The cornea-destroying enzyme of *Pseudomonas aeruginosa*

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The harvest media from five strains of Pseudomonas aeruginosa *collected from severe clinical infections did not contain a collagenase. Instead, an enzyme was isolated that could degrade the ground substance of the cornea. Partial purification of this enzyme indicated that it was the only enzyme in the harvest media that could destroy the rabbit cornea after intralamellar injection. Na_2EDTA completely inhibited this enzyme.*

The corneal infections from *Pseudomonas aeruginosa* are universally feared because of the rapid corneal dissolution accompanying these infections. A possible reason for the tissue destruction was offered by the studies of Fisher and Allen.[1] They showed that injection of the harvest media of *P. aeruginosa* into the rabbit cornea quickly resulted in stromal liquifaction. These harvest media showed caseinolytic activity and appeared to dissolve commercially prepared tendon collagen. The authors concluded that *P. aeruginosa* produced a collagenase which was responsible for these findings.

Morihara,[2] in 1960, also studied the proteolytic enzyme produced by *P. aeruginosa*. He showed that it operated at a neutral pH and although the purified protease could liquify gelatin, and degrade both casein and an artificial substrate whose amino acid composition was similar to collagen, it could not break down native collagen. This enzyme was inhibited by Na_2EDTA, 10^{-2}M, but not cysteine, 5×10^{-3}M. He suggested that the enzyme was metallodependent.

In 1966, Schoellmann and Fisher[3] isolated an enzyme from *P. aeruginosa* that degraded an artificial polypeptide whose amino acid composition was similar to collagen. They concluded that this enzyme was a collagenase.

Most recently, Wilson[4] showed, as did Fisher and Allen, that *Pseudomonas* harvest media quickly liquifies the cornea, but that destruction is prevented by the preincubation of the media with Na_2EDTA 10^{-2}M. These media also appeared to liquify commercially prepared collagen strips. Finally, Wilson prevented perforations of *P. aeruginosa*-infected rabbit corneas by topical treatment with Na_2EDTA and antibiotics.

Because of the lack of uniformity of the methods, controls, and results in the above

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studies of the production of a collagenase by *Pseudomonas*, the present investigation was undertaken to study the cornea-destroying enzyme produced by *P. aeruginosa*.

Methods

Five strains of *P. aeruginosa* were used in this study. All were taken from fulminating human corneal ulcers. These strains were grown separately in 1,000 c.c. of trypticase soy broth in a 6 L. flask mounted on a shaker and incubated at 37° C. for 48 hours. More of these organisms were also grown as above, except that collagen, 0.02 per cent, was added to the trypticase soy broth.

After 48 hours of incubation the cultures were centrifuged at 7,000 r.p.m. for 30 minutes and slowly filtered through 0.2 micron millipore filters to remove the organisms. The filtrates were then divided into two portions. The first was dialyzed overnight against 0.05M Tris HCl buffer, pH 7.4, containing 5 mM $CaCl_2$ and then lyophilized. The second portion was partially purified as in the following section.

Partial purification of* Pseudomonas *enzymes. A saturated ammonium sulfate solution, pH 7, was added to the filtered culture media so that the final saturation was 75 per cent. The precipitate was dissolved in 0.05M Tris HCl buffer, pH 7.4, containing 5mM calcium chloride, and dialyzed against this buffer for six hours. The solution containing approximately 25 mg. of protein was then applied to Sephadex G-200 columns which were equilibrated with 0.05 M Tris HCl, pH 7.4, containing 5mM $CaCl_2$. The flow rate was 15 ml. per hour and the temperature was maintained at 4° C. Three milliliter fractions were collected. The optical density of the fractions was monitored at 280 nm. on a Beckman DB-G Spectrophotometer. Every five fractions were pooled, dialyzed, lyophilized, and frozen.

Collagenase activity was tested in each step of purification by collagenase assays, examination for collagen breakdown products with disc electrophoresis, and via viscometry. The latter measurements were made in Ostwald viscometers with a water flow time of 49 to 53 seconds at 27° C. A solution of collagen, 0.1 per cent, was prepared for viscometry with 0.05M Tris HCl buffer, pH 7.4, containing 0.15M NaCl. Measurements were taken over a three hour period.

Viscosity measurements were also used to monitor proteoglycanolytic activity in each step of the purification procedure.[5] Solutions of corneal proteoglycan were prepared in a 1 per cent concentration with 0.05M Tris HCl buffer, with 0.15M NaCl, pH 7.3. The initial viscosity was approximately 5.0. A sample containing 7 μg from each fraction was added to the substrate. Measurements were taken over a four hour period. Control

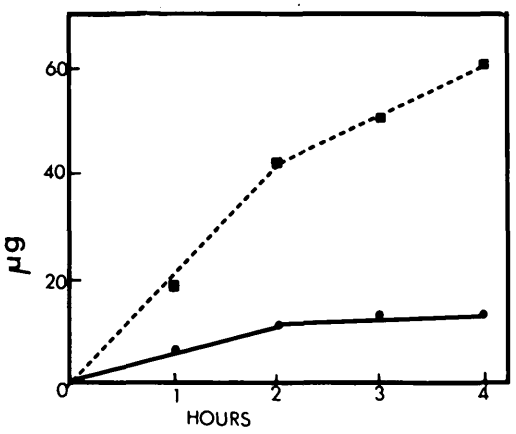

Fig. 1. Appearance of hexuronate containing material in "output" chamber after incubation of corneal proteoglycan with fraction 31-35 which is most active in reducing viscosity of the proteoglycan. Ordinate represents cumulative release at each time period. Solid line represents both of the control substances.

solutions for both viscosity studies were similar solutions of collagen or proteoglycan without the fractions and were carried out at the same time as the viscosity measurements with the reaction mixtures.

In order to substantiate the correlation of breakdown of proteoglycan with changes in viscosity a diffusion apparatus was constructed consisting of two lucite chambers separated by a millipore filter of 0.2 μ pore size. This is similar to a recently reported diffusion vessel which permits diffusion of chondroitin sulfate, but not the larger proteoglycan, across the multipore filter.[6] Measurements of uronic acid (hexuronate) in the "output" chamber indicated the amounts of proteoglycan degraded.

Corneal proteoglycan, 0.9 per cent, in 2 c.c. of 0.05M Tris HCl with 0.15M NaCl and 5 μg of that fraction which effected the most rapid and complete reduction in viscosity was placed in the input chamber. Seven cubic centimeters of 0.05M Tris HCl with 0.15M NaCl was placed in the output chamber. The diffusion vessel was then incubated at 37° C. for four hours. The uronic acid content of the output chamber was then estimated hourly by the carbozole reaction. The control substances for this study were two additional diffusion veselss with the same contents, except in one there was only 0.05M Tris HCl, pH 7.1, with 5mM $CaCl_2$ and in the other, the active fraction was added after it was warmed at 80° C. for 15 minutes.

At the end of the four hour incubation period there was 11 μg per cubic centimeter of hexuronate released into the output chamber of the control diffusion vessel without the active fraction,

Table I. Collagenolytic activity of *Pseudomonas* media

	Counts per minute solubilized
Control C^{14}-collagen gel	212
Control and trypsin (15 μg)	353
Harvest media-$(NH_4)_2SO_4$ pipetted (20 μg)	352
Harvest media (50 μg)	235

9 μg per cubic centimeter of hexuronate in the output chamber of the control vessel in the preheated fraction, and 61 μg per cubic centimeter of hexuronate released into the output chamber in the vessel with the active fraction (Fig. 1). This indicates the validity of the viscosity measurements to assay degradation of proteoglycan.

Radioactive collagen preparation and collagenase assay. Radioactive collagen was prepared from the skin of guinea pigs after intraperitoneal injection of 100 μCi of glycine 1-C^{14} six and twelve hours prior to death. Solubilized collagen was eluted from the skin with 0.4M NaCl, and purified by repeated salt precipitation. After dialysis the collagen was lyophilized and stored at -20° C. The specific activity of the labeled collagen was 20,000 d.p.m. per milligram of protein.

Plastic conical tubes containing 0.2 ml. of a 0.2 per cent glycine C^{14}-collagen solution, pH 7.6, were incubated at 37° C. for 15 hours to facilitate gelling. The fraction to be assayed for collagenase activity was lyophilized and hydrated with 0.2 ml. of distilled water, and 5.0 μl aliquots which contained 50 μg of protein were applied to the gels. The tubes were then incubated for 24 to 48 hours in a moist atmosphere at 37° C. Following incubation, 0.1 ml. of distilled water was added and the tubes were centrifuged at 44,000 × g for 10 minutes. Aliquots of the supernatant (0.25 ml.) were pipetted into vials containing glass filter papers and dried overnight. The radioactivity was counted in a liquid scintillation counter in 1.0 ml. of POPOP-1, 4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, PPO-2, 5-diphenyloxazole, and toluene mixture.

Disc electrophoresis of collagen components. Enzyme preparations having 150 to 200 μg of protein as determined by the Lowry method were mixed in 0.5 ml. of 0.07 per cent glycine-C^{14} collagen in Tris 0.005M, pH 7.4. After incubating for three hours at 27° C., the reactions were terminated with Na_2EDTA at a final concentration of 0.001M. The pH of the mixture was adjusted to 4.5 with acetic acid, and the collagen was denatured by heating at 48° C. for 15 minutes. Samples containing 100 to 200 μg of protein were analyzed by disc electrophoresis in polyacrylamide gel and stained with Amido-Schwartz according to the method of Nagai, Gross, and Piez.[7]

Estimation of molecular weight of the proteoglycanolytic enzyme. The molecular weight of the enzyme was calculated using the method of Andrews.[8] Aldolase, chymotrypsinogen A, ovalbumin, ribonuclease A, and blue dextran 2,000 (Pharmacia) were used as standards. The protein content (optical density) of the eluted standards was determined at 280 nm.

Inhibition of the proteoglycanolytic effect. Soybean trypsin inhibitor, 5 μg, epsilon amino caproic acid, 10^{-1}M and 10^{-2}M, cysteine 10^{-2}M, penicillamine 10^{-2}M and 10^{-1}M, NaEDTA 10^{-2}M and 10^{-3}M, and CaEDTA 10^{-2}M and 10^{-1}M were tested for inhibition of the reduction in viscosity of a proteoglycan solution by the crude, lyophilized crude, $(NH_4)_2SO_4$ precipitated crude, and the chromatographically purified harvest media.

Proteoglycan preparation. Proteoglycans were extracted from rabbit corneas with 4.0M guanidinium chloride. Fractions high in uronic acid content were obtained through the courtesy of Dr. John D. Gregory of Rockefeller University, New York, N. Y.

Corneal injection. Crude harvest media (0.05 c.c.) and 0.05 c.c. of each fraction eluted from the Sephadex G-200 columns were injected via a 27 gauge needle into the central corneal stroma of albino rabbits anesthetized with intravenous pentobarbital and topical 0.4 per cent benoximate hydrochloride.

Protease assay. The purified enzyme from the active fraction was assayed by casein digestion according to Kunitz.[9]

Enzyme solutions (0.2 ml.) containing 0.5 to 1.0 mg. of protein were incubated at 37° C. for two or three hours in 1 ml. of 0.6 per cent casein in 0.05M Tris HCl, pH 7.1, containing 0.001M CaCl, or in 1 ml. of 0.6 per cent casein in 0.1M sodium acetate, pH 4.5. The reaction was terminated by adding 2 ml. of 0.33M trichloroacetic acid and incubating for ten minutes at 37° C. The samples were centrifuged at 3,000 × g for five minutes and the turbidity was read at 280 nm. Trypsin standards were determined simultaneously.

Results

Neither the crude, lyophilized crude, nor dialyzed and concentrated crude harvest media from 5 strains of *P. aeruginosa* lysed collagen gels, solubilized radioactive collagen (Table I), reduced the viscosity of a collagen solution beyond the control values of 4 to 8 per cent, or degraded the collagen molecule as indicated by polyacrylamide

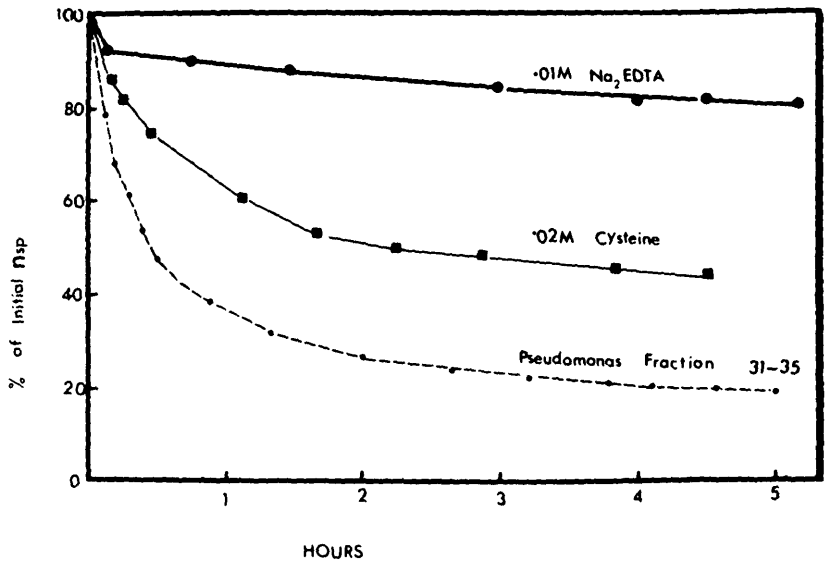

Fig. 2. Graph shows that the specific viscosity of corneal proteoglycans at pH 7.3 is reduced to 20 per cent of the initial value by incubation with fraction 31-35 eluted from Sephadex G-200. Cysteine, 0.02M, reduces the fall in viscosity by almost 50 per cent and Na_2EDTA 0.01M, completely prevents the reduction in viscosity.

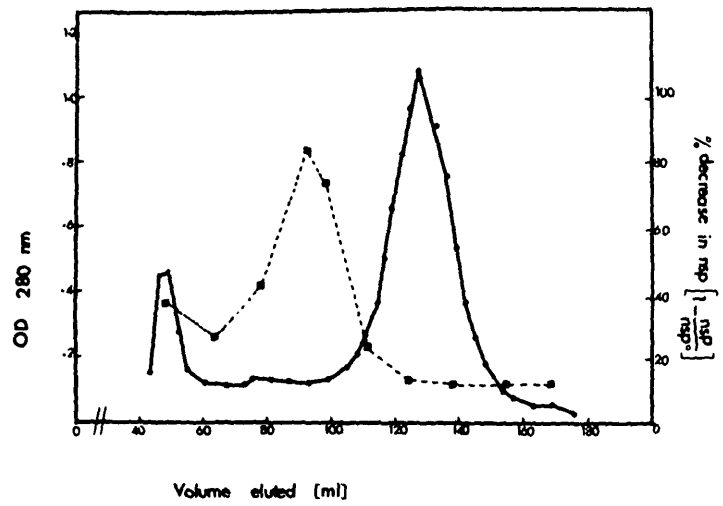

Fig. 3. Gel filtration of proteoglycanolytic enzyme *Pseudomonas* harvest media. The void volume was 40 ml. Optical density (●——●) was determined at 280 nm.; proteoglycanase activity (□-----□) is represented as per cent decrease in initial specific viscosity.

gel electrophoresis. Similar negative results were obtained after the proteins of the crude media had been concentrated by $(NH_4)_2SO_4$ precipitation or Sephadex G-200 chromatography.

Each of these harvest media ulcerated the rabbit cornea in vivo within six hours after intralamellar injection.

All of the above-mentioned harvest media quickly reduced the viscosity of corneal proteoglycan at pH 7.3 (Fig. 2). Partial purification of the crude harvest media by $(NH_4)_2SO_4$ and repeated chromatography showed a rather discrete single peak of proteoglycanolytic activity at both pH 7.3 and 4.3 (Fig. 3). When the various fractions eluted from the columns were injected into rabbit corneal stromas in vivo

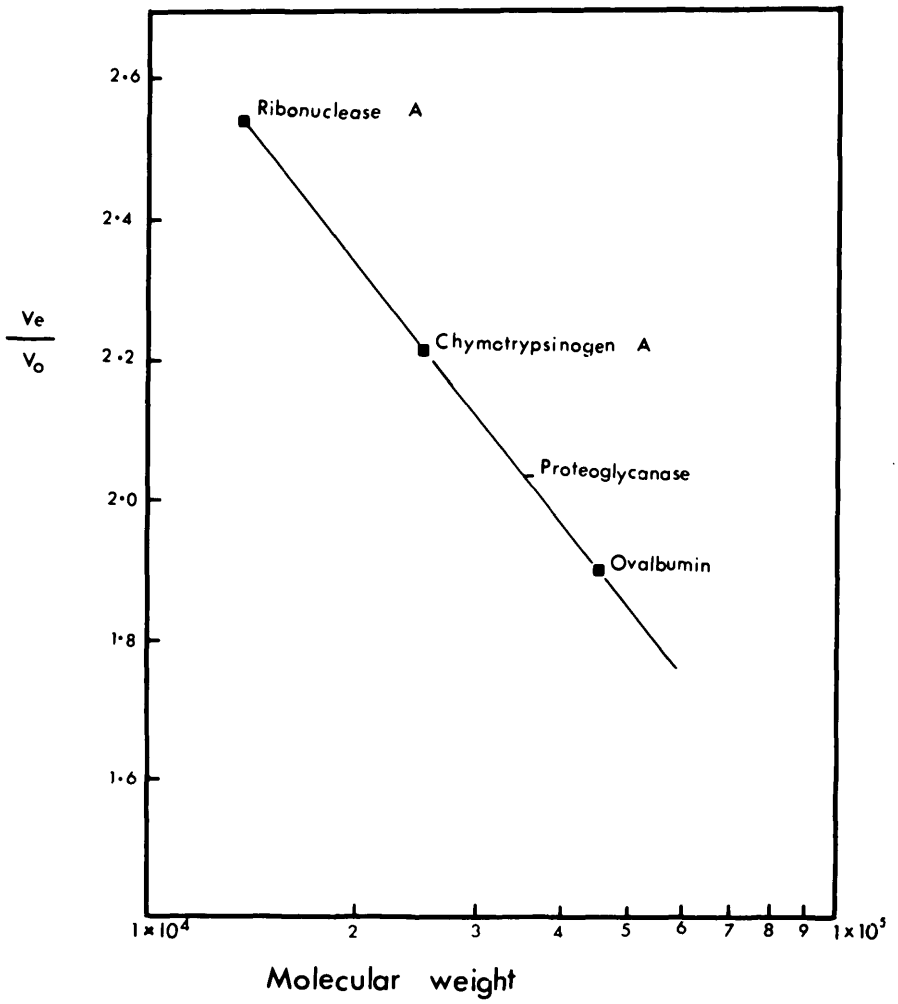

Fig. 4. Estimation of the molecular weight of the *Pseudomonas* proteoglycanolytic enzyme by gel filtration.

only the fraction with the peak activity (from 31-35) liquified the corneas.

The approximate molecular weight of the proteoglycanolytic enzyme in the active fraction was 33,500 (Fig. 4).

Neither soybean trypsin inhibitor, penicillamine, CaEDTA, nor epsilon amino caproic acid inhibited the proteoglycanolytic activity of the active fraction. Cysteine inhibited about 50 per cent of the proteoglycanolytic activity, penicillamine, 10^{-1}M, inhibited 27 per cent, while Na_2EDTA, 10^{-2}M, completely inhibited this activity. Na_2EDTA, 10^{-3}M, had no inhibitory effect. Cysteine, 10^{-2}M, and penicillamine, 10^{-1}M, without the active fraction, did not reduce the viscosity of the corneal proteoglycan.

The active fraction grossly liquified gelatin and showed caseinolytic activity (Fig. 5).

Discussion

The results of this study indicate that five strains of *P. aeruginosa* taken from fulminating corneal infections did not produce a neutral or acid collagenase when grown under generally optimal conditions, i.e., neither the crude, concentrated, nor partially purified harvest media from the tested strains could reduce the viscosity of a collagen solution, solubilize collagen fibrils, or degrade the tropocollagen molecule

as indicated by polyacrylamide gel electrophoresis. Earlier studies indicated that *P. aeruginosa* does produce a collagenase.[1-4] However, the substrates used in these studies to determine the activity of the enzyme were either artificial polypeptides or commercially prepared collagen that had not been tested against nonspecific proteases. The former has been shown to be inadequate for proof of collagenase activity and the latter must be shown to be able to resist nonspecific proteases for it to be a proper substrate for collagenase.[10]

Although the various crude harvest media tested did not contain a collagenase they were able to quickly liquify the rabbit cornea after intralamellar injection. Because the major noncollagen component of the dry cornea is proteoglycan, an enzyme capable of degrading this component was assayed for and found. Purification and characterization of this enzyme by chromatography showed that only the fraction highest in proteoglycanolytic activity could destroy the cornea when injected intralamellarly. The enzyme was active at a neutral pH, degraded casein, liquified gelatin, and had a molecular weight of 33,500. The proteoglycanolytic activity was not inhibited by epsilon amino caproic acid, CaEDTA, penicillamine, and soybean trypsin inhibitor. Cysteine, 10^{-2}M, partially inhibited while NaEDTA, 10^{-2}M, completely inhibited the proteoglycanolytic activity. It was interesting that sodium EDTA, 10^{-3}M, had no effect since this concentration is generally sufficient to inhibit most metallo-dependent enzymes in vivo. The significance of this finding is not certain as yet, and because the method of the enzyme's inhibition by sodium EDTA has obvious clinical importance, it will be the object of a future study.

The fraction with the highest proteoglycanolytic activity was the only fraction that destroyed the cornea after intralamellar injection. This fraction had a low protein content as indicated by optical density measurements. It is, therefore, likely that

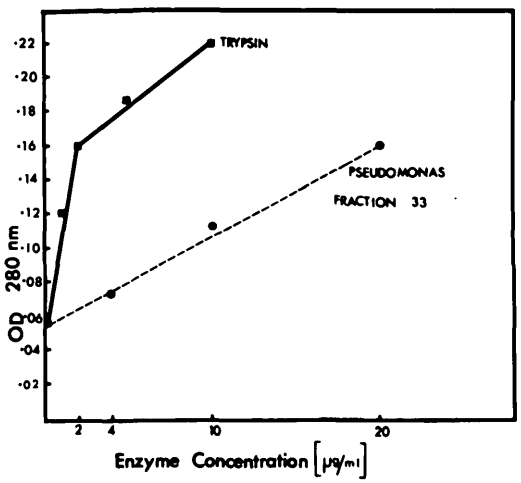

Fig. 5. This graph shows caseinolytic activity of active fraction (●-----●) versus trypsin controls (□——□).

the same enzyme is responsible for both actions. How the enzyme destroys the corneal stroma can only be speculated. It is possible that proteoglycan insulates and is responsible for maintaining the order and interfibrillar attachments of the collagen fibrils of the cornea. Destroying the proteoglycan might, therefore, free and disperse the collagen fibrils which could result in liquifying the cornea without destroying its collagen.

It is possible that *P. aeruginosa* can produce a collagenase when grown under different conditions or, that the quantity produced under the conditions of this study was too small to be detected. However, these possibilities probably have little relevance in the understanding of the cause of the destruction and liquifaction of the cornea infected by *P. aeruginosa*. The present studies indicate that, rather than a collagenase, a proteoglycanolytic enzyme produced by *P. aeruginosa* destroys the cornea.

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