Effect of *Pseudomonas aeruginosa* Elastase, Alkaline Protease, and Exotoxin A on Corneal Proteinases and Proteins

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**Purpose.** To determine the effects of exoproducts from the corneal pathogen *Pseudomonas aeruginosa* on corneal proteinases and proteins.

**Methods.** Whole rabbit corneas were cultured in the presence or absence of broths conditioned with *Pseudomonas aeruginosa*, elastase, alkaline protease, and exotoxin A. Protein synthesis was assayed by adding 35S-methionine during the last 6 hours of culture. Caseinolytic assays and zymography on sodium dodecyl sulfate polyacrylamide gels containing casein and gelatin were used in the presence and absence of inhibitors to quantify and identify corneal proteinases.

**Results.** The major proteinases released by the corneas were 92/89 kD (MMP9) and 65 kD (72 kD gelatinase, MMP2) gelatinases and a 97 kD caseinase. Minor proteinases observed included 184, 166, 156, 153, 126, 111, 102, 60, 57, and 43 kD gelatinases and 170, 136, 85, and 54 kD caseinases. *P. aeruginosa* elastase at 1 µg/ml cleaved the 92 kD gelatinase to yield a 77 kD active form and cleaved the 65 kD gelatinase to yield a 57 kD active form. At 25 µg/ml elastase, the gelatinases were degraded. *P. aeruginosa* alkaline protease had no effect on the 92 or 65 kD gelatinases. Both elastase and alkaline protease degraded the 97 kD caseinase. Proteinases other than elastase and alkaline protease in *P. aeruginosa*105- and *P. aeruginosa*01-conditioned broths also activated and/or degraded corneal proteinases. Exotoxin A inhibited the synthesis of the 92 kD gelatinase and most other proteins. The 72 kD gelatinase and the 97 kD caseinase were released in the presence of exotoxin A.

**Conclusions.** *Pseudomonas aeruginosa* exoproducts can contribute directly to keratitis caused by *Pseudomonas* organisms through toxic effects on corneal cells and degradation of corneal proteins and indirectly through the activation of corneal proteinases. Invest Ophthalmol Vis Sci 1993;34:2699-2712.

*Pseudomonas keratitis* is potentially blinding because it is often accompanied by degradation of the corneal stroma, which may result in perforation or severe scarring. *Pseudomonas aeruginosa* produces several proteinases and toxins, the best characterized being elastase, alkaline protease, and exotoxin A. These are all important factors in both the establishment of a bacterial infection and the amount of damage caused by the infection to the cornea. The relative amounts of the exoproducts produced by *P. aeruginosa* depends on the strain used and the culture conditions.

Genetic and biochemical studies of *P. aeruginosa* virulence determinants in animal model systems provide information about their role in pathogenesis. The proteinases, elastase and alkaline protease, interfere with host defense systems by degrading complement components, immunoglobulins, interferon, interleu-
kinds 1 and 2, and tumor necrosis factor. In vitro experiments show these proteinases inhibit polymorphonuclear leukocyte chemotaxis, natural killer cell function, and T lymphocyte function. In the Pseudomonas keratitis model, secretion of active P. aeruginosa proteinases is required for virulence. Proteinases contribute to the establishment of infection and affect the course of corneal healing primarily through degradation of the basement membranes and extracellular matrix (including laminin, proteoglycans, and various collagens). Exotoxin A, which is lethal to corneal cells through adenosine diphosphate-ribosylation of elongation factor 2, is also required with either elastase or alkaline protease to establish a Pseudomonas infection.

Exoenzyme S is another secreted adenosine diphosphate-riboyl transferase distinct from exotoxin A. The eukaryotic targets for exoenzyme S include the p21 product of Hras and Kras family and the intermediate filament protein, vimentin. Exoenzyme S appears to contribute to tissue damage and dissemination of the organism.

Host corneal proteinases may also contribute to the degradation of corneal matrix during Pseudomonas infections. Corneal cells under culture conditions are capable of producing the matrix metalloproteinases (MMPs), interstitial collagenase (MMP1), stromelysin (MMP3), a 72-kD gelatinase (MMP2, 65 kD under nonreducing conditions used for zymography) and a 92-kD gelatinase (MMP9). The 72-kD gelatinase is constitutively synthesized by corneal keratocytes whereas the 92-kD gelatinase requires induction. Cell and organ culture conditions can lead to the induction of the 92-kD gelatinase. The level of synthesis of these gelatinases is modulated by cytokines.

Matrix metalloproteinases are excreted in an inactive form. Activation occurs during infection and wound healing although the exact mechanisms are not known in vivo. In vitro, many proteinases can activate the 92-kD gelatinase but only P. aeruginosa elastase has been reported to cleave the 72-kD gelatinase to yield active forms. Chromatographic removal of the two molecules of tissue inhibitor of metalloproteinase 2 (TIMP2) associated with the 72-kD gelatinase, the enzyme can autoactivate to form active 62 and 43-kD fragments.

Other proteinases identified in the cornea include tissue plasminogen activator, urokinase, calpain, and the lysosomal enzymes, cathepsins B and D.

In this study, we describe the effects of the exoproducts from several P. aeruginosa strains, and P. aeruginosa elastase, P. aeruginosa alkaline protease, and P. aeruginosa exotoxin A on the synthesis, activation, and degradation of corneal proteinases released and/or secreted into corneal organ culture medium.

METHODS

Materials

Dr. B.H. Iglewski (University of Rochester School of Medicine and Dentistry, Rochester, NY) donated P. aeruginosa exotoxin A (purchased from Swiss Serum and Vaccine Institute, Berne, Switzerland), P. aeruginosa elastase and P. aeruginosa alkaline protease (purchased from Nagase Biochemicals Lt (Fukuchiyama City, Japan)), rabbit anti-P. aeruginosa exotoxin A and S and P. aeruginosa strains P. aeruginosa103 and P. aeruginosa388. Strain P. aeruginosa158 was donated by Dr. A.S. Kreger (Bowman Gray School of Medicine, Winston-Salem, NC). Rabbit eyes were obtained from a local abattoir. Mueller-Hinton broth and trypticase soy medium were purchased from Difco (Detroit, MI). Modified Eagle’s medium was obtained from Gibco-BRL (Grand Island, NY). All electrophoresis reagents were purchased from BioRad (Richmond, CA). Dibuthreitol, monosodium glutamate, fluorescein isothiocyanate, trichloroacetic acid (TCA), Chelex-100, with the addition of 0.05 M monosodium glutamate and 1% glycerol.26 The bacteria were cultured in sterile Mueller-Hinton broth supplemented with 0.9 mmol/l MgCl₂ and 1.3 mmol/l CaCl₂. Strains P. aeruginosa103 and P. aeruginosa388 were grown under conditions for exotoxin A production, using sterile trypticase soy medium that was deferrated with Chelex-100, with the addition of 0.05 M monosodium glutamate and 1% glycerol. The bacteria were cultured for 24 hours at 37°C from stock frozen at −80°C in 10% skim milk. The conditioned broth was harvested using centrifugation followed by filter sterilization using a 0.2 µm filter. The sterile broth was frozen in aliquot portions at −80°C. At harvest all cultures were in the stationary phase. Growth curves for these
strains are similar and within the variability of a given strain.

**Corneal Organ Cultures**

Corneas were dissected 1 mm internally from the limbus, rinsed with sterile modified Eagle’s medium and cultured individually with 1.5 ml MEMS-C serum-free medium (HEPES, modified Eagle's medium, trace elements and nutrients including vanadate) with 2% chondroitin sulfates at 37°C in 95% air/5% CO₂ for 24 hours according the method of Funderburgh et al²⁷ as modified by Cintron et al.²⁸ This medium was selected because of its ability to support near normal synthesis of corneal components including proteoglycans²⁷ and to retain normal form and structure.²⁸ Cultures with *P. aeruginosa* exoproducts were performed with the addition of broth conditioned with *P. aeruginosa* (1–25 μl) or purified *P. aeruginosa* exoproducts, exotoxin A (0.01–1 μg), elastase (1–10 μg) or alkaline protease (1–5 μg) in the presence or absence of corneas. The medium was collected and the supernatant stored at −80°C.

**Medium Incubation Experiments**

Cornea conditioned medium was incubated for 24 hours at 37°C under 95% air/5% CO₂ with 25, 5, or 2 μl of *P. aeruginosa*-conditioned medium, 0.1 or 0.2 μg *P. aeruginosa* exotoxin A, 1 or 2 μg *P. aeruginosa* elastase or 1 or 2 μg *P. aeruginosa* alkaline protease.

**Analysis of Proteinases**

Proteinases in the bacterial broth and corneal organ culture conditioned medium were resolved on 8% or 10% sodium dodecyl sulfate (SDS) polyacrylamide gels containing 0.1% casein or gelatin using a modified zymogram technique.²⁹ Most samples were electrophoresed on both 8% and 10% zymogram gels to more accurately determine the molecular weight of the proteinase bands, 8% for the high molecular weight bands and 10% for the low molecular weight bands. Samples (10–15 μl) were mixed with sample buffer, without reducing agents. Low and high molecular weight standards in the presence of diithiothreitol were electrophoresed on each gel for molecular weight determination. After electrophoresis, the gels were washed in 2.5% Triton X-100 and then incubated overnight at 37°C in either casein gel incubation buffer, 0.05 M tris pH 7.6, 0.1 M NaCl or gelatin gel incubation buffer, 0.05 M tris pH 8.0, 0.01 M CaCl₂, 1 mmol/l ZnCl₂, 0.15 M NaCl. The gels were stained with Coomassie brilliant blue R-250.

Proteinase characterization studies were performed by incubating proteinase samples with inhibitors for 10–120 minutes before electrophoresis and inclusion of the inhibitors in the gel washing and incubation solutions as follows: 4 mmol/l EDTA for metalloproteinases, 83 mmol/l pepstatin for aspartic proteinases, 83 mmol/l E-64 for cysteine proteinases or 17 mmol/l PMSF for serine proteinases. PMSF was used at 17 mmol/l to compensate for the short half-life of this inhibitor in aqueous solution. Control gels were incubated in the presence of the solvents used to solubilize the inhibitors, ethanol for pepstatin, and isopropanol for PMSF.

Caseinolytic activity in the culture supernatant fractions was assayed with fluorescein isothiocyanate-casein.³⁰ Fluorescence was measured on a Millipore 2300, 96-well plate fluorimeter (Millipore, Bedford, MA).

**Exotoxin A and S Assays**

The proteins in *P. aeruginosa*-conditioned medium were concentrated by ammonium sulfate precipitation, electrophoresed on 10% SDS-polyacrylamide gel electrophoresis gels and electrophoretically transferred to nitrocellulose.³¹ The toxins were immunologically detected using polyclonal antibodies to exotoxin A or exoenzyme S and either peroxidase-labeled second antibodies plus chemiluminescence (ECL kit) or ³²P]-labeled second antibodies. The concentration of exotoxin A was determined by densitometry.³¹

**Viability Studies of Cornea Tissue**

Seven-millimeter trephined rabbit corneas were incubated for 24 hours under organ culture conditions alone or in the presence of *P. aeruginosa* conditioned broth (2–10 μl), *P. aeruginosa* exotoxin A (0.01–1 μg), *P. aeruginosa* elastase (1–10 μg) or *P. aeruginosa* alkaline protease (1–5 μg). During the last 6 hours, 50 μg/cornea of ³⁵S-methionine was added to the medium. Control corneas were immediately placed in organ culture medium containing ³⁵S-methionine and cultured for 6 hours. After the culture period, the corneas were rinsed, freeze fractured in liquid N₂ with a percussion homogenizer, freeze thawed three times, and centrifuged. Aliquot portions of the supernatant fractions were mixed with sample buffer containing dithiothreitol, boiled and resolved on 10% SDS-polyacrylamide gel electrophoresis gels. The proteins were detected by autoradiography. Additional aliquot portions of the supernatant fractions were precipitated with 5% trichloroacetic acid, centrifuged, and the pellet was solubilized in 1 M NaOH. Nonincubated corneas were homogenized in the presence of ³⁵S-methionine to determine nonspecific binding and precipitation of the label. The corneal supernatant fractions and the TCA solubilized pellets were mixed with Optifluor and counted on a Packard (Downers Grove, IL) Tri-Carb 4640 scintillation counter.
RESULTS

Corneal Proteinases

Normal rabbit corneas were cultured for 24 hours in serum-free medium containing 2% chondroitin sulfate. The medium was collected and electrophoresed on zymogen gels containing gelatin or casein to detect proteinases. On gelatin gels loaded with 5 μl cornea conditioned medium, proteinase bands (Fig. 1) included a major 65 kD gelatinase band plus a doublet at 184-166, 126-92, 92-65, 43-1, and minor bands at 184, 166, 156, 153, 111, 102, 60, and 57 kD. The relative amounts of these enzymes varied from one cornea to another. These gelatinases were inhibited by 4 mmol/l EDTA. The control isopropanol gel for PMSF and the control ethanol gel for pepstatin were identical to those given for the respective inhibitors. The minor bands were inhibited by the organic solvents used to dissolve the inhibitors. The major bands at 126, 92, 65, and 43 kD were not inhibited by 0.083 mmol/l E-64, 17 mmol/l PMSF or 0.083 mmol/l pepstatin, confirming that these are metallo-proteinases. The 43- and 102-kD proteinases were inhibited by both EDTA and E-64. The EDTA-treated gels showed faint active bands at 97 and 85 kD.

Rabbit corneas cultured for 24 hours produced one major proteinase band on casein zymogram gels migrating at 97 kD (Fig. 2). This proteinase and 90/89 kD, and minor bands at 126 and 43 kD and faint bands at 184, 166, 156, 153, 111, 102, 60, and 57 kD. The relative amounts of these enzymes varied from one cornea to another. These gelatinases were inhibited by 4 mmol/l EDTA. The control isopropanol gel for PMSF and the control ethanol gel for pepstatin were identical to those given for the respective inhibitors. The minor bands were inhibited by the organic solvents used to dissolve the inhibitors. The major bands at 126, 92, 65, and 43 kD were not inhibited by 0.083 mmol/l E-64, 17 mmol/l PMSF or 0.083 mmol/l pepstatin, confirming that these are metallo-proteinases. The 43- and 102-kD proteinases were inhibited by both EDTA and E-64. The EDTA-treated gels showed faint active bands at 97 and 85 kD.
**Pseudomonas Exoproduct Effects on Corneal Proteinases**

Gelatin

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Strain PA

- PA158
- PA388
- PA01
- PA103

Elastase

- 163
- 83
- 70
- 53

Alkaline Prot.

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Casein

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Strain PA

- PA158
- PA388
- PA01
- PA103

Elastase

- 163
- 53

Alkaline Prot.

---

**FIGURE 3.** Proteinases present in *Pseudomonas aeruginosa* conditioned broth. Broths (5 μl for gelatin gels and 7.5 μl for casein gels) from four different strains of *P. aeruginosa* were electrophoresed on 8% polyacrylamide gels containing 0.1% gelatin or 0.1% casein and then processed for proteinase visualization. Purified elastase and alkaline proteinase are shown for reference.

mmol/l pepstatin, placing these proteinases in the serine class. The relative amounts of these enzymes also varied from one animal to another. The 97-kD band corresponds to the upper band observed on the gelatin gels after inhibition with EDTA (Fig. 1). Other faint bands were observed at >200, 170, 136 and 54 kD. The 54-kD band was sensitive to 4 mmol/l EDTA, placing it in the metalloproteinase class. This proteinase and other minor caseinases were inhibited in the presence of isopropanol, the solvent for PMSF, preventing examination for inhibition by this molecule.

**P. aeruginosa Exoproducts**

The *P. aeruginosa* strains used in this study were selected on the basis of the exoproducts each produced. The major proteinases produced by the strains of *P. aeruginosa* cleaved both gelatin and casein (Fig. 3). The 163-kD (*P. aeruginosa*158) and *P. aeruginosa*158 proteinase corresponded to the major band in commercial *P. aeruginosa* elastase. The 53-kD (*P. aeruginosa*158, *P. aeruginosa*388) proteinase corresponded to *P. aeruginosa* alkaline protease (Fig. 3). These bands were totally inhibited by EDTA (gel not shown). The relative amounts of each proteinase produced and visualized on substrate gels was characteristic for each strain. *P. aeruginosa*158 conditioned broth contained the most elastase and alkaline protease of the four strains examined, and the greatest activity toward fluorescein isothiocyanate-casein (Table 1). *P. aeruginosa*158 produced the greatest number of proteinases of any strain examined, in addition to elastase and alkaline protease, proteins were observed at >200, 97 and 83 kD. *P. aeruginosa*388 produced only the 53-kD, alkaline protease band. Faint bands at 70 kD and 53 kD were observed on gelatin gels from *P. aeruginosa*103 conditioned broth. A small amount of fluorescein isothiocyanate-caseinolytic activity was detected in this broth (Table 1). The *P. aeruginosa* proteinases preferred gelatin to casein as a substrate (Fig. 3).

When purified alkaline protease and elastase samples were electrophoresed on SDS-polyacrylamide gels and silver stained only the expected bands at 53 and 32 kD, were observed (gel not shown). Alkaline protease produced cleavage bands at 53 kD on both gelatin and casein zymogram SDS gels (Fig. 3). Elastase, however, has an observed cleavage band at a molecular weight of 163 kD on 8% zymogram casein or gelatin gels (Fig. 3).

**TABLE 1. Summary of Exoproducts Formed by Pseudomonas Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Elastase*</th>
<th>Alkaline Protease*</th>
<th>Other Proteinases</th>
<th>Total Proteinase (μg trypsin eq/ml)</th>
<th>Exotoxin A (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA158</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
<td>266.6</td>
<td>0</td>
</tr>
<tr>
<td>PA388</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>0.30</td>
<td>0.5</td>
</tr>
<tr>
<td>PA01</td>
<td>++</td>
<td>+++</td>
<td>97 and 83 kD</td>
<td>5.3</td>
<td>2.0</td>
</tr>
<tr>
<td>PA103</td>
<td>-</td>
<td>+</td>
<td>70 kD</td>
<td>0.10</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Estimate from zymogram gels.
Three Pseudomonas strains produced exotoxin A when cultured under conditions that stimulate exotoxin A production (Table 1). *P. aeruginosa* 103 conditioned medium contained the highest concentration of exotoxin A with strain *P. aeruginosa* 103 and *P. aeruginosa* 388 producing a much lower amount. *P. aeruginosa* 158 grown in Mueller-Hinton broth in the presence of additional Mg++ and Ca++ did not produce A. No exoenzyme S was detected in any of the four *P. aeruginosa* broths.

**Effects of P. aeruginosa Exoproducts on Corneal Proteinases**

The 92/89-kD corneal gelatinase doublet was observed in medium from corneas cultured alone (Fig. 4, lower right gel) but was not present in the medium from corneas incubated with 25 or 5 μl *P. aeruginosa* 158, *P. aeruginosa* 103 or *P. aeruginosa* 388 conditioned broth or 25 μl *P. aeruginosa* 388 conditioned broth. However, corneas incubated with 5 μl *P. aeruginosa* 388 broth synthesized and/or released the 92/89-kD gelatinase doublet. To determine whether this effect was attributable to proteolytic degradation and/or a toxic effect, corneas were individually incubated with purified *P. aeruginosa* alkaline protease, elastase, and exotoxin A. The 92/89-kD gelatinase doublet was lost on incubation with 1 or 10 μg elastase and 0.1 μg exotoxin A but not with 1 or 5 μg alkaline protease (Fig. 4, bottom). The loss of activity with purified exotoxin A was probably caused by the inhibition of proteinase synthesis by the toxin and not the small amount of contaminating alkaline protease or a 83 kD protein contaminant. These results suggest that the 92/89-kD gelatinase was a substrate for *P. aeruginosa* elastase but not alkaline protease.

To further explore the loss of the 92/89-kD doublet, corneal conditioned medium was mixed with the *Pseudomonas aeruginosa* conditioned broth or isolated *Pseudomonas* exoproteins. The mixtures were either incubated 24 hours at 37°C (Fig. 5 I) or electrophoresed immediately (Fig. 5 II). As in the case of the whole cornea, the 92/89-kD proteinases were not observed after incubation of the cornea conditioned medium in the presence of *P. aeruginosa* 158, *P. aeruginosa* 103 and *P. aeruginosa* 388 conditioned broth and elastase but not with *P. aeruginosa* 388 or alkaline pro-
Pseudomonas Exoproduct Effects on Corneal Proteinases

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FIGURE 5. Effect of Pseudomonas aeruginosa conditioned broth or purified exoproducts on gelatinases present in cornea conditioned medium. (I) Cornea conditioned medium (1 ml) was incubated for 24 hours in the presence or absence of the P. aeruginosa conditioned broth (25 or 12.5 μl) or purified exoproducts (2 or 1 μg elastase and alkaline protease and 0.2 or 0.1 μg exotoxin A). (II) Corneal conditioned medium (1 ml) was mixed with P. aeruginosa conditioned broth or purified exoproducts but not incubated. The amount of conditioned broth and purified exoproducts used were the same as that used for I. The medium (2 μl) was electrophoresed on 10% polyacrylamide gels containing 0.1% gelatin and then processed for proteinase visualization.

The 65-kD corneal gelatinase was either partially or totally lost on organ culture in the presence of P. aeruginosa elastase and the elastase containing P. aeruginosa158 and P. aeruginosa01 conditioned broths (Fig. 4). It was not significantly diminished with P. aeruginosa388 and P. aeruginosa103 conditioned broths, alkaline protease and exotoxin A. With the loss of the 65-kD gelatinase, a 60-kD band was observed in corneal cultures when 25 μg P. aeruginosa158 conditioned broth or 1 μg elastase were added. Also a major band at 57 kD was generated during culture with P. aeruginosa158 and P. aeruginosa01 conditioned broth and elastase, which was distinct from the 53-kD alkaline protease band. The 60 and 57-kD bands were probably active degradation products of the 65-kD gelatinase. At higher concentrations of elastase, this 65-kD gelatinase was lost. No additional bands were generated concurrent with the loss of the 65-kD gelatinase indicating further cleavage yielded inactive forms of the enzymes.
Incubation experiments with corneal conditioned medium plus either *P. aeruginosa*158 or elastase (Fig. 5 I) gave the same 65-kD gelatinase degradation products as those observed for the corneal organ culture experiments. Additional bands below 65 kD were observed for *P. aeruginosa*103, *P. aeruginosa*103, and exotoxin A, which were not observed in the respective corneal organ culture mediums. The presence of additional bands, not observed with elastase or alkaline protease, implies the presence of an uncharacterized proteinase(s) that can degrade the corneal proteinases. There was no loss of the 65-kD gelatinase activity when the samples were mixed and immediately electrophoresed (Fig. 5 II). These results indicate the loss of the 65-kD gelatinase and the appearance of lower molecular weight gelatinases are due to *P. aeruginosa* elastase and other uncharacterized proteinases.

The 97-kD corneal caseinase was either partially or totally lost on incubation of either corneas (Fig. 6) or corneal conditioned medium (Fig. 7) with *P. aeruginosa*158 or *P. aeruginosa*388 conditioned broth, *P. aeruginosa* elastase, and *P. aeruginosa* alkaline protease. These results imply that the proteinases do not affect synthesis of the caseinases but degrade the newly synthesized enzymes. Corneal organ culture with 25 μl *P. aeruginosa*103 conditioned broth or 1 μg exotoxin A resulted in the partial loss of the 97-kD caseinase (Fig. 6). Corneas cultured with *P. aeruginosa*388 produced this caseinase. Incubation of corneal conditioned medium with *P. aeruginosa*388 conditioned broth or exotoxin A resulted in an enhancement of the 97-kD band (Fig. 7). The mechanism for this is unclear.

The minor gelatinase and caseinase bands observed in Figures 1 and 2 were not observed in Figures 4–7 because the latter gels contained 20% of the sample used for Figures 1 and 2.

**Corneal Viability Studies**

The effect of organ culture on protein synthesis was determined using fresh corneas or corneas after 18 hours of culture that were placed in the presence of [35S]-methionine for 6 hours. After organ culture, the corneas were extracted. The extracts from corneas cultured for a total of 24 hours contained 1.4 × 10^6 to 1.6 × 10^6 dpm/cornea of TCA precipitable protein. This represented 85% of the TCA precipitable protein obtained from corneas incubated with [35S]-methionine for 6 hours immediately after dissection. When comparing the fresh vs the preincubated corneal extracts using electrophoresis, increases in the 73 and 49-kD protein staining bands (Fig. 8, top), and a decrease in the 58-kD [35S]-methionine labeled band (Fig. 8, bot-
Pseudomonas Exoproduct Effects on Corneal Proteinases

FIGURE 8. Effect of Pseudomonas aeruginosa conditioned broth or purified exoproducts on endogenous corneal proteins and newly synthesized corneal proteins under cornea organ culture conditions. Corneas were incubated for 6 hours with 50 μCi ³⁵S-methionine either immediately or after 18 hours of organ culture for a total of 24 hours in the presence or absence of P. aeruginosa conditioned broth or the purified exoproducts, elastase, alkaline protease and exotoxin A. The corneal proteins were extracted and electrophoresed (15 μl under reducing conditions on 10% polyacrylamide gels. The bands in the upper gels labeled "Protein" were stained with Coomassie brilliant blue. The bands in the bottom gels labeled "³⁵S-Methionine" were visualized by autoradiography.

results indicate that alkaline protease degrades proteins but the degradation products are still large enough to be TCA precipitable.

At 10 μg of elastase, 28% of the ³⁵S-methionine labeled corneal proteins were TCA precipitable (Table 2). Most proteins visible by staining were degraded, leaving only the 125 and 73-kD proteins without significant degradation (Fig. 8, top). Only a trace of several bands were noted on the autoradiogram of the electrophoresed proteins (Fig. 8, top). At 2 μg of elastase, the 69-kD protein band was degraded and a 110-kD degradation band was added (Fig. 8, top). Many ³⁵S-methionine labeled bands were lost (Fig. 8, bottom).

Incubation of corneas with 25 μl of P. aeruginosa 158 conditioned broth, resulted in a 90% decrease in precipitable proteins (Table 2). When the extracted, labeled proteins were electrophoresed, the Coomassie staining revealed that proteins were degraded with the exception of a 125-kD band (Fig. 8, top). A new 110-kD band was observed. Only trace amounts of ³⁵S-methionine labeled proteins were detected on the radioautograph (Fig. 8, bottom). At 2 μl of the broth 89% of the proteins were precipitable and very little effect was noted on the stained or labeled protein electrophoretic patterns. Because this medium does not contain exotoxin A, these effects are probably due to the major proteases present, alkaline protease and/or elastase. Both alkaline protease and elastase cleaved

<table>
<thead>
<tr>
<th>Exoproduct</th>
<th>Quantity Added</th>
<th>³⁵S Methionine TCA Precipitable* (%)</th>
</tr>
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<tbody>
<tr>
<td>None (24 hr culture)</td>
<td>None</td>
<td>23.34 ± 6.21</td>
</tr>
<tr>
<td>PA158 broth</td>
<td>25 μl</td>
<td>2.41 ± 1.79†</td>
</tr>
<tr>
<td></td>
<td>2 μl</td>
<td>20.71 ± 5.26</td>
</tr>
<tr>
<td>PA388 broth</td>
<td>25 μl</td>
<td>9.45 ± 0.99†</td>
</tr>
<tr>
<td></td>
<td>2 μl</td>
<td>11.48 ± 5.85</td>
</tr>
<tr>
<td>PA01 broth</td>
<td>25 μl</td>
<td>0.00 ± 0.00†</td>
</tr>
<tr>
<td></td>
<td>2 μl</td>
<td>7.15 ± 3.32†</td>
</tr>
<tr>
<td>PA103</td>
<td>25 μl</td>
<td>0.00 ± 0.00†</td>
</tr>
<tr>
<td></td>
<td>2 μl</td>
<td>3.50 ± 0.65†</td>
</tr>
<tr>
<td>PA elastase</td>
<td>10 μg</td>
<td>6.45 ± 1.51†</td>
</tr>
<tr>
<td></td>
<td>1 μg</td>
<td>18.70 ± 1.66</td>
</tr>
<tr>
<td>PA alkaline protease</td>
<td>5 μg</td>
<td>23.80 ± 1.09</td>
</tr>
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<td></td>
<td>1 μg</td>
<td>24.53 ± 2.00</td>
</tr>
<tr>
<td>PA exotoxin A</td>
<td>1.00 μg</td>
<td>0.00 ± 0.00†</td>
</tr>
<tr>
<td></td>
<td>0.10 μg</td>
<td>0.32 ± 0.67†</td>
</tr>
<tr>
<td></td>
<td>0.01 μg</td>
<td>4.99 ± 2.30†</td>
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* n = 3.
† P < 0.02 by Student’s t-test when compared to corneas incubated without P. aeruginosa exoproducts (None, 24 hour culture).
the major corneal proteins and gave similar degradation patterns to those observed for *P. aeruginosa*158 conditioned broth suggesting both proteinases contribute to the degradation of corneal proteins.

The major endogenous Coomassie staining corneal proteins were intact in corneas incubated with all levels of *P. aeruginosa*388, *P. aeruginosa*01, and *P. aeruginosa*103 broths and exotoxin A (Fig. 8). At 25 μl of *P. aeruginosa*103 and *P. aeruginosa*01 and at 0.01 to 1 μg exotoxin A added to the cultures, corneal protein synthesis was profoundly decreased (Table 2). No protein bands were noted on the autoradiogram (Fig. 8). When 2 μl of these conditioned broths were added, a few faint low molecular weight protein bands were observed. At both 2 and 25 μl of *P. aeruginosa*388 conditioned broths were added, the concentrations of newly synthesized proteins were reduced but not to the extent observed when *P. aeruginosa*103 or *P. aeruginosa*01 conditioned broths were added. The conditioned broths from *P. aeruginosa*388, *P. aeruginosa*01, and *P. aeruginosa*103 all contain exotoxin A but at widely differing concentrations (Table 1). In addition, the concentrations of proteinases differ; *P. aeruginosa*103 broth contains very low amounts of proteinases, *P. aeruginosa*388 broth contains only alkaline protease, and *P. aeruginosa*01 broth contains numerous proteinases (Fig. 3). These results also show protein synthesis was turned off by exotoxin A in this system. Further, the loss of corneal proteinases resulting from exotoxin A and high concentrations of elastase is a general phenomena. The lack of activity toward the corneal gelatinases by alkaline protease was relatively specific as many proteins are cleaved by this *P. aeruginosa* proteinase.

**DISCUSSION**

When whole rabbit corneas are cultured in serum-free medium, they produce two major metalloproteinases with molecular weights of 92 and 65 kD on nonreducing SDS-gelatin zymogram gels. These are probably the 92-kD (MMP9) and 65-kD (MMP2) gelatinases previously identified in the medium from corneas in organ culture and cultured cells using immunoblotting techniques. The 60 and 57-kD bands are probably activated forms of the 65-kD gelatinases detected in extracts of wounded corneas. Additional minor EDTA sensitive bands were observed on the gelatin zymograms that have not been previously reported in the medium of corneal organ cultures. The 126 kD and the main band >200 are probably forms of the 92-kD gelatinase with purified 92-kD gelatinase from neutrophils under nonreducing conditions. This 43-kD gelatinase is inhibited by both EDTA and the cysteine proteinase inhibitor E-64.

In addition, the cornea produces a major 97-kD serine caseinase and several minor caseinases. The 97-kD and the 85/90-kD doublet of proteinases were inhibited by PMSF but not EDTA, E-64, or pepstatin, which places it in the serine proteinase group. The isopropanol carrier for PMSF inhibited the minor caseinases resulting in the inability to determine whether these are serine proteinases. The 54-kD band was inhibited by EDTA, suggesting it is the metalloproteinase stromelysin (MMP3). Stromelysin has a similar molecular weight and cleaves casein to a greater extent than gelatin. Although the 85-kD band is similar in molecular weight to the 80-kD catalytic subunit of calpain, the 85-kD proteinase is not inhibited by EDTA as would be expected for calpain. The identity of the observed caseinases is not obvious based on their molecular weights. These enzymes need to be further characterized.

*P. aeruginosa* strains used in this study were chosen on the basis of their varying proteinase and exotoxin A exoproduction. In particular, we were interested in using strains that produced varying amounts of exotoxin A, alkaline protease and elastase, to determine the effects of each product on corneal proteinase production and/or degradation. These effects were compared to that of purified *Pseudomonas* proteinases and exotoxin A.

The major proteolytic band in purified *P. aeruginosa* elastase migrated to a position on the gelatin and casein zymogram gels under nonreducing conditions corresponding to 163 kD instead of the expected 33 kD. This high molecular weight could reflect a decrease in mobility on the gels caused by aggregation of the enzyme under the nonreducing conditions or direct interaction between the enzymes and the substrate copolymerized in the gels. Elastase was detected in *P. aeruginosa*01 and *P. aeruginosa*158, but not *P. aeruginosa*388 or *P. aeruginosa*103 conditioned broth on the zymogen gels in these studies. A low concentration of elastase has been reported in medium from *P. aeruginosa*388 cultured under different conditions than ours. *P. aeruginosa*103 has been reported as deficient in elastase production. This deficiency is not attributable to an alteration in the elastase structural gene but in the regulation of gene expression.

Purified alkaline protease was observed at 53 kD on the zymogen gels, which is within the range of reported molecular weights for alkaline protease (48–60 kD). This proteinase was observed in all four of the *P. aeruginosa* conditioned broths used. *P. aeruginosa*158 conditioned broth had a much higher concentration than the other strains.
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In addition to these two proteinases, *P. aeruginosa*01 conditioned broth contained a major band at 83 kDa and minor ones at 97 and >200 kDa. These do not match the molecular weights of the other reported *P. aeruginosa* proteinases. 37,38,40 These proteinase bands could be aggregates of alkaline protease and elastase or unique enzymes. Further studies are needed to elucidate the identity of these proteolytic bands.

*P. aeruginosa*03 conditioned broth contained high concentrations of exotoxin A, *P. aeruginosa*388 conditioned broth contained very low concentrations and *P. aeruginosa*01 contained intermediate concentrations. This agrees with previous reports. 3 Although there is only one exotoxin A structural gene present per genome, the amount produced varies considerably among strains because of differences in the control of protein synthesis and secretion of the toxin. 3 No detectable exotoxin A was found in *P. aeruginosa*158, however, this strain could be producing the toxin only to have it cleaved by the high concentrations of proteinases present. *P. aeruginosa* proteinases are known to cleave the toxin. 41

*Pseudomonas* elastase efficiently cleaved cornal gelatinases, caseinases and proteins in our system. This is consistent with the reported wide range of substrates for this enzyme. 5,7,8,42-44 Culturing corneas with elastase and incubating cornal conditioned medium with elastase resulted in the loss of both the 92 and 65-kDa gelatinases in the medium and the appearance of bands at 77, 60, and 57 kDa. The 77-kDa band is probably a proteolysis product of the 92-kDa band. Various active proteolytic products have been detected on incubation of this 92-kDa gelatinase isolated from several cell types with organomercurial compounds 45 and various proteinases 15-19 including 76 and 61-kDa molecular weight forms with cathepsin G. Cleavage of the N-terminal portion of latent matrix metalloproteinases results in an activation of the enzyme by destabilizing the bond between a cysteine in the activation peptide and the active site Zn++ bound to two histidine residues. 46

The 60- and 57-kDa gelatinase bands generated by incubation with *P. aeruginosa* elastase 46 probably correspond to the 60- and 58-kDa gelatinases shown by immunoblotting techniques to be activation products of the 65-kDa gelatinase produced during wound healing. 17 Unlike the other matrix metalloproteinases, the 65-kDa gelatinase (also called 72-kDa gelatinase, MMP2), cannot be activated by most proteinases including plasmin, thrombin, chymotrypsin, neutrophil elastase, cathepsin G and stromelysin. 18 The ability of *P. aeruginosa* elastase to activate this enzyme is significant. Because other organisms have enzymes similar to *P. aeruginosa* elastase 47 this may be a general characteristic of bacterial infections; the ability to induce degradation of extracellular matrix molecules by activating a major constitutively produced host enzyme.

*P. aeruginosa* alkaline protease cleaved the cornal caseinases but showed minimal activity toward cornal gelatinases. It also cleaved many of the cornal proteins but yielded proteolysis products that were still TCA precipitable.

Exotoxin A at all concentrations examined (0.01–1 µg) prevented the synthesis of the 92/89-kDa gelatinases and most of the other cornal proteins. This is consistent with the general mechanism of action for this toxin; the adenosine diphosphate ribosylation of elongation factor 2 inhibits protein synthesis. 48 This also suggests that the observed effects are not caused by the contaminating 83 kDa protein in the exotoxin A preparations. In these experiments, significant amounts of the 65-kDa gelatinase and the 97-kDa caseinase were found in the medium despite concentrations of exotoxin A, which almost totally inhibited protein synthesis. This would suggest that these enzymes are stored within the cornal cells or in the extracellular matrix and are released during the culture period by diffusion from the extracellular matrix, secretion from the cells or cell death. The 65-kDa gelatinase in cells has not been detected intracellularly except in the presence of monensin, a glycosylation inhibitor that traps the gelatinase in the golgi. 18

The results using purified *P. aeruginosa* exoproducts allowed us to interpret how the exoproducts interact and to determine whether other secreted factors play a role in the control of cornal proteinase activity. Incubation of either corneas or cornal conditioned medium with *P. aeruginosa*158 conditioned broth, containing no detectable exotoxin A but high concentrations of both elastase and alkaline protease, resulted in the loss of the 92/89 kDa and 65 kDa gelatinases with the concurrent production of smaller active forms of the enzymes. These active forms have the same molecular weights as those generated in the presence of elastase indicating the dominance of elastase over alkaline protease in the activation of cornal proteinases.

The 92/89-kDa doublet of gelatinases was no longer observed after cornal organ culture with *P. aeruginosa*03 or incubation of the cornal conditioned medium with *P. aeruginosa*03 conditioned broth. This strain contains high concentrations of exotoxin A, which could inhibit the synthesis of these enzymes under organ culture conditions. The absence of these proteinases in the cornal conditioned medium after incubation in the presence of *P. aeruginosa*03 was unexpected because the two identified exoproducts present, exotoxin A and alkaline protease, do
not directly degrade the 92/89-kD gelatinases. There are two possible mechanisms that can explain this result: an uncharacterized P. aeruginosa proteinase, such as the 70-kD gelatinase noted in this broth, could cleave the 92/89-kD proteinases or an inhibitor in the broth could form a covalent inhibitory complex with the enzymes. When cornea conditioned medium was mixed with P. aeruginosa 103 broth and immediately electrophoresed the 92/89-kD gelatinases were retained. The results of this mixing experiment rule out the presence of an inhibitor. These results suggest that an uncharacterized P. aeruginosa proteinase is responsible for the cleavage of the 92/89-kD gelatinase under incubation conditions with P. aeruginosao103.

Involvement of P. aeruginosa proteinases other than alkaline protease and elastase in the activation of corneal gelatinases was supported by the unique active bands observed in corneal medium generated in the presence of P. aeruginosa1. A 70-kD gelatinase and several bands below 50 kD were generated when the corneal conditioned medium was incubated with the conditioned broth. These bands were not observed in the presence of elastase or alkaline protease.

In addition to direct degradative effects of P. aeruginosa proteinases on corneal proteinases, indirect effects can occur via the degradation of cytokines, which influence the expression of matrix metalloproteinases. Because of the susceptibility of the corneal proteinases to direct cleavage by the P. aeruginosa proteinases, alterations in the expression of the proteinases could not be determined based on proteinase activity. To study the effect of P. aeruginosa proteinases on corneal proteinase expression, quantification of messenger RNA will be required.

Pseudomonas keratitis has a multifactorial pathogenesis. This keratitis progresses rapidly and is characterized by infiltration of inflammatory cells and tissue destruction (melting). This infection can lead to corneal perforation, intraocular infection, iris destruction, lens opacification and endophthalmitis. The virulence factors associated with Pseudomonas infections in the cornea and in other organs are the exoproducts studied here, alkaline protease, elastase, and exotoxin A, but also exonuclease S, phospholipase C, alkaline phosphatase, leukocidin, and alginate. The exoproducts secreted by the organism influence the initial characteristics of the P. aeruginosa infection. Strains that secrete high amounts of proteinases induce descemetocoele formation at a higher frequency than those which secrete lower amounts of these enzymes. The severity of the ulcer is also determined by the exotoxin A concentration and the resultant host response.

This study adds a dimension to the multifactorial pathology of Pseudomonas keratitis, namely the ability of Pseudomonas proteinases to activate corneal proteinases which can degrade the corneal matrix. The current study used sterile P. aeruginosa conditioned broth and purified P. aeruginosa exoproducts. Coculture of several P. aeruginosa strains with whole corneas resulted in the production of similar corneal proteinase activation bands.

This study has defined the gelatinases and caseinases released into corneal organ culture medium, in addition to the major gelatinases previously reported. The major 97-kD gelatinase was identified as a serine proteinase. Like other proteinases, P. aeruginosa alkaline protease cleaved the 92-kD gelatinase to an active form. However, unlike most proteinases, P. aeruginosa elastase cleaved the 58-5D gelatinase to a 58-kD active form. Although P. aeruginosa alkaline protease cleaved most corneal proteins and the corneal caseinases, it did not cleave the corneal gelatinases. Using conditioned broths from P. aeruginosao103 and P. aeruginosao1, P. aeruginosa proteinases other than the alkaline protease and elastase were detected, which can cleave cornea proteinases. Constitutively produced proteinases were released into the corneal organ culture medium even at concentrations of exotoxin A that almost totally inhibit protein synthesis. These results suggest that Pseudomonas exoproducts act in concert to activate host enzymes that contribute to the degradation of the cornea.

Key Words
proteinases, Pseudomonas aeruginosa, matrix metalloproteinases, exotoxin A, cornea

References
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