximately 39,000 in Fig. 3. There were also four to five faint, minor bands ranging below 21,000.

Two-dimensional electrophoresis. To correlate protein bands in SDS gels with protease activity, a two-dimensional electrophoresis system was developed in which the sample strip from a pH 9 gel was subsequently electrophoresed into an SDS slab, as shown in Fig. 4. Again, similar protein patterns were obtained with all three nonmucoid variants and the one mucoid isolate studied. Also noteworthy was the correspondence of the two-dimensional protein pattern to the banding in one-dimensional SDS gels, suggesting that most proteins migrated into the pH 9 gel.

Protease activities P1, P2, and P3 were each composed of more than one polypeptide with different molecular weights. Protease activities P4 and P5 each appeared as single polypeptides at molecular weights of approximately 22,000 and 60,000, respectively. P1, which migrated to the interface between the stacking and separating gels in pH 9 PAGE, diffused horizontally before migrating into the SDS gel and produced two bands perpendicular to the vertical streak of the majority of P1 material. The molecular weight of P1 was similar to that of P5.

Isoelectric focusing. A single culture supernatant from 24 h was isoelectrofocused. The protease assay revealed one major broad peak at pH 6.3 and a minor peak at pH 8.5. SDS-PAGE analysis of pooled dialyzed fractions from this experiment appear in Fig. 5. The overall separa-

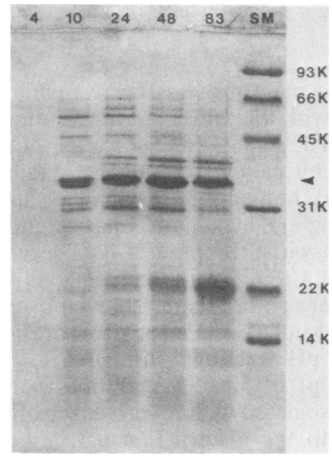

FIG. 3. Representative SDS-polyacrylamide gel of SC2v culture supernatant samples from various times (in hours). SM, Standard molecular weight marker proteins: phosphorylase B, 93,000; bovine serum albumin, 66,000; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 22,000; and lysozyme, 14,000. Equivalent amounts of protein (40 μg) were loaded for all samples except 48 h (45 μg). The anode was at the bottom. Generally, high-molecular-weight proteins decreased at later times and lower-molecular-weight proteins increased. The position of the major band produced by purified *P. aeruginosa* elastase is marked with an arrow.

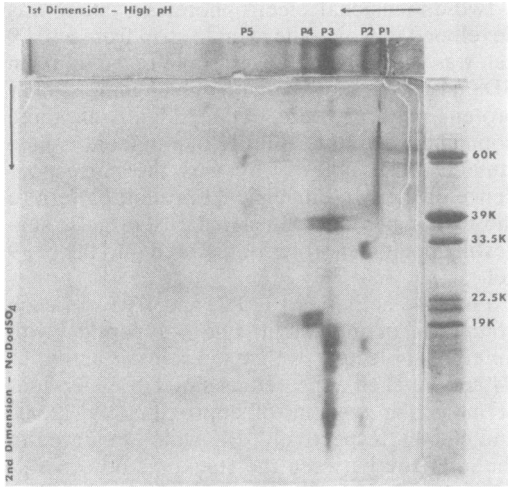

FIG. 4. Two-dimensional PAGE analysis of SC1v culture supernatant after 24 h of growth. The crude protein precipitate (65 μg) was electrophoresed into a pH 9 polyacrylamide gel in the first dimension, and the resulting gel strip was electrophoresed into an SDS ($NaDodSO_4$) polyacrylamide gel in the second dimension. Arrows indicate the direction of electrophoresis. A duplicate pH 9 gel strip stained for protein was placed across the top to indicate the positions of the main protease-active bands. A gel strip of the same sample run in SDS-PAGE alone was placed to the right, with the estimated molecular weights of the main protein bands as indicated. Protease-active bands P1, P2, and P3 appeared to contain several different molecular weight polypeptides.

tion of the main proteins corresponds remarkably well with that observed in two-dimensional analysis. P5, P4, P3, and P2 were not as well separated by the electrofocusing gradient as by pH 9 PAGE but still occurred in the same order.

The single 60,000-molecular-weight protein associated with P5 in Fig. 4 is observed in pH fractions 4.4 to 6.3 with an estimated pI of 5.3. By similar comparison, the approximately 22,000-molecular-weight protein of P4 is observed in pH fractions 5.3 and 6.3, giving an estimated pI of 5.8. The multiple polypeptide pattern associated with P3 protease activity corresponds to the pH fraction 6.3, although the 39,000-molecular-weight protein was spread across pH fractions 5.3 to 7.2. Finally, the tripolypeptide pattern associated with P2 protease activity was found in pH fraction 8.5. A basic protein corresponding to P1 in molecular weight and charge was not observed.

The three proteins associated with P2 in two-dimensional PAGE were found to migrate as a single band when the disulfide-reducing agent dithiothreitol was omitted from the sample buffer. The relative migrations of protein bands in the other fractions were not substantially altered, although they migrated slightly faster without dithiothreitol.

DISCUSSION

Experiments to examine possible time differences in the release of the various extracellular *P. aeruginosa* proteases revealed more separable protease activities (P1 to P5) than previously reported. Each of the five protease activities observed here occurred in all four strains studied, including a mucoid *P. aeruginosa* isolate and its nonmucoid variant. Different levels of each protease were produced by different *P. aeruginosa* strains, which corresponded to earlier findings (5, 18). In addition, our use of two-dimensional PAGE for the first time demonstrates the heterogeneity of proteins associated with charge-separated protease activities.

P2, P3, and P5, although varying in concentration, appeared to be released concurrently, reaching maxima at 24 to 48 h. P1 and P4 did not follow this pattern, but this may be due to factors other than a different schedule of release. Maximum elastase production by *P. aeruginosa* is observed in 24 to 72-h cultures, decreasing

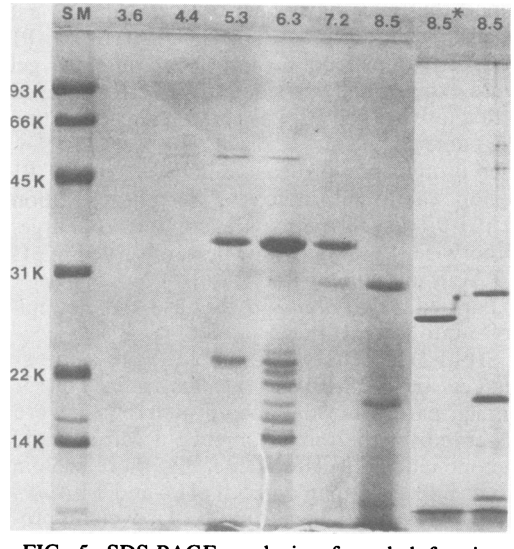

FIG. 5. SDS-PAGE analysis of pooled fractions from isoelectric focusing of SC1v culture supernatant after 24 h of growth. Numbers across the top indicate the mid-range pH value for pooled fractions. The two duplicate samples of the pH 8.5 fraction demonstrate this sample without dithiothreitol reduction (8.5*) and with reduction (8.5). Although the separation of the focused proteins was not as sharply defined as in the pH 9 gel dimension of two-dimensional PAGE, the overall protein pattern was remarkably similar. Only proteins corresponding to P1 were not observed. The three proteins observed in the pH 8.5 fraction migrated mainly as a single band if not reduced by dithiothreitol. SM, Standard molecular weight markers.

thereafter by one report (21) but showing no decrease by another (17).

Previously described *P. aeruginosa* protease activities can be correlated in part with our results. Morihara first separated three *P. aeruginosa* proteases by DEAE-cellulose chromatography (16). Subsequent work confirmed three classes based on net charge, with some variations: (i) a protease with a pI of 8.3 to 8.8 which does not bind to DEAE-cellulose at pH 7 to 8 (8, 16, 25) and has a molecular weight of 20,000 (25), (ii) a protease released in greatest quantity which hydrolyzes elastin and has a pI of 5.9 and a molecular weight of 39,500 (19), although other studies report a pI of 6.0 to 7.2 (6, 8, 25) and a molecular weight of 20,000 to 23,000 (9, 25), and (iii) an alkaline protease, so called because of its optimum pH for activity, with a pI of 4.1 to 5.0 (6, 8, 16, 25) and a molecular weight of 48,400 to 53,500 (6, 18), although again one report records a molecular weight of 20,000 (9). Table 2 summarizes and compares this information with our results as discussed below.

P2 had a pI of 8.5, which corresponded to that reported for Morihara's first type of *P. aeruginosa* protease eluted from DEAE-cellulose (17) but differed in molecular weight (25). The P2 protease appeared to be composed of three polypeptides bridged by disulfide bonds. Two possibilities exist: (i) the three proteins are subunits (1:1:1) of a 60,500 protein cross-linked by disulfide bonds, or (ii) the 31,500 protein represents an intact molecule, and the 18,000 and 11,000 are major proteolytic fragments which, if not reduced, migrate with the intact protein at approximately 29,000. The latter seems most likely due to the migration of the single unreduced protein. Further comparison is limited by lack of reported information for this *P. aeruginosa* protease. This protease has been generally neglected but was present in all clinical isolates of *P. aeruginosa* that we studied.

P3 was found to hydrolyze elastin, was released in largest amounts, had a pI of approximately 6.3 (range, 5.3 to 7.2), and migrated the same distance as purified *P. aeruginosa* elastase in pH 9 PAGE. These characteristics identify P3 as probably *P. aeruginosa* elastase. P3 was heterogeneous on two-dimensional PAGE; however, a protein at 39,000 and several proteins at 20,000 were observed which match those reported for *P. aeruginosa* elastase. The molecular weight of 39,000 has been attributed to dimerization of the 20,000 molecule (18). However, purified *P. aeruginosa* elastase in SDS-PAGE produced one major protein band at approximately 39,000 and only faint, minor bands at 21,000 and below, Thus, it would appear that pseudomonal elastase is not a noncovalent dimer at 20,000 but a single protein of 39,000 molecular weight. We suggest that hydrolysis may have occurred in our preparation and in those of other researchers (9, 25), and some of the lower molecular weight proteins may still be proteolytically active.

TABLE 2. Comparison of *P. aeruginosa* protease characteristics in literature with those reported here

Present report		Previous literature[a]		
Protease	Characteristic[b]	Protease	Characteristic[b]	Reference
P1	pI ND mw 57–60 K rm <0.01			
P2	pI 8.5 mw ND rm 0.07	I	pI 8.3–8.8 mw 20K	8, 16 25
P3	pI 6.3 mw 39 K 20 K rm 0.17	II ("elastase")	pI 5.9–7.2 mw 39.5K 20–23K rm 0.18	6, 8, 9, 19, 25 19 9, 25 5
P4	pI 5.8 mw 22 K rm 0.23			
P5	pI 5.3 mw 57–60K rm 0.42	III ("alkaline protease")	pI 4.1–5.0 mw 48–53K 20K rm 0.47	6, 8, 16, 25 6, 18 9 5

[a] The protease classification used here is that of Morihara (17) based on sequential elution from DEAE-cellulose.

[b] mw, Molecular weight; rm, relative migration in pH 9 PAGE; ND, not determined.

Alternatively, the lower-molecular-weight proteins in P3 may be unrelated to the elastase.

P5 migrated in a manner similar to "alkaline protease" in pH 9 PAGE (5), had a pI of approximately 5.3 (range, 4.4 to 6.3), did not hydrolyze elastin, and had a molecular weight of 57,000 to 60,000 in two-dimensional PAGE. P5 is tentatively identified as *P. aeruginosa* alkaline protease. P5 often appeared as a doublet, which Jensen et al. (5) attribute to autodigestion.

P1 demonstrated anomalous migratory behavior. It was not found in low pH PAGE and isoelectric focusing in a position consistent with its migration in pH 9 PAGE. In addition, variable amounts on pH 9 PAGE were observed at different time intervals of culture growth. The similar migration of P1 and P5 in two-dimensional gels strongly suggests that P1 may be an aggregate of P5 or a complex of P5 with other proteins or cellular material which is influencing migration in pH 9 gels. This is similar to the conclusions of Morihara and Tsuzuki (18).

P4 does not conform to the characteristics of previously described proteases. Its pI of 5.8 and molecular weight of 22,000 might warrant grouping with *P. aeruginosa* elastase, but it did not hydrolyze elastin, nor was it released in large quantity. In fact, its continued increase when P2, P3, and P5 were decreasing suggested that it may be an active degradation product of one of the proteases or, equally possible, a cellular protease released by autolysis of the culture in late stationary phase. The observed faint P4 protease activity in *P. aeruginosa* purified elastase may indicate that P4 is a degradation product of P3 or that it was a minor contaminant in this preparation.

Two-dimensional PAGE analysis of *P. aeruginosa* proteases now clarifies the confusion existing in the literature concerning the characteristics of the various *P. aeruginosa* proteases. Separation of these proteases first by relative charge and then by molecular weight shows the heterogeneity that is present in single protease bands, such as P3 in high pH gels. Thus, determinations of molecular weight for *P. aeruginosa* elastase of both 39,000 (19) and 20,000 (9) can obviously be obtained. As mentioned above, degradation products, including those with similar as well as altered charge, are a possibility. Likewise, separation by molecular weight first may yield proteases of 20,000 molecular weight which would then demonstrate different pIs, as in P3 and P4. Study of antigenic relationships will hopefully elucidate the relationships of the additional proteases described here. *P. aeruginosa* elastase and alkaline protease have been shown to be antigenically nonidentical (6), although the elastases of various *P. aeruginosa* strains are related (22).

The two-dimensional PAGE analysis we developed for identification of the proteins associated with extracellular protease activity from *P. aeruginosa* has wide applicability for study of proteases from other bacterial strains or even other enzyme systems. Although isoelectric focusing results confirmed those from two-dimensional PAGE analysis, the separation of protease activities obtained was not as clearly resolved.

Further study of *P. aeruginosa* protease interaction with host inhibitors and contribution to tissue damage must take into account the heterogeneity of active *P. aeruginosa* proteases demonstrated here. We have shown this heterogeneity by a refined, sensitive two-dimensional PAGE analysis. All clinical *P. aeruginosa* isolates studied by this method possessed the same protease heterogeneity, underscoring the importance of these proteases.

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