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In Vitro Inhibition of *Pseudomonas aeruginosa* Elastase by Metal-Chelating Peptide Derivatives

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Pseudomonas aeruginosa elastase is a zinc metalloendopeptidase, probably responsible for the tissue destruction observed during infections with this organism. The elastase of a virulent *Pseudomonas aeruginosa* strain (Habs serotype 1) was isolated and found to have a molecular weight of 35,000; it readily degraded elastin and cartilage proteoglycans. A series of amino acid and peptide derivatives containing the metal-chelating moieties hydroxamate, phosphoryl, or thiol were synthesized and tested as potential inhibitors of the enzyme. Inhibition constants (K_is) for the compounds were determined with the chromophoric substrate furylacryloyl-glycyl-L-leucyl-L-alanine. The hydroxamic acid derivatives of benzyloxycarbonyl-glycine, benzyloxycarbonyl-L-leucine and benzyloxycarbonyl-L-phenylalanine had inhibition constants in the range of 11 to 28 μM. The 2-mercaptoacetyl derivatives of L-leucyl-D-phenylalanine and L-leucyl-L-phenylalanine had K_i values of 34 and 1.5 μM, respectively, demonstrating the stereospecificity of the inhibition. The most potent inhibitors tested were 2-mercaptoacetyl-L-phenylalanyl-L-leucine and phosphoryl-L-leucyl-L-phenylalanine (K_i = 0.2 μM). Similar compounds lacking the metal-chelating moiety were about 3 orders of magnitude poorer inhibitors. When the inhibition of the enzyme activity towards azocasein, elastin, or cartilage was examined, inhibitor concentrations approximately 50-fold higher than the respective K_is were required to obtain 60 to 90% inhibition. Virtually complete inhibition was achieved with these substrates at inhibitor concentrations 500-fold higher than the respective K_is (0.1 to 14 mM). Although, 2-mercaptoacetyl-L-phenylalanyl-L-leucine and phosphoryl-L-leucyl-L-phenylalanine exhibited the same affinity to the enzyme, the latter was inferior in inhibiting cartilage proteoglycan degradation. 2-Mercaptoacetyl-L-phenylalanyl-L-leucine represents a class of potent elastase inhibitors that might prove useful in the management of *P. aeruginosa* infections.

Pseudomonas aeruginosa is a human pathogen that often causes fatal infections in burned and other compromised patients (10, 42). In addition, it is known as one of the leading bacterial causes of severe corneal ulcers (24). A variety of observations suggest that, in addition to exotoxin A, extracellular proteases play an important role in the pathogenesis of *Pseudomonas* infections (7, 8, 12, 13, 16, 19, 21, 26, 29, 41, 43). These proteases can digest structural components of the infected tissue and enhance the growth and invasiveness of the organisms. Indeed, in burned mice, highly proteolytic strains are significantly more invasive than strains that produce little or no protease activity, and the survival of the infected animals is enhanced by antiprotease serum (7, 16, 41, 43). The intracorneal injection of *Pseudomonas* proteases produces gross corneal damage and structural alterations, similar to those observed upon authentic infections (12, 14, 19, 21). These include the degeneration of corneal cells, the infiltration of polymorphonuclear leukocytes, the loss of staining of stromal proteoglycans, and the dispersal of collagen fibrils. *Pseudomonas* proteases readily degrade and solubilize corneal proteoglycans after incubation with rabbit stromas (6, 20).

Many strains of *P. aeruginosa* produce two distinct proteases, termed alkaline protease and elastase, the latter being favored as the major contributor to pathogenicity (30, 34, 36, 38, 41). This enzyme is a zinc metalloendopeptidase resembling thermolysin in many of its properties. It contains one catalytically essential zinc atom per mole, and it is inhibited by EDTA, 1,10-phenanthroline, or excess Zn^{2+}. It cleaves

specifically peptide bonds on the amino side of hydrophobic amino acid residues (31, 33, 36).

Highly efficient inhibitors for various zinc metalloproteases can now be prepared by the combination of a specific amino acid or peptide moiety and a transition metal ion-chelating moiety in the same molecule (17, 18, 39, 40). One such an inhibitor, phosphoramidon, is a naturally occurring peptide derivative that inhibits very effectively both thermolysin and *P. aeruginosa* elastase (2, 35). Other similarly efficient inhibitors for *Pseudomonas* elastase have recently been described (38).

In view of the possibility that the elastase is an important determinant in *Pseudomonas* infection, it is conceivable that inhibitors of the enzyme will reduce its destructive effects in vivo and might find clinical application in the future. As a step toward this end, we prepared several metal-chelating peptide derivatives and examined their inhibitory effects on an elastase we isolated from a virulent *P. aeruginosa* strain. Results obtained with the synthetic substrate furylacryloyl-glycyl-L-leucyl-L-alanine (FA-Gly-Leu-Ala) and with azocasein, as well as with the natural substrates elastin and cartilage, are presented in this paper.

MATERIALS AND METHODS

Bacterial strain and growth conditions. A virulent strain of *P. aeruginosa*, isolated from a human corneal ulcer and identified as Habs serotype I, was used throughout this study. Stock cultures were maintained on nutrient agar slants at 4°C. For enzyme production, bacteria were incubated with shaking at 37°C for 48 h in tryptic soy broth without dextrose (Difco Laboratories; 20).

Elastase purification. The enzyme was purified from the bacterial filtrate by ammonium sulfate precipitation (80% saturation) and DEAE-cellulose chromatography essentially as described elsewhere (20). The second DEAE-cellulose step was found unnecessary and therefore omitted. Solutions of the purified enzyme (2.5 to 10 mg/ml) in 0.02 M Tris-hydrochloride–0.5 mM $CaCl_2$ (pH 7.5) were stored in aliquots at −20°C. Under these conditions, the enzyme retained over 90% of its activity after 1 year of storage.

Substrates and inhibitors. Azocasein, elastin, benzyloxycarbonyl-L-leucine, (Z-Leu), and benzyloxycarbonyl-L-phenylalanine (Z-Phe) were from Sigma Chemical Co., and FA-Gly-Leu-Ala was prepared as previously described (5). Phe-Leu and Leu-Phe were prepared by coupling the *N*-hydroxysuccinimide esters (1) of Z-Phe and of Z-Leu with Leu and Phe, respectively, followed by catalytic hydrogenation with palladium over activated charcoal (Pd/C). The hydroxamates, Z-Gly-NHOH, Z-Leu-NHOH (37), and Z-Phe-NHOH were obtained by reacting the corresponding *N*-hydroxysuccinimide esters with NH_2OH. Phosphoryl-L-leucyl-L-phenylalanine (P-Leu-Phe) was obtained by the method of Kam et al. (18). The 2-mercaptoacetyl (HSAc) dipeptides, HSAc-Leu-Phe, HSAc-Phe-Leu, and HSAc-Leu-DPhe, were prepared by reacting the *N*-hydroxysuccinimide ester of S-acetyl-2-mercaptoacetic acid with the corresponding dipeptide methyl ester followed by hydrolysis with NaOH in aqueous methanol and acidification with HCl. Details of the synthesis of these inhibitors will be described elsewhere.

Enzyme assays. Proteolytic activity was determined with azocasein as the substrate. Reaction solutions (1 ml) containing 3 mg of azocasein and 0.005 to 0.02 U of enzyme in 0.05 M Tris-hydrochloride–0.5 mM $CaCl_2$ (pH 7.5) were incubated at 37°C for 15 min. Trichloroacetic acid (10%, 0.5 ml) was added to each reaction tube, and 30 min later, the tubes were centrifuged (16,000 × *g*, 20 min), and the absorbance at 400 nm of the supernatants was determined. One unit of activity is the amount of enzyme that causes an optical density increase of one unit per minute under the assay conditions. For inhibition studies with azocasein, Ca^{2+} concentration was reduced to 0.25 mM.

Elastolytic activity was determined with insoluble elastin as the substrate. Reaction suspensions (1 ml) containing 5 mg of elastin and 13 to 108 μg of purified enzyme in 0.02 M Tris-hydrochloride–0.25 mM $CaCl_2$ (pH 7.5) were shaken at 37°C for 2 to 9 h. EDTA (0.1 M [pH 7.5] 250 μl) was added to stop the reaction, and undigested elastin was removed by centrifugation (12,000 × *g*, 20 min). Solubilized elastin in the supernatants was determined by the method of Lowry et al. (27), with tyrosine as the standard. Added inhibitors were removed by dialysis against 0.02 M Tris-hydrochloride (pH 7.5) before the Lowry analysis.

Peptidase activity was assayed with the chromophoric substrate FA-Gly-Leu-Ala by monitoring the decrease in absorbance at 340 nm (A_{340}) due to the hydrolysis of the Gly-Leu bond (5, 11). Measurements were performed at 25°C with a Gilford 2400-S spectrophotometer, and the reaction solutions (2.5 ml) consisted of 0.1 to 0.2 mM substrate in 0.1 M NaCl–0.05 M Tris-hydrochloride–0.01 M $CaCl_2$ (pH 7.5); all were demetalized by dithizone extraction with CCl_4 and $CHCl_3$. The hydrolysis of the substrate was first order, and rates were determined from plots of ($A_t - A_\infty$) versus *t* (A_t and A_∞ represent absorbance at time *t* and after completion of the reaction, respectively). The strict first-order behavior of the hydrolysis at the low substrate concentration and the poor inhibitory potency of the products indicate that the observed first-order rates are true K_{cat}/K_m values. Inhibition constants (K_is) were derived from plots of K_0/K_i versus inhibitor concentration (K_0 and K_i are the observed rate constants in the absence and presence of inhibitors, respectively) (5). Under these conditions, competitive inhibition was indistinguishable from noncompetitive inhibition. Inhibition constants for the most potent inhibitors, P-Leu-Phe and HSAc-Phe-Leu, were derived from the initial rates of the hydrolysis at a low enzyme concentration (2.7×10^{-9} M).

Protease digestion of cartilage. Thin slices of cartilage were obtained from the tibia and femur of New Zealand albino rabbits (weighing 2 to 2.5 kg) by scraping with a scalpel immediately after sacrificing the animals. The cartilage slices were further cut into fine pieces (approximately 1 by 1 mm) and allowed to dry at 37°C for 1 h. Around 10-mg samples of the dried material were weighed out and each suspended in 1.5 ml of 0.05 M Tris-hydrochloride–0.25 mM $CaCl_2$ (pH 7.5) with or without inhibitors. The suspensions were

shaken at 37°C for 30 min before the addition of enzyme (0.04 U), and incubation was similarly continued for the specified times. Reactions were stopped by adding 0.5 ml of 0.1 M EDTA in 0.05 M Tris-hydrochloride (pH 7.5), insoluble tissue was removed by centrifugation (12,000 × g, 20 min), and the clear supernatants were analyzed for glucuronic acid (4) and for hydroxyproline (46). Supernatants containing inhibitors at concentrations higher than 1 mM were dialyzed against the reaction buffer before analyses.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was performed by the method of Laemmli (23) with 12% separating gels and 4% stacking gels. The samples were incubated (30 min) with 7.5 mM of 1,10-phenanthroline before the addition of SDS to avoid autodigestion during denaturation. Electrophoresis in the absence of SDS was performed with the buffer system of Davis (9), with 12% separating gels and 3% stacking gels. The gels were stained for proteins with Coomassie brilliant blue R-250, and proteolytic activity was located in the gels by the method of Arvidson and Wadström (3), except that casein was replaced by azocasein.

Protein determination. Protein concentrations were determined by the procedure of Lowry et al. (27), with bovine serum albumin as the standard.

Histochemical analyses. Freshly prepared cartilage slices (approximately 15 mg [wet weight]) extending from the cartilage surface to the bone, were incubated for 4 h with or without enzyme as described above. These slices were then fixed in alcohol-formaldehyde solutions (25), and after dehydration, they were embedded in paraffin and sectioned at 5 nm. The sections were stained by the Van Gieson technique to demonstrate collagen and by the colloidal iron method (15) and with alcian blue (44) to demonstrate acid mucopolysaccharides (MPS). Sulfated and nonsulfated MPS were differentiated by alcian blue staining at pH 1 and 2.5, respectively (28).

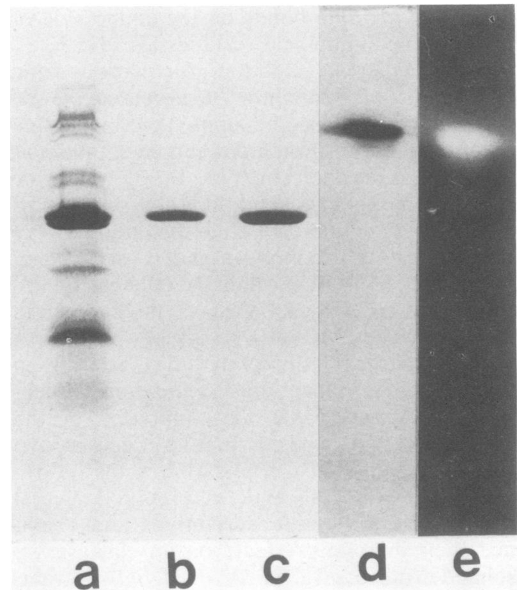

FIG. 1. Polyacrylamide slab gel electrophoresis of *Pseudomonas* elastase in the presence (lanes a, b, and c) or absence (lanes d and e) of SDS. Lane a, Crude enzyme (50 μg); lanes b through d, DEAE-cellulose-purified enzyme (7, 14, and 13 μg, respectively); lane e, a zymogram of the purified enzyme (8 μg) obtained by Coomassie blue staining of an azocasein-agar gel (1.25% agar; 3 mg of azocasein per ml) after its incubation (3 h, 37°C) over an unstained acrylamide gel, similar to lane d.

RESULTS

Purity and elastolytic activity of the protease preparation. The protease used throughout this work was purified from the growth medium of *P. aeruginosa* Habs serotype I (20). SDS-polyacrylamide slab gel electrophoresis of the purified enzyme revealed a single band (Fig. 1, lanes a, b, c). Based on its mobility relative to that of standard proteins (data not shown), we calculated a molecular weight of 35,000 for this band. Polyacrylamide slab gel electrophoresis in the absence of SDS (Fig. 1, lane d) resolved an additional faint band, migrating slightly ahead of the major protein band. Both components possessed proteolytic activity as judged by a zymogram analysis (Fig. 1, lane e). Further attempts to separate these species by several chromatographical techniques, including affinity chromatography on Sepharose-Gly$_3$-DPhe (5), were unsuccessful. This preparation was, therefore, used in all of the following experiments.

Among *Pseudomonas* proteases, the elastase is thought to be the main destructive factor in vivo. For this reason, we considered it crucial to verify that our enzyme has elastolytic activity. Insoluble elastin was incubated with enzyme, and elastin solubilization as a function of both enzyme concentration and time was followed. Practically all of the elastin was solubilized by 80 μg of the enzyme after 4 h of incubation.

Inhibition of hydrolysis of the synthetic substrate FA-Gly-Leu-Ala. Amino acid or peptide derivatives containing hydrophobic amino acid residues and hydroxamate, phosphoryl, or thiol moieties as the metal-chelating function (Table 1, V to XI) were synthesized, and their inhibition constants for the elastase were derived by measuring the inhibition of the enzymatic hydrolysis of the synthetic substrate FA-Gly-Leu-Ala. For comparison, the inhibition constants of similar derivatives which lack the metal-chelating function (Table 1, I to IV) were also determined.

The benzyloxycarbonyl derivatives (Table 1, I and II), which do not contain metal-chelating groups in their structure, were relatively poor inhibitors of the elastase, with inhibition constants in the millimolar concentration range. In contrast, the introduction of a hydroxamic met-

TABLE 1. Inhibitors of *Pseudomonas* elastase[a]

Compound	Concn (μM)	K_i (μM)
I. Z-Leu	1,000–8,000	6,200
II. Z-Phe	1,000–8,000	5,000
III. Leu-Phe	500–5,000	1,100
IV. Phe-Leu	250–2,000	400
V. Z-Gly-NHOH	10–100	28
VI. Z-Leu-NHOH	5–100	11
VII. Z-Phe-NHOH	8–80	21
VIII. P-Leu-Phe	0.1–2.0	0.2
IX. HSAc-Leu-DPhe	10–160	34
X. HSAc-Leu-Phe	0.5–10	1.5
XI. HSAc-Phe-Leu	0.1–0.8	0.2

[a] All reactions were carried out in 0.1 M NaCl–0.05 M Tris–0.01 M $CaCl_2$ (pH 7.5) at 25°C and at FA-Gly-Leu-Ala concentrations of 0.1 to 0.2 mM. Enzyme concentration was generally 55 nM, but it was reduced to 2.7 nM for the inhibition measurements of compounds VIII and XI (see the text).

al-chelating function into the molecule of the inhibitor (Table 1, V to VII) enhanced the degree of inhibition by 2 to 3 orders of magnitude. P-Leu-Phe and the 2-mercaptoacetyl dipeptides were very effective inhibitors, with K_i values in the micromolar concentration range or below (Table 1, VIII to XI). Again, the K_i values of these derivatives were 3 to 4 orders of magnitude lower than those of the corresponding free peptides Phe-Leu and Leu-Phe (Table 1, III and IV). The most potent inhibitors tested were P-Leu-Phe and HSAc-Phe-Leu (K_i = 0.2 μM).

Inhibition of proteolysis of natural substrates. The ultimate goal of this study was to inhibit *Pseudomonas* elastase activity towards natural substrates within the infected tissues. We therefore investigated the inhibition by selected inhibitors of the enzyme activity against the proteins azocasein and elastin and against cartilage as a model tissue. In this series of experiments, the concentrations of the different inhibitors were proportional to their respective inhibition constants (Table 1), namely, at fixed i/K_i ratios. Table 2 demonstrates that with the protein substrates azocasein and elastin, as opposed to the synthetic substrate FA-Gly-Leu-Ala, concentrations of inhibitors approximately 50-fold higher than their respective K_i values were required to obtain a significant degree of inhibition. This effect is likely to be due to a saturation of the enzyme by the substrates at the high protein concentrations used in the assay. Comparing the effectiveness of the inhibitors with the substrates azocasein and elastin, it is evident that all of the tested compounds were approximately equally effective when present at identical i/K_i ratios: at a ratio of 500, the enzyme activity was practically abolished, whereas at a ratio of 50, 70 to 90% inhibition of the enzyme activity was achieved. The degree of inhibition of elastin solubilization by HSAc-Phe-Leu at the lower concentration (10 μM; i/K_i = 50) was significantly lower than 70% (33%), probably due to a gradual loss of this inhibitor by oxidation during the prolonged incubation time (4 h).

Since the major structural components of cartilage are proteoglycans and collagen, we examined their susceptibility to the elastase. Up to a 100% of the total cartilage glucuronic acid and hexosamines (proteoglycan markers) were solubilized upon the incubation of cartilage slices with the enzyme, whereas less than 1% of the total cartilage hydroxyproline (collagen marker) were released under identical conditions. We therefore chose to express the inhibition of elastase activity against cartilage in terms of the percent inhibition of glucuronic acid solubilization. With cartilage as the substrate, the degree of inhibition caused by the hydroxamate, the

TABLE 2. Inhibition of elastase activity towards azocasein, elastin, and cartilage proteoglycans

Inhibitor	Concn[a] (mM)	% Inhibition[b]		
		Azocasein digestion[c]	Elastin[d] solubilization	Cartilage[e] glucuronic acid solubilization
Z-Gly-NHOH	14 (500)	98	95	95
	1.4 (50)	77	84	67
Z-Leu-NHOH	5.0 (500)	98	100	94
	0.5 (50)	76	93	57
P-Leu-Phe	0.1 (500)	98	94	98
	0.01 (50)	83	87	89
HSAc-Phe-Leu	0.1 (500)	96	97	97
	0.01 (50)	77	33	66

[a] Numbers in parentheses represent the ratio i/K_i.
[b] Each value represents the average of two to four independent experiments.
[c] Assay conditions were as described in the text, except that the Ca^{2+} concentration was 0.25 mM.
[d] Elastin was incubated with 30 μg of the elastase for 4 h as described in the text.
[e] Results obtained after 1 h of incubation as described in the text.

phosphoryl, and the 2-mercaptoacetyl derivatives was comparable to that observed with the protein substrates, namely, at an i/K_i ratio of 50, the degree of inhibition was 60 to 90%, and at a ratio of 500, virtually complete inhibition was observed (Table 2). The most efficient inhibitors, HSAc-Phe-Leu and P-Leu-Phe, were examined in further detail, by following the kinetics of the elastase-induced glucuronic acid release from the cartilage in the presence of equal concentrations (1 mM) of the inhibitors. As seen in Fig. 2, HSAc-Phe-Leu at 1 mM completely inhibited the enzymatic release of glucuronic acid from cartilage for at least 6 h. P-Leu-Phe at the same concentration was similarly effective for the first 3 h, but some of the glucuronic acid was released after longer periods of incubation, probably due to the limited stability of the phosphoryl-peptide linkage (48).

Cartilage slices incubated with the enzyme in the absence or the presence of HSAc-Phe-Leu (1 mM) were also analyzed histochemically by staining for acidic MPS and for collagen. No significant difference in staining for sulfated MPS was detected between control cartilage incubated with buffer only (Fig. 3a) and cartilage incubated with enzyme and HSAc-Phe-Leu (Fig. 3c). In contrast, cartilage slices incubated with the same amount of enzyme, but without the inhibitor, showed a dramatic loss of staining (Fig. 3b). The patterns of staining for total MPS, as well as that for the nonsulfated MPS, were essentially the same. Collagen staining of the enzyme-treated cartilage remained practically unchanged as compared with the control (data not shown). These histochemical findings are in agreement with the biochemical data and demonstrate that HSAc-Phe-Leu can protect the integrity of cartilage from the destructive effects of the elastase.

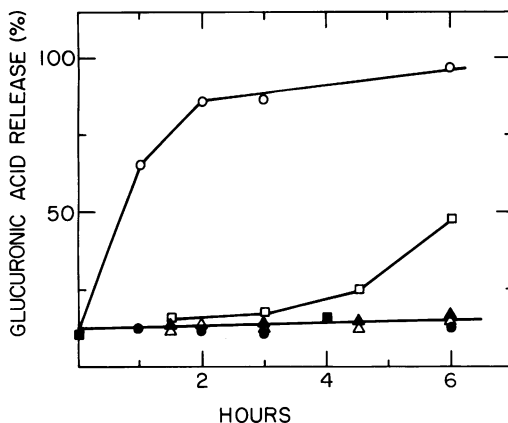

FIG. 2. Solubilization of cartilage glucuronic acid by *Pseudomonas* elastase and its inhibition by HSAc-Phe-Leu and by P-Leu-Phe. Cartilage slices (10 mg) were incubated (1.5 ml of 0.05 M Tris-hydrochloride–0.25 mM $CaCl_2$ [pH 7.5], 37°C) for various time intervals in the absence or presence of enzyme (0.04 U) and inhibitors (1 mM). Suspensions were made 12.5 mM in EDTA and centrifuged, and samples of the supernatants were analyzed for glucuronic acid. Symbols: ●, cartilage alone; ▲, cartilage plus HSAc-Phe-Leu; ■, cartilage plus P-Leu-Phe; ○, cartilage plus enzyme; △, cartilage plus enzyme in the presence of HSAc-Phe-Leu; □, cartilage plus enzyme in the presence of P-Leu-Phe.

DISCUSSION

The characterization of the inhibition properties of the compounds synthesized greatly benefits from the availability of purified *Pseudomonas* elastase. As a source for this enzyme, we selected a *Pseudomonas* strain that produces relatively large amounts of the enzyme (20). Gel electrophoresis patterns, (Fig. 1, lanes a to c) indicate that the enzyme accounts for at least 30% of the total proteins in the bacterial filtrate. The procedure required for isolating the enzyme from the growth medium was, therefore, simple and involved only one chromatographical step (DEAE-cellulose). The molecular weight of the purified enzyme was 35,000, essentially identical with that of thermolysin (45). This value is close to the value of 33,000, derived by Kreger and Gray from SDS-gels (21), and it is in good agreement with the value of 39,500, originally obtained by Morihara et al. by sedimentation studies (36). Gel-filtration experiments performed by several investigators have yielded considerably lower molecular weights for the enzyme, ranging between 20,000 and 25,000 (21, 22, 34, 47). The reason for this discrepancy is not clear. Kreger and Gray (21) proposed that gel-filtration estimates of the enzyme molecular weight are artificially low because the elastase is retarded on the columns due to interaction with the gel matrix, whereas Wretlind and Wadström (47) raised the possibility that the difference in molecular weights could also reflect differences in the strains of *P. aeruginosa* used.

The electrophoresis of the purified enzyme in the absence of SDS revealed two proteolytically active protein bands (Fig. 1, lanes d and e). Since both bands comigrated in SDS gels, they probably represent closely related forms of the same enzyme that differ from each other in charge. This idea is supported by the fact that the activity of the preparation could be completely inhibited (Table 2). A similar microheterogeneity of *Pseudomonas* elastase preparations was also observed by Kreger and Gray (21).

The purified enzyme is clearly an elastase since it degrades elastin readily. Furthermore, it resembles the *Pseudomonas* elastase originally described by Morihara et al. (36) in its pH

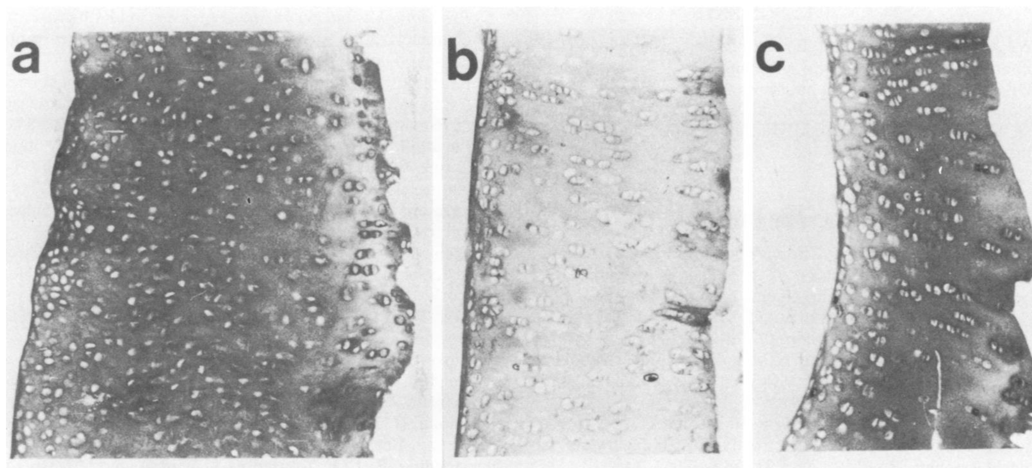

FIG. 3. Photomicrographs of rabbit articular cartilage sections stained with alcian blue to demonstrate sulfated MPS. (a) Cartilage incubated without enzyme (control); (b) cartilage incubated with elastase; (c) cartilage incubated with elastase and HSAc-Phe-Leu (1 mM). Magnification, ×160.

optimum, dependence on metal ions for activity, inhibition by chelating agents and by an excess of Zn^{2+} ions, as well as in its insensitivity to diisopropyl fluorophosphate (DFP) (20). This enzyme is, therefore, a typical *Pseudomonas* elastase, most suitable for the present study.

The majority of the inhibitors described herein contain metal-chelating groups, hydroxamate, phosphoramidate, and thiol. They also contain hydrophobic amino acid residues, as required by the specificity of *Pseudomonas* elastase (31). The metal-binding group contributes dramatically to the effectiveness of the inhibitors as exemplified by the respective K_i values of Z-Leu and Z-Leu-NHOH, Leu-Phe and P-Leu-Phe, or Phe-Leu and HSAc-Phe-Leu (Table 1). The K_i values of the most potent inhibitors (0.2 μM) are similar to the values reported by Holmquist and Vallee for the inhibition of thermolysin by analogous derivatives (17) and are comparable to the values of the elastase inhibitors described recently by Nishino and Powers (38).

Elastin and proteoglycans are connective tissue components and are susceptible to *Pseudomonas* elastase (6, 20, 36). We therefore examined the effect of the inhibitors on the solubilization of elastin and proteoglycans by the enzyme. The inhibition of the solubilization of corneal proteoglycans is of major clinical importance. The chemical structure of the cornea is similar to that of cartilage in that the major structural components of both tissues are collagen and proteoglycans. Yet, cartilage is available in larger quantities than the cornea, and its manipulation during preparation as a substrate for the enzyme is easier than that of the cornea. These reasons led us to employ cartilage as the model tissue in our experiments. The derivatives with the highest affinity for the enzyme, P-Leu-Phe and HSAc-Phe-Leu, were also most effective in preventing the hydrolysis of both elastin and cartilage. Thus, at a concentration of 0.1 mM, both inhibitors abolished the enzymatic hydrolysis of these substrates, as well as that of azocasein (Table 2). However, P-Leu-Phe, as a phosphoramidate, has only a moderate stability at a neutral pH and a very low stability at an acidic pH (48). Hence, upon prolonged incubation with the enzyme and cartilage, it gradually lost the inhibition efficiency as compared with HSAc-Phe-Leu (Fig. 2). The effectiveness of HSAc-Phe-Leu was also demonstrated by the histochemical analysis showing that at 1 mM, it prevented the destructive effect of the enzyme on cartilage (Fig. 3). It should be noted that the strong inhibition of cartilage digestion by HSAc-Phe-Leu pertains to the crude 80% ammonium sulfate-precipitated enzyme preparation, as well as to the purified enzyme (Kessler and Blumberg, unpublished data), indicating that the destructive effect by extracellular *Pseudomonas* enzymes is predominantly due to the elastase.

HSAc-Phe-Leu was highly inhibitory in vitro. It was also effective in inhibiting the elastase activity in rabbit eyes after intracorneal injection of the enzyme (unpublished data). Experiments to further characterize this compound (and others) and to test its effects in experimental models of *Pseudomonas* infections are currently under way.

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