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Properties of PASP: A *Pseudomonas* Protease Capable of Mediating Corneal Erosions

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Abstract

Purpose—To analyze PASP in terms of its gene distribution and expression, its corneal pathologic effects, its enzymatic properties, and the protectiveness of the immune response to this protease.

Methods—Twenty-five strains of *P. aeruginosa* were analyzed for the *PASP* gene and secreted protein by PCR and Western blot analysis, respectively. Active recombinant (r)PASP (10 μ g/20 μ L) or heat-inactivated rPASP was intrastromally injected into rabbit corneas. Pathologic changes were monitored by slit lamp examination (SLE) and histopathology. Purified rPASP was assayed for cleavage of collagens and susceptibility to TLCK. Rabbit antibody to rPASP was produced and tested for enzyme inactivation, and actively immunized rabbits were challenged by intrastromal injection of active rPASP (5 μ g).

Results—All 25 strains of *P. aeruginosa* analyzed were positive for the *PASP* gene and protein. SLE scores of eyes injected with active rPASP were significantly higher than control eyes at all postinjection times (PI; $P \leq 0.004$). Histopathologic studies documented the destruction of the corneal epithelial layer and portions of the corneal stroma at 9 hours PI, and polymorphonuclear (PMN) leukocyte infiltration into the cornea by 24 hours after active rPASP injection. PASP cleaved type I and IV collagens and was susceptible to TLCK inhibition. PASP was present in the cytoplasm and periplasm, but only secreted PASP was enzymatically active. A high antibody titer (ELISA titer $\geq 10,000$) was produced, but this antibody did not protect against active rPASP challenge.

Conclusions—PASP is a commonly produced *Pseudomonas* protease that can cleave collagens and cause corneal erosions.

The pathogen *Pseudomonas aeruginosa* is a Gram-negative bacterium that causes opportunistic infections, especially in patients with cancer, cystic fibrosis, and burns.^{1–4} It is also documented to cause severe corneal infections, most commonly in association with the use of contact lenses and, at a lesser frequency, after eye injury or surgery.^{5,6} *P. aeruginosa* continues to be the leading cause of contact lens-associated bacterial keratitis in the United States.^{7,8}

The virulence of *P. aeruginosa* is mediated by multiple mechanisms including the production of a wide array of extra-cellular proteases.⁹ There are several well characterized proteases—namely, elastase A and B, alkaline protease, and protease IV (PIV).^{10–13} Proteases produced by *Pseudomonas* can directly damage host tissues and can also indirectly damage the host by

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activating destructive host responses, such as host matrix metalloproteases.¹⁴ *Pseudomonas* elastase B (LasB) and alkaline protease (AP) are metalloproteases that can degrade a variety of host defense molecules, including complement and surfactant proteins.^{15–17} PIV, a serine protease, could be the most potent of the characterized *Pseudomonas* proteases and, unlike the other characterized proteases, is produced by essentially all clinical isolates.¹⁸ In contrast to the virulence retention of LasB- and/or AP-deficient mutant,^{19,20} the loss of the *PIV* gene has been shown to significantly reduce corneal virulence, and complementation of the mutated gene restored full virulence.²¹

Tissue damage caused by *Pseudomonas* proteases occurs independently of viable bacteria and can continue after bacteria are killed by antibiotic therapy. Thus, inhibition of these enzymes by chemical or specific immune therapy would be beneficial in protecting against corneal damage. Immunization against LasB and AP elicits neutralizing antibodies that have been shown to be protective, though to a limited degree, against the intrastromal challenge of whole bacteria.²² Efforts to develop an antibody capable of neutralizing PIV, however, have been unsuccessful, probably because of the low immunogenicity of this protease.²³

In addition to the well characterized proteases, *P. aeruginosa* produces two other proteases: modified elastase and *P. aeruginosa* small protease (PASP).²⁴ Modified elastase has been identified, but its biochemical properties or virulence potential have not been described. For PASP, one study of its molecular properties and possible importance to corneal virulence has been reported. PASP, as secreted into the culture medium, was found to have a molecular mass of 18.5 kDa. The *PASP* gene of *P. aeruginosa* strain PA103 is greater than 99% identical with a gene designated as *PA0423* of strain PAO1, a finding that could suggest conservation of the gene among *P. aeruginosa* strains. DNA sequences of no known function, yet homologous (80%–86%) to PASP, have been detected in *Escherichia coli*, *Pseudomonas putida*, and *Pseudomonas syringae*. Preparations of PASP were found to produce corneal epithelial erosions after injection into rabbit corneas.²⁴

The purpose of the present study was to determine the distribution of the *PASP* gene among *P. aeruginosa* strains, the production of this protease among clinical isolates, and the immunogenicity of PASP. Also included is a more detailed analysis of the effects of PASP on the rabbit cornea and its interaction with collagens. The results show that PASP is produced by all tested strains of *P. aeruginosa* and can cleave collagens and cause corneal erosions. The enzyme is shown to be in an inactive form in the cytoplasm and periplasm, but active after secretion.

Methods

Bacteria and Growth Conditions

The sources of *P. aeruginosa* strains used herein were described previously.^{18,24} Cultures were grown in M9 minimal medium containing 60 mM monosodium glutamate, 1 mM MgSO₄ and 1% glycerol at 37°C for 20 hours.¹³ The bacteria were removed by centrifugation at 5000g for 15 minutes. The supernatants were filtered through a 0.22-μm filter and concentrated 50-fold with an ultrafiltration cell with a 10-kDa molecular mass cutoff filter (Amicon; Millipore, Billerica, MA). Total protein concentrations of supernatants were determined by bicinchoninic acid (BCA) assay (Sigma-Aldrich, St. Louis, MO).

PCR Primers and Conditions

Genomic DNA from all *Pseudomonas* strains tested was purified with a genomic DNA isolation kit (Qiagen, Valencia, CA). Two sets of primers were designed based on the *PASP* sequence from *Pseudomonas* strain PA103. One set amplified the full-length *PASP* gene (573

bp, forward primer: 5'-ATGCTGAAGAAGACCCTTGCCGCG-3'; reverse primer: 5'-TTACTGGCGAAGCCTTCGACGGA-3') and the other amplified a portion of the gene (173 bp, forward primer: 5'-TCACCATCAAG-GCCAAGCTGATCGGCC-3'; same reverse primer). The PCR conditions were as follows: 100°C for 5 minutes; add *Taq* polymerase; 94°C for 1 minute; then 30 cycles of 94°C for 20 seconds, 55°C for 20 seconds, and 68°C for 1 minute. Products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

Recombinant PASP Production

The construct expressing rPASP was described by Marquart et al.²⁴ Briefly, the *PASP* gene from *Pseudomonas* strain PA103 was cloned into plasmid pHAT10 (Clontech, Mountain View, CA) with a histidine affinity tag (HAT) at the N terminus of the protein. The resulting pHAT10-*pasp* plasmid was expressed in *Escherichia coli*, and rPASP was purified from inclusion bodies on an affinity column (Talon; Clontech) under denaturing (8 M urea) conditions. The refolding of rPASP was achieved by gradual removal of urea from the suspension. The amount of refolded rPASP in the urea-free buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 7.0) was quantified by BCA assay and the activity was verified by zymography using gelatin as substrate as described by Caballero et al.²⁵

PASP Polyclonal Antibody Production

Heat-denatured rPASP from *E. coli* (100 µg) was injected SC into New Zealand White rabbits (Myrtle's Rabbitry, Inc., Thompson Station, TN) in a 1:1 (vol/vol) mixture with Freund's complete adjuvant. The rabbits ($n = 3$) were boosted twice by injecting 50 µg rPASP mixed with Freund's incomplete adjuvant. Mock-immunized rabbits ($n = 3$) received adjuvant only. The anti-sera were collected 7 to 10 days after the injections. Antibody titers were determined by ELISA, with rPASP as the plate-bound antigen.

Enzyme-Linked Immunosorbent Assay

ELISA was performed as previously described.²³ The antibody titer represented the midpoint of the titration curve and was expressed as the mean titer $\pm$ SEM.

Western Blot Analysis

Protein samples of culture supernatants (30 µL, 2 mg/mL) were electrophoresed on 12% SDS-PAGE gels, as described by Marquart et al.²⁴

Localization Study

Osmotic shock and cell lysis were performed according to the method of Engel et al.,¹³ with some modifications. PA103-29 was grown in 500 mL dialyzed tryptic soy broth at 37°C for 24 hours. Cell lysis was achieved by sonication. The culture supernatant was concentrated 50-fold. The total protein concentration was determined, and samples with a similar amount of total protein were assayed by both Western blot analysis and zymography.

Protease Activity Assays

Purified *P. aeruginosa* LasB and AP were purchased from Elastin Products Company, Inc. (Owensville, MO) and United States Biochemical (Cleveland, OH), respectively. LasA was obtained from Dennis Ohman (Medical College of Virginia Campus of Virginia Commonwealth University, Richmond, VA). LasA, LasB, AP, and rPASP activities were verified by zymography. Recombinant (r)PIV was purified as previously described.¹⁸ PIV activity was verified by the hydrolysis of the chromogenic substrate *N*-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate (Sigma-Aldrich).¹³ Fluorescein-conjugated substrates tested (Molecular Probes, Eugene, OR) included gelatin from pig skin, type I collagen from bovine skin, and type

IV collagen from human placenta. Each substrate was suspended in a buffer provided by the manufacturer and incubated with rPASP (6 μ g), rPIV, LasB, AP, or LasA (0.1 μ g each), at 37°C in a 96-well plate after the procedures recommended by the manufacturer. Fluorescence was measured at various time points in a fluorescence microtiter plate reader with excitation at ~495 nm and emission at ~515 nm. Background values derived from the enzyme-free control specimens were subtracted from the fluorescence measurements.

Inhibitor Study

Fluorescein-conjugated gelatin, as mentioned, was incubated with rPASP (10 μ g) alone or plus an inhibitor. Fluorescence was measured after 24 hours at 37°C. Controls included gelatin alone and inhibitor plus gelatin alone.

IgG Inhibition of PASP Activity

IgG antibodies to rPASP were purified from rabbit anti-sera with protein A agarose beads (Sigma-Aldrich). The purity of IgG was verified by SDS-PAGE analysis. Anti-PASP IgG (1.3 mg/mL) or IgG purified from sera of mock-immunized rabbits (1.2 mg/mL) was incubated with active rPASP (10 μ g/20 μ L) in a 1:1 (vol/vol) mixture at room temperature overnight. BODIPY FL casein (Molecular Probes) was then added to the mixture, and fluorescence was measured at the various time points.

Intrastromal Injection of PASP in Rabbits

Specific pathogen-free New Zealand White rabbits used in these studies were maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbits were anesthetized by SC injection of a mixture of xylazine (100 mg/mL; Butler Co., Columbus, OH) and ketamine hydrochloride (100 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA). Proparacaine hydrochloride (Bausch and Lomb, Tampa, FL) was topically applied to the eyes before intrastromal injections. Active rPASP (10 μ g/20 μ L) was injected to analyze pathologic changes, or an equal amount of heat-inactivated rPASP, prepared by boiling for 2 hours at 100°C, was injected as the negative control. Active rPASP (5 μ g/10 μ L) was also used to challenge actively immunized rabbits. Slit lamp examinations (SLEs) of rabbit corneas were performed by two masked observers at various postinjection (PI) times, with a scoring system ranging from 0 for normal eyes to 28 for a maximally inflamed eyes, as previously described.^{26,27} Corneal erosions were stained with fluorescein (Fluorets; Chauvin Pharmaceuticals, Aubenas, France) and examined under blue light. Photographs were taken with a slit lamp microscope (Topcon SL-7E; Koaku Kikai, Tokyo, Japan).

Histopathology

Rabbit corneas were intrastromally injected with 10 μ g in 20 μ L active rPASP or heat-inactivated rPASP (four eyes per group). Rabbits were killed via an intravenous overdose of pentobarbital (Sigma-Aldrich). Whole eyeballs were harvested at 9 ($n = 2$) and 24 ($n = 2$) hours PI and fixed in 10% neutral buffered formalin (EK Industries, Joliet, IL). After fixation, specimens were processed as previously described.^{28,29} Erosions in corneas injected with active rPASP were observed grossly and marked with dye. Microsections were made around the dyed area and stained with hematoxylin and eosin. The stained specimen slides were examined microscopically and photographed under both low and high magnifications.

Statistical Analysis

Mean and SEM were calculated with commercial statistical analysis software (SAS, Cary, NC). For comparison of SLE scores, nonparametric one-way analysis of variance (Kruskal-Wallis test) was performed. $P \leq 0.05$ was considered significant.

Results

Distribution of the *PASP* Gene among *P. aeruginosa* Strains

The gene sequence of *PASP* from *P. aeruginosa* strain PA103 has 99.5% identity to the sequence from strain PAO1 (*P. aeruginosa* genome project reference strain).²⁴ To extend the study of the *PASP* gene distribution, we analyzed various *P. aeruginosa* strains ($n = 25$) for the presence of the *PASP* gene by PCR. Two sets of primers were used: one set to amplify the full-length 573-bp gene and the other to amplify a 173-bp *PASP* gene fragment. The genomes of all the *P. aeruginosa* strains tested were found to be positive for the entire *PASP* gene (Fig. 1A, lanes 3–14, 16–28) and the *PASP* gene fragment (Fig. 1B, lanes 3–14, 16–28).

Secretion of *PASP* by *Pseudomonas* Strains

The presence of *PASP* in culture supernatants was tested by Western blot analysis. The polyclonal antibody to *PASP*, produced by immunizing the rabbits three times with heat-denatured r*PASP*, had an ELISA titer of $\geq 10,000$ (Fig. 2A). In contrast, the titer of the sera from mock-immunized rabbits was ≤ 120 . Sera from mock-immunized rabbits, unlike sera from immunized rabbits, failed to produce positive reactions in an immunoblot assay using r*PASP* as the antigen (data not shown).

Anti-*PASP* antibody recognized r*PASP* as a 27-kDa band and also detected some smaller bands (Fig. 2B, lane 2). The purified r*PASP* has a molecular mass larger than native *PASP* (18.5 kDa), because of the presence of the HAT tag and retention of the signal peptide. And the smaller band (23-kDa) in purified preparations of r*PASP* was previously shown to have the N-terminal sequence expected for *PASP*.²⁴ In all the *P. aeruginosa* strains tested, anti-*PASP* antibody recognized a protein band with the size of ~ 19 kDa, a size consistent with the molecular mass of native *PASP* (18.5 kDa; Fig. 2B, lanes 3–10, 12–20, 22–29). In some strains, the antibody reacted with proteins of lower molecular mass that were probably the breakdown products of *PASP*.

Localization of Active *PASP*

To determine whether active *PASP* is present inside the bacterial cell or periplasmic space, we tested *P. aeruginosa* strain PA103-29 for the *PASP* protein and proteolytic activity by Western blot analysis and zymography, respectively. The *PASP* protein was found in the periplasmic fraction as well as the cell lysate and culture supernatant (Fig. 3A). On zymogram, there was no protease activity present in the periplasmic fraction or cell lysate. Only the supernatant showed two species of protease activity, one at ~ 200 kDa and the other at ~ 80 kDa (Fig. 3B). The 200-kDa band corresponds to that reported for PIV and the 80-kDa band corresponds to *PASP*.²⁴

Pathologic Effects of *PASP* in Rabbit Eyes

Rabbit eyes (six per group) that were injected with 10 μ g of active r*PASP* demonstrated profound pathologic changes, whereas eyes injected with an equal amount of heat-inactivated r*PASP* lacked such extensive changes (Fig. 4A). At 5 hours PI, eyes injected with active r*PASP* showed corneal erosions, mild chemosis, and moderate iritis. Control eyes with heat-inactivated r*PASP* appeared normal.

At 9 hours PI, eyes injected with active r*PASP* showed even more severe pathologic changes, including the enlargement of erosions and development of mild corneal infiltrate. Control eyes at 9 hours PI demonstrated mild chemosis and injection.

At 24 hours after injection of active rPASP, the corneal infiltrate was more extensive, but corneal epithelial erosions were healing or had healed. In the control eyes injected with inactive rPASP, pathologic changes were limited and consisted of mild chemosis, iritis, and injection.

SLE scores at any time point measured after active rPASP injection were significantly higher than that of control eyes injected with heat-inactivated rPASP (Fig. 4B, $P \leq 0.004$). SLE scores increased consistently in eyes injected with active rPASP during the 24-hour experimental course. The rabbits were killed at 24 hours PI and the presence of contaminating bacteria was assayed. All corneas were free of bacterial contamination.

Histopathological analysis was performed on eyes injected with active or heat-inactivated rPASP (four eyes per group). At 9 hours PI, under low power, erosions were identified, and the continuum of the epithelial layer was found to be disrupted (Figs. 5A, 5B). Damage extending down into the corneal stroma was also observed (Fig. 5B). However, limited PMN infiltration was observed at this stage as examined under high power.

At 24 hours PI, histopathologic examination showed that the eyes injected with active rPASP had extensive PMN infiltration into the cornea (Fig. 5C). Active rPASP also induced PMN infiltration into the anterior chamber and limbus area (data not shown). The control eyes injected with heat-inactivated rPASP had very minimal PMN infiltration into the cornea (Fig. 5D), anterior chamber or limbus (data not shown), and the corneal structure appeared normal (Fig. 5D).

Collagen Susceptibility to PASP Digestion

Collagens are important structural proteins in the cornea. Type I collagen is the major type of collagen in human corneal stroma, whereas type IV collagen is found in the basement membrane of the corneal epithelium.³⁰ To test the susceptibility of type I and IV collagens to PASP digestion, fluorescence-based assays were performed. Fluorescein-conjugated gelatin and collagens were incubated with major known proteases of *P. aeruginosa*, including PASP, LasB, AP, LasA, and PIV (Fig. 6). All the proteases were able to digest gelatin, a hydrolyzed form of collagen, indicating their levels of enzymatic activity. PASP was shown to digest both type I and IV collagens. Among the other proteases, LasA, LasB, and AP demonstrated collagenase activity with at least one of the two collagens. PIV was found least efficient in digesting collagens.

Testing of PASP Neutralization by Antibody

The antibody produced by immunization with rPASP was tested for inhibition of rPASP activity. For the neutralization assay (Fig. 7A), 10 μ g of active rPASP was incubated with approximately 25 μ g of IgG purified from immune or mock anti-sera at room temperature overnight. The fluorescent casein substrate was added to the mixture, and then fluorescence was measured at various time points for 5 hours. The enzymatic activity of rPASP on casein was not inhibited by the anti-PASP IgG antibody. The IgG fraction of sera from mock-immunized rabbits also lacked an inhibitory effect on the enzymatic activity of rPASP.

The effect of PASP antibody on the activity of rPASP was also tested in vivo. The corneas of both mock- and rPASP-immunized rabbits (three rabbits per group) were intrastromally injected with 5 μ g of active rPASP and corneal pathology was examined. At 7 hours PI, both rPASP- and mock-immunized rabbits showed equivalent ocular pathology, including epithelial erosion, iritis, chemosis, and injection (data not shown). The corneal infiltrate of both groups of rabbits was profound at 24 hours PI (data not shown). There was no significant difference in SLE scores of the two groups of rabbits at either 7 ($P = 0.054$) or 24 ($P = 0.600$) hours PI.

(Fig. 7B). All injected corneas were healed with minimal scarring at 5 to 7 days PI (data not shown).

Chemical Inhibition of PASP

The metalloprotease inhibitor EDTA and the serine protease inhibitor TLCK were tested for their ability to block the hydrolysis of fluorescein-conjugated gelatin by rPASP. Only TLCK demonstrated dose-dependent inhibition of rPASP activity (Fig. 8).

Discussion

The present study shows that PASP can cleave collagens and cause corneal erosions and is secreted by all *P. aeruginosa* strains tested, including ocular and nonocular clinical isolates and laboratory strains.

The *PASP* gene has been identified only in *P. aeruginosa* strain PA103; however, the present study demonstrates that the *PASP* gene is commonly found in *P. aeruginosa* isolates. The presence of the *PASP* gene was confirmed by two PCR reactions targeting different portions of the gene. In addition to the distribution of the *PASP* gene among *P. aeruginosa* isolates, the secretion of PASP protein into *P. aeruginosa* culture supernatants was demonstrated by Western blot analysis of all the strains tested. The findings indicate that PASP, like PIV,¹⁸ is a product of a very high percentage of *P. aeruginosa* isolates, possibly all of them. The finding of PASP and PIV as products of possibly all strains is in contrast to LasB and AP, which are produced by a subset of *P. aeruginosa* strains.^{18,31,32} The strains tested in the present study varied in their geographic origins, having been isolated from multiple points of the United States. They also varied in terms of the anatomic site of the infection from which they were isolated. Of the 25 *P. aeruginosa* strains studied, there were eight nonocular clinical isolates, suggesting that PASP could have a role in *Pseudomonas* infections other than keratitis. Strain PAO1 and PA103 included in this study are known to be the prototype of the invasive and cytotoxic *P. aeruginosa* groups, respectively,³³ and both strains were found to produce PASP.

The active form of PASP was found only in the culture supernatant, not in the cytoplasm or the periplasmic space, similar to PIV.¹³ The PASP sequence of PA103 appears to have a signal peptide consistent with that needed for type II secretion.²⁴ The size of the PASP protein, as measured by SDS-PAGE, remains the same in the periplasm or the supernatant. The model of a larger proenzyme being processed into a smaller active enzyme does not apply to PASP. However, there could be some accessory molecules necessary for the maturation of PASP, especially as it exits the periplasmic space.

Purified active rPASP causes corneal erosions similar to native PASP.²⁴ The erosion is dependent on the enzymatic activity of rPASP because the heat-inactivated form did not cause an erosion. The destruction of corneal epithelium has been documented by fluorescein staining and histologic analysis. Histologic analysis has also demonstrated that rPASP can produce a corneal erosion that extends into a significant portion of the stroma. The epithelial erosion and ulcer in the stroma are consistent with the fact that rPASP cleaves type I and IV collagens. LasB was previously reported to cleave both type I and IV collagens, whereas AP cleaves type I collagen.³⁴ Our data confirmed these results in a different assay system. Also determined was that LasA can efficiently cleave both types of collagens whereas PIV cannot. The injection of rPASP into the cornea induced an inflammatory response that included the migration of PMNs into the corneal stroma, limbus, and anterior chamber. The mechanism by which PASP activates an inflammatory response is unknown; however, this could involve an interaction of PASP with a protease-activated receptor (PAR), as described for another *P. aeruginosa* protease.³⁵ In principle, the pathologic changes in the eye could be the direct effects of PASP or a combination of PASP with activated host matrix MMPs and neutrophil proteases.

The present immunization studies demonstrate that rPASP, unlike PIV, is very immunogenic and anti-rPASP antibody reacts with both native and recombinant PASP. However, this antibody does not interfere with the catalytic site on PASP to neutralize PASP activity. The antibody to PASP afforded no protection in the rabbit eye after challenge with active rPASP. PASP has not yet been well characterized as an enzyme; its cleavage sites on target proteins and its catalytic site have yet to be determined. Amino acid sequence analysis and a structural computer model of PASP offer little information about the active site of PASP (data not shown). The preliminary inhibitor study presented herein indicates that PASP may be a serine protease because TLCK completely inhibits PASP activity. However, sequence homology search relates PASP to YceI-like proteins in the Gram-negative bacteria that are involved in the metabolism of polyisoprenoids. PASP does not have sequence homology to any of the serine proteases. There is a possibility that, in addition to the proteolytic activity, PASP is also involved in the metabolism of polyisoprenoids.

In the present study, the correlation between PASP and corneal virulence has been further strengthened. Among the major *Pseudomonas* proteases, LasB has been associated with corneal virulence during *Pseudomonas* keratitis, although multiple clinical isolates, including ocular isolates, are deficient in LasB.^{18,36} Also, Kernacki et al.³⁷ showed that LasB was not detected in corneas infected with *P. aeruginosa*. Based on deletion mutant studies, both LasA and AP are not required for corneal virulence.^{19,20,38–40} PIV, like PASP, is commonly produced by *P. aeruginosa* strains and is associated with corneal virulence.^{21,26} The production of PIV and PASP by numerous *P. aeruginosa* strains suggests that the two proteases could have important roles in keratitis. PIV can cleave a wide spectrum of host proteins (e.g., IgG, complement, surfactants)^{13,41} and thus help protect the bacteria from host defenses. However, PIV is not efficient in cleaving collagens and does not cause erosions when expressed in the nonpathogenic *P. putida* during experimental keratitis.⁴² Preliminary studies of PASP indicate this enzyme is not very efficient in cleaving a variety of host proteins (e.g., IgG) (Tang A et al. *IOVS* 2007; 48:ARVO E-Abstract 725), but as shown herein, can readily cleave collagens and cause corneal erosions. It is possible that, for a typical strain of *P. aeruginosa*, PIV helps protect the bacterium from host defenses and PASP could directly contribute to the destruction of ocular tissue during *Pseudomonas* keratitis.

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Figure 1.

PCR analysis of the *PASP* gene among multiple *P. aeruginosa* isolates. Two sets of primers were used: one to amplify the full-length *PASP* gene (573 bp, **A**) and the other to amplify a portion of the gene (173 bp, **B**). *Pseudomonas* genomic DNA (0.5–1.0 μ g) was used as the template in PCR reactions. *Lanes 1 and 15*: DNA standard (100-bp ladder); *lane 2*: negative control; *lanes 3 to 14*: PAO1, PA103, PA103-29, ATCC 19660, PA23, PA24, PA70, PA178, PA13003, PA13004, PA13010, and PA13014, respectively; *lanes 16 to 28*: PA13036, PA30009, PA30069, PA30099, PA30132, PA30244, PA51003, PA51005, PA51036, PA51037, PA51131, PAALC2, and PAALC3, respectively.

Figure 2.

Production and reactivity of antibody to PASP. **(A)** Antibody titers in immunized rabbits measured by ELISA. Anti-sera were collected after three injections of heat-denatured rPASP. Antibody titers were determined as the midpoint of the titration curve and expressed as the average $\pm$ SEM. **(B)** Western blot analysis of *Pseudomonas* culture supernatants for the presence of PASP protein. Concentrated *P. aeruginosa* culture supernatants (30 μ L, 2 mg/mL) were electrophoresed and then probed with rabbit polyclonal anti-PASP antibody (1:1000). Lanes 1, 11 and 21: prestained molecular mass standard; lane 2: rPASP; lanes 3 to 10: PAO1, PA103, PA103-29, ATCC 19660, PA23, PA24, PA70, and PA178, respectively; lanes 12 to 20: PA13004, PA13010, PA13014, PA30009, PA30069, PA30099, PA30132, PA30244, and

PA51003, respectively; *lanes 22 to 29*: PA51005, PA51036, PA51037, PA51131, PA13036, PAALC 2, PAALC 3, and PA13003, respectively; *lane 30*: negative control (*E. coli* M15 supernatant).

Figure 3.

Localization of active PASP. **(A)** Western blot analysis of the cell lysate, periplasm and culture supernatant of PA103-29 for the presence of PASP. Samples, each containing approximately 60 μ g of total protein, were electrophoresed on a 15% SDS-polyacrylamide gel. *Lane 1*: molecular mass standard; *lane 2*: rPASP; *lane 3*: cell lysate; *lane 4*: periplasm; and *lane 5*: supernatant. **(B)** Gelatin zymography for the presence of active PASP in PA103-29. An equal amount of samples (60 μ g each) was electrophoresed on a 10% SDS-polyacrylamide gel containing 0.1% gelatin. *Lane 1*: cell lysate; *lane 2*: periplasm; and *lane 3*: supernatant. Only the supernatant sample (*lane 3*) showed PASP enzyme activity.

Figure 4. Effects of PASP in rabbit eyes. **(A)** Appearance of eyes. Ten micrograms of active (I) or heat-inactivated (II) rPASP was intrastromally injected into rabbit corneas (six eyes per group). Pathologic changes were monitored at 5, 9, and 24 hours PI. Corneal erosions were stained with fluorescein and photographed under blue light (I). Shown are two representative eyes from the two groups. **(B)** SLE of PASP-injected rabbit eyes. The eyes were scored by SLE at 5, 9, and 24 hours PI. The mean score $\pm$ SEM per group was calculated. SLE scores of active rPASP injected eyes were significantly higher than the ones injected with heat-inactivated enzyme at any time point ($P \leq 0.004$).

Figure 5.

Histopathologic analysis of PASP effects in rabbit eyes. Ten micrograms of active (A–C) or heat-inactivated (D) rPASP was intrastromally injected into rabbit corneas. Eyes were harvested at 9 (A, B) and 24 (C, D) hours after injection. At 9 hours PI, eyes with active rPASP showed corneal erosions (A, B) and an erosion extending into the stroma (B). Intense corneal infiltrate in the eye with active rPASP was present at 24 hours PI (C). Eyes with heat-inactivated rPASP showed normal corneal structure (D). Magnifications: (A–C) $\times 40$ and $\times 200$; (D) $\times 40$ and $\times 400$.

Figure 6.

Fluorescence assays of gelatin and collagen digestion by *P. aeruginosa* proteases. Gelatin, type I or type IV collagen was incubated with either 6 μg of rPASP or 0.1 μg of LasB, AP, LasA, and rPIV individually. Fluorescence was measured at 1, 3, 5, and 24 hours after incubation. Background fluorescence, determined by no-enzyme reactions, was subtracted from each value. Data are expressed as the mean $\pm$ SEM. Experiments were performed in triplicate and repeated once.

Figure 7.

In vitro and in vivo reactions of PASP with anti-PASP antibody. **(A)** In vitro casein fluorescence assay. Active rPASP (10 μ g in 20 μ L) was incubated with anti-PASP IgG (20 μ L; 1.3 mg/mL) antibody overnight. Fluorescent casein was then added to test the enzymatic activity. Fluorescence was measured at various time points. IgG from mock anti-sera (20 μ L; 1.2 mg/mL) served as the control. Data are expressed as the mean $\pm$ SEM. Experiments were performed in triplicate and repeated once. **(B)** SLE scores of rPASP-challenged rabbit eyes. Active rPASP (5 μ g in 10 μ L) was used to challenge the rabbits by intrastromal injection. Mock-immunized rabbits ($n = 3$) did not produce significantly higher SLE scores in the eye than did rPASP-immunized rabbits ($n = 3$) at either 7 or 24 hours PI ($P \geq 0.05$). Data are expressed as the mean SLE score $\pm$ SEM.

Figure 8. Inhibition of PASP activity by TLCK. Fluorescein-conjugated gelatin was incubated with rPASP (10 μ g) alone or with rPASP plus various concentrations of TLCK. Fluorescence was measured after 24 hours. Assays were performed in triplicate. Data are expressed as mean fluorescence value $\pm$ SEM.