Purification and Properties of a Protease with Elastase Activity from *Pseudomonas aeruginosa*

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The isoelectric points of three proteases (I, II and III), separated from culture supernatants of *Pseudomonas aeruginosa* strain PAKS-1 by isoelectric focusing, were 8.5, 6.6 and 4.5 respectively. Collagenase activity was not detected. More than 75% of the extracellular protease activity of this strain was due to protease II. This enzyme also possessed elastase activity. When purified by ammonium sulphate precipitation, isoelectric focusing and gel chromatography, protease II showed one band on disc electrophoresis and one band on conventional immunoelectrophoresis. The pH optimum, stability and effect of inhibitors and substrate concentration were examined. The molecular weight was 23000 ± 5000 . Protease II was lethal for mice when injected intraperitoneally at a high dose (minimum lethal dose 0.1 mg). Dermonecrosis and subcutaneous haemorrhages were produced in new-born mice upon subcutaneous injection of 10 µg protease II. A sensitive test for cytotoxicity showed no evidence of cytoplasmic membrane damage to HeLa cells or human diploid embryonic lung fibroblasts by protease II. Morphological changes similar to those produced by trypsin were found.

INTRODUCTION

Pseudomonas aeruginosa is a micro-organism with a low virulence for humans. However, it may cause severe infections in patients debilitated by burns, malignant tumours or chronic diseases (Young & Armstrong, 1972; Flick & Cluff, 1976). Strains of *P. aeruginosa* produce several extracellular proteins, some of which are believed to be virulence factors (Wretling *et al.*, 1973; Liu, 1974). Morihara (1964) showed that *P. aeruginosa* produced three proteases with different isoelectric points, substrate specificities and pH optima, and that one of these enzymes had elastase activity. Kreger & Griffin (1974) separated three cornea-damaging proteases from the culture supernatant of a strain that was capable of producing keratitis in rabbits. These proteases seemed to correspond to those described by Morihara (1964).

One of the features of *Pseudomonas* septicæmia is the destruction of the arterial elastic lamina (Margaretten, Nakai & Landing, 1961). There is evidence that this vascular lesion is precipitated by an elastolytic protease (elastase) (Mull & Callahan, 1965). A correlation between the ability to produce protease in broth and virulence for mice was found for strains of *P. aeruginosa* (Muszyński, 1973). The above-mentioned investigations indicate a role

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for the protease in the pathogenesis of *Pseudomonas* infections and that the proteases might be important factors contributing to the virulence of the organism.

The present study was undertaken to purify and characterize the protease with elastase activity on a scale large enough to allow studies on the physicochemical and biological properties of the enzyme, since conflicting reports on this subject have appeared (Liu, 1974).

METHODS

Bacterial strain and growth conditions. *Pseudomonas aeruginosa* strain PAKS-1 was isolated from a human urine specimen at the Karolinska Hospital in 1970. The strain produced pyocyanin and was identified as *Pseudomonas aeruginosa* according to the methods described previously (Wretling *et al.*, 1973). This strain was the best producer of extracellular protease activity among 30 strains tested. It was stored in foetal calf serum at -20°C and grown in: (i) brain heart infusion broth (BHI) supplemented with 50 mM-glucose; (ii) minimal medium of Davis & Mingioli (1950) supplemented with tryptone (5 g l⁻¹), yeast extract (2.5 g l⁻¹), glucose (10 mM) and CaCl₂ (1 mM) (MTYG); (iii) minimal medium supplemented with yeast extract (2 g l⁻¹), glucose (50 mM) and CaCl₂ (1 mM) (MYG); and (iv) MYG medium supplemented with Casamino acids (10 g l⁻¹) (M CYG). The different components were autoclaved separately.

Cultures were grown at 37°C for 14 to 16 h in 125 ml medium in 1 l indented Erlenmeyer flasks (Arvidson, Holme & Wadström, 1971) with rotary shaking (140 rev. min⁻¹, rotation radius 25 mm). Polypropylene glycol was used as an antifoaming agent. In later experiments, cultures were grown in a 3 l fermenter (FL-103, Biotec, Stockholm, Sweden), as described by Arvidson *et al.* (1971), for 7 h at 37°C . The impeller speed was 600 rev. min⁻¹, aeration was at 1 l min⁻¹, and pH was maintained at 7.0 by automatic addition of 1 M-HCl or 1 M-NaOH. Tributyl phosphate was used as an antifoaming agent. The inoculum was prepared by centrifuging (4000g, 20 min) 300 ml of an overnight culture in the same medium as in the fermenter and resuspending the bacteria in the fresh medium.

Assays. Protease activity was assayed according to Kunitz (1946/1947) using heat-denatured casein as substrate. The extent of proteolysis was determined by reading the absorbance of perchloric acid-soluble degradation products at 280 nm. The reaction mixture consisted of casein (10 g l⁻¹) in 0.05 M-sodium phosphate pH 7.4 supplemented with CaCl₂ (1 mM). The sample (0.2 ml) was added to 4 ml of the mixture, pre-incubated at 37°C , and the reaction was terminated after 30 min by adding 3 ml 1 M-perchloric acid. The precipitate was removed by centrifugation. A blank was prepared for each sample tested. One unit of activity was defined as an increase in $E_{280\text{ nm}}^{10\text{ mm}}$ of 1.0 in 30 min at 37°C . The reaction was linear up to $E_{280\text{ nm}}^{10\text{ mm}} = 0.5$.

Elastase activity was estimated by the elastin-congo red method of Sachar *et al.* (1955). Elastin-congo red was suspended in 0.1 M-Tris/maleate buffer. One unit of elastase activity released soluble dye from 1 mg elastin-congo red in 2 h at 35°C , corresponding to $E_{496\text{ nm}}^{10\text{ mm}} = 0.25$. A similar assay with elastin-orcein was used in a few experiments but was less sensitive.

Collagenase activity was estimated by the collagen disc assay described by Smyth & Arbuthnott (1974). Protein was determined according to Lowry *et al.* (1951) with bovine serum albumin as standard. The samples were dialysed against distilled water before analysis.

Isoelectric focusing. Electrofocusing was done in an LKB column of 440 ml capacity according to Vesterberg *et al.* (1967). The focusing was run for 48 h and 10 ml fractions were collected. A pH gradient of 3 to 10 was used consisting of Ampholine 3-5 (1 ml), Ampholine 6-8 (2 ml) and Ampholine 3.5-10 (8.2 ml). A density gradient of glycerol [0 to 60% (w/v)] was used in all experiments. Columns of 110 ml capacity were used in a few experiments (for analytical purposes). A shallow gradient of pH 5 to 8 was used for refocusing of protease II; it consisted of Ampholine 6-8 (0.8 ml) and Ampholine 5-7 (1.5 ml). A column of 110 ml capacity was used and 2 ml fractions were collected.

Gel chromatography and molecular weight determination. Protease II was separated after isoelectric focusing by gel chromatography on columns of Biogel P-60 or Sephadex G-75 (1 m × 25 mm). The columns were calibrated at 4°C with Dextran Blue 2000, cytochrome c, myoglobin, ovalbumin and haemoglobin (Wadström & Hisatsune, 1970). The molecular weight was determined by the method of Andrews (1964).

Production of antisera. Antisera against culture supernatants were produced in rabbits. Culture supernatants were concentrated to one-tenth of their original volume by dialysis against polyethylene glycol and centrifuged at 100000g at 4°C for 4 h. The pellet was discarded. The rabbits were injected four times at 3-week intervals with supernatants corresponding to 2 to 5 mg protein mixed with Freund's complete adjuvant, followed by injection of the same amount of antigen intravenously 1 week before collection of blood.

Immuno-electrophoresis and disc electrophoresis. Immuno-electrophoresis was done according to Grabar & Williams (1953). Disc electrophoresis on polyacrylamide gel was carried out as described by Wadström & Möllby (1971) in a continuous Tris/glycine buffer at pH 8.5. Samples containing 100 µg protein were applied

to each gel. Two gels were run with purified protease: one gel was stained with Coomassie blue, and the other was sliced longitudinally and applied to an agar plate containing 15% (v/v) skimmed milk. Protease activity was detected as a clearing of the skimmed milk agar.

Purification of extracellular proteases. The culture supernatants (2 to 3 l) were centrifuged (6000g, 20 min, 4 °C) and concentrated to approximately one-third of their original volume by dialysis against polyethylene glycol. Two volumes of saturated $(\text{NH}_4)_2\text{SO}_4$ (4 °C, pH 7) were added dropwise with constant stirring and left for 24 h. The precipitate was collected by centrifugation, dissolved in 0.05 M-sodium phosphate buffer pH 7.0, dialysed against the same buffer overnight and then dialysed against distilled water. The pH was adjusted to 8.0 with 0.05 M-NaOH. Negative adsorption was performed by adding 3 g diethylaminoethyl-Sephadex (DEAE-Sephadex A-25) per litre of original culture supernatant (Wretling, Möllby & Wadström, 1971). The DEAE-Sephadex was removed by centrifugation and the supernatant was dialysed against 0.13 M-glycine, concentrated by dialysis against polyethylene glycol and subjected to isoelectric focusing. After electrofocusing, the peak fractions containing protease activity were pooled and chromatographed on Biogel P-60 or Sephadex G-75. The purified protease was concentrated 10-fold by ultrafiltration (Diaflo ultrafiltration membrane PM10, Amicon, Lexington, Massachusetts, U.S.A.) and stored at -60 °C.

Cytotoxicity tests. For determining membrane-damaging activity, a quantitative assay was used (Thelestam, Möllby & Wadström, 1973). Human diploid embryonic lung fibroblasts and HeLa cells were incubated for 2 h in medium containing [^3H]uridine ($1 \mu\text{Ci ml}^{-1}$, 20 Ci mm^{-1}). The cells were then exposed for 30 min to the protease diluted in Eagle medium to a final volume of 1 ml. The medium was removed and centrifuged, and the supernatant was transferred to a scintillation vial. Maximum release was measured after lysing the cells with 0.06 M-sodium borate buffer pH 8.6. The samples were run in duplicate, and uninoculated controls were included.

Animal experiments. Female mice, strain NMRI (Anticimex, Stockholm, Sweden; 16 to 18 g) were used for toxicity tests. The enzyme was injected intravenously or intraperitoneally. For a few experiments, NMRI mice (1 to 2 days old), were injected subcutaneously with protease diluted in phosphate-buffered saline. The mice were observed for 1 week.

Chemicals. L-[(Toluene-4-sulphonamido)-2-phenyl]ethyl chloromethyl ketone, *N*- α -toluene-4-sulphonyl-L-lysine chloromethyl ketone hydrochloride, elastin-congo red, elastin-orcein, cytochrome *c*, myoglobin, ovalbumin, horse haemoglobin and Tris were obtained from Sigma; *N*- α -toluene-4-sulphonyl-L-arginine methyl ester hydrochloride from BDH; Dextran Blue 2000 from Pharmacia; agarose (Indubiose A 37) from L'Industrie Biologique Française, Gennevilliers, France; acrylamide and bis-acrylamide from Eastman Organic Chemicals; vitamin-free casein from Nutritional Biochemicals, Cleveland, Ohio, U.S.A.; bovine serum albumin from AB Kabi, Stockholm, Sweden; polypropylene glycol P-200 from Dow Chemical Co., Midland, Michigan, U.S.A. and polyethylene glycol 20M (mol wt 20000) of technical grade from KEBO, Stockholm, Sweden.

RESULTS

Influence of cultivation conditions

Pseudomonas aeruginosa strain PAKS-I produced protease activity in all broth media tested. The yields during cultivation in shaking flasks in MTYG, MCYG and BHI plus glucose media were 20 to 30 u. ml^{-1} . Cultivation in the fermenter yielded protease activities of 30 to 60 u. ml^{-1} in MCYG and MTYG media and 20 to 30 u. ml^{-1} in MYG medium. Collagenase activity was not detected in supernatants of cultures grown in BHI plus glucose medium.

Isoelectric focusing of culture supernatants

Culture supernatants from different media were subjected to isoelectric focusing. Three proteases were partially separated from MYG medium but only two proteases were found in MCYG medium (Fig. 1). The peak fractions of the three proteases were at pH 8.5 (protease I), pH 6.0 to 7.2 (protease II) and pH 4.5 (protease III). Proteases I and II were detected in all media used, but protease III was not detected in MCYG medium and was sometimes absent in BHI plus glucose medium. Refocusing of protease II in a shallow pH gradient gave peak fractions of activity at pH 6.2 to 6.3 (Fig. 2). The isoelectric point of protease II could not be determined accurately, though based on 12 electrofocusing experiments a pI of 6.6 ± 0.6 was calculated. The isoelectric points of the other proteases (8.5 and 4.5) were reproducible.

Fig. 1. Separation by isoelectric focusing, in a pH gradient of 3 to 10 (●) at 4 °C, of three proteases from crude culture supernatants of strain PAKS-I grown in MYG medium (△) and MCYG medium (○). The cultivations (in fermenters) and electrofocusing experiments were run in parallel.

Fig. 2. Isoelectric focusing, in a shallow pH gradient at 4 °C, of protease II (pI 6.6 ± 0.6) from the experiment shown in Fig. 1. Symbols as in Fig. 1.

Purification of protease II

For purification of proteases, MTYG medium was chosen since all three proteases were produced in this medium and the protease activity was higher than in MYG medium. After growth in MTYG medium in a fermenter, protease II was purified from the culture supernatant by $(\text{NH}_4)_2\text{SO}_4$ precipitation, negative adsorption with DEAE-Sephadex, isoelectric focusing and gel chromatography (Table 1). Based on electrofocusing experiments, 75 to 90% of the total protease activity of the culture supernatant was calculated to be due to protease II. The final yield was thus 3 to 4% after correction for the activities of proteases I and III in the supernatant. Similar results were obtained after growth of cultures in MCYG, MYG and BHI plus glucose media. The molecular weight of protease II as determined by gel chromatography on Sephadex G-75 or Biogel P-60 was $23\,000 \pm 5\,000$ (five experiments; Fig. 3). Disc electrophoresis of the purified protease showed one band in gels containing 7.5% (w/v) polyacrylamide (Fig. 4). However, in gels containing 5% and 10% polyacrylamide two faint extra bands were visible. The zymogram on agar containing skimmed milk showed that the main band was associated with protease activity. Immuno-electrophoresis gave one line of precipitate. The protease did not migrate during electrophoresis.

Table 1. Purification of protease II from a culture supernatant of *Pseudomonas aeruginosa*

The culture supernatant was from strain PAKS-I grown in a tryptone-containing broth (MTYG) at 37 °C in a 3 l fermenter.

Fraction	Volume (ml)	Protein		Protease activity		Yield (%)	Specific activity (u. mg ⁻¹)
		(mg ml ⁻¹)	total (mg)	(u. ml ⁻¹)	total (u.)		
Culture supernatant	2700	0.3	810	38	102 000	100	127
(NH ₄) ₂ SO ₄ precipitation and DEAE-Sephadex adsorption	61	4.0	244	1020	62 000	61	255
Isoelectric focusing	30	2.2	66	370	11 000	11	170
Sephadex G-75 chromatography	40	0.2	8	70	2 800	3	350

Fig. 3

Fig. 4

Fig. 3. Determination of the molecular weight of protease II by chromatography on Sephadex G-75: 1, cytochrome *c*; 2, myoglobin; 3, pseudomonas protease II; 4, ovalbumin; 5, haemoglobin.

Fig. 4. Analytical polyacrylamide gel electrophoresis of protease II. From left to right: culture supernatant; enzyme after isoelectric focusing; purified protease after chromatography on Sephadex G-75. The gels contained 7.5% polyacrylamide.

Properties of purified protease

With casein as substrate, protease II had a pH optimum at 8.0 (Fig. 5) in both sodium phosphate and Tris/HCl buffers. Elastase activity of protease II was 14 times higher in Tris/maleate than in sodium phosphate buffer, and the pH optimum was also affected by the buffer, being 6.5 in Tris/maleate and 8.0 in phosphate buffer (Fig. 5).

The rate of hydrolysis of casein was determined in phosphate buffer containing 1 mM-CaCl₂. The action of protease II followed Michaelis-Menten kinetics when determined by the method of Lineweaver & Burk (1934). A *K_m* value of 1.5 g casein l⁻¹ and a *V_{max}* value of 0.4 Δ*E*_{280 nm}^{10 min} (30 min)⁻¹ μg⁻¹ (pH 7.4) based on a specific activity of 350 u. mg⁻¹ were calculated from these data.

Of the different ions and potential inhibitors of caseinolytic activity tested (Table 2), heavy metal ions, dithiothreitol and EDTA were inhibitory.

Purified protease II was rapidly degraded at 4 °C with an estimated half-life of 3 to 5 days. The enzyme activity was stable for more than a year at -60 °C, but lost 30 to 50% of its activity after 4 months at -20 °C.

Fig. 5. Effect of pH on protease (caseinolytic) and elastase activity of protease II. Protease activity in phosphate buffer (□); elastase activity in Tris/maleate buffer (△); elastase activity in phosphate buffer (○). All buffers were supplemented with 1 mM-CaCl₂. To each tube was added 0.5 µg protease for the caseinolytic assay and 8 µg protease for the elastase assay.

Toxicity studies

The minimum lethal dose in mice for protease II was 0.1 mg after intravenous or intraperitoneal injection. Subcutaneous injection of 10 µg into newborn mice caused local dermonecrosis and subcutaneous haemorrhages. The lesions healed after 1 week but no hair growth appeared in the damaged area.

Cytotoxicity tests showed no evidence of membrane damage as indicated by lack of leakage of [³H]uridine when HeLa cells or human embryonic lung fibroblasts were treated with 10 to 100 µg purified protease II ml⁻¹. However, morphological changes were seen with rounding of the cells, similar to those produced by trypsin (Thelestam *et al.*, 1973).

DISCUSSION

Moriyama (1964) separated three proteases from *P. aeruginosa* by ion exchange chromatography which seem to correspond to those obtained in this study as judged by similarities in pH optima, isoelectric points and, in the case of protease II, elastase activity. However, Moriyama *et al.* (1965) estimated the molecular weight of fraction II (corresponding to protease II in this study) to be 39 500 by determining the sedimentation coefficient by ultracentrifugation. The molecular weight we found for protease II (23 000) agrees with the results of Kreger & Griffin (1974) and Scharmann & Balke (1974). The reason for this discrepancy is not known but differences in the strains of *P. aeruginosa* used, in the methods for purifying the enzymes and in the methods for determining molecular weight are probably important.

The isoelectric points of the various proteases do, however, agree. Although the pI of protease II of PAKS-1 varied between 6.0 and 7.2 when culture supernatants were subjected to isoelectric focusing, these differences did not depend on the media used. The lack of reproducibility is therefore possibly due to post-synthetic modification such as autodigestion of the enzyme. Similar results have been described for several bacterial enzymes and toxins, e.g. staphylococcal proteins (Arbuthnott, McNiven & Smyth, 1975). Suss *et al.* (1969) reported the existence of two distinct elastases in strains of *P. aeruginosa* which showed complete immunological identity and similar properties except for isoelectric points, indicating the existence of two molecular forms with different charge properties.

Scharmann & Balke (1974) found only one protease in culture supernatants of their

Table 2. *Effect of metal salts, reducing and chelating agents and protease inhibitors on the activity of purified protease II*

Protease activity was assayed according to Kunitz (1946/1947) using casein as substrate in 0.05 M-Tris/HCl pH 7.4. The enzyme was pre-incubated with the various agents for 16 h at 4 °C. Results are expressed as a percentage of the control activity which was 1.6 u._gml⁻¹.

Agent tested	Concn (mM)	Protease activity (%)
None (control)	—	100
CaCl ₂	1	100
	10	100
CdCl ₂	1	30
	10	22
CuCl ₂	1	11
	10	1
MgCl ₂	1	89
	10	61
MnCl ₂	1	44
ZnCl ₂	1	42
	10	8
HgCl ₂	1	2
	10	0
CoCl ₂	1	111
	10	85
Sodium cacodylate	1	100
	10	100
EDTA	1	31
	10	4
Dithiothreitol	1	57
	10	53
	10	100
<i>N</i> - α -Toluene-4-sulphonyl-L-arginine methylester.HCl		
L-[(Toluene-4-sulphonamido)-2-phenyl]ethyl chloromethyl ketone	10	100
<i>N</i> - α -Toluene-4-sulphonyl-L-lysine chloromethyl ketone.HCl	10	100

strain of *P. aeruginosa* which contrasts with our findings. Their result, however, may be due to using complex media containing amino acids as we, like Morihara (1964), found that protease fraction III (corresponding to protease III) was not produced in such media. Alternatively, different strains may produce different protease profiles. Indeed, proteases with properties different from the enzymes described in this paper have been characterized from other isolates of *P. aeruginosa*. Schoellman & Fisher (1966) found a collagenase active against a synthetic substrate and Carrick & Berk (1975) described another, probably different, collagenolytic enzyme which was not identical to any of the enzymes produced by strain PAKS-1.

The toxicity of our purified protease II is consistent with previous reports (Meinke *et al.*, 1970; Kawaharajo *et al.*, 1975*b*). The minimum lethal dose for mice (0.1 to 0.5 mg depending on the route of inoculation) indicates a low toxicity compared with bacterial toxins, such as exotoxin A from *P. aeruginosa* with an LD₅₀ for mice of 140 ng (Callahan, 1976). As protease II is inhibited by α_2 -macroglobulin in serum (Hochstrasser, Theopold & Brandl, 1973), it probably cannot contribute to the ability of the micro-organism to produce lethal

infections. Studies on the virulence of protease-deficient mutants of strain PAKS-I for mice again indicate that the role of these enzymes in bacteraemic infections has been overestimated (B. Wrettlind and T. Kronevi, unpublished observations). However, the dermo-necrotic and cornea-damaging effects are produced by a few micrograms of purified protease (Kreger & Griffin, 1974; Kawaharajo *et al.*, 1974, 1975*a*), which suggests a role in localized infections where serum protease inhibitors may not be present in sufficient amounts.

Although Botzenhart & Ebel (1973) and Pflüger, Scharmann & Blobel (1975) found a cytotoxic effect of protease from *P. aeruginosa*, we failed to detect a membrane-damaging effect in HeLa cells and fibroblasts, using a sensitive method, beyond that elicited by trypsin (Blumberg & Robbins, 1975). The tissue damage caused by pseudomonas protease II probably depends more on the effect on elastin or the ground substance of connective tissue and cornea than on cytotoxic effects in infected patients or animals.

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