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Production of Protease and Elastase by *Pseudomonas aeruginosa* Strains Isolated from Patients

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Using 20 strains of *Pseudomonas aeruginosa* isolated from patients, production of protease, elastase, and collagenase was determined by shaking culture in either complex or semisynthetic medium. No collagenase was produced by any strain of *P. aeruginosa*. According to their production of protease and elastase in different media, the *P. aeruginosa* strains were divided into three groups: the first group can produce elastase in complex medium and both protease and elastase in semisynthetic medium; the second group cannot produce any proteolytic enzymes in complex medium but can produce them in semisynthetic medium; and the third group cannot produce any proteolytic enzymes in either medium. In spite of the differences in the ability of the strains to produce the enzymes, depending upon the origin of the strains, the proteases or elastases produced in different broths were regarded as identical.

Pseudomonas aeruginosa has become an important pathogen in recent years due to its antibiotic resistance. Although it is customary to explain the pathogenesis of gram-negative bacteria as being due to the endotoxin, which is the lipopolysaccharide-protein complex of the cell wall, this may not be the case for *P. aeruginosa* infection; the bacteria can elaborate many extracellular substances that are much more toxic than its lipopolysaccharide (20). Numerous reports have accumulated on the protease or elastase elaborated by the bacteria as one of the components of the toxins. Injection of protease or elastase of *P. aeruginosa* into the skin of animals induces hemorrhagic lesions within a few minutes, and within 24 h these lesions become necrotic (15, 18). Hemorrhagic skin lesions have been considered to be pathogenic infections of *P. aeruginosa*. Hemorrhages of internal organs, particularly the lungs, are common in systematic infections with *P. aeruginosa*; protease or elastase is probably responsible for the pathogenesis of such lesions (4, 14, 21). The protease or elastase is also most likely the factor responsible for destruction of corneal tissues when *P. aeruginosa* infects the eye (5-7, 11, 16). Interestingly, an elastase-positive *P. aeruginosa* (IFO 3455) showed much more cornea-damaging activity than an elastase-negative one (IFO 3080) (T. Sasaki, N. Tanaka, S. Miyaji, T. Ozaki, and E. Gotoh, Abstr. Annu. Meet. *P. aeruginosa* Jpn. Sci., 9th, 1975, p. 141-144; 12).

We have shown (23) that the IFO 3455 strain can produce elastase in complex medium and both elastase and protease (non-elastolytic) in synthetic or semisynthetic medium containing Ca ion, whereas the IFO 3080 strain can produce little elastase in either media but can produce protease in synthetic or semisynthetic medium containing Ca ion. In 1963 (22), we isolated the protease as crystals from the broth of the IFO 3080 strain cultured in semisynthetic medium, and in 1965 (32) we isolated elastase from the broth of the IFO 3455 strain cultured in bouillon. We have conducted a detailed study on the crystalline protease and elastase of *P. aeruginosa*, and their properties are given below.

The protease is most active at a slightly alkaline pH but does not hydrolyze elastin (22, 23); it might therefore be called an "alkaline proteinase." In this paper, we call it simply "protease." Its molecular weight is 48,400, and its isoelectric point is at pH 4.1 (9, 33). This enzyme is inactivated by various metal chelators but not by the other usual protease inhibitors, such as diisopropyl phosphofluoridate (DFP), *p*-chloromercuribenzoic acid, tosyl-L-phenylalanine chloromethyl ketone, and ethylenediaminetetraacetate (EDTA) (22, 25). The enzyme is inactivated by *o*-phenanthroline in the presence of a high concentration of Ca ion (28), suggesting that the enzymatic activity is related not to Ca ion but to heavy metal ions. Among various metal ions tested, Co^{2+} was effective in promoting hydrolysis (28). As we have not succeeded in preparing the apo-enzyme (25, 28), we cannot state that the enzyme is a Co^{2+}-enzyme, although the above data suggest this.

The specificity of the protease was deter-

mined using either oxidized insulin B chain (26) or various synthetic substrates (31), and the results indicated that the specificity is determined by the amino acid residues distant from the splitting point (e.g., secondary interaction) rather than by those on either side of it (e.g., primary specificity). Therefore, the specificity against oxidized insulin B chain appears to be very broad.

The elastase is most active in the neutral pH range, and since it is active on elastin it is called "elastase" (23). Its molecular weight is 39,500, and its isoelectric point is at pH 5.9 (32). Due to the difference in the isoelectric points of elastase and protease, these enzymes produced in the same broth are separable by column chromatography on diethylaminoethyl-cellulose (23). The elastase is a typical metal chelator-sensitive neutral proteinase (24, 27, 29, 30), which requires Zn^{2+} for appearance of enzymatic activity and shows specificity to bulky or hydrophobic amino acid residues at the amino side of the splitting point. In affinity chromatography, with Sepharose-ε-aminocaproyl-D-phenylalanine methyl ester, the elastase behaved (29) in the same manner as thermolysin and *Bacillus subtilis* neutral proteinase.

Many others have studied protease and elastase, especially elastase produced by strains isolated from patients. The results were not always consistent with ours. The differences are summarized as follows: (i) the elastase from strain 158 is sensitive to DFP as well as EDTA (2); (ii) the molecular weights of elastases from strains 158 and 5-31 (human corneal infection) are 22,300 (37) and 20,000 (16), respectively, according to gel filtration; and (iii) two or three types of elastases are produced with different isoelectric points but with the other properties remaining the same (37, 39). A strain of *P. aeruginosa* can produce a collagenase that attacks native collagen (3). On the other hand, very few reports on the protease of *P. aeruginosa* exist.

The above indicates that *P. aeruginosa* can produce various types of elastase or protease in addition to collagenase, depending upon the strain used. We studied the enzymes produced by various strains isolated from patients.

MATERIALS AND METHODS

Organisms. Twenty *P. aeruginosa* strains isolated from patients were kindly donated by M. Mayama of this laboratory. Two strains (IFO 3080 and 3455) were from the Institute for Fermentation, Osaka. PA-103 (a non-proteolytic strain that can produce exotoxin A; 19), NC-5, and N-10 were supplied through the courtesy of J. Y. Homma from the Institute of Medical Science, Tokyo University.

Culture. Bouillon (1% meat extract, 1% peptone, 0.5% NaCl, and tap water; pH 7.0) was as the complex medium. The following was used (23) as the semisynthetic medium: 7% glucose, 1% $(NH_4)_2HPO_4$, 1% $Na_2HPO_4 \cdot 12H_2O$, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% yeast extract, and 2.5% $CaCO_3$. Strains were cultured in 100 ml of medium in a 500-ml flask and were shaken at 130 rpm at a 7-cm amplitude in an incubator at 28°C.

Substrates and inhibitors. Casein (Hammarsten) was obtained from Wako Chemical Industries, Ltd., Osaka. Elastin (C grade) was purchased from Calbiochem. Carbobenzoxy-glycyl-L-leucine amide (Cbz-Gly-Leu-NH_2) was supplied by The Protein Research Foundation, Osaka. DFP and EDTA were obtained from L. Light and Co. and Wako Pure Chemical Industries, Osaka, respectively. Fresh rat tail tendon was used as native collagen.

Estimation of enzymatic activity. Proteolytic activity was determined by digestion of 1 ml of 2% casein solution (pH 7.4) with 1 ml of enzyme solution, pH 7.4 (suitably diluted with 0.1 M phosphate, pH 7.4), at 40°C for 10 min, and the liberated tyrosine was estimated by the Folin-Ciocalteu reagent, as described previously (22). The absorbancy at 670 nm was read. The specific activity (protease units) was expressed as milligrams of tyrosine released per minute per milliliter of enzyme solution.

Elastolytic activity was determined according to the plate assay method (36); an elastin agar containing 1% elastin, 2% agar, and 0.03 M tris(hydroxymethyl)aminomethane buffer, pH 8.0, was used. The activity was determined by measuring the clear zone produced on the elastin agar by enzyme impregnated on a round filter paper of 1 cm in diameter, with incubation at room temperature.

For determination of collagenase activity, fresh rat tail tendon (ca. 10 mg) was suspended in 5 ml of the respective broth; toluene was added, and the mixture was kept at room temperature for about 1 week to determine whether the tail tendon would dissolve or not.

The peptidase activity against Cbz-Gly-Leu-NH_2 was determined as follows. A reaction mixture (1 ml) containing an appropriate concentration of enzyme (showing 0.25×10^{-3} protease units), 10 mM substrate, and 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 8) was kept at 40°C for 1 h. The increased ninhydrin value was determined according to the method of Yemm and Cocking (41). The sites of action of the substrate were deduced by paper chromatography of the hydrolysate in comparison with authentic compounds.

Gel filtration chromatography. Chromatography was performed with a column of Sephadex G-100 (medium, Pharmacia) equilibrated with 0.1 M KCl at approximately 4°C. Enzyme concentrate (3 to 5 ml) was applied to the column (3.4 by 97 cm) and eluted at a flow rate of 50 ml/h with the equilibrating buffer. The enzyme concentrate was prepared by salting out the cultural filtrate of the organism used with $(NH_4)_2SO_4$ (0.6 saturation). Fractions (10 ml) were collected and assayed for protease and elastase activity. Apparent molecular weight was determined by using the method of Andrews (1).

Isoelectric focusing. Electrofocusing in sucrose

density gradients was performed as described by Vesterberg (40). Focusing was done using the enzyme concentrate at about 4°C for approximately 46 h in a 110-ml electrolysis column (LKB Instruments) with a final potential of 900 V for the pH 3 to 10 gradient. The pH of each fraction (1 ml) was determined at 4°C, and the fractions were assayed for protease and elastase activity.

RESULTS

Production of protease and elastase by various strains of *P. aeruginosa*. The production of protease or elastase by 20 *P. aeruginosa* strains isolated from patients was studied by shaking culture on either complex or semisynthetic medium. Inhibition with EDTA or DFP was also examined with the protease or elastase produced. The results are summarized in Table 1, with those of strains IFO 3080 and 3455 added for comparison. The table indicates that there are three groups that produce protease and elastase, depending upon the medium used; the first group (IFO 3455) produces protease and/or elastase abundantly in either complex or synthetic medium. The six strains (no. 4, no. 20, TM13, TM14, TM49, and TM97) included in this group can therefore liquefy gelatin by stab culture in bouillon-gelatin.

The second group (IFO 3080) cannot produce protease and elastase in complex medium but can produce both in synthetic or semisynthetic medium. Most of these strains (no. 3, no. 6, no. 15, no. 17, no. 18, no. 19, no. 33, TM92, and TM95) can produce little elastase, like IFO 3080, but three strains (no. 24, no. 40, and TM98) can produce it. The last group produces very little protease and elastase in complex, semisynthetic, and synthetic media; this group includes strains TM16 and TM87. A similar case was observed with PA-103 (a non-proteolytic strain that can produce exotoxin A; 19), NC-5, and N-10, as has been described previously (12).

All the proteases or elastases produced were sensitive to EDTA but not to DFP. No collagenase was produced by any of these strains in either complex or semisynthetic medium.

Properties of proteases and elastases produced by various *P. aeruginosa* strains. To compare the proteases and elastases produced by various strains of *P. aeruginosa*, their molecular weights and isoelectric points were determined by gel filtration and electrofocusing, respectively. A typical example is shown in Fig. 1, where the enzyme concentrate prepared by salting out with $(NH_4)_2SO_4$ (0.6 saturation) from the broth of IFO 3455 cultured in semisynthetic medium was used. In gel filtration with Sephadex G-100 (Fig. 1A), two peaks showing casein digestion activity appeared; the first peak corresponded to a molecular weight of 49,000 and the second one to a molecular weight of 23,000. Elastase activity was not detected in the first peak, but was detected in the second peak. The peptidase activity against Cbz-Gly-Leu-NH_2 was negligible in the first peak and marked in the second peak, and the internal peptide bond was attacked.

In electrofocusing (Fig. 1B), two peaks showing casein digestion activity again appeared; the isoelectric point of the first peak corresponded to pH 5.1 and that of the second peak corresponded to pH 6.9. Both elastase and peptidase (against Cbz-Gly-Leu-NH_2) activities were detected in the second peak but not in the first. Also, the fractions showing isoelectric points of pH 5.1 and 6.9 corresponded to molecular weights of 49,000 and 23,000, respectively; this was found by gel filtration of the respective fractions differing in electrostatic charge, as above.

According to the method described above, the molecular weights, isoelectric points, and other properties of proteases and elastases produced by several strains were determined. The results indicated that all the proteases tested had a molecular weight of 49,000 and an isoelectric point at pH 4.6 to 5.6 and were not active to elastin and Cbz-Gly-Leu-NH_2, whereas the elastases had a molecular weight of 23,000 and an isoelectric point at pH 6.1 to 7.2 and were active to both substrates. The results are summarized in Table 2, where the activity ratio of both enzyme fractions in gel filtration are calculated for the respective strains cultured in either complex or semisynthetic medium. The table indicates that the strains belonging to the first group cannot produce protease but can produce elastase in bouillon. In the semisynthetic medium, this group can produce both protease and elastase, although the activity ratio of the latter enzyme is much greater. In the second group of strains, the activity ratio of protease and elastase differs depending upon the strain used.

DISCUSSION

Mull and Callahan (34) have shown that 56 strains (86%) revealed elastolytic activity among 65 *P. aeruginosa* strains isolated from infections processes in humans and cultured in brain heart infusion (Difco). Our study, however, indicates that the percentage of elastolytic strains in complex medium is considerably smaller (30%). This might be ascribed to the fact that the strains used here had been isolated 3 years ago and, therefore, were not fresh iso-

TABLE 1. *Production of protease and elastase by various strains of P. aeruginosa*

Group	Strain[a]		Proteolytic activity (PU/ml, × 10^3)	Elastolytic activity[b]		Inhibition of proteolytic activity (%)[c]	
				24 h	48 h	EDTA	DFP
I	IFO 3455	(C)	8.0	++	+++	100	0
		(S)	6.0	++	+++	100	0
	No. 4	(C)	3.0	+	+++	100	0
		(S)	3.6	+	+++	63	0
	No. 20	(C)	4.2	++	+++	100	0
		(S)	2.2	+	+++	85	16
	TM 13	(C)	7.0	++	+++	100	0
		(S)	6.0	++	+++	86	0
	TM 14	(C)	6.6	+	+++	100	0
		(S)	5.7	+	+++	86	0
	TM 49	(C)	7.3	++	+++	100	0
		(S)	7.4	+	+++	48	0
	TM 97	(C)	0.9	±	++	100	0
		(S)	No growth				
II	IFO 3080	(C)	0	−	−		
		(S)	2.0	−	±	100	0
	No. 3	(C)	0	−	−		
		(S)	1.5	−	+	100	0
	No. 6	(C)	0.1	−	−		
		(S)	1.7	±	+	100	0
	No. 15	(C)	0	−	−		
		(S)	2.3	−	+	100	0
	No. 17	(C)	0	−	−		
		(S)	1.6	±	+	100	0
	No. 18	(C)	0.3	−	±		
		(S)	1.1	−	−	82	0
	No. 19	(C)	0	−	−		
		(S)	3.3	−	±	100	7
	No. 24	(C)	0.1	−	−		
		(S)	2.7	±	+++	100	0
	No. 33	(C)	0.1	−	−		
		(S)	1.9	−	±	100	0
	No. 40	(C)	0	−	−		
		(S)	1.7	+	++	100	0
	TM 92	(C)	0.1	−	−		
		(S)	1.3	−	±	100	0
	TM 95	(C)	0	−	−		
		(S)	1.2	−	±	100	0
	TM 98	(C)	0	−	−		
		(S)	2.2	±	++	100	0
III	TM 16	(C)	0.2	−	−		
		(S)	0.2	−	−		
	TM 87	(C)	0.1	−	−		
		(S)	0.1	−	−		

[a] The medium used is given in parentheses: C, complex medium; S, semisynthetic medium.

[b] The degrees of elastase activity are as follows: +++ > ++ > + > ± (a slight formation of clear zone) > − (no formation of clear zone).

[c] Suitably diluted solution (2.5×10^{-3} protease units [PU]) was mixed with inhibitor (final concentration: EDTA, 10^{-2} M; or DFP, 10^{-3} M), and the pH of the mixture was adjusted to 7. The mixture was kept at 40°C for 1 h. The remaining activity was determined by the casein digestion described in the text.

lates. It is well known (23) that most strains of stock cultures of *P. aeruginosa* show negligible elastolytic activity in complex medium.

In spite of the marked difference in production of protease and/or elastase by *P. aeruginosa*, depending upon their origin, the respective enzymes produced by various strains of the bacteria may be regarded as identical. The concept is supported not only by our previous report (23) that four proteases (non-elastolytic)

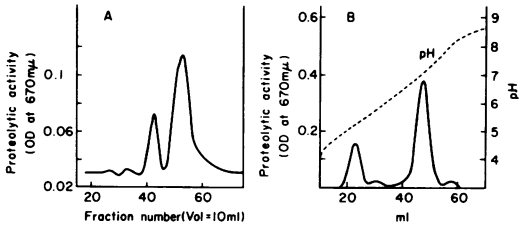

FIG. 1. *Sephadex G-100 gel filtration (A) and electrofocusing (B) of protease and elastase from P aeruginosa IFO 3455 (semisynthetic medium). Column of Sephadex: 3.4 by 97 cm, 0.1 M KCl. Electrofocusing: 110-ml column, pH 3 to 10 carrier ampholite.*

from different origins showed identical immunospecificity, but also by our present study, except for the fact that the isoelectric points of proteases or elastases from different origins were not always the same. Concerning the isoelectric point of elastases, Suss et al. (39) have shown previously that two elastases from different origins differed in electrostatic charges, although both possessed approximately the same molecular weights and proteolytic properties and were immunologically indistinguishable. Scharmann and Balke (37) have also reported that strain 158 produces two elastases with isoelectric points at pH 6.45 (main component) and 5.7, respectively.

Our present study indicates that the isoelectric points of the proteases or elastases produced by various origins differ by about one pH unit. This may mean that the respective enzymes form complexes with impurities present in the broth, which cannot always be removed by electrofocusing. Depending upon the kinds of impurities, the electrostatic charges of the respective enzymes may differ. Therefore, the real isoelectric points should be determined using pure preparations, and the values of proteases or elastases must be identical to that of the crystalline protease from IFO 3080 (22) or the crystalline elastase from IFO 3455 (32), respectively.

In the preceding papers (9, 32), the molecular weights of crystalline protease from IFO 3080 and elastase from IFO 3455 were determined by examining both sedimentation and diffusion coefficients. The molecular weight (48,400) of the former enzyme was almost the same as those (49,000) of proteases from various origins determined by the gel filtration method in this study. On the other hand, all the elastases tested in this experiment had a molecular weight of 23,000 being almost one-half the value (39,500) obtained previously by sedimentation and diffusion. The former value is similar to those in the literature (16, 37), which were determined by gel filtration. We consider that elastase has a molecular weight of approximately 20,000 and dimerizes under certain conditions to yield an apparent molecular weight of 39,500.

TABLE 2. *Molecular weights and isoelectric points of proteases or elastases produced by various strains of P. aeruginosa*

Strain[a]	Activity ratio[b] (Protease [MW 49,000]/ Elastase [MW 23,000])	pI	
		Protease	Elastase
IFO 3455 (C)	0/100		6.8
No. 4 (C)	0/100		7.2
No. 20 (C)	0/100		7.1
TM13 (C)	0/100		6.1
IFO 3455 (S)	25/75	5.1	6.9
No. 4 (S)	10/90	4.6	6.9
No. 20 (S)	30/70	4.8	6.8
TM13 (S)	20/80	5.6	6.4
IFO 3080 (S)	85/15	5.2	7.1
No. 19 (S)	90/10	4.9	6.1
TM98 (S)	30/70	4.6	6.9

[a] The medium used is given in parentheses: C, complex medium; S, semisynthetic medium.

[b] The enzymatic activities corresponding to protease and/or elastase by column chromatography of Sephadex G-100 were determined using casein as substrate (see Fig. 1A), and the activity ratio of both enzymes was calculated. MW, molecular weight.

Balke and Scharmann (2) have shown that the elastase from strain 158 was active against Cbz-Pro↑-Leu-Gly-Pro-DArg (the arrow shows the bond split). Our present study indicates that all the elastases tested are active against Cbz-Gly↑-Leu-NH_2 and are metal chelator-sensitive neutral proteinases, which show their specificity to hydrophobic amino acid residues at the amino side of the splitting point. As the elastase from IFO 3455 has been shown to be active against Cbz-Gly-Pro↑-Leu-Gly-Pro (30), similarly to other neutral proteinases, the proline residue at the carboxyl side of the splitting point probably does not inhibit hydrolysis by the neutral proteinases. Therefore, it could be assumed that the elastase from strain 158 had a specificity identical to that of the usual neutral proteinases thus far examined. However, the enzyme has been shown (2) to be sensitive to DFP as well as EDTA. Our present study indicates that all the proteases or elastases tested are sensitive to EDTA but not to DFP, as has been observed by others (10, 17).

A collagenase-like enzyme was produced by strain 7 in peptone-rich medium (38). The enzyme, purified by column chromatography

on diethylaminoethyl-Sephadex, was active against Cbz-Gly-Pro-Gly↑-Gly-Pro-Ala (a synthetic substrate for clostridial collagenase), casein, and gelatin but was not active against native collagen. We have shown (31) that the protease from IFO 3080 is active against the above hexapeptide at the same splitting point. Nevertheless, our previous (23) and present experiments indicate that the enzyme is produced only in synthetic or semisynthetic medium. Usual neutral proteinases cannot attack the hexapeptide; therefore, a similar case might be observed with elastases from *P. aeruginosa*, which are produced in complex medium. To clarify the conflict, a more detailed study would be required on the collagenase-like enzyme from strain 7.

Strain L-1, isolated from a patient, produced a collagenase in peptone-Trypticase soy broth, and the collagenase was purified in the homogeneous form (3). However, in our experiment using 20 strains isolated from patients, no collagenolytic enzyme was detected in any broth. The collagenase from strain L-1 had a molecular weight of 17,500 and dimerized under certain conditions. The other properties were similar to those of the elastase from IFO 3455, except that the collagenase was active against native collagen. For example, they are inactive against Cbz-Gly-Pro-Gly-Gly-Pro-Ala, and amino acid compositions of both enzymes are markedly similar (Table 3), when the composition is calculated assuming that the molecular weight of both are identical.

We conclude that *P. aeruginosa* can produce at least two proteolytic enzymes, such as protease and elastase, that are identical notwithstanding the difference in their origin.

TABLE 3. *Amino acid analyses of elastase and collagenase*[a]

Amino acid	Elastase (ref. 32)	Collagenase (ref. 3)
Asp	12.2	12
Thr	5.0	5
Ser	6.3	7
Glu	5.0	6
Pro	3.0	4
Gly	10.0	11
Ala	8.0	8
Val	5.0	5
Met	2.2	2
Ile	2.4	3
Leu	3.7	4
Tyr	6.0	6
Phe	4.7	5
Lys	3.0	4
His	1.8	3
Arg	4.0	4
CysSH	1.1	1.0

[a] The compositions (number of residues per molecule) were calculated assuming that the molecular weight is 9,900.

This conclusion is supported by Homma et al. (8), who established a method for estimating antibodies of protease and elastase of *P. aeruginosa* by passive hemagglutination tests using fixed sheep erythrocytes coated with each of the two enzymes; the crystalline protease from IFO 3080 (22) and the crystalline elastase from IFO 3455 (32) were used as the enzyme sources. High antibody titers of protease and elastase were found in the hemagglutination tests on sera from some patients suffering from *P. aeruginosa* infection.

It has also been shown (12, 35) that *P. aeruginosa* strains (such as IFO 3455) that can produce protease and elastase are much more pathogenic than those that cannot produce either of the enzymes. Therefore, antibodies or vaccines of both enzymes should be useful medically for *P. aeruginosa* infection. Experiments along this line have been done (13) and more are now in progress (E. Honda, J. Y. Homma, C. Abe, K. Tanamoto, H. Noda, and R. Yanagawa. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg., in press).

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