Identification of a Novel Secreted Protease from *Pseudomonas aeruginosa* that Causes Corneal Erosions

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Purpose. The purpose of this study was to identify a new *Pseudomonas* protease and determine its possible role in keratitis.

Methods. Concentrated culture supernatants of the *Pseudomonas aeruginosa* strains PA103 and ATCC 19660 were analyzed by zymography. *P. aeruginosa* small protease (PASP) was purified from strain PA103, and modified elastase B (LasB) was purified from strain ATCC 19660. SDS-PAGE and Western blot analysis were performed on purified PASP and modified LasB. PASP was further analyzed by mass spectrometry and amino-terminal sequencing. The *Pasp* gene was cloned and expressed, affinity-purified in denatured form from inclusion bodies, and refolded by removal of the denaturant. Purified recombinant PASP was analyzed by zymography for protease activity. PASP and heat-inactivated PASP were injected into rabbit corneas, and the corneas were monitored for erosions caused by protease activity.

Results. Each strain produced a protease with a molecular mass of 80 kDa on zymograms. LasB antiserum identified the ATCC 19660 protease as modified LasB. Mass spectrometry defined the PA103 protease as having a molecular mass of 18.5 kDa. Amino-terminal sequencing and analysis of the *P. aeruginosa* genome sequence determined that the PA103 *Pasp* gene sequence was >99% identical with the PA0423 sequence of strain PAO1. Recombinant PASP was proteolytic, with a zymogram mass of 50 kDa. PASP purified from PA103 produced extensive corneal epithelial erosions, whereas heat-inactivated PASP produced no erosions.

Conclusions. PASP is a protease that has not been previously identified. It causes corneal epithelial erosions, indicating its likely activity as a virulence-promoting factor in *Pseudomonas* keratitis. (*Invest Ophthalmol Vis Sci.* 2005;46:3761–3768) DOI:10.1167/iovs.04-1483

The pathogen *Pseudomonas aeruginosa* is responsible for a variety of diseases, including lung infections associated with cystic fibrosis,[1] burn wound infections,[2] hospital-acquired pneumonia,[3] and keratitis.[4] Major virulence factors produced by this opportunistic pathogen include secreted proteases that damage host tissues.[5] Several *P. aeruginosa* proteases have been isolated and shown to be involved in pathogenesis.[5]

One of the best characterized *Pseudomonas* proteases is elastase B (LasB). This 33-kDa metalloprotease degrades elastin and is inhibited by chelators such as EDTA.[6–9] LasB can cleave and activate the proforms of host matrix metalloproteases[10–12] and can inactivate host inflammatory cytokines such as tumor necrosis factor-α and interferon-γ.[13] This protease is also key in facilitating epithelial cell invasion by inhibiting negative regulators of type III secretion.[14] LasB is associated with corneal damage during *Pseudomonas* keratitis[10,15–19] and has been shown to be produced in clinical ocular isolates.[20] However, a LasB-negative *P. aeruginosa* mutant has been shown to be virulent in the eye,[21] and Caballero et al.[22] demonstrated multiple clinical strains of *P. aeruginosa* to be deficient in LasB.

Protease IV is a virulence factor that has been associated with keratitis.[23–25] This protease was named by Toder and Gambello,[26] and later was characterized by Engel et al.[27] Protease IV, a 26-kDa lysine-specific protease inhibited by the serine protease inhibitor tosyl-L-lysine chloromethylketone (TLCK), degrades fibrinogen, plasminogen, IgG, the complement proteins C1q and C3,[27] and surfactants.[28] Protease IV has been shown to be a virulence factor in *P. aeruginosa* corneal infections.[22,29] Its production is augmented with increased calcium or magnesium in the growth medium.[30]

Alkaline protease is a metalloprotease of *P. aeruginosa*. The role of this protease in keratitis has not been fully elucidated. Increased amounts of alkaline protease have been detected in clinical ocular isolates compared with clinical isolates from other types of infections,[20] and mice resistant to *Pseudomonas* keratitis had lower amounts of alkaline protease isolated from their corneas.[31] In contrast, alkaline protease–deficient mutants have been demonstrated to retain wild-type virulence in the eye,[21,32] and multiple clinical strains do not produce alkaline protease.[22] Alkaline protease has, however, been shown to degrade cytokines in vitro,[33] indicating a possible role for this protease in inhibiting the host inflammatory response to infection. This 49.5-kDa metalloprotease is iron regulated[34] and cleaves polylysine.[35]

Another characterized protease secreted by *P. aeruginosa* is elastase A (LasA), an enzyme possessing both elastolytic[36–38] and staphylolytic activities. LasA is able to cleave the pentaglycine bonds in the peptidoglycan of *Staphylococcus aureus*.[39–42] This protease has been projected to be an ocular virulence factor[43]; however, more recent research, based on the high virulence of LasA deletion mutants, has shown it not to be necessary for corneal virulence.[21,44,45] LasA is responsible for shedding of the host cell surface proteoglycan syndecan-1.[46]

The most recent *P. aeruginosa* protease to be described is a 56-kDa secreted leucine aminopeptidase.[47] The activity of this heat-stable protease is dependent on the presence of zinc and is inhibited by dithiothreitol and 1,10-phenanthroline, but not by serine protease inhibitors. The host proteins subject to digestion by this protease have not yet been identified, and the possible function of this enzyme in pathogenesis is not known.

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The purpose of this study was to investigate two *P. aeruginosa* proteases that have not been previously analyzed. One interest of this study was modified LasB, an enzyme that is recognized by antibodies to classic LasB but has a different migration on zymograms than that of classic LasB (Twining SS, et al. *IOVS* 1998;39:ARVO Abstract 3058). Purification of modified LasB from *P. aeruginosa* led to the discovery and purification of a new protease that has never been reported. The identification, properties, and activity of this additional protease, PASP, are described in this report.

METHODS

Bacterial Strains and Growth Conditions

The *P. aeruginosa* strains used in this study, PA103 and ATCC 19660, were purchased from the American Type Culture Collection (Manassas, VA). *P. aeruginosa* colonies were isolated on tryptic soy agar (Difco TSA; BD Biosciences, Sparks, MD) and then grown at 37°C with aeration in deferrated tryptic soy broth (Difco TSB; BD Biosciences) that had been dialyzed to remove high-molecular-mass proteins and supplemented with 50 mM monosodium glutamate (Sigma-Aldrich, St. Louis, MO) and 1% glycerol. The *Escherichia coli* strain used for recombinant protease expression was DH5α PRO (BD-Clontech, Palo Alto, CA), which was resistant to 50 μg/mL spectinomycin and was isolated and grown at 37°C in Luria-Bertani medium (Difco LB; BD Biosciences) containing this antibiotic.

Zymography

Zymography was performed by a modification of the method of Twining et al.[10] A 30-μL aliquot of each 50-fold concentrated culture supernatant or purified native or recombinant protease, which was not boiled or treated with reducing agent, was electrophoresed at 4°C and 20 mA through a 10% SDS-polyacrylamide gel containing 0.1% gelatin. SDS was removed by washing twice for 20 minutes with 2.5% Triton X-100. Each gel was washed and incubated in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM $CaCl_2$, and 1 μM $ZnCl_2$ at 37°C for 48 hours. The gel was stained for 20 minutes in 0.5% Coomassie brilliant blue R-250 in glacial acetic acid-isopropanol-dH_2O (1:3:6). Washing with dH_2O revealed clear bands, where proteolysis of gelatin occurred, on a blue background. Molecular mass determinations were made by running protease standards with known zymogram migrations (alkaline protease, LasB, and protease IV) and also by running a lane of prestained molecular mass standards that were cut from the gel before staining.

Purification of Protease

P. aeruginosa strain PA103 or ATCC 19660 was grown in deferrated dialyzed TSB at 37°C with vigorous aeration for 24 hours. Bacteria were removed by centrifugation at 5000*g* for 10 minutes and subsequent filtration with a 0.22-μm filter. Extracellular proteins were concentrated 50-fold (from 5 L to 100 mL) in a stirred cell (Amicon; Millipore, Billerica, MA) containing a 3000-Da molecular mass cutoff filter. Concentrated proteins were dialyzed in 50 mM Tris-HCl (pH 8.0) and applied to a diethylaminoethyl (DEAE)-cellulose matrix (Bio-Rad, Hercules, CA) equilibrated in the same buffer. The matrix was washed in 50 mM Tris-HCl (pH 8.0) and then fractions were eluted with a pH gradient generated by slow addition of 100 mM Tris-HCl (pH 6.0) to the wash buffer at the top of the matrix. Further purification was achieved with gel filtration chromatography (Sephacryl S-300; GE Healthcare, Piscataway, NJ). Fractions containing the protease were identified by gelatin zymography.

Extraction and Analysis of Proteins from Zymogram Bands

The 80-kDa protease band in a gelatin zymogram was excised and analyzed by SDS-PAGE to confirm that this protease was the same as the purified 25-kDa protein from strain PA103. A modification of a manufacturer's method for purifying proteins from polyacrylamide gels was used (Pierce, Rockford, IL). Three lanes of the purified protease were subjected to zymography on the same gel at the same time. The first lane was stained and destained as described earlier, whereas the second and third lanes were removed and kept unstained. The 80-kDa protease band was located in the stained gel and used as a marker for the unstained lanes. The areas corresponding to 80 kDa in the unstained lanes were then excised from the gel with a razor blade and crushed with a pestle in 0.5 mL elution buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1 mM EDTA). This suspension was incubated for 2 hours at 37°C with mixing at 100 rpm. The sample was centrifuged at 13,000*g* for 15 minutes, to remove the acrylamide from the proteins, and the supernatant was subjected to SDS-PAGE and silver staining. As a control, a band of equivalent size was excised from a lane of the gelatin zymogram that was applied with SDS-PAGE sample buffer only, and this control band was treated in the same manner as the 80-kDa band.

LasB Polyclonal Antibody Production

Purified *P. aeruginosa* LasB was purchased from Elastin Products Company, Inc. (Owensville, MO). Specific pathogen-free (SPF) New Zealand White rabbits (Myrtle's Rabbitry, Inc., Thompson Station, TN) were each injected subcutaneously with 100 μg LasB mixed 1:1 (vol/vol) with Freund's complete adjuvant. Rabbits were given three monthly booster injections of immunogen in Freund's incomplete adjuvant. Antisera were collected 7 to 10 days after booster injections. Antibody titers were determined by ELISA.[48] Antibodies (IgG) were purified from serum on agarose coupled with protein A (Sigma-Aldrich).[49]

SDS-PAGE and Western Blot Analysis

SDS-PAGE using boiled and reduced protein samples was performed according to the method of Sambrook et al.[50] Polyacrylamide gels were either stained with Coomassie brilliant blue, or the proteins were transferred to PVDF membranes (Bio-Rad) using a semidry transfer cell (Bio-Rad). After transfer, nonspecific protein binding sites were blocked with 5% skim milk for 1 hour. Polyclonal antiserum to LasB (diluted 1:1000), generated as described earlier, or 5 μg/mL protein A-purified anti-LasB IgG was incubated with the membranes for a minimum of 2 hours. Membranes were washed for 1 hour in Tris/NP-40 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, and 0.05% Nonidet P-40). Secondary antibody (1:10,000 peroxidase-labeled anti-rabbit IgG) was allowed to bind to the primary antibody for 1 hour. Membranes were washed for 1 to 2 hours in Tris/sarkosyl buffer (50 mM Tris-HCl [pH 7.5], 1 M NaCl, 5 mM EDTA, and 0.4% N-laurylsarcosine). All steps were performed at room temperature on a rocking platform. Chemiluminescent detection of the secondary antibody was then performed (ECL kit; GE Healthcare).

Mass Spectrometry and Amino Acid Sequence

Mass spectrometry and amino-terminal sequencing of the protease were performed at the University of Texas Medical Branch Biomolecular Resource Facility (Galveston, TX). The purified PA103 protease was dialyzed in PBS and submitted in liquid form (50 μg in 0.1 mL) for mass spectrometry. The mass spectrometry method was matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF; voyager-DE STR Biospectrometry Workstation; Applied Biosystems Inc. [ABI], Foster City, CA). Protein sequencing was performed on a commercial system (494/HT, controlled by Procise software and a 610/A data analysis system; ABI). The purified recombinant protease (described in the next section) was electroblotted onto PVDF membrane, and the 23- and 27-kDa bands were cut from the membrane and submitted for sequencing.

Cloning and Sequencing of the PA103 Protease

The DNA sequence of the protease was obtained by a search of the *Pseudomonas* Genome Project database (www.pseudomonas.com,

GenBank Accession #AE004091) using the N-terminal amino acid sequence of the purified protease. PCR primers were constructed to amplify the gene with *Eco*RI and *Bam*HI restriction sites flanking 5′ and 3′ ends, respectively. The forward primer was 5′-CGGAATTCCATGCTGAAGAAGACCCTT-3′ and the reverse primer was 5′-GGATCCTTACTGGCGAATGCCTTC-3′. The gene was amplified using *P. aeruginosa* strain PA103 genomic DNA as the template, 50 picomoles of each primer, 2 mM $MgCl_2$, *Taq* polymerase (Promega, Madison, WI), 10× buffer, 10 mM dithiothreitol, 5% glycerol, 0.01% BSA, and 0.2 mM each of dATP, dCTP, dGTP, and dTTP. The PCR reaction was performed 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.

The 573-bp PCR product was purified from a 1% agarose gel with a gel-extraction kit (Qiagen, Valencia, CA). The purified product was ligated into vector pCR2.1 and transformed into *E. coli* TOP10F′ cells, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Transformants were analyzed by restriction digestion of purified plasmid DNA.

Plasmid pHAT10 (BD-Clontech) was chosen as the expression vector. Plasmids pCR2.1 harboring the cloned protease gene and pHAT10 were digested with *Bam*HI and *Eco*RI. The digested protease gene and pHAT10 were gel-purified using the gel-extraction kit. The protease gene was ligated into pHAT10, resulting in plasmid pHAT10-*Pasp*, and transformed into chemically competent *E. coli* DH5αPRO cells. Colonies that grew on 50 μg/mL ampicillin were chosen for plasmid DNA analysis. Sequencing of the *Pasp* gene in pHAT10 was performed by the LSUHSC Genomics Core Facility (Louisiana State University Health Sciences Center, New Orleans, LA). The sequencing primer was 5′-AGGCTTTACACTTTATGCTTCCGGCTCGTA-3′, located 189 nucleotides upstream of *Pasp* in pHAT10.

Expression and Purification of Recombinant PASP

E. coli containing pHAT10-*Pasp* was grown to the logarithmic phase in 1 L Luria-Bertani broth containing 50 μg/mL ampicillin and 50 μg/mL spectinomycin. This culture was induced with 1 mM IPTG (isopropyl-β-d-thiogalactopyranoside) and grown for an additional 5 hours. Cells were pelleted and resuspended in a binding buffer (Talon; BD-Clontech) containing 5.4 M guanidine-HCl according to the manufacturer's instructions. The cells were lysed by sonication and centrifuged at 15,000*g* for 30 minutes to separate cell lysate and debris. SDS-PAGE was used to determine the cellular location of the recombinant protease.

Recombinant PASP was purified from inclusion bodies according to the manufacturer's instructions (BD-Clontech). Briefly, the cell debris was resuspended in the binding buffer containing 5.4 M guanidine-HCl, allowed to bind to the affinity resin (Talon; BD-Clontech), washed with the binding buffer containing 5.4 M guanidine-HCl, and eluted with the elution buffer (Talon; BD-Clontech) containing 5.4 M guanidine-HCl and 100 mM imidazole. Eluted fractions containing the recombinant PASP were combined and dialyzed overnight at 4°C against the binding buffer containing 3 M guanidine-HCl, to remove the imidazole and decrease the concentration of guanidine-HCl. After dialysis, the sample was placed in a stirred-cell concentrator equipped with a 3-kDa molecular mass cutoff filter. The binding buffer, devoid of guanidine-HCl, was added to the sample in a 1:1 (vol/vol) ratio, and the sample was concentrated to half the starting volume. Addition of the binding buffer in a 1:1 ratio was repeated until the guanidine-HCl was essentially eliminated, and the sample was concentrated to a final volume of 2 mL. The purified, concentrated recombinant PASP was analyzed for purity, amino acid sequence, and proteolytic activity.

Injection of PASP into Rabbit Corneas

New Zealand White rabbits were used in these studies and maintained according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbits were anesthetized by subcutaneous 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 was topically applied to each eye before intrastromal injection. *P. aeruginosa* PASP (7 μg in 10 μL) was injected into the stroma of the right corneas of three rabbits. Heat-inactivated PASP (7 μg in 10 μL), prepared by boiling for 1 hour at 100°C, was injected in the stroma of the left corneas of the same three rabbits. The corneas were examined periodically for 48 hours by two observers, corneal erosions were stained with fluorescein and measured, and photographs were taken with the aid of a slit lamp microscope.

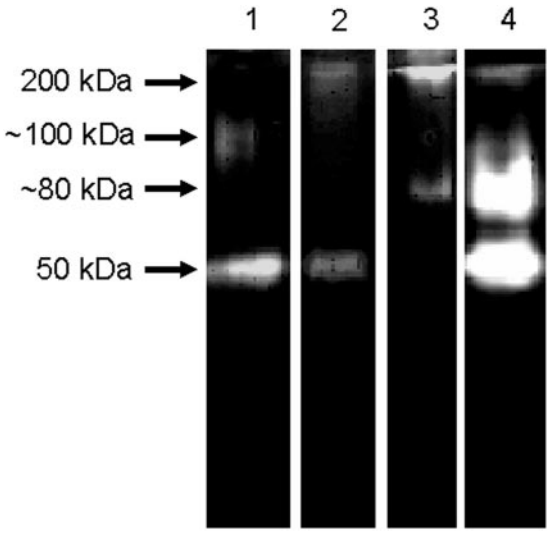

Figure 1. Gelatin zymography of *P. aeruginosa* proteases. *Lane 1*: 1 μg purified LasB (100 kDa) and 0.1 μg alkaline protease (50 kDa); *lane 2*: 5 μg purified protease IV (>200 kDa) and 0.1 μg alkaline protease (50 kDa); *lane 3*: concentrated culture supernatant from strain PA103 (15 μg total protein); and *lane 4*: concentrated culture supernatant from strain 19660 (15 μg total protein). LasA (100 kDa) is not shown.

Results

Proteases of Strains PA103 and ATCC 19660

Gelatin zymography was performed on concentrated culture supernatants from *P. aeruginosa* strains PA103 and ATCC 19660. Both strains produced protease IV, which migrated to >200 kDa (Fig. 1, lanes 3 and 4). The culture supernatant of strain ATCC 19660 contained alkaline protease (50 kDa, lane 4), whereas that of PA103 did not (lane 3). Each strain additionally produced a protease of approximately 80 kDa (lanes 3 and 4).

Purification of the Protease from Each Strain

The protease with a mass of 80 kDa on gelatin zymography was purified for each of the two *P. aeruginosa* strains by chromatography. Purity was determined by SDS-PAGE (Fig. 2, lanes 1 and 3) and the purified enzymes were analyzed by zymography (Fig. 2, lanes 2 and 4). On SDS-polyacrylamide gels, after boiling and reduction of the samples, the protease purified from strain PA103 appeared as a pair of bands at 25 kDa (lane 1). The protease bands at 25 kDa have not been reported. In contrast to the protease of PA103, the protease purified from strain ATCC 19660 appeared as a single band at 36 kDa (lane 3), a molecular mass equivalent to that reported earlier (Twining SS, et al. *IOVS* 1998;39:ARVO Abstract 3058) for modified LasB. The yield of the purified 25-kDa protease from PA103 was 53

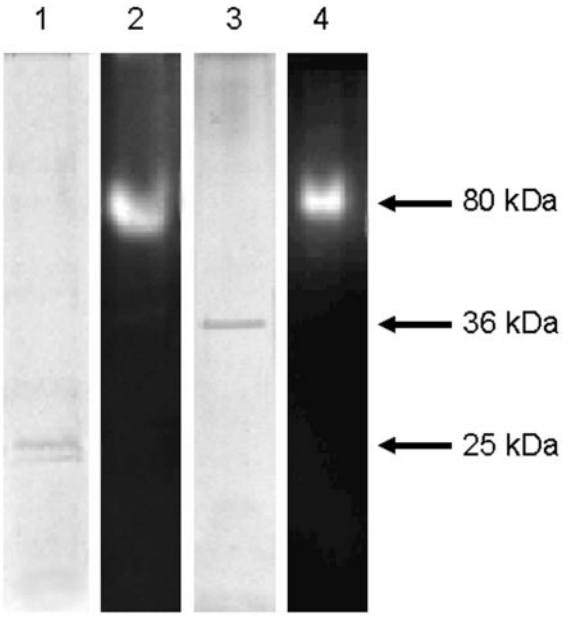

FIGURE 2. SDS-PAGE and gelatin zymography of purified proteases from strains PA103 and 19660. *Lane 1*: SDS-PAGE of the purified novel protease (1 μg, boiled) from strain PA103; *lane 2*: zymogram of purified protease from strain PA103 (1 μg); *lane 3*: SDS-PAGE of purified protease (1 μg, boiled) from strain 19660; *lane 4*: zymogram of purified protease from strain 19660 (1 μg).

μg/L, and the yield of the purified 36-kDa protease from ATCC 19660 was 133 μg/L.

Analysis of Zymogram Bands

To confirm that the purified 25-kDa protein from strain PA103 was indeed the same protease as the 80-kDa protease observed in gelatin zymograms, the 80-kDa protease band was removed from the gelatin zymogram. The band was subjected to an extraction method to remove the acrylamide from the proteins. As a control, a sample of the gelatin zymogram gel of equivalent size was removed from a lane that was applied with sample buffer only, and this negative control was treated in the same manner as the 80-kDa band. The negative control sample contained numerous protein bands corresponding to gelatin proteins (Fig. 3, lane 1), whereas the sample from the 80-kDa zymogram band contained the 25-kDa protein in addition to the gelatin proteins (Fig. 3, lane 2). The presence of the 25-kDa protein extracted directly from the 80-kDa protease band confirms that these two proteins are one in the same.

Western Blot Analysis of the Protease from Each Strain

The reactivity with polyclonal antibody to *P. aeruginosa* LasB of the purified 25- and 36-kDa proteases from strains PA103 and ATCC 19660, respectively, was tested by Western blot analysis (Fig. 4). The 25-kDa protease from strain PA103 was not recognized by LasB antibody (lane 2), whereas the 36-kDa protease from strain ATCC 19660 reacted with the antibody to LasB (lane 3).

Sequence Analysis of the Protease from Strain PA103

The purified 25-kDa protease from strain PA103 had an N-terminal amino acid sequence of ADYKIDKEGQ. The molecular mass, as determined by mass spectrometry, was 18,525.2 Da. The determined N-terminal sequence was a 100% match to

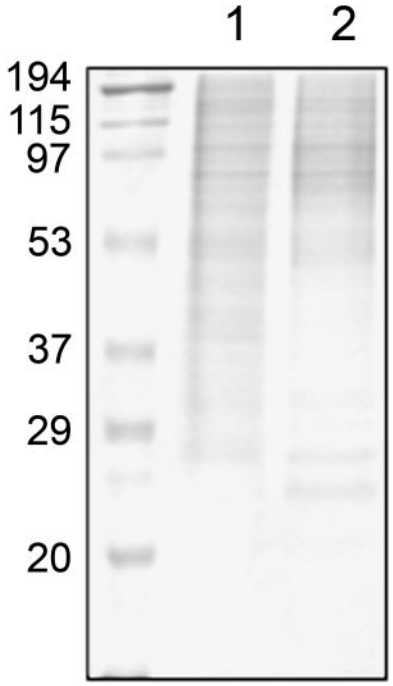

FIGURE 3. SDS-PAGE and silver staining of proteins extracted from bands in gelatin zymograms. Proteins were extracted by removal of bands from gelatin zymograms followed by treatment with EDTA and centrifugation. *Lane 1*: gelatin proteins from a lane that was loaded with SDS-PAGE sample buffer (control lane); *lane 2*: proteins extracted from the 80-kDa protease lane depicted in Figure 2, *lane 2*. *Leftmost lane*: molecular mass standard.

the sequence of a protein of unknown identity produced by *P. aeruginosa* strain PAO1 with a beginning nucleotide position at 470,662 on the PAO1 genome (gene PA0423, GenBank Accession Number AE004479). The PAO1 gene appears to have an open reading frame that codes for a protein of 18.5

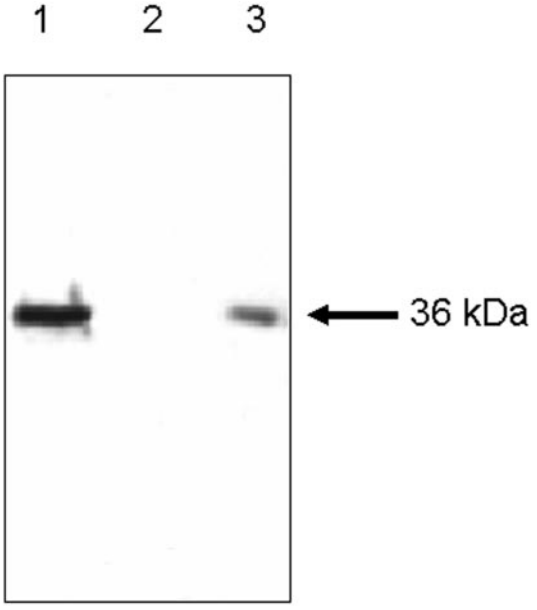

FIGURE 4. Western blot analysis of *P. aeruginosa* proteases using polyclonal antiserum to LasB. *Lane 1*: commercial LasB (1 μg); *lane 2*: purified protease from strain PA103 (1 μg); and *lane 3*: purified protease from strain 19660 (1 μg).

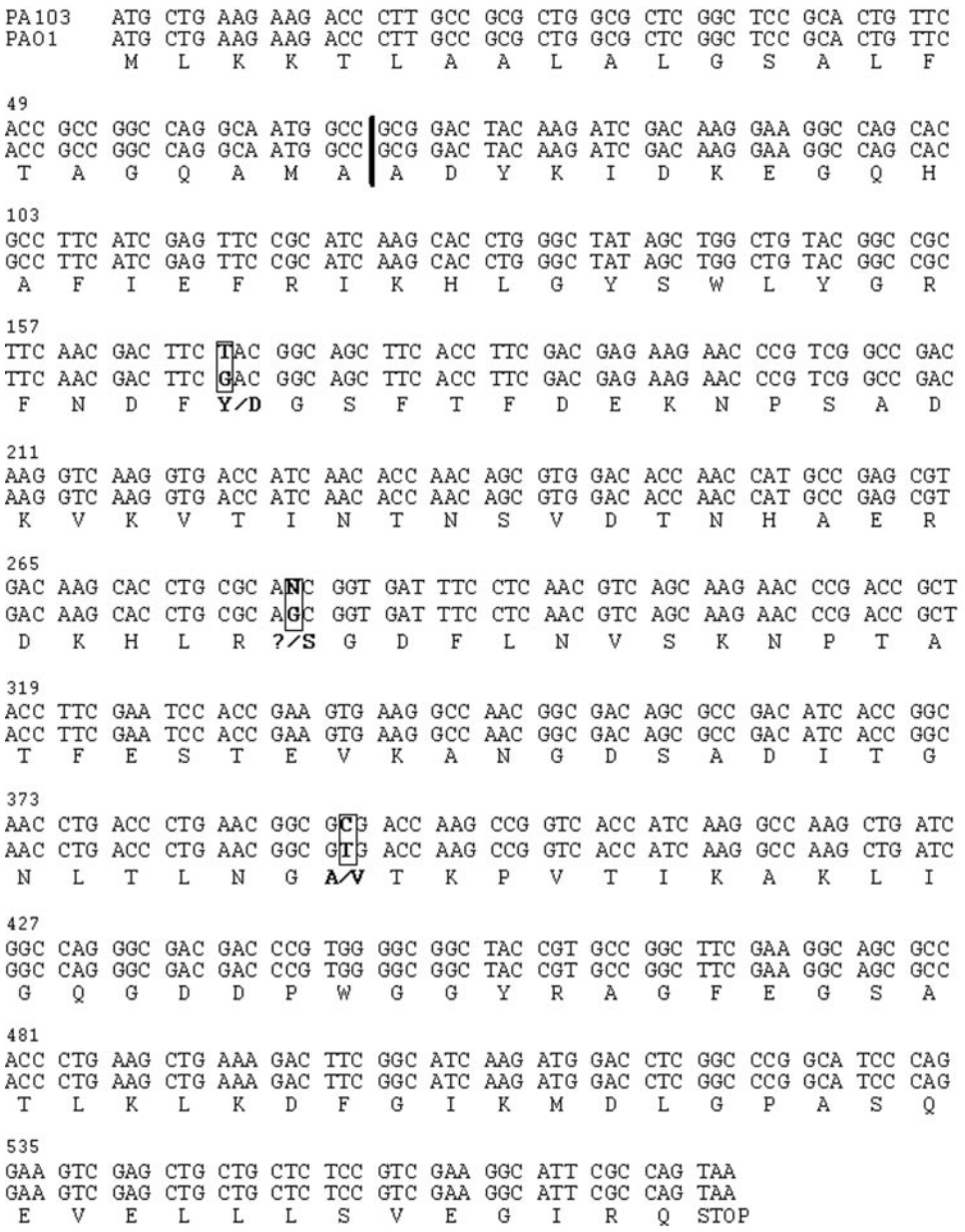

FIGURE 5. Nucleotide sequence alignment (+ strand) of strain PA103 *Pasp* with PA0423 from strain PAO1. The amino acid sequence is given *below* each codon, and nucleotides that differ between the two strains are in *boxes*. The cleavage site of the type II secretory signal sequence is indicated by a *vertical line*.

kDa, a mass identical with the molecular mass determined herein for the protease purified from PA103. The *Pasp* gene in either PAO1 or PA103 appears to have a signal peptide consistent with type II secretion.

Cloning and Sequence Analysis of PASP in *E. coli*

The *Pasp* gene was cloned in vector pHAT10, which contained the *Lac* promoter and placed a tag of 19 amino acids with 6 histidine residues interspersed (for affinity purification) on the N terminus of the recombinant protease. The resultant pHAT10-*Pasp* was purified and the inserted DNA sequenced. The *Pasp* sequence has GenBank Accession number AY744142. The DNA sequence of PA103 *Pasp* (Fig. 5) was determined to be identical with the sequence of gene PA0423 from strain PAO1 in 570 of the 573 nucleotides (99.5% identical). The differences in the *Pasp* gene sequence for PA103 and the corresponding sequence in PAO1 results in a predicted two- to three-amino-acid difference in the expressed proteases (98.4%–99.0% identity). The first difference is at nucleotide 169, which in PAO1 translates to aspartate, a polar amino acid. The nucleotide at position 169 in PA103, however, translates to tyrosine, a nonpolar amino acid. A second possible difference may exist at nucleotide 281, as this nucleotide was not determined in PA103. The final difference is at nucleotide 392, which in the PAO1 sequence translates to valine and in the PA103 sequence translates to alanine, both of which are nonpolar. The sequence of the *Pasp* gene site of PAO1 (gene PA0423) is identical with that of strain PA14.[51] The PA103 PASP protein sequence, like that of the PAO1 sequence, contains a putative type II secretion signal sequence immediately preceding the amino acid sequence obtained for its N terminus. The putative cleavage site is between amino acids 23 and 24 (Fig. 5).

The full *Pasp* nucleotide sequence was also found to be 82% identical with an *E. coli* K-12 gene designated *YceI* (GenBank Accession Number U00096). The *E. coli* protein YceI has been shown to be upregulated by alkaline pH during bacterial growth,[52] but no other attributes have been determined for this protein. In regard to *Pseudomonas* species other than *P. aeruginosa*, *Pasp* was found to be 86%

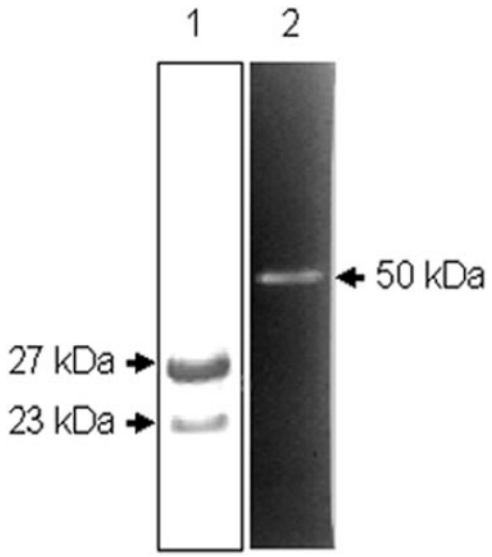

FIGURE 6. Analysis of recombinant PASP after purification and refolding. *Lane 1*: SDS-PAGE of purified recombinant PASP (8 μg, boiled); and *lane 2*: gelatin zymogram of purified recombinant PASP (8 μg).

identical with the gene coding for a conserved hypothetical protein produced by *Pseudomonas putida* strain KT2440 (GenBank Accession Number AAN70547), as well as 80% identical with the gene coding for a YceI-like family protein from the *Pseudomonas syringae* strain DC3000 (GenBank Accession Number AE016874).

Analysis of the Purified and Refolded Recombinant PASP

Recombinant PASP was expressed in *E. coli* DH5α PRO cells, but the protein was located predominantly in inclusion bodies. The recombinant protease from the inclusion bodies was denatured with guanidine-HCl and subsequently purified and refolded. The purified recombinant and refolded PASP appeared as two bands on SDS-PAGE located at 27 and 23 kDa (Fig. 6, lane 1). The sequence of the N terminus of the 27-kDa band was determined to be AMITPSLKDH, which corresponds to the pHAT10 multiple cloning site sequence 103 nucleotides upstream of the cloned *Pasp* gene and includes the first three codons (coding for KDH) of the affinity tag. (The theoretical mass of the recombinant protein with tag is approximately 25 kDa.) The sequence of the N terminus of the 23-kDa band was determined to be TLAALALGSA, which corresponds to the sequence of *Pasp* without the first four residues. Thus, the 27-kDa band represents recombinant PASP and the 23-kDa band represents a breakdown product. Gelatin zymography of this recombinant PASP revealed protease activity at 50 kDa (Fig. 6, lane 2); however, it is unknown which protein (23 or 27 kDa) is responsible for the enzymatic activity, or whether both are active.

Effect of PASP on Rabbit Corneas

Active PASP caused erosions in rabbit corneas by 4 hours after injection. At 5 hours after injection, erosions ranged from 1 to 2.5 mm in diameter (Figs. 7A, 7B). By 10 hours after injection, an erosion of 6 mm was observed (Fig. 7 B). All erosions were healed by 48 hours after injection. Heat-inactivated PASP caused no erosions whatsoever (Fig. 7C, 7D).

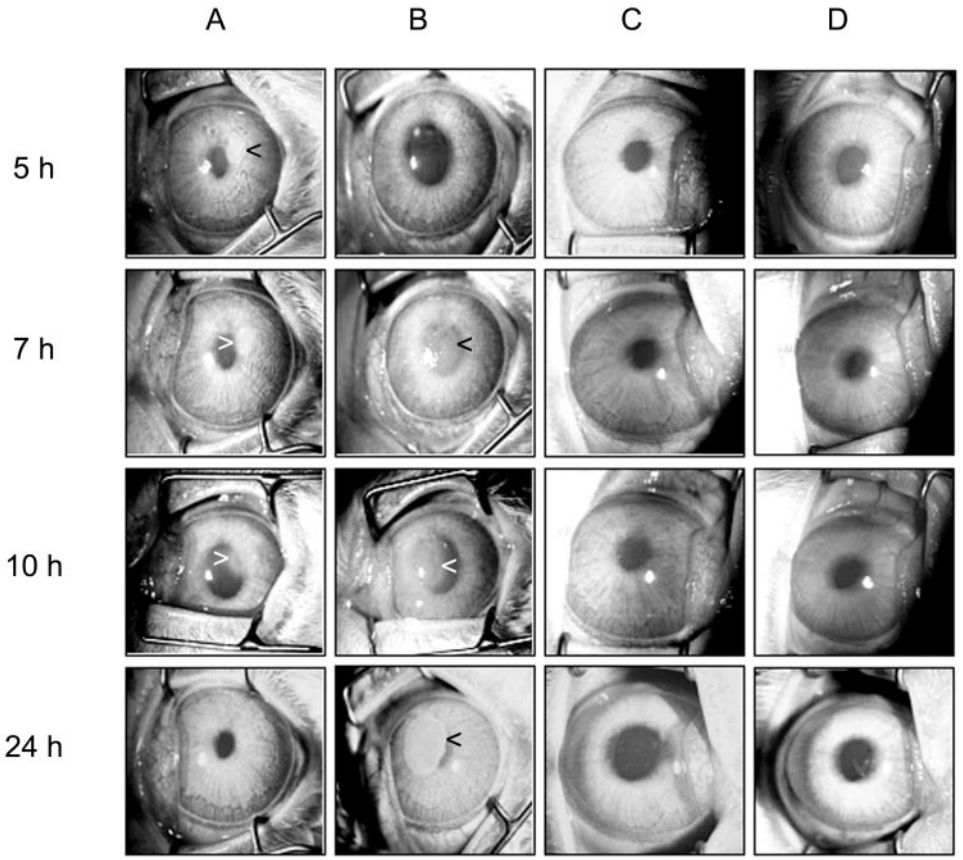

FIGURE 7. Rabbit corneas at 5, 7, 10, and 24 hours after intrastromal injection of active PASP (**A**, **B**) or heat-inactivated PASP (**C**, **D**). <, epithelial erosions. Two representative corneas from each group were chosen for photography.

Discussion

The results presented herein show that *P. aeruginosa* can secrete a previously unrecognized protease, designated PASP, that causes corneal epithelial erosions. PASP has an apparent molecular mass of 18.5 kDa and on zymograms migrates with an apparent molecular mass of approximately 80 kDa. This protease is distinct from all other bacterial proteases, including the modified LasB of strain 19660, which has the same migration on zymograms. The sequence of the gene for PASP and the DNA sequence of strain PAO1 (gene designation PA0423) are 99.5% identical, indicating that PAO1 can produce this protease. Nouwens et al.[53] determined that the gene at PA0423 produces a secreted protein of unknown function. The sequence of the PASP protease is homologous (80%–86%) to proteins of undefined function in *E. coli*, *P. putida*, and *P. syringae*. Analysis of the sequence of PASP revealed the presence of a putative N-terminal signal sequence that was probably cleaved before secretion and purification of the protease. The presence of this signal sequence suggests a type II secretion mechanism for PASP.

The proof that the PA0423 gene codes for the proposed PASP protease is derived from three experiments: the enzymatic activity of the product of the cloned gene, the presence of the 25-kDa protein in the 80-kDa band on zymograms, and the sequence of the protease extensively purified for *P. aeruginosa* PA103 culture supernatants. The great sequence similarity (>99%) between the PA0423 gene of strain PAO1 and that of the gene isolated and sequenced from strain PA103 suggest that the PASP protease gene is conserved among *P. aeruginosa* strains.

PASP has a molecular mass of 18.5 kDa; however, its native mobility on gelatin zymograms is approximately 80 kDa, possibly because of multimer (trimer or tetramer) formation in the presence of SDS and the omission of a reducing agent and heating. PASP expressed from the cloned *Pasp* gene on plasmid pHAT10 produced a 50-kDa band in gelatin zymograms, which could be a dimer of the recombinant protease. The recombinant form of PASP has an affinity tag comprising 19 amino acids with 6 interspersed histidine residues that could prevent the formation of an 80-kDa trimer or tetramer and allow only the formation of a 50-kDa dimer.

The aggregation of PASP to high-molecular-mass forms on zymograms is not unexpected. LasB and protease IV also have larger molecular masses on zymograms than their monomeric masses.[35] Zymogram analyses by different research groups have shown the presence of LasB to vary, depending on the method and percentage of acrylamide in the gel.[20] Reported herein are migrations of 100 kDa for LasB, 80 kDa for either modified LasB or PASP, and 50 kDa for alkaline protease. Further distinction between modified LasB and PASP was obtained by Western blot analysis using LasB antiserum.

Strain PA103 is a clinical strain originally reported to be completely deficient in protease activity,[54] but was later shown to produce zones of lysis in skim milk-containing agar.[55] Strain PA103 is known to be virulent in the cornea[23] and lung.[56] The injection of purified PASP into rabbit corneas caused the rapid destruction of the epithelium. This effect was dependent on the enzymatic activity of PASP and was not due to a host response to the protein per se, as demonstrated by the absence of erosions after injection of heat-inactivated PASP. PASP was injected at a concentration of 7 μg, whereas in a previous study, protease IV caused epithelial erosions at concentrations of 50 to 100 ng. The units of enzyme activity per milligram protein have not been determined for PASP because a specific assay for this protease is not available; therefore, the relative ability of PASP in causing corneal damage cannot yet be determined. *Pseudomonas* keratitis is characterized by a rapid destruction of the cornea, an event that is probably mediated by the action of bacterial proteases. PASP appears to be one factor with the activity to mediate corneal damage rapidly. Further characterization of this protease with respect to its proteolytic mechanism, specific protein targets, and role in virulence should be undertaken. This novel protease joins the list of proteases of unknown catalytic mechanism.

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