Enzymatic Activities Associated with Opacification Factors from Pseudomonas aeruginosa

Marilou G. Gagalac-Nicolas*, Salvador R. Salceda*
Lourdes J. Cruz**

*Institute of Ophthalmology, Health Sciences Center
University of the Philippines, Manila
**Department of Biochemistry and Molecular Biology
College of Medicine, University of the Philippines, Manila

ABSTRACT

Extracellular protein concentrate from the culture filtrate of Pseudomonas aeruginosa *was fractionated in Cellex D, an anion exchanger. Four opacification factors were resolved. Determination of enzymatic activity and lyophilization studies showed that a protease may have contributed to effecting corneal injury, but for* P. aeruginosa *grown in 10% peptone broth, the primary enzyme involved in the opacification process was a hyaluronidase-like enzyme.*

INTRODUCTION

Some extracellular substances in the culture filtrate of *Pseudomonas aeruginosa* had been observed to cause corneal damage, manifested by loss of transparency or opacification. These manifestations are believed to be generally effected by enzymatic hydrolysis.

Various researches on the cornea-damaging substances suggest that the enzyme involved in corneal destruction is a collagenase and/or a protease. Fisher and Allen (1,2), Wilson (3) and Salceda (4) attribute corneal perforation characteristic of *P. aeruginosa* infection to collagenase — a conclusion derived from the culture filtrate's action on synthetic collagen substrates and the prevention of that action and of corneal damage by EDTA and cysteine. Other workers (5,6), however, believe that the cornea-damaging enzyme is a more general protease. Brown and co-workers (7) and Kessler et al (8), found that this cornea-damaging protease acts on the stromal proteoglycans. In addition, histochemical and ultrastructure studies by Gray and Kreger (9) of *P. aeruginosa*-infected rabbit corneas showed that collagen fibers remain intact while proteoglycans were degraded from these corneas. The protease, therefore, digests the proteoglycan matrix, thereby dispersing collagen fibers and allowing entry of water, with consequent opacification.

However, because of variations in media and experimental methods used, the exact nature and mechanism of the proteolytic enzyme has not been elucidated. To complicate matters further, recent researches attribute some toxicity effects of bacterial exudates

(eg. necrosis), to chemotactic factors (10). More recently, Buenaflor et al. (11) developed an in vitro assay method which eliminated the effects of host response. It was found that the extracellular substances from a *P. aeruginosa* culture filtrate could cause corneal injury in vitro, but with no accompanying necrosis as observed in vivo. However, results from in vivo and in vitro assays correlate very well as far as location of active fractions are concerned. Two fractions responsible for corneal damage were resolved. The higher molecular weight fraction coincided with protease activity. β-galactosidase activity was detected but it did not coincide with the opacification activity fractions.

This investigation was done to elucidate the mechanism of corneal destruction due to the *P. aeruginosa* exudates. Since opacification is believed to be a product of enzymatic activities associated with these exudates, four other enzyme activities: protease, collagenase, elastase, and hyaluronidase, were studied for their role in corneal damage.

MATERIALS AND METHODS

Reagents and equipment. Electrophoresis reagents were obtained from Sigma Chemical Company, St. Louis, Missouri, and the disc gel electrophoresis done using the Canalco Research Disc Standard apparatus. Concentration gradient was checked using a Hach Conductivity Meter. Lyophilization was done in a Virtis Lyophilizer. All other reagents and equipment used were as previously stated (11).

Organism and biological activity assays. The *Pseudomonas aeruginosa* strain was that used in a previous study of cornea-damaging substances (11). This strain was isolated in 1972 at the Philippine Eye Research Institute from a 3-year old boy. In vivo opacification activity determination was done as previously reported (11).

Ion exchange chromatography. The culture filtrate and extracellular protein concentrate (EPC) were prepared as in the previous report (11). An aliquot of the EPC containing approximately 90 mg protein was applied to a 3 x 17 cm Cellex D column pre-equilibrated with 0.01 M Tris-HCl, pH 7.4. Elution was carried out using a linear gradient with 500 mL 0.01 M Tris-HCl, pH 7.4, and 500 mL of 0.01 M Tris-HCl-1.5 M NaCl, pH 7.4, as limiting buffers. Fractions were collected at a flow rate of 10-15 mL/5 min. Collagenase (E.C. 3.4.24.3), protease, elastase (E.C. 4.2.2.1) were monitored. Protein content was determined according to the method of Lowry et al. (12), using bovine plasma albumin (BPA) as standard.

Enzymatic analysis. All enzyme assays were tested with known enzyme standards. The method for the determination of proteolytic (6) and collagenolytic (13) activities were as stated in the previous study (11). A unit of proteolytic activitiy is equivalent to the amount (mg) of azocasein digested to give an OD change of 1.0 unit per mg protein in 5 min at 37°C.

Elastolytic activity was determined following a modification of the method used by Sacher (16) and Shotton et al (17). Two hundred microliter aliquots of a 1.0 mg/mL Congo red-elastin suspension in 0.01 M Tris, pH 7.4, were placed in tubes and preincubated at 37°C for five minutes. To each of the tubes was added 100 μL of test enzyme solution and the mixture was shaken vigorously for 30 seconds. The solutions were incubated at 37°C for 12 hours. For controls, the substrate, Congo red-elastin, was omitted, and the tubes treated similarly. The undigested substrate was removed by centrifugation at 12,000 x G for 20 minutes and the absorbance of the supernate was read at 495 nm.

A standard curve was prepared using varying concentrations of substrate

completely digested with elastase. One unit of activity is equivalent to milligrams of Congo red-elastin digested per mg protein in 12 hours at 37°C.

Disc gel electrophoresis. The solutions, gels and electrophoretic method used were those described by the CANALCO Industrial Corporation for research disc standard (RDS) as given in their technical bulletin (18).

After electrophoresis, the proteins were stained in 0.1% Coomassie Blue Black R in 30% methanol-10% acetic acid-60% H_2O, and destained by diffusion in acetic acid-methanol-H_2O mixture.

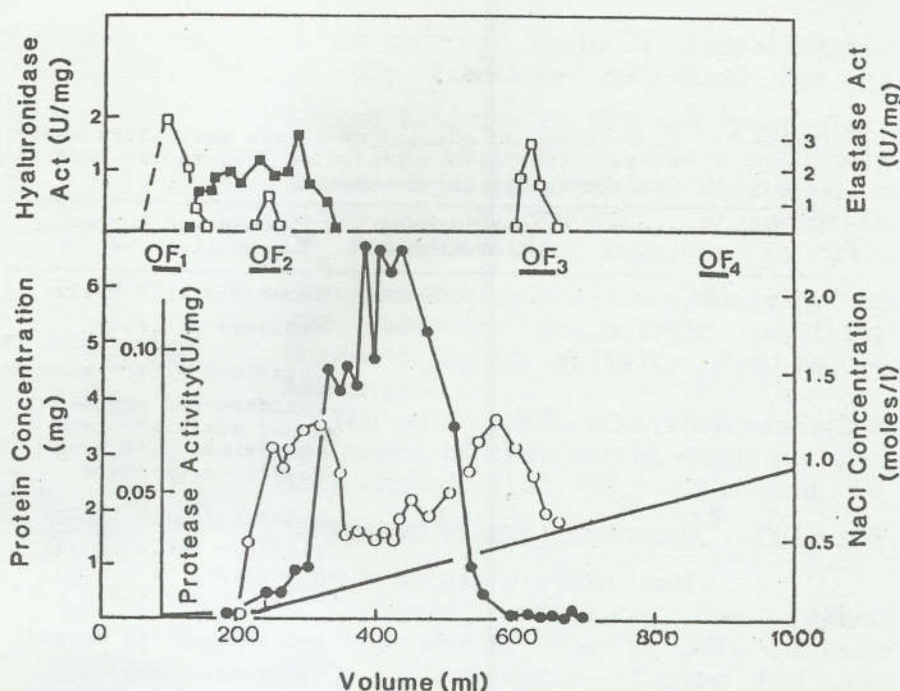

Fig. 1. DEAE-cellulose chromatography of extracellular proteins from *Pseudomonas aeruginosa* grown in 10% peptone broth.

The upper graph shows the elastolytic (close squares) and hyaluronidase (open squares) activities. The lower graph shows the protein profile (close circles) and proteolytic activities (open circles). The degree of opacification vary for different runs and for this particular run, the degrees of opacification are: OF_1(+1), OF_2(+1), OF_3(+1), and OF_4(+2).

RESULTS AND DISCUSSION

The enyzmes investigated belong to two general types according to the corneal substrate: those that effect protein hydrolysis (protease, collagenase, elastase), and one that caused carbohydrate degradation (hyaluronidase). A fifth enzyme (β-galactosidase) had already been previously investigated and its activity reported.

Studies by other investigators (1-9) dealt with the probably collagenolytic, elastolytic and/or proteolytic natures of the cornea-damaging substances. Previous work (11) done in this laboratory showed that the fractions which caused cornea damage contained proteolytic activity but not collagenolytic activity. The report also showed that galactosidase, an enzyme involved in the cleavage of terminal galactose units, and detected in the crude culture filtrate, is not involved in corneal opacification.

Enzyme activities of the Cellex D fractions from the crude concentrate were 3.5 x 10^{-4} U/mg for elastase, 0.022 U/mg for hyaluronidase and 0.0345 U/mg for protease. Collagenase activity was not detectable.

With the fractionation technique used, four opacification factors were resolved. The typical chromatographic pattern is shown in Figure 1 and the properties of the opacification factors summarized in Table 1. Three of the

Table 1. Properties and summary of enzyme activities associated with the opacification factors resolved by DEAE-cellulose chromatography.

Opacification Factors	Ionic Strength of elution, u	Observed Enzyme Activities
OF_1	0.06	Coincided with hyaluronidase activity
OF_2	0.09	Coincided with hyaluronidase and proteolytic activities and overlapped with elastolytic activities
OF_3	0.51	Coincided with hyaluronidase activity and overlapped with proteolytic activity
OF_4	0.81	Had none of the enzyme activities assayed

opacification factors (OF_1, OF_2, OF_3) were associated with hyaluronidase activity. Only OF_2 ccincided with a peak of proteolytic activity, which is probably contributory to the observed corneal damage it caused as shown by the lyophilization experiments (Table 2). On the other hand, OF_3 merely overlapped with another proteolytic activity, hence this enzyme may not be important in as far as corneal damage due to OF_3 is concerned. OF_1 showed no proteolytic activity. OF_4 did not exhibit any of the enzyme activities assayed. Among all the enzymes tested, hyaluronidase seemed to be consistently associated with opacification activity.

Some studies (5,8) have reported that elastase is the protease responsible for the observed corneal opacification. Chromatographic results showed that the elastolytic activity levels are very low and are spread over a volume of about 200 mL (about 15 fractions). Opacification factor 2 is within this spread, but this factor did not coincide with the peaks of highest elastolytic activities. Thus, although the literature (5,8,9) deals exhaustively with elastase and equates opacification activity with elastolytic activity, our study shows that the elastolytic activity observed could be just a general protease effect. In confirming this, the ratio of specific activities of elastase to protease (E:P) was determined in the crude filtrate and compared with the ratio in a pronase preparation at the same reaction conditions. E:P ratio of the elastase activity in the crude filtrate was found to be 0.01:1 which is tenfold less than the 0.1:1 E:P ratio determined for pronase. The implication is that the elastolytic activity observed could very well be due to less specific proteases.

The in vivo cornea-damaging activities of the different opacification factors are also indicated in Figure 1 for this particular run. The degree of opacification varied from one batch to another. This is probably a result of variations in the amount of protein injected. The extent of corneal damage produced by the different opacification factors in live rabbits is illustrated in Figure 2.

Lyophilization of crude extracts was shown to decrease cornea-damaging activity. The lability of the enzyme activities associated with OF_2 and OF_3 was therefore similarly tested.

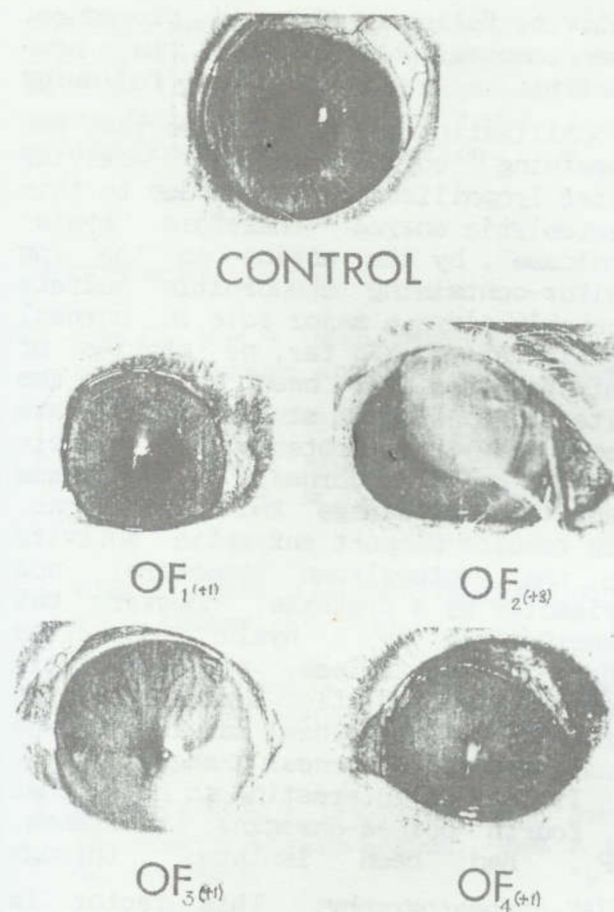

Fig. 2. Corneal damage in live rabbits produced by the opacification factors from DEAE-cellulose chromatography.

As shown in Table 2, lyophilization of OF_2 fractions decreased hyaluronidase activity by 92% and proteolytic activity by 23%. Correspondingly, cornea-damaging activity decreased by about half in intensity. Freeze-drying OF_3 fractions also resulted in 99.7% decrease in hyaluronidase activity and 63% loss of proteolytic activity. Cornea-damaging activity was reduced markedly.

The disc gel electrophoresis(DGE) patttерns of the cornea-damaging factors showed that OF_1, OF_2, and OF_3 contained several proteins. Only OF_4 gave a single protein band.

Collagen constitutes the highest fraction among the stromal proteins (19). Thus early studies had been focused on the possible involvement of a collagenolytic enzyme in corneal damage due to *P. aeruginosa*. In this study, however, collagenase activity was not detected in the crude, unconcentrated and chromatographic fractions. This finding supports the observations of several workers that collagenase is not an extracellular product expressed by *P. aeruginosa* grown in vitro. The ultrastructure study of Gray (9) showed that collagen remained undigested in *P. aeruginosa*-infected rabbit corneas, while the stromal proteoglycans were degraded in these corneas.

It would seem, then, that both specific proteases, collagenase and elastase are unimportant in effecting corneal opacification. As far as degradation of pro-

Table 2. Effect of lyophilization on the enzyme and cornea-damaging activities *in vivo* of OF_2 and OF_3.

ENZYMATIC ACTIVITIES	SPECIFIC ACTIVITIES, U/mg					
	Fresh		Lyophilized		% Decrease	
	OF_2	OF_3	OF_2	OF_3	OF_2	OF_3
Protease, x 10^3	6.5	11.0	5.0	4.1	23.0	63.0
Hyaluronidase	3.4	5.4	0.3	0.02	92.0	99.7
Cornea-damaging activity	+2	+1	+1	negligible		

tein structures of the corneal stroma is concerned therefore, only a non-specific protease, or a protease which has not been characterized as yet, is involved. Its involvement is probably secondary as demonstrated by the lyophilization experiments shown in Table 2.

The corneal stroma does not contain hyaluronic acid. However, this substrate was used to determine acid mucopolysaccharide lyase or "hyaluronidase" activity because of the presence in the cornea of a low sulfur-containing chondroitin sulfate analogue which has similar chemical properties as hyaluronic acid. This analogue is composed of equimolar amounts of N-acetylhexosamine and uronic aid. Meyer (20) had termed this structure "chondroitin", which is an analogue of chondroitin sulfate A or C but it is very low in sulfate concentration. It can be hydrolyzed by bacterial and testicular hyaluronidase at a rate comparable to that of hyaluronic acid, releasing a product with the same electrophoretic mobility as that produced from hyaluronic acid.

The assumption that a hyaluronidase or hyaluronidase-like enzyme specifically acts on the stromal proteoglycans is substantiated by the finding that three of the four opacification factors resolved by DEAE-chromatography are associated with hyaluronidase activity. One of these, OF_1, did not have any of the other enzyme activities determined. However, the possibility of the presence of other enzymes which were not assayed could not be ruled out. Nevertheless, if corneal opacification due to OF_1 is the result of an enzymatic hydrolysis of corneal structures, then it is probably due to the hyaluronidase-like enzyme.

Although OF_2 and OF_3 have proteolytic activities aside from hyaluronidase, hyaluronidase activity was consistently more sensitive to lyophilization and could be correlated with the decrease in opacification activity following the said procedure. The concommitant decrease in proteolytic activity of OF_2 following lyophilization was only 23% so that the remaining cornea-damaging activity after lyophilization may be due to this proteolytic enzyme. Therefore "hyaluronidase", by its action on the low sulfur-containing chondroitin sulfate probably plays a major role in corneal opacification. So far, no mention of this role has ever been made in the literature. Recent studies (7-9) have concluded that protease, by their action on the corneal proteoglycans cause corneal damage and ulcerations. Our results support enzymatic activity on the proteoglycan structure, not primarily by a protease, however, but specifically by a hyaluronidase-like enzyme. Nevertheless, the hydrolytic action of proteolytic enzymes on the proteins of the corneal matrix could be contributory to corneal damage.

It is also interesting to note that a fourth cornea-damaging substance, OF_4, had been isolated through DEAE-chromatography. This factor is not associated with any of the enzymes assayed for. The role of this opacification factor has to be investigated further. A low molecular weight component which exhibited no enzyme activity had also been isolated from Sephadex G-75 and reported in a previous paper (11). However, no correlation between this latter component with OF_4 could be made until further studies is performed.

In summary, the corneal damage observed due to at least three of the opacification factors resolved is the result of an enzymatic action on the carbohydrate portion of the stromal proteoglycans. One of these factors also showed presence of an enzyme that acts on the protein portion thereby causing corneal damage. The fourth factor did not exhibit any of the enzymatic activities tested. The mechanism for the overall opacification process is complex and uncertain but

the data and arguments presented show that for *Pseudomonas aeruginosa* grown outside the eye, hyaluronidase activity seems to be the most significant cause of the observed opacification. ■

REFERENCES

1. Fisher E Jr, Allen JH. Corneal ulcers produced by cell-free extracts of *Pseudomonas aeruginosa*. Am. J. Ophthalmol. 1958; 46:21.

2. Fisher E Jr, Allen JH. Mechanism of corneal destruction by *Pseudomonas* proteases. Am. J. Ophthalmol. 1958; 46 Pt II:249.

3. Wilson LA. Chelation in experimental *Pseudomonas* keratitis. Brit. J. Ophthalmol. 1970; 54:587.

4. Salceda SR, Villamora EA. Cysteine in experimental *Pseudomonas* corneal infection. Phil. J. Ophthalmol. 1972; 4(1):5.

5. Kawaharajo K, Chiyogi A. Corneal ulcers caused by protease and elastase from *Pseudomonas aeruginosa*. Japan J. Exp. Med. 1974; 44:435.

6. Kreger A, Griffin OK. Physicochemical fractionation of extracellular cornea-damaging proteases of *Pseudomonas aeruginosa*. Infect. Immun. 1974; 9:828.

7. Brown SI, Bloomfield SE, Tam WI. The cornea-destroying enzyme of *Pseudomonas aeruginosa*. Invest. Ophthalmol. 1974; 13:174.

8. Kessler E, Kennah HE, Brown SI. *Pseudomonas* proteases: purification, partial characterization and its effect on collagen, proteoglycan, and rabbit corneas. Invest. Ophthalmol. Visual Sci. 1977; 16:488.

9. Gray LD, Kreger AS. Rabbit corneal damage produced by *Pseudomonas aeruginosa* infection. Infect. Immun. 1975; 12:419.

10. Adler J. Chemotaxis in bacteria. Annual Rev. Biochem. Snell EJ, Boyer PD, Meister A, Richardson CC, eds. California: Annual Reviews Inc. 1975; 44:341-356.

11. Buenaflor GG, Gagalac-Nicolas MG, Salceda SR, Tan RV, Cruz LJ. The cornea-damaging substances from *Pseudomonas aeruginosa*. Phil. J. Ophthalmol. (In press).

12. Lowry OH, Rosebrough JJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J. Biol. Chem. 1951; 193:265.

13. Mandl I, MacLennan JD. Isolation and characterization of proteinase and collagenase from *Clostridium histolyticum*. J. Clin. Invest. 1953; 32:1323.

14. Tolksdorf S. The turbidimetric assay of hyaluronidase. J. Lab. Clin. Med. 1949; 34:74.

15. Kass EH, Seastone CV. The role of the mucoid polysaccharide (hyaluronic acid) in the virulence of Group A hemolytic septicemia. J. Exp. Med. 1944; 79:319.

16. Sacher LA. Photometric method for estimation of elastase activity. Proc. Soc. Exp. Med. 1955; 90:323.

17. Shotton LM, Hartley BS. Evidence for the amino acid sequence of porcine pancreatic elastase. Biochem. J. 1973; 131:643.

18. Research Disc Standard Electrophoresis. CANALCO Technical Bulletin. 1969.

19. Maurice DM, Riley MV. The cornea. Biochemistry of the eye, Graymore CN, ed. Academic Press, Inc. 1970; 1-95.

20. Davidson EA, Meyer K. Chondroitin, a new mucopolysaccharide. J. Biol. Chem. 1954; 211:605.

P. C. Ocampo Sporting Goods

258-G Teresa St.
Sta. Mesa, Manila

Tel. No. 60-83-71

Pedro C. Ocampo
Proprietor