Disruption of Respiratory Cilia by Proteases Including Those of *Pseudomonas aeruginosa*

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Pseudomonad proteases disrupted the function and structure of demembranated cilia (axonemes) extracted from porcine tracheae. Proteolytic degradation by the two pseudomonad proteases elastase and alkaline protease and by trypsin and subtilisin impaired motility of ATP-activated axonemes. In addition, electron microscopic observation of negatively stained axonemes indicated that exposure to proteases caused dissociation into individual doublet or singlet microtubules. Inhibition of motility and axonemal fraying occurred when axonemes were treated with <5 U of proteolytic activity of any of the four proteases tested. When the effects of 2 U of each protease were compared, trypsin and subtilisin were able to produce immotility in less time than pseudomonad elastase and alkaline protease, while alkaline protease and subtilisin caused the most axonemal fraying in 10 min. Proteolytic digestion of axonemal proteins was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All four proteases cleaved dynein proteins (proteins necessary for motility), though treatment with trypsin resulted in the most extensive solubilization of axonemal proteins. Trypsin and subtilisin both produced more changes in the protein profiles of treated axonemes, using fewer units of proteolytic activity, than the pseudomonad proteases. However, the limited alteration of only a few axonemal proteins by pseudomonad proteases indicates that cleavage need not be extensive to produce dysfunction. Thus, ciliary axonemes are susceptible to proteolytic attack. Degradation of axonemal proteins by pseudomonad proteases, which are released during active infection, may contribute to the impaired ciliary function associated with pseudomonad colonization of the respiratory tract.

Certain components of cilia and flagella of organisms that are members of primitive phyla, including algae, protozoans, and echinoderms, are particularly sensitive to proteolytic disruption and, as a consequence, become rapidly dysfunctional (5, 29, 32, 33, 36, 39). We investigated whether proteases in mammalian airways could similarly attack selected structures of respiratory cilia.

In inflammatory states, proteases are present in airway secretions as a result of release from bacterial sources and elicited host granulocytes (6, 25, 30, 31, 34). *Pseudomonas aeruginosa*, which chronically infects the respiratory tract of most patients with cystic fibrosis, produces an elastase as well as an alkaline protease (14, 17, 19). These proteases can be detected in sputum and evoke antibody response in patients colonized with *P. aeruginosa* (9, 10, 18, 22). Both observations indicate that pseudomonad proteases are released in vivo. Because naturally occurring inhibitors are inactivated by pseudomonad proteases (20, 27), the ciliated epithelium is exposed to and unprotected from pseudomonad proteolytic activities.

Instillation of purified pseudomonad proteases produces necrotic lesions of mammalian respiratory epithelium and inflammatory cell infiltrates similar to those in human pseudomonad pneumonia (12, 21, 23). Inoculation of hamster tracheal explants with protease-producing strains of *P. aeruginosa* caused extensive damage and exfoliation of the ciliated epithelium (2). In a model of chronic lung infection utilizing agar beads embedded with *P. aeruginosa*, protease production was required for maximal virulence and histopathologic changes (40). Such whole animal or organ systems (4, 7, 8, 28), however, are complex and provide limited quantitation. Moreover, they prohibit analysis of the impact

of these substances on the molecular architecture of respiratory cilia.

We have developed a system whereby cilia are extracted from mammalian tracheae and isolated free of cellular contaminants (13). These structurally intact, demembranated cilia, referred to as axonemes, retain functional ability, as they resume beating on the addition of ATP. Thus quantitation of dysfunction in the presence of inhibitory factors is possible, analysis of alterations to constituent proteins can be made by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and ultrastructural changes can be evaluated by electron microscopy. These criteria were employed to investigate the ability of pseudomonad proteases to disrupt ciliary structure and function. To further elucidate the proteolytic sensitivity of mammalian cilia, we selected trypsin, which has narrow specificity, and subtilisin, which has broad specificity, for comparison with the pseudomonad proteases.

MATERIALS AND METHODS

Materials. Purified elastase and alkaline protease from *P. aeruginosa* were a gift from K. Morihara, Shionogi & Co., Ltd., Osaka, Japan. The following materials were obtained from the indicated commercial firms: trypsin, soybean trypsin inhibitor, lima bean trypsin inhibitor, azocasein, and subtilisin (Sigma Chemical Co., St. Louis, Mo.), phenylmethylsulfonyl fluoride (PMSF; Bethesda Research Laboratories, Gaithersburg, Md.), and ATP (Calbiochem-Behring Corp., La Jolla, Calif.).

Isolation of tracheal cilia. Cilia were removed from tracheae of freshly exsanguinated pigs by a modification of the method described by Anderson (1), as described previously (13). Briefly, cilia were extracted with a solution of 20 mM Tris hydrochloride (pH 7.5), 0.05 M NaCl, 10 mM CaCl₂, 1

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FIG. 1. CBF of control cilia (○) and cilia treated with 2 U of elastase (●), alkaline protease (▲), trypsin (□), or subtilisin (■). Each point represents the mean CBF $\pm$ standard deviation of active axonemes at a given time interval, as described in the text.

mM EDTA, 7 mM 2-mercaptoethanol, and 0.1% Triton X-100 and were pelleted by subsequent differential centrifugation. PMSF was added to the extraction buffer to a final concentration of 0.5 mM (diluted from a 100 mM stock solution in isopropanol) to inhibit serine protease activity during extraction. The pellet of axonemes was suspended in a solution containing 20 mM Tris hydrochloride (pH 8.0), 50 mM KCl, 4 mM MgSO₄, 1 mM dithiothreitol, and 0.5 mM EDTA (resuspension buffer) to an approximate concentration of 0.5 mg/ml and then rinsed with resuspension buffer (RB) to remove possible traces of Triton X-100 remaining after extraction. In experiments involving trypsin, axonemes were suspended in RB alone. Otherwise, axonemes were suspended in RB containing 0.1 mg of soybean or lima bean trypsin inhibitor per ml. The cilia suspension was assessed by phase-contrast microscopy for activity and contamination. Some variation was observed between axoneme preparations in the extent of the number of axonemes reactivated by ATP, usually 20 to over 50%. Only those preparations which appeared to have at least 50% active axonemes, i.e., one of every two beating, were subsequently used in motility assays.

Protease assay. Isolated axonemes were treated with a solution of the pseudomonad proteases elastase or alkaline protease, subtilisin, or trypsin in RB. Proteolytic activity of these enzyme solutions was measured by the method of Fisher and Allen (11), using azocasein as substrate and a trypsin standard. A range of trypsin concentrations was examined for inhibition of ciliary motility. One or two units were found to be effective. Comparable units of proteolytic activity of subtilisin and the pseudomonad proteases were tested, and an appropriate range of concentrations was selected accordingly for further studies. A 0.5-ml volume of protease solution was added to 0.5 ml of 0.5% azocasein in RB and incubated for 30 min at 37°C. Proteolysis was stopped by the addition of 3.5 ml of 5% trichloroacetic acid, undigested azocasein was removed by centrifugation (1,400 $\times$ g) for 10 min, and 2.0 ml of the supernatant was neutralized with 2.0 ml of 0.5 N NaOH. The A₄₂₀ was read on a Stasar III automated spectrophotometer (Guilford Instru-

ments, Oberlin, Ohio), and proteolytic activity was calculated from a standard curve derived using 0.5 to 2.0 μ g of trypsin per ml in RB. One unit of proteolytic activity was defined as the activity equivalent to 1 μ g of trypsin per ml. One unit of subtilisin, pseudomonad elastase, and pseudomonad alkaline protease contained approximately 0.2, 0.1, and 1.0 μ g of enzyme per ml, respectively.

Protein assay. Samples of isolated axonemes (300 μ l) were treated with an equal volume of protease solution for 10 min at 37°C. Trypsin activity was stopped by the addition of 600 μ l of 0.1 mg of soybean or lima bean trypsin inhibitor per ml in RB. Subtilisin and the two pseudomonad proteases were inhibited by the addition of 600 μ l of 1.0 mM PMSF and 0.2 M EDTA in RB, respectively. The total protein concentration of control and protease-treated samples was determined by the microdilution method with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.), with ovalbumin as the standard. The axonemes were then pelleted and saved for SDS-PAGE, and the protein concentration of the supernatant was determined. The percentage of total protein present in the supernatant was calculated for each sample.

Inhibition of reactivated cilia by proteases. A 20- μ l volume of cilia suspension was mixed with an equal volume of a solution containing 10 mM ATP, 20 mM Tris hydrochloride (pH 8.0), 0.4 M potassium acetate, 6 mM MgSO₄, 0.5 mM EDTA, and 1 mM dithiothreitol, and 40 μ l of either RB or protease diluted in RB. Forty microliters was placed on a microscope slide and viewed (at 37°C) by oil immersion phase-contrast optics at $\times$ 1,500 magnification on a Zeiss microscope. Video recordings were made with a video camera with a Newvicon image tube (Dage MTI, Inc., Michigan, Ind.) and a Beta-format recorder (NEC Home Electronics Inc., Elk Grove Village, Ill.). Axonemes were recorded at timed intervals until they were inactive. Beat frequency was determined by timing beating during slow motion replay (13). The calculated beats per second were multiplied by the ratio of recorded time at reduced speed to real time. Beat frequency was determined on one or two fields per sample per time period.

Electron microscopy. Ten minutes after mixing axonemes, ATP, and RB or protease solutions for motility assays, the 40 μ l of sample not placed on the microscope slide for observation of ciliary beating was fixed for electron microscopy by the addition of 40 μ l of 37% formaldehyde diluted 5:1 with RB. Approximately 10 μ l of this axoneme sample was placed on a Formvar, carbon-coated grid for 30 s. Fluid was then drawn off with filter paper, and a drop of 2% potassium phosphotungstic acid was applied to the grid. After 30 s this was removed with filter paper, the grid was air dried and examined with an Hitachi H-600 electron microscope at 75 kV and at instrumental magnifications ranging from $\times$ 12,000 to $\times$ 50,000.

The extent of axonemal fraying was quantitated by placing

TABLE 1. Inhibition of motility by proteases

Sample ^a	Time to immotility (min)	
	Mean ^b	Range
Control	>30	>20->40
Alkaline protease	11	8-15
Elastase	12	7-15
Subtilisin	7	6-7
Trypsin	5	3-7

^a Protease-treated samples were treated with 2 U of proteolytic activity.

^b Mean of three experiments for each protease.

FIG. 2. Effect of proteases on axonemal ultrastructure. Isolated axonemes were treated for 10 min with either resuspension buffer (control cilia) (A) or pseudomonas elastase (B) in the presence of ATP. Bar, 0.5 μ m.

a grid (8 by 10 inches [about 20.3 by 25.4 cm], 1-inch [2.54-cm] squares) over each micrograph and scoring any axonemes lying below the intersection of two grid lines as intact, fraying, or single microtubules. Axonemes were enlarged to a final magnification of $\times 36,000$. Sixty-three grid intersections were considered for each micrograph, and 10 micrographs were counted for each sample. Micrographs were randomized and scored without knowledge of sample treatment. Experiments in which controls had a frequency of intact axonemes of less than approximately 0.6 were discounted.

SDS-PAGE. The axoneme pellet obtained as described above (see protein assay) was suspended in 100 μ l of a sample buffer solution of 0.1 M Tris hydrochloride (pH 6.8), 2% SDS, 1% dithiothreitol, 20% glycerol, and 0.004% bromophenol blue, heated at 100°C for 5 min, and loaded onto a vertical slab gel. The separating gels were either 4 to 6% or 4 to 15% gradient gels. Electrophoresis was performed under standard SDS-dissociating buffers and conditions (24), and gels were stained with silver stain (26). Densitometer scans of the stained gels were made with a soft laser densitometer (Biomedical Instruments, Inc., Chicago, Ill.). Areas of se-

TABLE 2. Decrease in frequency of intact axonemes following treatment with 2 U of proteolytic activity for 10 min

Sample	Frequency (range) of ^a :	
	Intact axonemes	Damaged axonemes ^b
Control	0.75 (0.58–0.87)	0.25 (0.14–0.47)
Alkaline protease	0.16 (0.01–0.32)	0.84 (0.68–0.98)
Elastase	0.34 (0.28–0.50)	0.66 (0.50–0.72)
Subtilisin	0.18 (0.03–0.49)	0.82 (0.50–0.97)
Trypsin	0.40 (0.17–0.52)	0.60 (0.48–0.83)

^a Frequency of observation at grid transcripts, as described in the text, given as mean and range from four experiments.

^b Fraying axonemes or individual microtubules.

lected peaks were measured with a planimeter and expressed as a percentage of the total area measured for their respective gel lanes.

RESULTS AND DISCUSSION

Motility. To test the differential effects of pseudomonad proteases on the function of isolated respiratory cilia, the motility of axonemes reactivated with ATP was monitored in the presence or absence of proteases. Ciliary beat frequency (CBF), a measure of motility, was consistent in different axoneme preparations. The CBF of control cilia from four motility assays was 11.0 ± 1.6 Hz at 37°C.

A representative motility assay is shown in Fig. 1. The beat frequency of control cilia ranged from 10 to 11 Hz for at least 20 min, was slightly less at 30 min, and was 0 by 40 min. Axonemes treated with 2 U of elastase, alkaline protease, trypsin, or subtilisin had initial beat frequencies similar to or only slightly less than those of untreated control axonemes (9 to 10 Hz), before becoming precipitously immotile in less than 15 min. Therefore, inhibition of ciliary activity by proteases was observed as a complete cessation of active axonemes over time, rather than a gradual slowing of CBF.

Typically, 2 U of either trypsin or subtilisin caused immotility within approximately 5 min, whereas a similar concentration of the two pseudomonad proteases inhibited axonemal beating by 10 to 15 min (Table 1). Thus, pseudomonad proteases inhibited ciliary activity but were less active than either trypsin or subtilisin on the basis of equivalent hydrolysis of azocasein.

Ultrastructure. Treated and control axonemes were negatively stained and examined by electron microscopy to assess structural alterations correlating with the inhibition of ciliary function, as described above. The majority of control axonemes were intact (Fig. 2A), though some dissociation into individual doublet microtubules that comprise the axoneme was observed (Table 2). Figure 2B shows a typical effect of proteases, in this case elastase, on a cilia preparation in which damaged axonemes were in the process of fraying or had completely dissociated into microtubules. Axonemal fraying following exposure to proteases was quantitated, and the frequencies of intact and frayed axonemes in control and protease-treated samples are shown in Table 2. The different axoneme preparations varied in their proportion of intact control axonemes and the degree of fraying following exposure to proteases. However, in all preparations, 2 U of proteolytic activity of any of the four proteases tested produced a measurable increase in damaged axonemes. At equivalent units of proteolytic activity, alkaline protease and subtilisin typically caused more axonemal fraying than elastase or trypsin. This dissociation, or fraying, of intact axonemes into their constituent microtubules dem-

FIG. 3. SDS-PAGE of control axonemes (RB) or axonemes treated for 10 min with alkaline protease (AP; 2.7, 5.0, and 7.0 U), elastase (E; 1.0, 3.5, and 5.8 U), trypsin (T; 1.0 and 2.0 U), or subtilisin (S; 1.1 and 2.4 U). Proteins were electrophoresed into a 4 to 15% gradient gel and stained with silver stain. Molecular weight standards (in thousands [K]) are shown in lane M.

onstrates that pseudomonad proteases degrade ciliary components that are necessary for maintaining the axoneme structure.

There was little difference in the frequency of intact control axonemes in the presence or absence of ATP. Protease-treated axonemes had lower frequencies of intact axonemes in the presence of ATP than in its absence. Thus, ATP was required for maximum fraying.

Loss of motility did not strictly coincide with loss of structural integrity. While the relative activities of elastase and subtilisin were consistent, those of alkaline protease and trypsin were not. In the presence of ATP, alkaline protease was more active than trypsin at causing axonemal fraying, though it was less active than trypsin in disrupting function. Therefore, we conclude that these proteases damage different axonemal components, some of which result in dysfunction without observable structural alteration.

Axonemal analysis by SDS-PAGE. To examine the correlations that exist between proteolytic digestion of specific proteins and dysfunction in protease-treated axonemes, we used SDS-PAGE to detect changes in the protein profiles of treated and untreated axonemes. A 4 to 15% gradient gel, resolving a wide molecular weight range of proteins, re-

TABLE 3. Solubilization of axonemal proteins by proteases

Sample	Units	Difference in percent protein in supernatants of control and treated axonemes		No. of expts
		Mean (%)	Range (%)	
Alkaline protease	2–4	7.7	5.0–13.9	4
Elastase	2–3	5.8	1.4–8.5	3
Subtilisin	1–2	4.8	4.3–5.3	2
Trypsin	1–2	24.1	11.6–42.1	5

FIG. 4. SDS-PAGE of control axonemes (RB) or axonemes treated for 10 min with alkaline protease (AP; 2.7, 5.0, and 7.0 U), elastase (E; 1.0, 3.5, and 5.8 U), trypsin (T; 1.0 and 2.0 U), or subtilisin (S; 1.1 and 2.4 U). Proteins were electrophoresed into a 4 to 6% gradient gel and stained with silver stain. Molecular weight standards (in thousands [K]) are shown in lane M. The three major dynein proteins, indicated by lines to the right (1, 2, and 3), migrated with molecular weights of 308,000 to 330,000. Protease-induced bands 3a and 4 are best observed as the two bands beneath the band 3 region of subtilisin (2.4 U)-treated axonemes.

vealed relative resistance to digestion of many of the major protein bands (Fig. 3). In addition, 2 U of trypsin solubilized more of the total protein from the axonemes than did the other three proteases (Fig. 3).

Approximately 40 to 45 distinct protein bands could be distinguished in control axonemes electrophoresed into 4 to 15% gradient gels. Treatment with 2 U of trypsin or subtilisin altered the migration of greater than one-half or one-fourth of these bands, respectively. On the other hand, treatment with 2 to 4 U of elastase or alkaline protease affected the migration of less than 5 of the protein bands detected in control axonemes. Thus, trypsin in particular, and subtilisin to a lesser extent, cleaved more axonemal proteins, with the same or lower units of proteolytic activity, than did the pseudomonad proteases. This is consistent with the results of motility experiments in which trypsin and subtilisin appeared to be more active per unit of proteolytic activity at inhibiting axonemal beating than were elastase and alkaline protease.

These results were supported by quantitation of the amount of protein solubilized from the axoneme. The increase in the percentage of protein in the supernatant of treated axonemes minus that in the control is shown in Table 3. Treatment with alkaline protease and elastase, at 2 to 4 U of proteolytic activity, and subtilisin, at 1 to 2 U of proteolytic activity, produced less than a 10% change in the proportion of total protein present in the supernatant. Trypsin solubilized more axonemal protein, representing a 24% increase in supernatant protein when compared with that in buffer controls (Table 3). As indicated by both SDS-PAGE

and the results with elastase, alkaline protease, and subtilisin, minimal digestion of peptides from a few key proteins of this complex structure apparently is sufficient to disrupt ciliary structure and function.

We have previously identified dynein protein bands by SDS-PAGE (13) and looked here for protease-induced changes in these proteins. Dynein proteins cross-bridge adjacent doublet microtubules and are responsible for generating the force necessary for motility through hydrolysis of ATP (36, 37). Dynein proteins were resolved on 4 to 6% gradient gels as four major protein bands (the middle two are referred to as a doublet, band 2), migrating with apparent molecular weights of 308,000 to 330,000 (Fig. 4). The first band (band 1), the lower band of the doublet (band 2), and the third band (band 3) have been shown previously to be associated with ATPase activity and thus are dynein proteins (13). It is still unclear whether the upper doublet band (band 2) is also a component of the dynein arms.

All four proteases cleaved dynein proteins (Fig. 4). The pattern of dynein band cleavage seen in the gel shown in Fig. 4 was quantitated from planimetry measurements of densitometer scans (Fig. 5 and 6). The total amount of protein in bands 1 to 3, and protease-induced bands 3a and 4, appearing as two bands beneath band 3 (represented by the total area measured per gel lane; Fig. 5) decreased with increasing units of proteolytic activity, indicating a progressive cleavage of all dynein proteins into smaller molecular weight fragments. Bands 1 and 3 were both susceptible to proteolytic cleavage (Fig. 4 and 6). The loss of protein from these two bands coincided with the appearance of bands 3a

FIG. 5. Relative change in the total amount of protein in dynein bands 1 to 3 and cleavage products (bands 3a and 4) following a 10-min treatment with increasing units of proteolytic activity. The amount of protein in bands 1 to 4 is represented by the total area for these four protein bands, as measured by planimetry from densitometer scans of the 4 to 6% gradient gel shown in Fig. 4. Axonemes were treated with alkaline protease (○), elastase (●), trypsin (□), or subtilisin (■).

and 4, presumably cleavage products, with the exception of alkaline protease-treated axonemes, in which band 4 did not appear. The increase in the relative amount of protein associated with band 2 (Fig. 6) with increasing protease concentrations implied that loss of protein from this band lagged somewhat behind the loss of protein from bands 1 and 3. Thus, the doublet appeared to be relatively more resistant to proteolytic cleavage than did the other dynein proteins. The four proteases tested all attacked dynein proteins, though cleavage of dynein required more units of proteolytic activity of the pseudomonad proteases than either trypsin or subtilisin, which is consistent with results from motility assays.

The digestion of dynein proteins by pseudomonad elastase and alkaline protease, trypsin, and subtilisin showed similar high-molecular-weight cleavage products (bands 3a and 4). Thus, certain terminal regions of these particular proteins appeared more susceptible to proteolytic attack, leaving larger protease-resistant cores. A similar observation was made by Bell and Gibbons (3) in their investigation of the

proteolytic cleavage of dynein proteins isolated from sea urchin sperm flagella.

We conclude that pseudomonad proteases impair function without degrading many of the major axonemal proteins. Furthermore, several proteases with broad or narrow specificities inhibited motility, though our data indicate that despite similarities, different targets were cleaved by different proteases. This is an expected result of the combined multiplicity of axonemal proteins and protease specificities.

While trypsin and subtilisin appeared better able to inhibit motility and digest axonemal proteins than the pseudomonad proteases per unit of proteolytic activity, when activities were compared per unit weight, pseudomonad elastase (0.1 $\mu\text{g/ml} = 1 \text{ U}$) and subtilisin (0.2 $\mu\text{g/ml} = 1 \text{ U}$) might be considered more active against axonemes than alkaline protease (1.0 $\mu\text{g/ml} = 1 \text{ U}$) or trypsin (1.0 $\mu\text{g/ml} = 1 \text{ U}$). Even so, it would seem that any protease acquiring access to axonemal structure, whether of bacterial or host origin, could produce rapid dysfunction.

The importance of pseudomonad proteases in respiratory infections of this microorganism is accepted. Furthermore, synergism between the proteases and other exoproducts of *P. aeruginosa* has been indicated for maximal virulence (12, 16, 40). We have previously reported that *P. aeruginosa* produces phenazine derivatives and alkyl-4-hydroxyquinolines which can inhibit ciliary activity without obvious ultrastructural damage (15). The rhamnolipid of *P. aeruginosa* disrupts ciliated cell membranes (15). During initial stages of pseudomonad colonization of the airways, proteases may act in combination with these cilioinhibitory substances to produce localized ciliary dysfunction. The impairment of mucociliary clearance even locally with consequent inadequate removal of these factors and other toxic substances both from *P. aeruginosa* and elicited inflammatory cells would promote the tissue destruction observed as endpoints of pseudomonad infections in experimental animal or tracheal organ model systems (2, 12, 16, 40).

Although the interaction of various pseudomonad products to effect ciliostasis is plausible, there is also evidence that proteases alone can inhibit ciliary activity and cause disruption of cell contact in the respiratory epithelium (30, 35, 38). The *in vivo* situation is complex, as it contains additional protease-sensitive targets as well as protective barriers. Given this complexity, the contribution of each

FIG. 6. Change in relative amounts of each dynein protein band (bands 1 to 3) and cleavage product (bands 3a and 4) following a 10-min treatment with increasing units of proteolytic activity. The relative amount of protein per band is determined as its percentage of total area measured from densitometer scans of the 4 to 6% gradient gel shown in Fig. 4. Axonemes were treated with alkaline protease (○), elastase (●), trypsin (□), or subtilisin (■).

component requires assessment for complete understanding of protease interaction with respiratory clearance. This study represents an initial step in such studies.

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