Cornea-damaging proteases of

*Serratia marcescens*

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Fractionation of the culture supernatant fluids of *Serratia marcescens*, strain BC, by ammonium sulfate precipitation, isoelectric focusing, ion-exchange chromatography, hydroxypatite adsorption chromatography, and gel filtration failed to separate the rabbit cornea-damaging activity and, the in vitro protease activity of the preparations. Two proteases having similar molecular weights (44,000), estimated by gel filtration, and isoelectric points of approximately 5.0 and 5.3 were obtained free of detectable amounts of other known extracellular *Serratia* enzymes. Heating a mixture of the two proteases for 15 minutes at 60° C. resulted in complete loss of protease and cornea-damaging activities. Production of protease and cornea-damaging activities was inhibited by ammonium sulfate. The results support the conclusion that extracellular proteases produced in vitro by *S. marcescens* can elicit rapid and extensive damage to the rabbit cornea.

Key words: cornea-damaging proteases, *Serratia marcescens* proteases.

*Serratia marcescens*, initially considered to be an avirulent saprophytic bacterium, is known to be an opportunistic pathogen capable of producing clinically significant infections in many human tissues and organ systems. In the ophthalmologic literature, this organism has been identified as the cause of lacrimal duct infection, conjunctivitis, corneal ulcers, and endophthalmitis. The mechanism(s) by which *S. marcescens* produces ocular pathology is unclear. Salceda, Lapuz, and Vizconde showed that a sterile culture filtrate and a cell-free extract of the bacterium could, following intravitreal injection, produce endophthalmitis in rabbits. The crude composition of the injected preparations made it difficult, however, to ascribe the observed pathology to a particular *Serratia* enzyme or toxin.

The present study was initiated as a first step toward elucidating the nature and properties of the extracellular cornea-damaging products of *S. marcescens*. Data are presented to show that extracellular proteases produced in vitro by this bacterium can elicit rapid and extensive damage to the rabbit cornea.
Materials and methods

Bacteria and culture media. S. marcescens, strain BC, was obtained from the culture collection of the Department of Microbiology of the Bowman Gray School of Medicine. This strain produced severe corneal infections in rabbits following intracorneal injection. The bacteria were cultivated in tryptone-yeast extract-glucose broth (TYE broth, pH 7.2) containing 0.5 per cent tryptone (Difco), 0.25 per cent yeast extract (BBL), and 0.1 per cent glucose. Liu and Hsieh 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 per cent weight per volume, pH 7.2), sterilized by membrane filtration, to the medium.

Preparation of extracellular protease concentrate. Two-liter flasks containing 200 ml. of TYE broth were inoculated with 0.1 ml. of a stationary-phase culture of S. marcescens, strain BC. The medium was incubated for 20 to 24 hours at 30° C. on a gyrotatory shaker (Model C25, New Brunswick Scientific Co., New Brunswick, N. J.) operating at 200 cycles per minute, and the cells were removed by centrifugation at 5° C. The culture supernatant fluid was clarified in the supernatant fluid was saturated with ammonium sulfate (Schwarz/Mann, ultra-pure grade) and, after standing overnight at 5° C, the precipitate was recovered by centrifugation and mixed with sufficient 0.1 M ammonium bicarbonate (pH 7.8) to yield an extract 1/20 the volume of the initial culture supernatant fluid. The extract was clarified by centrifugation and membrane filtration (0.45 μm pore size) and was dialyzed against the buffer required for the various fractionation procedures. The extract was stored at -40° C, until use.

Assays. Protease activity was assayed as previously described. Protein was estimated by the method of Lowry and co-workers with crystalline bovine albumin as the standard. Protease preparations obtained by isoelectric focusing were examined for the presence of hexapeptidase, collagenase, leucine C, and desosylthromocinase, lipase, and esterase activities. Enzymes, used as positive controls, and substrates were obtained from the Sigma Chemical Co. (St. Louis, Mo.), 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 (30 gauge by 0.5 inch needles, Storz Surgical Instruments, St. Louis, Mo.) of samples (30 dilutions) in 3 to 5 pound New Zealand white rabbits. Prior to injection, the rabbits were anesthetized with ether and topical 0.5 per cent tetracaine hydrochloride. The corneas were examined for gross damage at various time intervals for two days after injection.

Isoelectric focusing. Electofocusing in sucrose density gradients was performed with the equipment and methodology described by Vesterberg. Focusing was done at 4° C. for approximately 46 hours in a 110 ml. electrolysis column (LKB Instruments, Rockville, Md.) 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 4 to 6 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 dialyzed, prior to injection, against 0.1 M ammonium bicarbonate (pH 7.8) and were tested for cornea-damaging activity.

Ion-exchange chromatography. Diethylaminoethyl agarose (DEAE Bio-Gel A, Bio-Rad Laboratories, New York, N. Y.) was equilibrated with 0.02 M Tris (hydroxymethyl) aminomethane (Tris)-hydrochloride (pH 8) and packed in a water-cooled (5° C.) column (2.6 by 40 cm.). After addition of the extracellular protease concentrate (approximately 1,000 protease units and 34 mg. of protein in 17 ml. of equilibrating buffer) to the column, the column was washed with 387 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 8) and 0.6 M NaCl which fed into a constant-volume mixing chamber containing 270 ml. of equilibrating buffer. The constant-volume mixing chamber discharged into the column at a rate of approximately 20 ml. per hour. Fractions (5 ml.) were collected and assayed for protease activity and for 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-1082, Beckman Instruments, Cedar Grove, N. J.).

Hydroxyapatite adsorption chromatography. Hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories, New York, N. Y.) was equilibrated with 0.02 M potassium phosphate buffer (pH 7) and packed in
Fig. 1. Isoelectric focusing of extracellular proteases of S. marcescens in a pH 3.5 to 10 gradient. The gradient was prepared with (1) a less dense solution consisting of 44.2 ml of water, 0.8 ml of 40 per cent (weight per volume) anipholine (pH 3.5 to 10, LKB Instruments, Rockville, Md.), and 10 ml of extracellular protease concentrate (304 protease units, 8.5 mg of protein) in 1 per cent glycine and 10^{-3} M calcium chloride, and (2) a more dense solution consisting of 38.8 ml of water, 1.7 ml of 40 per cent anipholine (pH 3.5 to 10), and 25 cm. of sucrose (Schwarz/Mann, ultrapure grade). Fractions (4 ml.) were examined for protease activity (O) and for pH at 4° C. (△).

Gel filtration chromatography. Gel filtration was performed with water-cooled (5° C.) columns (2.6 by 32 cm.) of Sephadex C-75 and G-100 regular (Pharmacia) equilibrated with 0.1 M ammonium bicarbonate (pH 7.8). Extracellular protease concentrate (approximately 408 protease units and 8.4 mg of protein in 6 ml of equilibrating buffer) was applied to the columns and eluted, in the upward flow mode, at a flow rate of 12 ml per hour (3.3 ml per square centimeter per hour) with the equilibrating buffer. Fractions (3 ml.) were collected and assayed for protease activity and for absorbance at 280 nm. The peak fractions of protease activity were 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 (1.5 to 2 protease units per milliliter, 0.37 mg of protein per milliliter) of the extracellular protease concentrate in 0.02 M Tris-hydrochloride (pH 7.5) for 15 minutes at various temperatures, followed by assaying for residual protease and cornea-damaging activity.

Results

Isoelectric focusing. One peak of protease activity having an isoelectric pH (pI) of approximately 5.1 was observed in the pH 3.5 to 10 gradient system (Fig. 1). In the pH 4 to 6 gradient system (Fig. 2), this peak was resolved into a major (pI 5.3) and a minor peak (pI 5.0) accounting for approximately 85 and 15 per cent, respectively, of the recovered activity. Solutions (1 protease unit per milliliter) of the two protease peak fractions produced extensive colliquative necrosis of rabbit corneas two to four hours after intracorneal injection. The necrosis usually progressed to des-
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Ion-exchange chromatography. No protease activity was eluted during the washing of the column with 0.02 M Tris-hydrochloride (pH 8, conductivity at 25°C of 900 millimhos per centimeter). During the application of the salt gradient (Fig. 4), a minor peak of protease activity was eluted at a conductivity of approximately 9,400 millimhos per centimeter, and a major peak was eluted at approximately 12,400 millimhos per centimeter. Solutions (1 protease unit per milliliter) of the two protease peak fractions produced extensive corneal damage similar to that elicited with the proteases isolated by electrofocusing.

Hydroxyapatite adsorption chromatography. A small and broad band of protease activity was eluted during the washing of the column with 0.02 M potassium phosphate buffer (pH 7, conductivity at 25°C of 2,650 millimhos per centimeter). The activity was well separated from a large peak of nonproteolytic 280 nm.-absorbing material which was eluted at approximately the column void volume. During the application of the phosphate gradient (Fig. 5), a large symmetrical peak of protease activity was eluted at a conductivity of approximately 8,700 millimhos per centimeter.
Solutions (0.5 to 1 protease unit per milliliter) of the two proteases produced extensive corneal pathology similar to that elicited with the proteases isolated by electrofocusing and ion exchange chromatography.

**Gel filtration chromatography.** A symmetrical peak of protease activity, accounting for approximately 95 per cent of the applied activity, was observed (Fig. 6). The peak was well separated from small amounts of nonproteolytic 280 nm-absorbing material. The elution volume of the peak of protease activity corresponded to an apparent molecular weight of 44,000 (Fig. 7). A sample of a diluted solution (1 protease unit per milliliter) of the protease peak elicited corneal damage similar to that produced with the proteases isolated by electrofocusing, ion-exchange chromato-
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Fig. 6. Sephadex G-100 gel filtration of extracellular protease concentrate of S. marcescens. The column dimensions were 2.6 by 60.5 cm, and the column void volume was approximately 110 ml. Fractions (3 ml.) were assayed for protease activity (○) and for absorbance at 280 nm. (●).

Fig. 7. Estimation of the apparent molecular weight of serratia proteases by Sephadex G-75 and Sephadex G-100 gel filtration. The Sephadex G-75 column dimensions were 2.6 by 57.5 cm. (void volume 105 ml.) and the Sephadex G-100 column dimensions were 2.6 by 61.5 cm. (void volume 116 ml.).

tography, and hydroxyapatite adsorption chromatography.

Heat inactivation. Heating the extracellular protease concentrate for 15 minutes at 60° C. resulted in complete loss of protease and cornea-damaging activities. Heating for 15 minutes at 45° C. caused a 60 to 70 per cent reduction in protease activity. The heated sample diluted with 3 parts of 0.02 M Tris-hydrochloride (pH 7.5) did not produce extensive corneal damage by six hours after injection; however, the unheated sample diluted in the same manner produced extensive corneal damage by two hours after injection.

Inhibition of production of protease and cornea-damaging activities by ammonium sulfate. Sterile culture filtrates obtained from cultures grown in TYE broth contained approximately 6 protease units per milliliter and, even when diluted with 7 parts of 0.02 M Tris-hydrochloride (pH 7.5), produced rapid and extensive corneal pathology similar to that elicited with protease preparations isolated by the various fractionation procedures. Sterile culture filtrates obtained from cultures grown in TYE broth supplemented with 4.7 per cent ammonium sulfate contained approximately 0.6 protease units per milliliter and, when
diluted with 7 parts of 0.02 M Tris-hydrochloride (pH 7.5), failed to produce corneal pathology by six hours after injection. The addition of ammonium sulfate (final concentration 4.7 per cent) to a sterile cornea-damaging culture filtrate prepared from a TYE broth culture did not measurably inhibit the protease or cornea-damaging activity of the filtrate.

Examination for other enzymatic activities. Hexapeptidase, collagenase, lecithinase C, desoxyribonuclease, lipase, and esterase activities were not detected in cornea-damaging protease preparations obtained by isoelectric focusing.

Discussion

Liu' found that crude extracellular antigen preparations of Serratia sp. could produce dermonecrosis in rabbits. In addition, Salceda, Lapuz, and Vizconde7 produced endophthalmitis in rabbits by the intravitreal injection of the sterile culture filtrate or cell-free extract of a strain of S. marcescens causing human endophthalmitis. The heterogeneous nature of the preparations used in both studies makes it difficult, however, to determine the role of the various extracellular products of S. marcescens in eliciting the observed pathology. Four observations described in this communication support the conclusion that extracellular proteases produced in vitro by a cornea-virulent strain of S. marcescens can elicit rapid and extensive damage to the rabbit cornea. First, the cornea-damaging and protease activities were inseparable when the culture supernatant fluids of a cornea-virulent strain of S. marcescens were fractionated by ammonium sulfate precipitation, isoelectric focusing, ion-exchange chromatography, hydroxyapatite adsorption chromatography, and gel filtration. Second, the cornea-damaging protease preparations were free of detectable amounts of other known extracellular serratia enzymes. Third, heating the proteases for 15 minutes at 60° C. caused complete loss of protease and cornea-damaging activities. Fourth, in vitro production of protease and cornea-damaging activities was inhibited by ammonium sulfate.

The knowledge that S. marcescens 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 Gram-negative endotoxin causes extensive and persistent corneal opacification.22 This substance does not, however, elicit the marked colliquative necrosis and corneal perforation characteristically induced by the serratia proteases. Studies are planned to determine if other known extracellular serratia products possess cornea-damaging activity similar to the serratia proteases.

Proteases of serratia origin do not appear to be unique in their ability to damage the mammalian cornea. Trypsin, papain, Pronase, proteases of Pseudomonas aeruginosa, and a crude protease preparation obtained from polymorphonuclear leukocyte lysosomes have been reported11-26 to possess cornea-damaging activity. Brown, Bloomfield, and Tam24 observed that a cornea-destroying pseudomonal protease preparation obtained by sequential ammonium sulfate precipitation and Sephadex G-200 gel filtration could degrade the isolated proteoglycan ground substance of the rabbit cornea. They speculated that if the proteoglycan is responsible for maintaining the order and interfibrillar attachments of the corneal collagen fibrils then degrading the proteoglycan might disperse the fibrils and result in corneal liquefaction without destroying its collagen. Light and electron microscopy studies are currently in progress in our laboratory to attempt to determine the sequence of ultrastructural alterations occurring in the rabbit cornea following the administration of cornea-damaging proteases.

Several investigators27-31 have studied the various proteases of Serratia sp. Work is in progress in our laboratory to obtain large amounts of homogeneous cornea-damaging proteases of S. marcescens. The physico-
Cornea-damaging proteases will be compared with those reported for the Serratia proteases previously isolated by others workers. Obtaining large amounts of homogeneous cornea-damaging proteases will also enable us to commence studies designed to determine (1) the mechanism(s) by which the enzymes produce corneal pathology, and (2) the enzymes' role(s) in the pathogenesis of Serratia corneal infections.

REFERENCES


