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Purification and Characterization of an Active Fragment of the LasA Protein from *Pseudomonas aeruginosa*: Enhancement of Elastase Activity

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A 22-kilodalton protein purified from the culture supernatant fraction of *Pseudomonas aeruginosa* (strains PA220 and PAO1) was found to enhance the elastolytic activity of purified *P. aeruginosa* elastase. N-terminal sequence analysis identified the protein as a fragment of the *lasA* gene product (P. A. Schad and B. H. Iglewski, J. Bacteriol. 170:2784–2789, 1988). However, comparative analysis with the reported LasA sequence indicated that the purified LasA fragment is longer than the deduced sequence reported. The purified LasA fragment had minimal elastolytic and proteolytic activity and did not enhance the proteolytic activity of purified elastase, yet enhanced the elastolytic activity more than 25-fold. The LasA fragment was found to also enhance the elastolytic activities of thermolysin, human neutrophil elastase, and proteinase K. The results presented here suggest that the LasA protein interacts with the elastin substrate rather than modifying elastase.

Pseudomonas aeruginosa elastase is recognized as one of several secreted proteins associated with the virulence of this opportunistic pathogen (20). The majority (74 to 99%) of *Pseudomonas* isolated derived from hospital patients produce the enzyme (10, 20, 27). It is significant that the elastolytic and proteolytic activities of elastase have been demonstrated to inactivate several serum proteins (5, 11, 18, 28) and to digest connective tissues (14, 16, 20). In cystic fibrosis patients, *P. aeruginosa* elastase and neutrophil elastase have been implicated in the tissue destruction associated with this disease as a result of chronic infection by this organism (3, 13). The *P. aeruginosa* elastase structural gene, *lasB*, has been cloned and sequenced (1, 6) and the gene product has been expressed in an *Escherichia coli* host, with resultant elastolytic activity (1).

Recently, a second gene associated with elastolytic activity, *lasA* has been cloned and sequenced (8, 26). The *lasA* gene product complements the mutation in a *P. aeruginosa* strain (PAO-E64) which has been shown to be devoid of elastolytic activity but otherwise expresses normal proteolytic activity (21). Expression of the *lasA* gene in *E. coli* has resulted in the reporting of gene products with molecular weights of 31,000 (9) and 40,000 (26). In each case, cell extracts from *lasA*-transformed *E. coli* expressing the *lasA* gene product enhanced the elastolytic activity of culture supernatant fractions from the mutant *P. aeruginosa* PAO-E64 (26) or other LasA$^-$ mutants (9). Proposed mechanisms for activation include the mediation of optimal folding of the elastase protein by the LasA protein (26) or involvement of the LasA protein in the efficient secretion of elastase (8).

In this report, we describe the purification and characterization of an active fragment (22 kilodaltons [kDa]) of the LasA protein secreted by *P. aeruginosa* and show that this protein enhances the elastolytic activity of *P. aeruginosa* elastase as well as other proteases, including human neutrophil elastase. We purified the LasA fragment and elastase from culture supernatants of *P. aeruginosa* PAO1 and PA220 and determined that the LasA fragment has minimal elastolytic activity but specifically enhances the elastolytic activity of purified *P. aeruginosa* elastase more than 25-fold. Our results showed that the purified LasA fragment is encoded by a *lasA* sequence which differs from the published version and suggest that the purified LasA protein enhances elastolytic activity by interacting with the elastin substrate instead of altering the elastase protein.

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MATERIALS AND METHODS

Bacterial strains. The studies described in this report were performed with two strains of *P. aeruginosa* which have previously been characterized. Strain PAO1 is a wild-type prototrophic strain (12) upon which the *Pseudomonas* genetic map is based and has been described previously with regard to elastase production (21). Strain PA220 is derived from a clinical isolate selected for its high production of extracellular proteins (22).

Elastase purification. Elastase was purified from the culture filtrate of *P. aeruginosa* PA220 and PAO1 by methods which have previously been described (19). Essentially, the enzyme was purified from 3 liters of culture filtrate by sequential ammonium sulfate and acetone fractionations, followed by DEAE-Sephacel (Pharmacia, Inc., Piscataway, N.J.) chromatography with a 2.5- by 80-cm column. Purified elastase was obtained after elution with 0.1 M NaCl in 0.02 M sodium phosphate (pH 8.0). Elastase-containing fractions were pooled, concentrated by ultrafiltration, and stored at −70°C. Elastase remained stable and active (>90%) for more than 1 year when stored under these conditions. Elastase purified in this manner was compared with commercially available elastase produced by Nagase Chemical Ltd. (Fukuchiyoma, Japan). No apparent differences were detectable.

LasA purification. Essentially, the LasA active fragment was purified as a by-product of the elastase purification described above. Initially, the LasA protein was separated from elastase as the pass-through fraction of the DEAE-Sephacel column. Fractions containing LasA were pooled and concentrated by ammonium sulfate precipitation. The resulting precipitate was suspended in 0.025 M diethanolamine (pH 9.5) and dialyzed extensively before being applied to a Mono-P column (Pharmacia) at a flow rate of 1 ml/min. Under these conditions, the LasA protein passed through

the column and was essentially pure. The purified LasA was dialyzed against 0.02 M sodium phosphate (pH 8.0) and stored at −70°C.

Enzyme activity determinations. Elastolytic activity was determined by a modification of the method described by Ohman et al. (21), using measured quantities of enzyme suspended in 3 ml of 0.1 M Tris-maleate buffer (pH 7.0)–1 mM $CaCl_2$. Elastin digestion was measured with insoluble bovine nuchal ligament elastin-Congo red (ICN Biochemicals, Cleveland, Ohio) as the substrate. Reaction suspensions (3 ml) containing 10 mg of insoluble elastin in Tris-maleate buffer, purified protease, and LasA were incubated at 37°C for 2 to 15 h with rapid shaking. The reaction was terminated by adding 2 ml of 0.7 M sodium phosphate buffer (pH 6.0). Solubilized elastin in the supernatant fraction was determined by measuring the A_{495} after filtration with glass microfiber filters (Whatman International Ltd., Maidstone, England). Milligrams of elastin digested were determined by use of a standard curve. Elastolytic specific activity was expressed as milligrams of elastin digested per milligram of enzyme per hour. All assays were run in duplicate with equivalent results. Thermolysin, proteinase K, trypsin, and pronase were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Proteolytic activity was determined with Azocoll (Calbiochem-Behring, San Diego, Calif.) following the instructions of the manufacturer. Essentially, 500 ng of the enzyme to be assayed was added to 0.1 M sodium phosphate buffer (pH 7.0) to a final volume of 5 ml. Azocoll (50 mg) was added, and the mixture was incubated at 37°C for 30 min with rapid shaking. The absorbance of the filtrate was monitored at 520 nm. Proteolytic activity was measured as the increase in absorbance per 30-min incubation. All determinations were run in duplicate with equivalent results.

PAGE and related procedures. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (PAGE) was done by the method described by Laemmli (15) with 15% resolving gels and 4% stacking gels. The isoelectric point (pI) of the purified LasA fragment was determined by an isoelectric focusing procedure which has been previously described (7). Immunoblotting analysis was done on samples of purified elastase and LasA proteins first separated by sodium dodecyl sulfate-PAGE and then transferred to nitrocellulose by a previously described procedure (4).

Protein determinations. Protein concentrations were determined by the Bradford method (2) with bovine serum albumin as the standard.

Antiserum preparation. Essentially, New Zealand White rabbits were initially immunized by intradermal injection with 200 μg of protein suspended in Freund complete adjuvant (Sigma). This was followed by three booster injections (intramuscular, 10-day intervals) of 100 μg of protein in phosphate-buffered saline (pH 7.2). Serum was collected 5 days after the last immunization.

RESULTS

Purification of elastase and LasA. These studies began with the purification of elastase from the culture supernatant fraction of *P. aeruginosa* PA220, a known high producer of elastase (22). However, upon final purification by a previously published procedure (19), elastase appeared to lose a substantial degree of specific activity after elution from a DEAE-Sephacel column. Before passage over the column, the dialyzed acetone-precipitated fraction had a specific activity of 18.3 U (measured as milligrams of elastin-Congo red digested per milligram of protein per hour), whereas the purified elastase eluted from the column had a very low specific activity of 2.0 U. A comparable loss of elastolytic specific activity was found with elastase purified from strain PAO1, with the activity decreasing from 17.5 to 2.0 U. Subsequent analysis of the purified elastase indicated the protein to be highly purified and free from degradation by-products (Fig. 1). Significantly, the final specific activity (both elastolytic and proteolytic) was comparable to that of commercially available elastase.

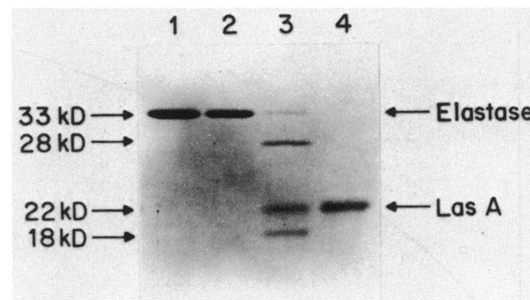

FIG. 1. Sodium dodecyl sulfate-15% PAGE (Coomassie blue stain) of fractions from DEAE-Sephacel chromatography and fast protein liquid chromatofocusing purification. Lane 1, PAO1 elastase; lane 2, PA220 elastase; lane 3, PA220 pass-through; lane 4, fast protein liquid chromatofocus-purified LasA from PA220. All samples contain 5 μg of total protein. kD, Kilodaltons.

In an effort to find an explanation for the dramatic loss of elastolytic specific activity upon final purification of elastase, we analyzed the DEAE-Sephacel pass-through fractions for elastolytic activity and/or ability to enhance the elastolytic activity of purified elastase. The DEAE-Sephacel pass-through fraction (from *P. aeruginosa* PA220 or PAO1) contained significantly higher (11-fold) elastolytic activity than elastase eluted from the column. Sodium dodecyl sulfate-PAGE analysis of the DEAE-Sephacel pass-through fraction revealed the presence of several proteins, including a trace amount of elastase (Fig. 1, lane 3). Subsequent fractionation of these proteins by fast protein liquid chromatofocusing provided a highly pure 22-kDa protein (Fig. 1, lane 4) which was found to be associated with the ability to enhance the elastolytic activity of purified elastase. Figure 2 demonstrates the increasing specific elastolytic activity with a fixed amount of purified elastase incubated with increasing quantities of this 22-kDa protein in the presence of excess elastin substrate. These results indicate a 25-fold increase in specific elastolytic activity of purified *P. aeruginosa* elastase in the presence of the highly purified 22-kDa protein. Interestingly, identical results were consistently obtained in repeat assays with elastase purified from strain PAO1 or PA220 when analyzed with the 22-kDa protein isolated from either strain (data not shown).

Identification of 22-kDa protein. In an effort to identify the 22-kDa protein, N-terminal sequence analysis of the purified protein with an Applied Biosystems 470A Protein Sequencer revealed the following sequence: N-Ala-Pro-Pro-Ser-Asn-Leu-Met-. A computer-based homology search indicated an exact match with the deduced amino acid sequence of the *lasA* gene product reported by Schad and Iglewski (26), beginning at amino acid 237. Based on this information and the proposed function of the *lasA* gene product, the purified 22-kDa protein appears to be an active fragment of the *lasA* gene product. Furthermore, comparison of the purified 22-kDa protein with the proposed sequence of the *lasA* gene product indicates a probable error in the reported sequence

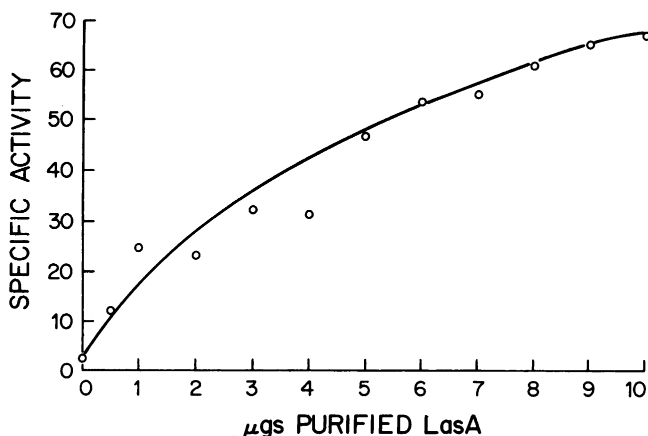

FIG. 2. LasA concentration-dependent enhancement of elastolytic activity with 10 μg of PAO1 elastase. Incubation was for 5 h.

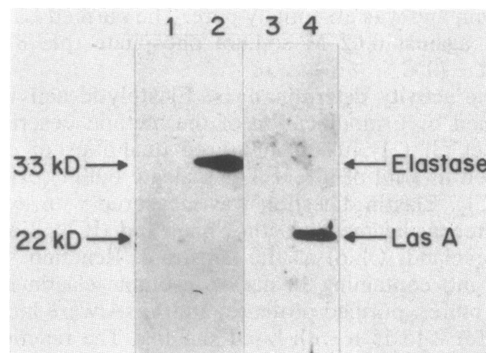

FIG. 3. Immunoblot analysis of purified elastase and LasA with antielastase (lanes 1 and 2) and anti-LasA (lanes 3 and 4). Lane 1, 20 μg of LasA; lane 2, 1 μg of PAO1 elastase; lane 3, 10 μg of PAO1 elastase; lane 4, 1 μg of LasA. kD, Kilodaltons.

since the proposed sequence falls short of the actual size of the purified protein. We propose that the published *lasA* sequence has an additional base inserted between bases 1126 and 1292. If a base is deleted in this region, a new stop codon is generated beginning at base 1468 of the published sequence. Estimation of the molecular mass of the resulting LasA fragment with the additional amino acids is approximately 22.3 kDa. Equally significant is a projected shift of the isoelectric point to a value greater than 10. We determined the isoelectric point of the LasA fragment to be greater than 9.5. This analysis of the published LasA sequence is consistent with our research findings with the purified LasA fragment. On the other hand, if the published sequence was correct, the resulting LasA fragment would have a molecular mass of 15,297 daltons and an isoelectric point of 7.48. Upon further inspection, it was noted that the N terminus of the purified 22-kDa LasA fragment, when aligned with the proposed LasA sequence (Ala-Ala-Leu → Ala-Pro-Pro-Ser), identifies a cleavage site specificity of elastase at the arrow (17). Thus, it is possible that elastase itself may cleave the full-length LasA protein at this site. Obviously, this suggests the probable existence of a LasA precursor molecule and suggest a possible step in the mechanism for the LasA-mediated enhancement of elastolytic activity.

Other studies. To carefully analyze the enzymatic properties associated with these two proteins, each of which may possible affect the other, it was important to determine that the purified proteins did not contain minute traces of the other protein. Specific antisera were separately prepared against the purified elastase and LasA proteins and were used in immunoblotting analysis to detect whether the purified proteins were mutually contaminated. The results in Fig. 3 indicate that purified preparations of LasA do not contain any trace of elastase and that purified elastase does not reveal any trace of the LasA protein. Furthermore, there appeared to be no evidence of fragmentation of the purified proteins, indicating their stability upon purification.

Subsequent comparative analysis of the elastolytic and proteolytic activities associated with purified elastase and the LasA fragment indicated that the LasA 22-kDa fragment has some elastolytic activity. Elastolytic activity assays with 1 to 10 μg of purified LasA incubated with 10 mg of elastin-Congo red in the absence of any elastase released a small quantity of Congo red dye estimated to be equivalent to the digestion of 0.5 mg of elastin during a 15-h incubation. This reaction does not result in the degradation of elastin, yet appears to be limited by the availability of substrate (data not shown). LasA has little, if any, proteolytic activity (Table 1). Additional experiments revealed that the addition of cysteine (2 mM) plus EDTA (5 mM) to the LasA fragment did not enhance its proteolytic activity, suggesting that LasA is not a cysteine protease (Table 1).

It is in studies in combination with other proteases that the LasA fragment appears to demonstrate its activity. For example, the purified LasA fragment was found to be capable of enhancing the elastolytic activity of thermolysin, human neutrophil elastase, and proteinase K; however, the LasA fragment had no similar effect on trypsin or pronase (Table 2). Significantly, the LasA fragment did not enhance the proteolytic activity of elastase (Table 1). Thus, the LasA 22-kDa fragment appears to affect only the elastolytic activity of numerous proteolytic enzymes. It is interesting that the final LasA-enhanced specific elastolytic activity for each of these proteases was roughly comparable (Table 2).

DISCUSSION

In this study, we described the isolation and purification of a 22-kDa protein from the culture supernatant fraction of two separate strains of *P. aeruginosa* (PA220 and PAO1) and demonstrated that this protein enhances the specific elastolytic activity of purified *P. aeruginosa* elastase. N-terminal sequence analysis revealed that this protein is encoded within the published *lasA* sequence (26), thus identifying the 22-kDa elastase-enhancing protein as an active fragment of LasA and defining its location outside the cell along with the mature elastase protein. Interestingly, comparison of the purified LasA fragment with the proposed *lasA* gene product sequence indicated that (i) the 22-kDa protein is an active fragment of the *lasA* gene product, revealing that the entire *lasA* gene product is not required for enhancement of elastolytic activity; (ii) the published *lasA* sequence is incon-

TABLE 1. Proteolytic activity of purified elastase and LasA

Enzyme (μg)	Activity[a]
PA220 elastase (0.5)	1.753
LasA (20)	0.068
LasA (10) + 2 mM cysteine + 5 mM EDTA	0.049
PA220 elastase (0.5) + LasA (0.5)	1.797

[a] Proteolytic activity equals increase in A_{520} per 30-min incubation.

TABLE 2. Enhancement of elastolytic activity of different proteases (10 μg) by LasA (3.0 μg)

Protease	Elastolytic activity[a]	
	− LasA	+ LasA
PAO1 elastase	3.0	32.3
Thermolysin	12.0	23.0
Proteinase K	8.0	15.4
Human neutrophil elastase	22.0	45.0
Trypsin	0	<3
Pronase	0	<3

[a] Elastolytic activity equals milligrams of elastin digested per milligram of enzyme per hour.

sistent when compared with the purified LasA fragment; (iii) the initial *lasA* gene product must exist in a larger precursor form than previously described; and (iv) the *lasA* gene product or some fragment of it is secreted by the cell.

Using the purified LasA fragment, we demonstrated that it specifically increases the elastolytic activity of purified *Pseudomonas* elastase but does not increase the proteolytic activity of this enzyme (Tables 1 and 2). A significant finding reported in these studies is the fact that the LasA fragment enhances the elastolytic activity of several proteases, including human neutrophil elastase, a fact that is potentially of importance to lung pathology associated with chronic *Pseudomonas* infections.

Other proteases presented in this report exhibited initial elastolytic activities greater than those of *Pseudomonas* elastase (Table 2). This fact probably reflects the initial specificity of each of these proteases for the nonmodified elastin substrate. Yet, LasA-mediated enhancement of elastolytic activity in each case resulted in nearly the same level of final specific activity (Table 1).

Previously published reports of LasA-containing cell extracts have proposed that the LasA protein directly modifies *Pseudomonas* elastase to enhance its elastolytic activity and have even suggested that the *lasA* gene product activates a proposed inactive proelastase which cannot be distinguished from elastolytically active elastase (9, 25). The results reported here do not support those interpretations. Since the LasA fragment enhances elastolytic activity of a number of proteases of differing enzymatic classification, it would seem unlikely that LasA interacts directly with each of these enzymes. Preliminary experiments with preincubation of elastin with purified LasA fragment (followed by removal of LasA by washing the insoluble elastin) result in the enhancement of elastolysis upon subsequent exposure to purified elastase (data not shown). It is thus possible that the elastin has been nicked or modified when exposed to LasA. Separate analysis of the isolated LasA fragment revealed that it does exhibit minimal digestion of elastin but appears to be devoid of any proteolytic activity. The low amount of elastolytic digestion detected when elastin is exposed to the purified LasA fragment is suggestive of some nicking action on the elastin substrate, potentially yielding exposed monomeric sequences of elastin for subsequent hydrolysis. Examination of the elastin sequence reveals multiple sites where repetitive alanine residues (four to nine residues) are found between desmosine and isodesmosine cross-links (23). It is possible that LasA-mediated modification of elastin results in the exposure of these polyalanine segments to the proteolytic specificity of elastase, which would then result in the more rapid degradation of elastin. This possibility is supported by the finding that the proteolytic activity of elastase has been shown to result in the rapid digestion of tetra-alanine and penta-alanine sequences (24). A reasonable interpretation of the data presented in this report suggests that the LasA protein interacts with and modifies the elastin substrate in such a way as to increase its susceptibility to subsequent proteolytic digestion by a number of proteases. Evidence in support of this interpretation includes the following: (i) the broad spectrum of proteases which display increased elastolytic (but not proteolytic) activity in the presence of LasA (Table 2), thus suggesting that LasA does not result in an allosteric modification of the enzyme; (ii) LasA-treated elastin is more readily degraded upon subsequent exposure to elastase; and (iii) LasA-treated elastin undergoes minimal digestion consistent with a possible nicking action by LasA.

Finally, it is noteworthy that a fragment of the proposed LasA protein displays functional activity. Of particular interest is the N-terminal sequence of the LasA fragment, which suggests the possibility that the mature LasA protein is cleaved by elastase. Elastase has been shown to have high proteolytic activity directed to the sequence N-Ala-Ala-Leu-Ala with hydrolysis occurring on the C-terminal side of leucine (17, 24). Inspection of the proposed LasA sequence reveals a site which would produce the 22-kDa fragment described in this report, but the remaining sequence of the active fragment does not contain any additional sites for proteolysis by elastase. Thus, it is possible that elastase activates the LasA protein which in turn cooperates in the subsequent proteolysis of elastin. Preliminary results with LasA-specific antisera have revealed the existence of a larger protein in *P. aeruginosa* extracts which appears to be the mature LasA gene product (data not shown). Further developments in this area will require analysis of purified full-length LasA protein.

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