Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions

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Pulmonary surfactant is synthesized and secreted by alveolar type II cells and is composed of 90% lipids (~80% phospholipids) and 10% proteins, the latter consisting primarily of four surfactant-associated proteins designated SP-A, SP-B, SP-C, and SP-D (9). Pulmonary surfactant has two distinct functions within the lung: reduction of surface tension at the air–liquid interface and participation in innate host defense. Both functions are dependent on surfactant-associated proteins. Pseudomonas aeruginosa is primarily responsible for respiratory dysfunction and death in cystic fibrosis patients and is also a leading pathogen in nosocomial pneumonia. P. aeruginosa secretes a number of proteases that contribute to its virulence. We hypothesized that P. aeruginosa protease IV degrades surfactant proteins and results in a reduction in pulmonary surfactant host defense and biophysical functions. Protease IV was isolated from cultured supernatant of P. aeruginosa by gel chromatography. Incubation of cell-free bronchoalveolar lavage fluid with protease IV resulted in degradation of surfactant proteins (SP)-A, -D, and -B. SPs were degraded in a time- and dose-dependent fashion by protease IV, and degradation was inhibited by the trypsin-like serine protease inhibitor Nε-p-tosyl-L-lysine-chloromethyl ketone (TLCK). Degradation by protease IV inhibited SP-A- and SP-D-mediated bacterial aggregation and uptake by macrophages. Surfactant treated with protease IV was unable to reduce surface tension as effectively as untreated surfactant, and this effect was inhibited by TLCK. We speculate that protease IV may be an important contributing factor to the development and propagation of acute lung injury associated with P. aeruginosa via loss of surfactant function within the lung.

PULMONARY SURFACTANT IS SYNTHESIZED and secreted by alveolar type II cells and is composed of 90% lipids (~80% phospholipids) and 10% proteins, the latter consisting primarily of four surfactant-associated proteins designated SP-A, SP-B, SP-C, and SP-D (9). Pulmonary surfactant has two distinct functions within the lung: reduction of surface tension at the air–liquid interface and participation in innate host defense against inhaled pathogens (3, 9, 16).

Surfactant is stored within type II cells as lamellar bodies and secreted into the air space to form tubular myelin, the precursor to the surfactant surface film. Tubular myelin and other large surfactant aggregates (LA) can be isolated via centrifugation and are biophysically active; i.e., they effectively lower surface tension (32, 37, 42). Upon change in surface area with respiration, LA are converted into small lipid vesicles termed small aggregates (SA) that are biophysically inactive (32, 37, 42) and are either taken back up into the type II cell or cleared by alveolar macrophages (14, 40). SP-A and SP-B are important for LA function and integrity, and increased conversion of the functional LA fraction is directly proportional to the loss of SP-A and SP-B (12, 36, 38).

SP-A and SP-D are members of a family of innate immune molecules known as collectins that have an NH2-terminal collagen-like region and a COOH-terminal lectin domain that has preferential binding sites for non-host oligosaccharides, such as those found on bacterial and viral surfaces. SP-A and SP-D are expressed by epithelial cells lining the respiratory tract. In humans, these include type II cells, nonciliated respiratory epithelial cells, and subsets of cells in submucosal glands (24, 34). Collectins opsonize bacteria and viruses and enhance their phagocytosis by macrophages and neutrophils. In addition, collectins regulate a variety of immune cell functions, including production of cytokines and radicals and chemotaxis (3, 16). In vivo studies have shown that SP-A- and SP-D-null mice have an increased susceptibility to various pulmonary pathogens, including Pseudomonas aeruginosa (19–21).

P. aeruginosa is a common environmental gram-negative bacillus that is associated with corneal infections, bacteremia associated with severe burns and both acute and chronic lung infection. P. aeruginosa infection is quite rare in otherwise healthy individuals, and nearly all clinical cases occur in immunocompromised hosts (23). Chronic pulmonary infection with P. aeruginosa is a major cause of morbidity and mortality in cystic fibrosis patients and is also a leading cause of nosocomial pneumonia (23, 35). This opportunistic pathogen has a number of different survival mechanisms that may also be detrimental to the host. For example, P. aeruginosa produces a mucoid film that provides a physical barrier to clearance (33), lipopolysaccharide that can help the organism evade host defense mechanisms (10), and a variety of secreted enzymes that can degrade biological proteins (5, 18).

We have previously observed that P. aeruginosa degrades SP-A and SP-D (25). This degradative activity was attributed to two different proteins. One protein was definitively identified as P. aeruginosa elastase, and the second protein could not be definitively identified.
be conclusively identified at that time. Since this initial observation, we have determined that this additional secreted protein is the recently described *P. aeruginosa* protease IV, a secreted serine protease that is regulated in a manner distinct from the other *P. aeruginosa* proteases (5, 39). Protease IV has been shown to be a significant virulence factor in keratitis caused by *P. aeruginosa* (6, 28). The significance of protease IV in lung virulence of *P. aeruginosa* has not been investigated. We hypothesize that protease IV degrades surfactant proteins, decreasing both the host defense and biophysical properties of pulmonary surfactant, therefore suggesting that protease IV may be a significant virulence factor in the development of acute lung injury associated with *P. aeruginosa*.

**MATERIALS AND METHODS**

**Materials and animals.** All chemicals were purchased from Sigma (St. Louis, MO) except where noted. All experiments utilizing bronchoalveolar lavage fluid (BALF) and alveolar macrophages were conducted with pathogen-free male Sprague-Dawley rats (Taconic, Germantown, NY) ranging from 250 to 350 g. All animal procedures were approved by the Duke University Medical Center Institutional Animal Care and Use Committee.

**Purification of *P. aeruginosa* protease IV.** Protease IV was initially purified from concentrated culture supernatants of *P. aeruginosa* strain PA103-29 using ion exchange and molecular sieve chromatography as previously described (5). Additional protease IV was isolated from culture supernatants of PAO1-B1 (kindly provided by Dr. B. Iglewski; Univ. of Rochester, Rochester, NY) by another chromatography method. Briefly, *P. aeruginosa* PAO1-B1 was grown in 2-liter cultures of nutrient broth (Difco, Detroit, MI), shaken (250 rpm) for 24 h at 37°C, and subsequently centrifuged at 7,000 g for 20 min to pellet the cells. Resulting supernatant was filtered over a 0.45-μm Durapore membrane filter (Millipore, Coralville, MA) and concentrated by ultrafiltration with tangential flow using a Pellicon XL device with a 10-kDa cut-off membrane (Millipore) to a volume of 200 ml. The supernatant was applied to a DEAE Sepharose CL-6B column (50 × 1.5 cm) in 30 mM Tris-Cl, pH 8.3, and proteins were eluted with a continuous linear gradient of NaCl (0–0.5 M) in the same buffer. Protease IV-positive fractions were pooled and applied to a CM Sepharose Fast Flow column (Amersham Biosciences) in 10 mM ammonium acetate, pH 6.8, and proteins were eluted with a gradient formed by the addition of 10 mM ammonium acetate buffer at pH 9 to 10 mM ammonium acetate buffer at pH 6.8. Purity of the protease was determined by electrophoresis on 12.5% SDS-PAGE gels and visualized by silver staining.

**Colorimetric substrate assay for protease IV activity.** Protease IV activity was determined by the hydrolysis of the chromogenic substrate Chrozmoxyl PL (tosyl-Gly-Pro-Lys-p-nitroanilide) as described previously by O’Callaghan et al. (28). Protease IV activity was measured as an increase in optical density (OD) at 410 nm. End-point reactions were incubated for 30 min at 37°C, and kinetic analysis was performed at 25°C by reading the optical density every 2 min for a 30-min time period.

**Amino acid analysis and protein sequencing.** Protein sequence analysis was performed at the Harvard Microchemistry Facility as previously described (25). In brief, two purified proteins from column fractions exhibited degradative activity toward SP-A. These two proteins were separated by SDS-PAGE and treated in-gel by reduction, S-carboxamidomethylation, and tryptic digestion. The resulting mixture was analyzed by micropipillary reverse-phase high-performance liquid chromatography nanoelectrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer. The MS/MS spectra were correlated with known sequences in the National Center for Biotechnology Information nr and dbest databases using the algorithm Sequest and programs developed at the Harvard Microchemistry Facility and confirmed by manual inspection.

**Purification of human SP-A.** SP-A was purified from BALF of alveolar proteinosis patients as previously described (41). SP-A preparations were treated with polymyxin agarose to reduce endotoxin contamination, dialyzed against 5 mM Tris, and centrifuged at 100,000 g for 30 min before storage in 5 mM Tris, pH 7.4. Aliquots of each preparation were tested for the presence of endotoxin by the Limulus amebocyte lysate assay (Bio-Whittaker, Walkersville, MD), and only samples containing <0.5 pg of endotoxin per microgram of protein were used.

**Purification of recombinant rat SP-D.** Recombinant rat SP-D was purified from the media of a stably transfected Chinese hamster ovary (CHO) cell line (4). Briefly, CHO cells were grown in serum-free media for 10–12 days, and the culture supernatant was collected and dialyzed against maltose loading buffer (50 mM Tris, 150 mM NaCl, and 5 mM CaCl2, pH 7.8) to remove glucose. The dialyzed supernatant was then incubated with maltose-Sepharose beads overnight at 4°C. The maltose-Sepharose beads were then poured into a column and washed with maltose loading buffer to remove unbound proteins. SP-D was then eluted from the column with maltose elution buffer (50 mM Tris, 150 mM NaCl, and 2 mM EDTA, pH 7.8).

**Degradation of purified SPs.** Alveolar proteinosis SP-A and rat recombinant SP-D were incubated with purified protease IV at varying times and concentrations (as noted in figure legends) in 30 mM Tris, pH 7.75, at a final volume of 100 μl at 37°C. Control samples replaced protease IV with reaction buffer. The protease was inactivated by the addition of SDS-PAGE sample buffer containing 100 mM diethiothreitol followed by boiling for 5 min. Degradation was determined by electrophoresis of equal volumes of reaction mixture (5 μl) on 15% SDS-PAGE under reducing conditions and analyzed by Western blot using well-characterized anti-human SP-A antibody (kindly provided by ALTANA Pharma, Konstanz, Germany) and anti-rat SP-D antibody (26). Inhibition studies were performed in an identical manner as the degradation assays except protease IV was incubated with 1 μM Nα-p-tosyl-L-lysine-chloromethyl ketone (TLCK) for 30 min at room temperature before the addition of the SPs.

**Degradation of SPs in BALF.** Normal rat BALF was obtained by a single whole lung lavage using 150 mM NaCl with 0.2 mM EDTA. The resultant BALF was spun at 150 g for 10 min to remove alveolar cells. Cell-free BALF (0.15 mg phospholipid/ml) was incubated with purified protease IV (5 μg/ml) for 24 h at 37°C. Control samples replaced protease IV with an equal volume of 30 mM Tris, pH 7.75. For analysis of SP-A and SP-D, equal volumes of reaction mixture (30 μl) were incubated by 15% SDS-PAGE under reducing conditions and analyzed by Western blot using anti-human SP-A antibody (kindly provided by ALTANA Pharma) and anti-rat SP-D antibody. For analysis of SP-B, equal volumes of reaction mixture (30 μl) were resolved by 15% SDS-PAGE under nonreducing conditions and analyzed by Western blot using an anti-sheep SP-B antibody.

**Aggregation of bacteria by SP-A and SP-D.** Twenty-four hours before the aggregation assay, SPs were incubated in the presence or absence of protease IV at 37°C. SP-A at a concentration of 50 μg/ml was incubated with 5 μg/ml of protease IV. SP-D at a concentration of 12.5 μg/ml was incubated with 5 μg/ml of protease IV. *Salmonella typhimurium* was grown overnight on Luria-Bertani (LB) agar plates and resuspended into 1 ml of LB broth. Colony-forming units (cfu) were determined by measurement of absorbance at 660 nm using a calculated extinction coefficient. A volume equal to 10⁶ cfu of bacteria was suspended in 1 ml of Tris-buffered saline (TBS) containing 2 mM CaCl2, pH 7.6. SP-A and SP-D reaction mixtures (100 μl) were added to 5 × 10⁷ cfu of *S. typhimurium* with 1 mM CaCl2 and brought up to a final volume of 500 μl with TBS. Aggregation was observed as a decrease in the absorbance (660 nm) as bacterial aggregates precipitated out of solution. Absorbance was measured at 15, 30, and 60 min after the addition of the intact and degraded SPs. Additional controls...
were performed which included bacteria plus an equivalent amount of protease IV that would have been associated with the SP degradation assays.

Bacterial association with alveolar macrophages. Escherichia coli strains K12 (rough) and HB101 were grown overnight in 5 ml of LB broth, collected at 2,000 g for 10 min, and resuspended in 1 ml of NaCO3, pH 9.0. Bacteria were labeled with FITC by adding 10 μl of FITC (Molecular Probes) at a concentration of 10 mg/ml in dimethylformamide for 1 h at room temperature. Excess dye was removed by washing the bacteria three times with PBS. Bacteria concentration was determined by measurement of absorbance at 660 nm utilizing known extinction coefficients.

SPs were incubated in the presence or absence of protease IV for 24 h at 37°C before the bacterial association assay was performed. SP-A at a concentration of 50 μg/ml was incubated with 5 μg/ml of protease IV. SP-D at a concentration of 12.5 μg/ml was incubated with 5 μg/ml of protease IV.

Alveolar macrophages from normal rats were isolated by whole lung lavage, and resultant lavage fluid was centrifuged at 150 g for 10 min. The cell pellet was resuspended in PBS plus 2 mM CaCl2 plus 0.1% BSA, and cell numbers were determined by counting on a hemocytometer. Purity of isolated cells was determined by cytoospin and hemacolor staining.

Eppendorf tubes were initially incubated with PBS plus 1% BSA (1 ml) at 4°C for 1 h before addition of macrophages and bacteria to block nonspecific binding sites. Freshly isolated alveolar macrophages (5 × 10⁷) were then incubated with FITC-labeled bacteria (5 × 10⁸ cfu) in 250 μl of PBS plus 1 mM CaCl₂ plus 0.1% BSA for 1 h at 37°C. For SP-A experiments, a volume of the degradation assay resulting in a final concentration of 5 μg/ml was added to the macrophages and E. coli K12. For SP-D experiments, a volume of the degradation assay resulting in a final concentration of 1 μg/ml was added to the macrophages and E. coli HB101. Assay was terminated by addition of 1 ml of ice-cold PBS plus 0.1% BSA. The alveolar macrophages were washed twice with ice-cold PBS buffer (150 g, 10 min, 4°C), fixed in 300 μl of 1% formaldehyde in PBS, and analyzed for fluorescence by fluorescence-activated cell sorting (FACS). An additional control was performed that included alveolar macrophages plus bacteria plus an equivalent amount of protease IV that would be associated with the SP degradation assay.

Surface activity analysis. In vitro surface tension measurements of LA were performed using a pulsating bubble surfactometer (Electronetics, Amherst, NY) as described by Enhorning (8). LA were pelleted after a 15-min, 40,000 g centrifugation of cell-free BALF recovered from healthy rats. LA were prepared to obtain a final concentration of 2.5 mg of phospholipids per milliliter in 0.15 M NaCl and 1.5 mM CaCl₂, and frozen for later use. Three experimental conditions were analyzed: LA were incubated for 72 h at 37°C without protease IV, LA were incubated with protease IV (10 μg/ml), and LA were incubated with protease IV in the presence of 1 mM TLCK. A 72-h incubation period was chosen on the basis of preliminary studies showing SP degradation in the concentrated LA samples. During this time period, the LA were kept in suspension with gentle agitation. After the incubation period, the samples were immediately frozen for later analysis, at which time the samples were incubated for 90 min at 37°C before surface activity was measured. Briefly, a bubble was created in the suspension containing the LA fraction. Surfactant adsorption kinetics was measured during the initial 10 s after bubble formation. Subsequently, the bubble was pulsed for a period of 5 min between a minimum radius of 0.44 mm and a maximum radius of 0.55 mm at a rate of 20 pulsations/min and a temperature of 37°C. Pressure was monitored across the air-liquid interface by a pressure transducer, and surface tension was calculated at the minimum and maximum bubble radii. All samples were analyzed at the same time, and the investigator was blinded to the experimental group.

Statistics. Data are expressed as means ± SD, and values between groups were compared using an unpaired Student’s t-test. A probability level of P < 0.05 was considered statistically significant.

RESULTS

Identification of P. aeruginosa protease IV. Previously, we determined that the supernatant collected from cultures of P. aeruginosa degrades SP-A (25). After separation of the secreted proteins in the cultured supernatant on an anion exchange column, two separate pooled fractions were determined to contain degradative activity toward SP-A. One of the fractions contained P. aeruginosa elastase; we were unable at that time to identify definitively the protein responsible for the degradation in the second fraction (25). Subsequently, Edman degradation analysis showed that the sequence of the peptide fragments (Fig. 1) corresponds to the recently described P. aeruginosa virulence factor, protease IV (5, 39). Protease IV has been determined to be a serine protease that demonstrates activity for the COOH side of lysine-containing peptides (5).

SPs are degraded in the presence of protease IV. To characterize the degradation of SPs by purified protease IV, we initially examined the time dependence of degradation. Figure 2A shows SP-A is degraded in a time-dependent manner by protease IV. Reduced human alveolar proteinosis SP-A migrates as both the dimer and monomer. Within 1 h of incubation, there is a decrease in the amount of both the SP-A dimer and monomer and the appearance of an intermediate band of ~65 kDa. After a 12-h incubation period, approximately one-half of the immunoreactive SP-A is degraded, and within 24 h, no immunoreactive protein can be detected. Figure 2B demonstrates that SP-D degradation is also time dependent. Incubation of recombinant rat SP-D with protease IV for 1 h results in the loss of any intact SP-D protein (~49 kDa) and produces a SP-D fragment of ~33 kDa. With increasing incubation time, this SP-D fragment is progressively degraded until no immunoreactive SP-D fragment is observed after a 24-h incubation period.

The effect of protease IV dose on the degradation of these proteins was investigated. Figure 3A demonstrates that incubation of SP-A with increasing concentrations of protease IV for 24 h results in increased degradation of the protein. At the lowest dose of protease IV (2.5 μg/ml), we observe degra-
tion of both the monomer and dimer of SP-A and the appearance of the ~65-kDa intermediate band. The highest dose of protease IV tested (10\(\mu\)g/ml) resulted in almost complete degradation of both SP-A forms. To implicate a specific effect of protease IV, the ability of TLCK, an irreversible inhibitor of the serine protease trypsin and many trypsin-like serine proteases, to inhibit degradation was analyzed. When protease IV was preincubated with TLCK, there was complete inhibition of the degradation of SP-A. Figure 3B demonstrates that degradation of SP-D by protease IV is also dose dependent, similar to SP-A degradation. The lowest dose of protease IV tested (2.5 \(\mu\)g/ml) results in the complete loss of the intact proteins and the appearance of the ~33-kDa SP-D fragment. Increasing the concentration of protease IV resulted in the complete degradation of this SP-D fragment. As demonstrated with SP-A, preincubation with TLCK resulted in the complete inhibition of protease IV activity against SP-D.

SPs are degraded by protease IV in the presence of surfactant lipids. Pulmonary surfactant is primarily composed of phospholipids (~80%), whereas the proteins comprise 10% by weight. SP-A and SP-D are hydrophilic proteins; SP-A avidly binds dipalmitoylphosphatidylcholine, the most abundant surfactant phospholipid (17), and SP-D binds phosphatidylinositol, a minor component of surfactant (29). The other SPs, SP-B and SP-C, are hydrophobic proteins that are tightly associated with surfactant lipids. To determine whether protease IV can degrade SPs in whole pulmonary surfactant, BALF was obtained from normal healthy rats and incubated with or without protease IV. Immunoreactive SP-A, SP-D, and SP-B were detected in aliquots of the reaction mixture as shown in Fig. 4, A–C, respectively. In Fig. 4A, rat SP-A migrates as a monomer (~38 kDa), and after incubation of BALF with protease IV, there is a considerable loss of immunoreactive SP-A. Figure 4B demonstrated that degradation of SP-D in the presence of surfactant lipids also results in the appearance of an ~33-kDa SP-D fragment similar to the isolated SP-D experiments. Non-reduced SP-B is displayed in Fig. 4C and demonstrates that this hydrophobic SP is also susceptible to degradation by protease IV.

Degradation of SPs by protease IV inhibits bacterial aggregation. Aggregation of bacteria by SP-A and SP-D has been shown to be a function of these innate immune molecules. To test a functional consequence of degradation of SPs by protease IV, isolated SP-A and SP-D were incubated with and without protease IV for 24 h at 37°C and subsequently tested for its...
ability to aggregate *S. typhimurium*. Bacterial aggregation was measured by the decrease in absorbance (OD 660 nm) as the aggregated bacteria precipitate out of solution.

Figure 5A displays the effect of SP-A (10 µg/ml) on bacterial aggregation over a 60-min time course for three independent experiments. *S. typhimurium* alone did not self-aggregate as shown by stable absorbance readings over the 60-min experimental time period. Incubation of *S. typhimurium* with intact SP-A (not incubated with protease IV) resulted in aggregation of the bacteria as demonstrated by a significant decrease in absorbance at the 30- and 60-min time points compared with the *S. typhimurium* control (*P < 0.01*). In contrast, incubation of *S. typhimurium* with degraded SP-A (incubated with protease IV for 24 h before) inhibited bacterial aggregation as demonstrated by a significantly higher absorbance at the 30- and 60-min time points compared with the intact SP-A (*P < 0.01*). There was also no significant difference in absorbance compared with the control *S. typhimurium* group at any time point. Incubation of *S. typhimurium* with protease IV alone did not affect bacterial aggregation (data not shown). Figure 5B displays an individual bacterial aggregation experiment with the corresponding degree of SP-A degradation shown by Western blot.

Figure 6A displays the effect of SP-D (2.5 µg/ml) on the mean absorbance (OD ± SD) of a bacterial suspension over a 60-min time course for three independent experiments. *S. typhimurium* alone did not result in self-aggregation as shown by a nonsignificant decrease in absorbance over the 60-min experimental time period. Incubation of *S. typhimurium* with intact SP-D (not incubated with protease IV) resulted in aggregation of the bacteria as demonstrated by a significant decrease in absorbance at the 30- and 60-min time points compared with the *S. typhimurium* control (*P < 0.05*). In contrast, incubation of *S. typhimurium* with degraded SP-D (incubated with protease IV for 24 h before) reduced bacterial aggregation as demonstrated by a significantly higher absorbance at the 30- and 60-min time points compared with the intact SP-D (*P < 0.05*). There was also no significant difference in absorbance compared with the control *S. typhimurium* group at any time point. Incubation of *S. typhimurium* with protease IV alone did not affect absorbance of the bacterial suspension (data not shown). Figure 6B displays an individual bacterial aggregation experiment with the corresponding degree of SP-D degradation shown by Western blot. Interestingly, even in the presence of the SP-D degradation fragment formed after incubation with protease IV, bacterial aggregation was still abolished. Therefore, initial degradation of SP-D to the fragment would abrogate this immunological function.

**Fig. 4.** PIV degrades SP-A, SP-D, and SP-B in bronchoalveolar lavage fluid (BALF). Rat BALF was incubated in the absence or presence of PIV (1 µg) for 24 h at 37°C. A: SP-A was measured in equal volumes of reaction mixture, resolved by 15% SDS-PAGE under reducing conditions, and analyzed by Western blot. B: SP-D was measured in equal volumes of reaction mixture, resolved by 15% SDS-PAGE under reducing conditions, and analyzed by Western blot. C: SP-B was measured in equal volumes of reaction mixture, resolved by 15% SDS-PAGE under nonreducing conditions, and analyzed by Western blot.

**Fig. 5.** Degradation of SP-A by PIV inhibits bacterial aggregation. SP-A (50 µg/ml) was incubated in the presence or absence of PIV (5 µg/ml) at 37°C for 24 h. Reaction mixture was incubated with *Salmonella typhimurium* (5 x 10^7 colony-forming units (cfu)), and aggregation was measured as a decrease in absorbance at 660 nm over 60 min. A: the mean and SD of 3 independent experiments for the 3 experimental groups: *S. typhimurium* control (*S. typh*), *S. typhimurium* with intact SP-A (*S. typh + SP-A*), and *S. typhimurium* with degraded SP-A (*S. typh + D SP-A*). B: individual experiment with the corresponding SP-A Western blot displaying the degree of degradation of SP-A in the presence of PIV. *P < 0.05* vs. *S. typh* and *S. typh + D SP-A*.
Degradation of SPs by protease IV inhibits their ability to enhance bacterial association with alveolar macrophages. SP-A and SP-D can act as opsonins within the lung to promote bacterial clearance by alveolar macrophages. To investigate the functional consequence of protease IV on this host defense function of SP-A and SP-D, the uptake of FITC-labeled bacteria with alveolar macrophages in the presence of intact or protease IV-degraded SPs proteins was investigated by FACS.

Figure 7A displays the effect of SP-A (5 μg/ml) on the association of FITC-labeled E. coli with alveolar macrophages as measured by mean fluorescence (± SD) and displayed as a percentage of the alveolar macrophages plus E. coli. Addition of intact SP-D (not incubated with protease IV) to the alveolar macrophage-bacteria suspension resulted in a significant increase in the association of E. coli with this cell type (P < 0.01). In contrast, degraded SP-D (incubated with protease IV for 24 h before assay) did not enhance bacterial association as did intact SP-D (P < 0.01). Of note, incubation of alveolar macrophages and E. coli with protease IV alone did not have an effect on the mean fluorescence level associated with alveolar macrophages after the experimental period (data not shown). Figure 7B displays an individual bacterial association experiment with the corresponding degree of SP-A degradation shown by Western blot.

Figure 8A illustrates the effect of SP-D (1 μg/ml) on the association of FITC-labeled E. coli with alveolar macrophages as measured by mean fluorescence (± SD) and displayed as a percentage of the alveolar macrophages plus E. coli control group. Addition of intact SP-D (not incubated with protease IV) to the alveolar macrophage-bacteria suspension resulted in a significant increase in the association of E. coli with this cell type (P < 0.01). Of note, incubation of alveolar macrophages and E. coli with protease IV alone did not have an effect on the mean fluorescence level associated with alveolar macrophages after the experimental period (data not shown). Figure 8B displays an individual bacterial associ-
in which protease IV was inhibited with TLCK. Degradation of SP-A by protease IV and inhibition of SP-A degradation by TLCK confirmed SP degradation in these samples (Fig. 9B). Unfortunately, we could not conclusively demonstrate SP-B degradation in these LA samples after the incubation time period. Of note, we were able to clearly resolve SP-B in preparations that had not undergone the 72-h incubation period, but resolution of the SP-B dimer after incubation was extremely poor in all samples. We cannot provide an explanation for the loss of SP-B resolution, but based on the Western blots, we speculate that it may be due to aggregate formation. Importantly, protease IV had no effect on surfactant phospholipids as total surfactant phospholipid levels were unchanged after incubation with protease IV (data not shown).

**DISCUSSION**

The original observation that *P. aeruginosa* degraded SP-A and SP-D was made by Mariencheck et al. (25) when investigating binding interactions between SP-A and *P. aeruginosa*. In these initial experiments, it was shown that two separate pooled fractions obtained by separation of *P. aeruginosa* culture supernatants on an anion exchange column degraded SP-A and SP-D. At that time, *P. aeruginosa* elastase, a zinc metalloprotease, was positively identified as one of the degradative proteins; however, the second protein could not be identified.

As presented in this paper, the other degradative protein has now been identified by Edman degradation as *P. aeruginosa* protease IV (lysyl endopeptidase; EC 3.4.21.50; Fig. 1). In this paper, it is demonstrated in vitro that protease IV degrades SP-A and SP-D in a time- and concentration-dependent manner (Figs. 2 and 3) and, moreover, degrades SP-A, SP-D, and SP-B in BALF (Fig. 4). The degradation of SP-A and SP-D resulted in the inhibition of their ability to aggregate bacteria and enhance bacterial uptake by alveolar macrophages (Figs. 5–8). Moreover, incubation of surfactant with protease IV impacted the biophysical function of surfactant such that protease IV inhibited the surface tension-lowering function of LA (Fig. 9).

This is the first paper to demonstrate the effect of a *P. aeruginosa* protease, namely protease IV, on both the innate host defense functions and the biophysical function of pulmonary surfactant.

*P. aeruginosa* secretes a number of virulence factors that aid in its survival and are crucial in the pathogenesis of infection. Four secreted proteases are a component of these factors and include the aforementioned elastase (LasB protease), a LasA protease, alkaline protease, and the recently described protease IV (5, 18). Protease IV is a serine protease that cleaves on the COOH side of lysine-containing peptides as determined by substrate susceptibility test. This 26-kDa protease has now been identified by Edman degradation as *P. aeruginosa* protease IV (lysyl endopeptidase; EC 3.4.21.50; Fig. 1). In this paper, the other degradative protein has now been identified by Edman degradation as *P. aeruginosa* protease IV (lysyl endopeptidase; EC 3.4.21.50; Fig. 1). In this paper, it is demonstrated in vitro that protease IV degrades SP-A and SP-D in a time- and concentration-dependent manner (Figs. 2 and 3) and, moreover, degrades SP-A, SP-D, and SP-B in BALF (Fig. 4). The degradation of SP-A and SP-D resulted in the inhibition of their ability to aggregate bacteria and enhance bacterial uptake by alveolar macrophages (Figs. 5–8). Moreover, incubation of surfactant with protease IV impacted the biophysical function of surfactant such that protease IV inhibited the surface tension-lowering function of LA (Fig. 9).

This is the first paper to demonstrate the effect of a *P. aeruginosa* protease, namely protease IV, on both the innate host defense functions and the biophysical function of pulmonary surfactant.

*P. aeruginosa* protease IV reduces its biophysical activity. The ability of surfactant to reduce surface tension within the lung is dependent on the interactions of surfactant phospholipids and primarily the hydrophobic SPs. To investigate the effect of protease IV on the biophysical function of surfactant, the ability of LA to reduce surface tension after an incubation period with protease IV was analyzed on a pulsating bubble surfactometer. Figure 9A displays the surface tensions of normal rat LA, rat LA incubated with protease IV, and rat LA incubated with protease IV and TLCK. Surface tensions after the 10-s adsorption period (0 pulsation) and at minimum surface tension (100 pulsations) were significantly greater in the protease IV group compared with both the control LA group and the inhibited protease IV group (protease IV + TLCK; *P* < 0.01). The decrease in biophysical function was specifically due to protease IV as there was no difference in surface tensions over the entire experimental period between the control group and the group

![Graph](image-url)

**Fig. 8.** Degradation of SP-D by PIV inhibits bacterial association with AM. SP-D (12.5 μg/ml) was incubated in the presence or absence of PIV (5 μg/ml) at 37°C for 24 h. Subsequently, freshly isolated rat AM (5 × 10⁵) were incubated with FITC-labeled *E. coli* strain HB101 (5 × 10⁷ cfu) and SP-D degradation reaction volume (10 μl) equaling a final concentration of 1 μg/ml of SP-D for 1 h at 37°C. Bacterial association was measured by FACS analysis and expressed as the mean fluorescence of the FITC-positive macrophages: A: mean and SD of 3 independent experiments as a percentage of AM + *E. coli* (AM + EC) for the AM + *E. coli* + SP-D (AM + EC + SP-D) and AM + *E. coli* + degraded SP-D (AM + EC + D SP-D). B: individual experiment with the corresponding degree of SP-D degradation shown by Western blot.

**Incubation of pulmonary surfactant with protease IV reduces its biophysical activity.** The ability of surfactant to reduce surface tension within the lung is dependent on the interactions of surfactant phospholipids and primarily the hydrophobic SPs. To investigate the effect of protease IV on the biophysical function of surfactant, the ability of LA to reduce surface tension after an incubation period with protease IV was analyzed on a pulsating bubble surfactometer. Figure 9A displays the surface tensions of normal rat LA, rat LA incubated with protease IV, and rat LA incubated with protease IV and TLCK. Surface tensions after the 10-s adsorption period (0 pulsation) and at minimum surface tension (100 pulsations) were significantly greater in the protease IV group compared with both the control LA group and the inhibited protease IV group (protease IV + TLCK; *P* < 0.01). The decrease in biophysical function was specifically due to protease IV as there was no difference in surface tensions over the entire experimental period between the control group and the group

![Graph](image-url)

**Fig. 8.** Degradation of SP-D by PIV inhibits bacterial association with AM. SP-D (12.5 μg/ml) was incubated in the presence or absence of PIV (5 μg/ml) at 37°C for 24 h. Subsequently, freshly isolated rat AM (5 × 10⁵) were incubated with FITC-labeled *E. coli* strain HB101 (5 × 10⁷ cfu) and SP-D degradation reaction volume (10 μl) equaling a final concentration of 1 μg/ml of SP-D for 1 h at 37°C. Bacterial association was measured by FACS analysis and expressed as the mean fluorescence of the FITC-positive macrophages: A: mean and SD of 3 independent experiments as a percentage of AM + *E. coli* (AM + EC) for the AM + *E. coli* + SP-D (AM + EC + SP-D) and AM + *E. coli* + degraded SP-D (AM + EC + D SP-D). B: individual experiment with the corresponding degree of SP-D degradation shown by Western blot.

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![Graph](image-url)
epithelial damage within 3 h after injection into the corneal stoma, and *Pseudomonas* strains that produce protease IV are highly damaging to rabbit and mouse corneas, whereas strains that are deficient in protease IV have reduced corneal virulence. To date, the effect of protease IV on other organ systems has not been investigated.

The current study demonstrates that protease IV degraded the pulmonary innate host defense molecules SP-A and SP-D in a time- and dose-dependent manner. Incubation of SP-A with protease IV resulted in qualitatively decreased concentrations of SP-A dimer and monomer with the appearance of an intermediate SP-A species as early as 1 h after incubation with protease IV. Previously, neutrophil elastase and *P. aeruginosa* elastase were shown to degrade SP-A in a similar manner (22, 25, 30); however, *P. aeruginosa* elastase resulted in the appearance of a 20-kDa SP-A fragment, whereas degradation of SP-A by protease IV resulted in only very small peptide fragments as confirmed by silver staining (data not shown). From the complete degradation of SP-A, it would be assumed that its immunological functions would be abrogated. To confirm this speculation, we examined two different SP-A functions after degradation by protease IV by utilizing two different types of bacteria. Degradation of SP-A after incubation with protease IV impaired its ability to aggregate *S. typhimurium* and also abolished SP-A-mediated increased uptake of *E. coli* by alveolar macrophages. In vivo, this would translate into decreased clearance of bacteria from the lung. Of note, SP-A null mice, when inoculated with *P. aeruginosa*, have impaired clearance of the bacteria, further enforcing the importance of SP-A in the protection from this pathogen (20). Therefore, this opportunistic organism may secrete protease IV to aid in its survival and colonization by degrading a component of the innate host defense within the lung.

Recently, there has been a series of reports describing the degradation of SP-D by different biologically produced proteases. *P. aeruginosa* elastase, a zinc metalloprotease, degraded SP-D by cleaving in the COOH-terminal carbohydrate recognition domain, resulting in an ~35-kDa fragment (1). In addition, three active neutrophil serine proteases: neutrophil elastase, proteinase-3, and cathepsin G also degraded SP-D within the conserved subregion of the COOH-terminal lectin domain, resulting in a relatively stable 35-kDa fragment (15). In the current study, it was demonstrated that *P. aeruginosa* protease IV also degraded SP-D to an ~35-kDa fragment within 1 h of incubation time. However, in the presence of protease IV, this fragment was relatively unstable and degraded over time, whereas the degradation by the previous proteases resulted in a relatively stable fragment. Interestingly, all of these biologically relevant proteases degraded SP-D to a similar-sized fragment; however, more specifically, *P. aeruginosa* protease IV degraded SP-D to a slightly larger fragment than *P. aeruginosa* elastase as observed by Western blot analysis (unpublished observation). These data suggest that there may be a region of SP-D that is highly accessible to different proteases resulting in comparable sized fragments but with distinct cleavage sites.

As with SP-A, we investigated the functional significance of SP-D degradation by protease IV. As seen with SP-A, degraded SP-D failed to aggregate *S. typhimurium* and no longer enhanced *E. coli* uptake by alveolar macrophages. Interestingly, when examining the degree of SP-D degradation that corresponded with individual aggregation and bacterial uptake assays, we discovered individual experiments in which the 35-kDa fragment was still present after 24 h of incubation with protease IV. In these experiments, as shown in Figs. 6B and 8B, the SP-D fragment formed by protease IV had lost its host defense functions, which is in agreement with observations from the previous studies (1, 15). Of particular importance, this SP-D fragment was observed within the first hour of incubation with protease IV, and together with the previous observations, this suggests that protease IV can rapidly inhibit the host
defense function of SP-D, which could be an important initiating step in colonization of the lung by P. aeruginosa.

Investigations of pulmonary surfactant recovered from cystic fibrosis patients and patients with severe pneumonia have documented decreased lavage levels of SP-A and SP-D, unchanged phospholipids levels but diminished surface tension-reducing properties (11, 13, 27, 31). In the current study, P. aeruginosa protease IV decreased the adsorption kinetics and increased minimum surface tension of isolated LA. This effect was attributed to protease IV since specific inhibition of its proteolytic activity by TLCK abrogated any decrease in biophysical function of the LA. SP-A was degraded in the LA incubated with protease IV, and this degradation was absent when TLCK inhibited protease IV activity. Unfortunately, it was not possible to convincingly demonstrate that SP-B was degraded in these samples and therefore we cannot definitively conclude whether impaired biophysical properties are due to degradation of SP-A alone or SP-A and SP-B. Importantly, a study by Liau et al. (22) reported that degradation of SP-A, SP-B, and SP-C resulted in higher adsorption rates and higher minimum surface tension values compared with controls, whereas degradation of SP-A alone resulted in only altered adsorption rates. These previous findings in combination with the detectable degradation of SP-B in our BALF experiments (Fig. 4C) suggest that there may have been a nondetectable degradation of SP-B in our LA samples. Nevertheless, we have documented that P. aeruginosa protease IV specifically alters the biophysical function of pulmonary surfactant by impairing adsorption and increasing minimum surface tension. Importantly, protease IV had no effect on the concentration of phospholipids, and the restoration of LA function when protease IV was inhibited with TLCK indicates the observed decrease in function was not the consequence of nonspecific protein inhibition (2). In this context, proteolytic degradation of SPs, such as that reported in the current study, may explain the loss of surfactant function even when overall phospholipid levels are unchanged in a situation of acute lung injury.

In conclusion, protease IV can be included with elastase as P. aeruginosa proteases that degrade SP-A and SP-D. Importantly, though, production of protease IV can be regulated in a different manner than P. aeruginosa elastase (39), demonstrating redundancy in the P. aeruginosa repertoire, undoubtedly contributing to its success as an opportunistic pathogen. In the present study, we have demonstrated that P. aeruginosa protease IV degrades SP-A and SP-D, resulting in reduction in host defense functions of these SPs, which may contribute to colonization of the lung. Additionally, protease IV alters the biophysical function of surfactant by inhibiting the surface tension-reducing property of this material, thus potentially contributing to decreased lung function. Together, these in vitro observations suggest that P. aeruginosa protease IV may play a significant role in both colonization of the lung and the progression of the associated acute lung injury. Future in vivo studies are important to determine the contribution of protease IV to the pathophysiology of the lung injury associated with P. aeruginosa.

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