Physicochemical Fractionation of Extracellular Cornea-Damaging Proteases of *Pseudomonas aeruginosa*

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Fractionation of the culture supernatant fluids of a cornea-virulent strain of *Pseudomonas aeruginosa* by ammonium sulfate precipitation, dialfiltration, isoelectric focusing, ion-exchange chromatography, gel filtration, and sucrose density gradient centrifugation failed to separate the rabbit cornea-damaging activity and the in vitro protease activity of the preparations. Three proteases having similar molecular weights (approximately 20,000) and isoelectric points of approximately 4.6, 5.8, and 8.8 were obtained free of detectable amounts of other known extracellular pseudomonal enzymes. Heating a mixture of the three proteases for 15 min at 80°C resulted in complete loss of protease and cornea-damaging activities. The sterile culture filtrate of a nonproteolytic but lethal toxin-producing strain of *P. aeruginosa* did not contain cornea-damaging activity. Cultivation of the proteolytic strain in broth containing 4.7% ammonium sulfate yielded a culture supernatant fluid free of protease and cornea-damaging activities. The results obtained support the conclusion that a cornea-virulent strain of *P. aeruginosa* can produce, in vitro, at least three different extracellular proteases capable of eliciting rapid and extensive damage to rabbit corneas. 

*Pseudomonas aeruginosa* produces an acute and severe corneal infection which progresses rapidly, is difficult to treat, and often results in extensive corneal scarring and visual impairment (10, 19). The rapidly developing corneal destruction caused by this bacterium also may result in corneal perforation with its attending complications of panophthalmitis. In addition, the infection may become systemic in premature infants and cause their death (8).

Despite the fact that this bacterium is currently one of the leading causes of central corneal ulcers of bacterial etiology (19), the mechanism(s) by which it produces corneal pathology is still unclear. Fisher and Allen (11, 12) and Wilson (40) showed that sterile culture filtrates of cornea-virulent strains of *P. aeruginosa* could, if intracorneally injected, produce damage grossly similar to that observed during corneal infection. The culture filtrates possessing this pharmacological activity were not extensively examined for the presence of the many known extracellular products of *P. aeruginosa* (16, 41); however, they were reported to contain protease and hexapeptidase activities, to digest rat tendon collagen, and to degrade a crude corneal extract thought to contain collagen (11, 12, 35, 40). The cornea-damaging activity was nondialyzable, could be precipitated with ammonium sulfate or ethanol, and was inhibited by disodium ethylenediaminetetraacetate. The active component was believed to be a pseudomonal protease or a collagenase also possessing hexapeptidase activity. However, in view of the fact that past investigators examined relatively crude preparations, which could have contained many different pseudomonal products, the observed pathology could not be rigorously ascribed to a particular enzyme or toxin.

The present study was initiated as a first step toward elucidating the nature and properties of the extracellular products of *P. aeruginosa* which elicit the fulminating corneal destruction characteristic of pseudomonal corneal infections. Data are presented to show that a cornea-virulent strain of *P. aeruginosa* produces, in vitro, three extracellular proteases capable of causing rapid and extensive damage to rabbit corneas.

**MATERIALS AND METHODS**

**Bacteria and culture media.** *P. aeruginosa*, strain 5-31, isolated from a human corneal infection, was kindly supplied by J. Gerke (14). *P. aeruginosa*, strain PA-103, was supplied through the courtesy of P. V.
Liu. This strain produces the pseudomonal lethal toxin in vitro but is a very poor producer of protease (21). The bacteria were cultivated in tryptone-yeast extract-glucose broth (pH 7.2) containing 0.5% tryptone (Difco), 0.25% yeast extract (Difco or BBL), and 0.1% glucose. Preliminary experiments showed that the 5-31 strain produced an optimal amount of extracellular protease activity when cultivated in this medium. Liu and Hsieh (22) have shown that the presence of ammonium sulfate in the medium inhibits protease production by various bacteria. During our investigation, the ability of ammonium sulfate to inhibit the production of protease and cornea-damaging activities was examined by adding various amounts of a solution of ammonium sulfate (52% wt/vol, pH 7.2), sterilized by membrane filtration, to the medium.

Preparation of extracellular protease concentrate. Two-liter flasks containing 400 ml of tryptone-yeast extract-glucose broth were inoculated with 0.1 ml of a stationary-phase culture of P. aeruginosa, strain 5-31. The medium was incubated for 20 to 24 h at 35°C on a gyratory shaker (model G25, New Brunswick Scientific Co., New Brunswick, N.J.) operating at 200 cycles/min, and the cells were removed by centrifugation at 5°C. The culture supernatant fluid was saturated with ammonium sulfate (Schwarz/Mann, ultrapure grade) and, after standing overnight at 5°C, the precipitate was recovered by centrifugation (18,000 × g, 20 min) and mixed with sufficient 0.05 M ammonium bicarbonate (pH 7.8) to yield an extract 1/4th the volume of the initial culture supernatant fluid. The extract was clarified by centrifugation and membrane filtration (0.45 μm pore size) and diafiltered in a TCF 10 ultrafiltration unit, equipped with a PM 10 membrane (Amicon Corp., Lexington, Mass.), against 5 volumes of 0.05 M ammonium bicarbonate (pH 7.8). The diafiltered extract was lyophilized and stored at 0°C.

Assays. Protease activity was assayed by a modification of the method used by Fisher and Allen (11). The reaction mixture containing 0.5 ml of an aqueous solution (5 mg/ml) of azocasein (lot 32C-3630, Sigma Chemical Co., St. Louis, Mo.), 0.5 ml of 0.2 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5), 0.4 ml of water, and 0.1 ml of enzyme solution diluted with 0.02 M Tris-hydrochloride (pH 7.5) was incubated for 10 min at 37°C. The reaction was stopped by the addition of 3.5 ml of 5% trichloroacetic acid, the precipitate was removed by centrifugation, and 1 volume of 0.5 M NaOH was added to the supernatant fluid containing the diazotized trichloroacetic acid-soluble peptides. The absorbance of the mixture at 440 nm (A440) was measured with 10-mm light-path cuvettes in a Beckman DB-GT spectrophotometer. A linear rate of increase was obtained up to an A440 of approximately 0.2. One unit of activity was defined as that amount which yielded, under the conditions described, a mixture having an absorbance of 1 at 440 nm.

Pseudomonal protease preparations obtained by isoelectric focusing were examined for the presence of elastase (31), hexapeptidase (15), collagenase (33), lecithinase C (18), esterase (23), lipase (36), lipo- protein lipase (3), deoxyribonuclease (25), ribonuclease (28), alkaline phosphatase (13), hemolysin with rabbit and sheep erythrocytes (6), and bacteriolytic (7) activities. The methods used are those described in the appropriate references. Enzymes, used as positive controls, and substrates were obtained from the Sigma Chemical Co., Worthington Biochemical Corp. (Freehold, N.J.) or Schwarz/Mann (Orangeburg, N.Y.).

Preparations sterilized by membrane filtration (0.2 μm pore size) were examined for cornea-damaging activity by intracorneal injection of samples (30 μlitters), with needles (30 gauge by 0.5 inch) (Storz Surgical Instruments, St. Louis, Mo.), into 3- to 5-lb (about 1.4 to 2.3 kg) New Zealand white rabbits. Prior to injection, the rabbits were anesthetized with ether and their corneas were anesthetized with a sterile solution (0.5%) of tetracaine hydrochloride (Pontocaine HCl, Winthrop Laboratories, New York, N.Y.). The corneas were examined for gross damage at various time intervals for 2 days after injection.

Isoelectric focusing. Electrofocusing in sucrose density gradients was performed with the equipment and methodology described by Vesterberg (38). The gradient was prepared with (i) a less dense solution consisting of 51.7 ml of water, 0.8 ml of 40% (wt/vol) amphetamine (pH 3.5 to 10) or ph 5 to 7; LKB Instruments, Rockville, Md.), and 25 ml of sucrose to make a density of 1.4 (about 30% sucrose) and (ii) a more dense solution consisting of 38.8 ml of water, 1.7 ml of 40% (wt/vol) amphetamine (pH 3.5 to 10 or pH 5 to 7), and 25 g of sucrose (Schwarz/Mann, ultrapure grade). Focusing was done at 4°C for approximately 46 h in a 110-ml electrolysis column (LKB Instruments) with a final potential of 600 V for the pH 3.5 to 10 gradient, and a final potential of 900 V for the pH 5 to 7 gradient. The pH of each fraction (4 ml) was determined at 4°C, and the fractions were assayed for protease activity. The peak fractions of protease activity were tested for cornea-damaging activity. They were dialyzed, prior to injection, against 0.1 M ammonium bicarbonate (pH 7.8).

Ion-exchange chromatography. Diethylaminoethyl agarose (DEAE Bio-Gel A, Bio-Rad Laboratories, New York, N.Y.) was equilibrated with 0.02 M Tris-hydrochloride (pH 8) and packed in a water-cooled (5°C) column (2.6 by 45 cm). After addition of the extracellular protease concentrate (40 mg dissolved in 8 ml of equilibrating buffer) to the column, the column was washed with 411 ml of equilibrating buffer. Gradient elution was performed with a linear gradient maker (Pharmacia Fine Chemicals, Piscataway, N.J.) consisting of a reservoir containing 0.02 M Tris-hydrochloride (pH 7) and 0.5 M NaCl which fed into a constant-volume mixing chamber containing 260 ml of 0.02 M Tris-hydrochloride (pH 7). The constant-volume mixing chamber discharged into the column at a rate of approximately 20 ml/h. After gradient elution, the column was washed with 350 ml of 0.02 M Tris-hydrochloride (pH 7) containing 1 M NaCl. Fractions (5 ml) were collected and assayed for protease activity and absorbance at 280 nm. The peak fractions of protease activity were tested for cornea-
damaging activity. The elution gradient was measured with a conductivity bridge (model RC-16B2, Beckman Instruments, Cedar Grove, N.J.).

**Gel filtration chromatography.** Gel filtration was performed with water-cooled (5°C) columns of Sephadex G-75 regular and superfine (Pharmacia) equilibrated with 0.1 M ammonium bicarbonate (pH 7.8). Extracellular protease concentrate (30 mg dissolved in 3 ml of equilibrating buffer) was applied to the columns and eluted, in the upward flow mode, at a flow rate of 12 ml/h (2.3 ml per cm² per h) with the equilibrating buffer. Fractions (3 ml) were collected and assayed for protease activity and absorbance at 280 nm. The peak fractions of protease activity were tested for cornea-damaging activity. Blue dextran was used to determine the column void volume and the homogeneity of column packing. Apparent molecular weight was estimated by the method of Andrews (2).

**Sucrose density gradient centrifugation.** Rate zonal centrifugation in a sucrose density gradient was performed by the method of Martin and Ames (24). Samples (0.1 ml) were layered onto 5-ml preformed linear gradients of 5 to 20% sucrose (wt/vol) in 0.02 M Tris-hydrochloride (pH 7.5) contained in cellulose nitrate tubes (0.5 by 2.0 inches; ca. 1.3 to 5.1 cm) and were centrifuged (SW-50 rotor, 40,000 rpm, 24 h, 5°C) in a Beckman L2-65B ultracentrifuge. The extracellular protease concentrate (1 mg) and reference proteins (2 mg) were centrifuged in separate tubes, in triplicate, and the contents of each of the tubes were collected in five-drop fractions. The concentrate fractions were assayed for protease activity, and the reference proteins were located by their absorbance at 280 nm. The peak fractions of protease activity were dialyzed against 0.1 M ammonium bicarbonate (pH 7.8) and tested for cornea-damaging activity.

**Heat inactivation.** The ability of the protease and cornea-damaging activities to resist heat inactivation was examined by heating solutions (0.2 mg/ml; 3 to 4 protease units/ml) of the extracellular protease concentrate in 0.02 M Tris-hydrochloride (pH 7.5) for 15 min at various temperatures, followed by assaying for residual protease and cornea-damaging activity.

**RESULTS**

**Isoelectric focusing.** Two peaks of protease activity were observed in the pH 3.5 to 10 gradient system (Fig. 1). A major peak containing 95% of the recovered activity focused at pH 5.9, and a minor peak focused at approximately pH 8.3 to 8.8. In the pH 5 to 7 gradient system (Fig. 2), the major peak was resolved into two peaks. Three peaks of protease activity having isoelectric points (pl) of approximately 4.6, 5.8, and 8.8 accounted for about 10, 86, and 4%, respectively, of the recovered activity. The major protease (pl 5.8), but not the other two proteases, also degraded orexin-elastin. Solutions (1 to 2 protease units/ml) of the three protease peak fractions produced extensive colliquative necrosis of rabbit corneas 3 to 6 h after intracorneal injection. The necrosis usually progressed to descemetocoele formation and corneal perforation by 12 to 24 h postinjection. Injection of the control buffer (0.1 M ammonium bicarbonate, pH 7.8) did not produce any grossly observable corneal pathology. The lesion produced by the major protease, 18 h postinjection, is shown in Fig. 3.

**Ion-exchange chromatography.** Three proteases were separated by ion-exchange chromatography (Fig. 4). One small peak of protease activity was eluted during the washing of the column with 0.02 M Tris-hydrochloride (pH 8, conductivity at 25°C of 900 mmhos/cm). During the application of the elution gradient, a major peak was eluted at a conductivity of 8,000 mmhos/cm, and a minor peak was eluted at approximately 13,500 mmhos/cm. Solutions (1 to 2 protease units/ml) of the three protease peak fractions produced extensive corneal dam-
age similar to that elicited with the proteases isolated by electrofocusing.

**Gel filtration chromatography.** A symmetrical peak of protease activity, accounting for approximately 95% of the applied activity, was observed (Fig. 5). The peak was well separated from large amounts of nonproteolytic 280 nm-absorbing material and from a small amount of protease activity which was eluted slightly after the column void volume. Analysis of the large protease peak by isoelectric focusing in a pH 5 to 7 gradient showed the same distribution pattern of three proteases observed when the extracellular protease concentrate was fractionated by electrofocusing. The elution volume of the large peak of protease activity corresponded to an apparent molecular weight of 20,000 (Fig. 6). A sample of a diluted solution (1 protease unit/ml) of this peak, and of the protease peak eluting near the void volume, produced corneal damage similar to that elicited with the pro-

**FIG. 3.** Corneal damage produced in rabbits by intracorneal injection of extracellular protease (pH 5.8) of P. aeruginosa. (A) Front view, (B) side view. Results shown are 18 h postinjection of a sample (30 µliters) containing 1 protease unit/ml. Note the protruding descemetoceles (d).

**FIG. 4.** Ion-exchange chromatography of extracellular proteases of P. aeruginosa. Fractions (5 ml) were assayed for protease activity (○) and absorbance at 280 nm (●).

**FIG. 5.** Sephadex G-75 (superfine) gel filtration of extracellular protease concentrate of P. aeruginosa. The column dimensions were 2.6 by 62.5 cm and the column void volume was approximately 116 ml. Fractions (3 ml) were assayed for protease activity (○) and absorbance at 280 nm (●).

**FIG. 6.** Estimation of apparent molecular weight of pseudomonal proteases by Sephadex G-75 gel filtration. The column dimensions were 2.6 by 57.5 cm and the column void volume was approximately 105 ml.
tases isolated by electrofocusing and ion-exchange chromatography.

Sucrose density gradient centrifugation. The protease activity migrated as one band to a distance approximately 17.5 mm from the meniscus. This corresponded to a sedimentation coefficient of approximately 3.3 (Fig. 7). A solution (1 to 2 protease units/ml) of the protease activity peak produced corneal damage similar to that elicited with the proteases isolated by electrofocusing, ion-exchange chromatography, and gel filtration.

Heat inactivation. Heating the extracellular protease concentrate for 15 min at 80°C resulted in complete loss of protease and cornea-damaging activities. Heating for 15 min at 60°C caused a 50% reduction in protease activity; the heated sample produced a smaller lesion by 2 h postinjection than did the unheated preparation.

Protease production and cornea-damaging activity of culture filtrates. Sterile culture filtrates of the proteolytic 5-31 strain contained 1.4 to 1.8 protease units/ml and produced extensive corneal damage similar to that elicited with protease preparations isolated by the various fractionation procedures. Sterile culture filtrates of the PA-103 strain did not contain protease or cornea-damaging activity. When the 5-31 strain was grown in broth containing 4.7% ammonium sulfate, the sterile culture filtrate did not contain protease or cornea-damaging activity.

Examination for other pseudomonal products. Hexapeptidase, collagenase, lecinthinase C, esterase, lipase, lipoprotein lipase, deoxyribonuclease, ribonuclease, alkaline phosphatase, hemolysin, and bacteriolytic activities were not detected in cornea-damaging protease preparations obtained by isoelectric focusing.

DISCUSSION

Partially purified protease preparations of P. aeruginosa have been reported to damage several mammalian tissues (11, 20, 27); however, the heterogeneous nature of the preparations has made it difficult to determine whether all the reported in vivo activities are properties of a pseudomonal protease. Five observations described in this communication support the conclusion that a cornea-virulent strain of P. aeruginosa can produce, in vitro, at least three different extracellular proteases capable of eliciting rapid and extensive damage to rabbit corneas. First, physicochemical fractionation of the culture supernatant fluid of a cornea-virulent strain of P. aeruginosa isolated three cornea-damaging proteases having markedly different pl. Second, the proteases were free of detectable amounts of other known extracellular pseudomonal enzymes. Third, heating a mixture of the three proteases for 15 min at 80°C caused complete loss of protease and cornea-damaging activities. Fourth, the sterile culture filtrate of a nonproteolytic but lethal toxin-producing strain did not contain cornea-damaging activity. Fifth, in vitro production of protease and cornea-damaging activities was inhibited by ammonium sulfate.

At the present time, we do not know whether the small amount of protease activity which was eluted near the Sephadex G-75 column void volume is indicative of a fourth protease in the preparation, or represents a small amount of one of the other three proteases in an active aggregated or carrier-bound form. Studies are planned to resolve this question.

The knowledge that P. aeruginosa produces cornea-damaging proteases should not, at this time, be construed as suggesting that this bacterium cannot, under the appropriate in vitro or in vivo conditions, synthesize other cornea-damaging products. For example, intracorneal injection of pseudomonal endotoxin causes extensive and persistent corneal opacification (26). This substance does not, however, elicit the marked colligative necrosis and corneal perforation characteristically induced by the pseudomonal proteases. Intracorneal injection of Clostridium histolyticum collagenase has been reported (29) to result in degradation of corneal collagen and in corneal perforation; however, the question of whether P. aeruginosa produces a collagenase is unsettled. Adamcic and Clark (1) described the adaptive collagenolytic activity of two pigmented pseudomonads grown in a medium containing soluble chicken skin collagen. The bacteria studied were not identified, however, as strains of P.
Cornea-damaging P. aeruginosa proteases. Morihara et al. (31) did not, however, detect collagenase activity in their pseudomonal elastase preparation. The knowledge that nonspecific proteolytic enzymes active at neutral pH are capable, in vitro, of readily dissolving highly cross-linked tissues such as skin and tendon (34), and the assay procedure of Kučera and Lysenko suggests that these workers were, in fact, detecting dissolution of collagen fibrils rather than hydrolysis of the collagen backbone structure. Schoellmann and Fisher (35) showed that a strain of P. aeruginosa could produce an extracellular enzyme capable of hydrolyzing a synthetic hexapeptide substrate in a manner identical to the cleavage of the substrate by C. histolyticum collagenase. Waldvogel and Swartz (39) reported, however, that this hexapeptidase was not active againstundenatured collagen. Diener et al. (9) reported the production of collagenase by a strain of P. aeruginosa. The enzyme was incubated for 18 h with insoluble bovine tendon collagen, followed by assaying for the release of free amino group-containing peptides (but not hydroxyproline or proline) from the substrate. A number of common proteases are known to produce slow and nonspecific hydrolysis of peptide linkages located at the nonhelical terminal portions of the triple-stranded collagen helix (34), and thus cause the release of free amino group-containing peptides without attacking the characteristic helical regions of the collagen molecule. Diener et al. may have been detecting this phenomenon with their enzyme preparation, and further kinetic studies and analysis of the reaction end products of their system should be performed before the enzyme can be definitively classified as a true collagenase. We will be examining the possibility that the corneadamaging pseudomonal proteases elicit corneal pathology via an indirect rather than a direct mechanism of corneal collagen degradation. For example, perhaps the enzymes (i) damage one or more of the corneal cell types and cause them to release collagenase, or (ii) activate an extracellularzymogen form of collagenase normally present in the corneal stroma. Studies are also planned to determine if other known extracellular pseudomonal products possess corneadamaging activity similar to the pseudomonal proteases.

Proteases of pseudomonal origin do not appear to be unique in their ability to damage the mammalian cornea. Fisher and Allen (11) did not observe damage after intracorneal injection of trypsin; however, other investigators (5, 29) subsequently reported that trypsin, papain, Pronase, and a crude protease preparation obtained from polymorphonuclear leucocyte lysosomes possessed cornea-damaging activity.

Several investigators (4, 17, 30–32, 37) have partially characterized the physicochemical properties of some of the multiple proteases of P. aeruginosa. Work is currently in progress to obtain large amounts of homogeneous cornea-damaging proteases. The physicochemical properties of these proteases will be compared with those reported for the pseudomonal proteases previously isolated by other workers. Obtaining large amounts of homogeneous cornea-damaging proteases will also enable us to commence studies designed to determine (i) the mechanism(s) by which the enzymes produce corneal pathology, and (ii) the enzymes’ role(s) in the pathogenesis of pseudomonal corneal infections.

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Addendum in proof

Shortly after this paper was submitted and accepted for publication, Brown et al. (S. I. Brown, S. E. Bloomfield, and W. I. Tam. 1974. The cornea destroying enzyme of Pseudomonas aeruginosa. Invest. Ophthalmol. 13:174–180.) reported on the partial purification, by ammonium sulfate precipitation and Sephadex G-200 gel filtration, of a cornea-damaging extracellular protease of P. aeruginosa. The enzyme preparation did not possess collagenase activity but degraded gelatin, casein, and the isolated proteoglycan ground substance of rabbit corneas. The enzyme was inhibited by cysteine and by sodium ethylenediaminetetraacetic acid, and was estimated, by Sephadex G-200 gel filtration, to have a molecular weight of approximately 33,500.

Literature Cited