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Purification and Partial Characterization of an Elastolytic Serine Protease of *Prevotella intermedia*

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Elastolytic strains of *Prevotella intermedia* were isolated from pus samples of adult periodontal lesions. Elastase was found to associate with envelope, and it could be solubilized with guanidine-HCl. The enzyme was purified to homogeneity by sequential procedures including ion-exchange chromatography, gel filtration, and hydrophobic interaction chromatography. This elastase was a serine protease, and its mass was 31 kDa. It hydrolyzed elastin powder, but collagen and azodye-conjugated proteins were not degraded by this enzyme. Both synthetic substrates for human pancreatic (glutaryl-L-alanyl-L-alanyl-L-prolyl-L-leucine *p*-nitroanilide) and leukocyte elastase (methoxy succinyl-L-alanyl-alanyl-L-prolyl-L-valine *p*-nitroanilide) were hydrolyzed.

Of many species which reside in the gingival crevice and developing periodontal pockets, black-pigmented anaerobic rods including *Porphyromonas gingivalis* and *Prevotella intermedia* have been considered putative pathogens of periodontitis (24, 30, 32, 39). Pathogenicity and virulence factors of *P. gingivalis* have been extensively studied (15, 20, 31, 33). However, those of *P. intermedia*, a closely related species to *P. gingivalis*, have not been as extensively analyzed. We isolated black-pigmented elastin-hydrolyzing strains from clinical materials of adult periodontal lesions which were identified as *P. intermedia* and then purified elastase from one of these strains. This activity is thought to be important in degradation of gingival tissue, as Cergneux et al. demonstrated in vitro breakdown of gingival tissue by human leukocyte elastase in electron microscopic studies (6). We describe in the present report the production, purification, and partial characterization of *P. intermedia* elastase.

MATERIALS AND METHODS

Bacterial strains and cultivation methods. First, pus samples taken from the lesions of five adult periodontal patients who visited our college hospital were inoculated to 2.5% heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.2% yeast extract (Difco), 5% horse erythrocytes, hemin (5 μg/ml), menadione (0.5 μg/ml), and bovine neck ligament elastin (Nakarai Tesque, Kyoto, Japan) and incubated at 37°C for 5 days in an anaerobic glove box filled with a mixture of gases (N_2, H_2, and CO_2 [85:10:5]). Seven isolates from the three patient pus samples showed elastolytic zones around the colonies on the plates. To test the reproducibility of the elastolytic activity of these strains, each elastolytic strain was picked up and inoculated to an elastin plate containing the same ingredients as above and incubated. All the strains tested were confirmed again to be elastolytic. These organisms were streaked on agar plate of general anaerobic medium (GAM; Nissui Seiyaku Co., Tokyo, Japan) supplemented with hemin (5 μg/ml), menadione (0.5 μg/ml), and 1% (wt/vol) bovine neck ligament elastin and incubated for 5 days as described above (Fig. 1). Liquid culture for cell harvest was carried out in hemin and menadione-supplemented GAM anaerobically as described above. Cell growth was monitored turbidimetrically at 660 nm.

Identification of bacterial species. Determination of species of isolated elastolytic organisms were carried out according to the descriptions of Shah and Collins (29). Immunological properties were examined by the methods of Okuda et al. (27).

Assay of enzyme activity. (i) Elastase. Routine assay of elastolytic activity was done with a chromogenic synthetic substrate for pancreatic elastase (7), glutaryl-L-alanyl-L-alanyl-L-prolyl-L-leucine *p*-nitroanilide (pNA) (Peptide Institute Inc., Osaka, Japan). Principles of assay method were those of Erlanger et al. (8). Specific conditions were the same as described in our earlier report (10). Buffer used in the assay system was 0.05 M Tris-HCl buffer (pH 8.0). One unit of activity was defined as the liberation of 1 μmol of pNA per min. Hydrolytic activity of the purified elastase against other pNA derivatives of the peptides, such as succinyl (Suc)Ala-Ala-Ala-pNA (Peptide Institute Inc.), Suc-Ala-Pro-Ala-pNA (Peptide Institute Inc.), Methoxy succinyl (Metsuc)-Ala-Ala-Pro-Val-pNA (Sigma Chemical Co., St. Louis, Mo.), and Suc-Ala-Ala-Pro-Phe-pNA (Sigma Chem. Co.) was also examined.

(ii) Protease. Hydrolysis of azocasein (Sigma) and azoalbumin (Sigma) was tested by the method of Leighton et al. (17). Degradation of type I Collagen (Sigma) was tested by ninhydrin reaction (22) as described earlier (10). Activity against elastin powder was detected with agar plates containing elastin as follows. Bovine neck ligament elastin (Nakarai Tesque) was embedded in an 0.8% agar plate (pH 8.0) at a concentration of 1%. The test samples were put onto the surface of the elastin-agar plates and incubated at 37°C for 18 h. Degradation of elastin was judged by formation of clear zones.

Protein determination. Protein was estimated by the method described by Lowry et al. (19).

Electrophoresis. Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (14) for examination of purity of the samples and molecular mass determination by using about 5 μg of purified sample. Molecular mass markers were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa)

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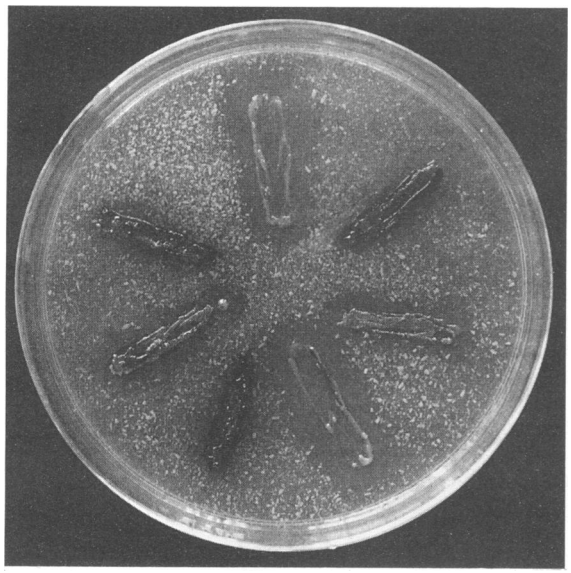

FIG. 1. Hydrolysis of elastin by the isolates on elastin containing GAM agar.

(Pharmacia LKB Biotechnologies, Uppsala, Sweden). Gels were stained with Coomassie brilliant blue R250.

Sonication and preparation of envelope. Cells were harvested by centrifugation at 10,000 × *g* for 10 min and washed twice with a 0.15 M NaCl solution. The cells were disrupted with Insonator model 200M (Kubota Co., Tokyo, Japan) at 9 kHz for 20 min. The sonicated materials were referred to as whole-cell sonicates. To separate the envelope, whole-cell sonicates were centrifuged at 10,000 × *g* for 10 min to remove unbroken cells. The supernatant was then centrifuged at 100,000 × *g* for 90 min, and the pellet (envelope) was collected and washed with 0.05 M Tris-HCl buffer (pH 7.2).

Solubilization of elastase. The envelope samples containing 0.50 U of elastase was suspended in 0.05 M Tris-HCl buffer (pH 7.2), and then the following reagents were added separately and stirred at 4°C overnight: *N*-lauroylsarcosine (1%), Emulgen 109P (polyethylene glycol laurylether, 1%), Triton X-100 (1%), NaCl (0.5 and 1.2 M), and guanidine-HCl (4 and 6 M). Each mixture was then centrifuged at 100,000 × *g* for 60 min. Elastase obtained in the supernatant was considered to represent the solubilized enzyme. The degree of solubilization of elastase was expressed as the amounts of the solubilized enzyme per activity in the untreated envelope (0.50 U).

Purification. Extract of envelope with 6 M guanidine-HCl was used as starting material for elastase purification. When this extract was dialyzed against 0.05 M Tris-HCl buffer (pH 7.2), a significant amount of precipitate occurred in the dialysis tube; this precipitate was then removed by centrifugation at 40,000 × *g* for 15 min. However, since 85% of the elastase activity was recovered in the supernatant, it was applied to a column (2.6 by 20 cm) of Q-Sepharose fast flow (Pharmacia) equilibrated with 0.05 M Tris-HCl buffer (pH 7.2). When the column was washed with 0.05 M Tris-HCl buffer (pH 7.2) containing 1 M NaCl, large amounts of proteins without elastolytic activity eluted from the column. After the A_{280} of the eluates became less than 0.05, the column was eluted with 0.05 M Tris-HCl buffer (pH 7.2) containing 6 M guanidine-HCl, which performed the elution of the proteins with elastase activity. The active fractions were collected, combined, dialyzed against 0.05 M Tris-HCl buffer (pH 7.2) briefly, concentrated in vacuo, and dialyzed against 0.05 M Tris-HCl buffer (pH 7.2) containing 0.15 M NaCl and 3 M guanidine-HCl. This sample was applied to a column (2.4 by 95 cm) of Sephacryl S-300 (Pharmacia) equilibrated with this solution. When the column was eluted with the same solution, elastase eluted as a single peak with a minor protein peak. The active fractions were dialyzed against 0.05 M Tris-HCl buffer (pH 7.2) and further applied to a column (1.6 by 10 cm) of phenyl Sepharose CL-4B (Pharmacia) equilibrated with this buffer (pH 7.2) containing 50% ethylene glycol; no elastase activity was detected in the washings of the column with this solution. However, when the column was eluted with 0.05 M Tris-HCl buffer (pH 7.2) containing 6 M guanidine-HCl, the enzyme eluted in a single peak coincident with a protein peak. The active fractions were collected and dialyzed against Tris-HCl buffer (pH 7.2). This was the purified elastase sample.

Test for heat stability. The purified elastase was heated from 20 to 70°C for 10 min in 0.05 M Tris-HCl buffer (pH 7.2), and the residual activities were assayed.

Effect of pH on the activity. Effect of pH on the activity was tested with different buffers in the assay system of this enzyme activity. Employed buffers were acetate buffer (pH 5 to 6), Tris-maleate buffer (pH 6 to 7.5), Tris-HCl buffer (pH 7 to 9), and borate buffer (pH 9 to 10). Each buffer was used at a final concentration of 0.1 M.

Examination of various reagents on the enzyme. Effects of various reagents on activity were examined at pH 7.5. The purified enzyme samples were preincubated with each reagent at 25°C for 30 min, and the activity was measured. The activity was expressed as the percentage of the values for the control experiment without testing reagents.

RESULTS

Properties and identification of elastolytic organisms. Degradation of elastin by seven isolated strains is illustrated in Fig. 1. All of these seven elastolytic isolates presented jet-black colonies on the blood agar, and interestingly, their main properties were the same; they were gram-negative rods, and fermentation of glucose and sucrose and production of indole were positive. Production of catalase, hydrolysis of esculin, fermentation of lactose and cellobiose, hemagglutination of sheep erythrocytes, and trypsinlike activity were negative. On the basis of these biological properties, these elastin-hydrolyzing strains were identified as *P. intermedia*. Cellular antigen preparations of these strains formed precipitin lines specifically with an antiserum against *P. intermedia* (ATCC 25611), but they did not react to antisera against other black-pigmented anaerobic rods. One of the strains (EL2-1) which exhibits a large elastolytic zone on the elastin-containing plates was chosen as a producer strain of elastase.

Time course of elastase production, cell growth, and cellular location of elastase. Ten milliliters of the whole culture of *P. intermedia* EL2-1 in liquid GAM were taken every day, and turbidity and elastase activity of the samples were estimated. Elastase activity was not detected in the culture supernatant, but it was found in the whole-cell sonicates containing soluble and envelope fractions of the cell. The production of elastase started from day 2, and the amount of the enzyme increased linearly until day 4. The pattern of the cell growth curve was found to be about the same as that of elastase

TABLE 1. Purification of elastase

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)
Guanidine-HCl extract	92.0	7.96	0.09
Q-Sepharose fast flow	14.6	4.22	0.29
Sephacryl S-300	2.8	2.86	1.02
Phenyl-Sepharose CL-4B	0.2	1.85	9.25

production; the times of commencement and cessation of the enzyme accumulation coincided with those of the cell growth. When the whole sonicates containing elastase activity was centrifuged at 100,000 × *g* for 60 min to separate soluble and insoluble (envelope) materials, 84% of the activity was recovered in the envelope, and the rest was found in the cytoplasmic fraction.

Extraction of elastase. From the results mentioned above, elastase is primarily associated with the cell envelope; therefore, we attempted to solubilize the enzyme with various agents. Effective solubilization with the detergents could not be observed. They released at most 16% of the elastase from the envelope. No significant amount of the activity was dissolved with NaCl solutions. Contrarily, 4 and 6 M guanidine-HCl dissolved 64 and 100% of the activity, respectively.

Purification, purity, and molecular weight. Elastase was purified from the guanidine-HCl-dissolved fraction of the envelope 103-fold, with a recovery rate of 23%. Purification is summarized in Table 1. As seen in Fig. 2, the purified enzyme appeared to be homogeneous on SDS-PAGE. Mass of elastase was calculated to be 31 kDa from SDS-PAGE.

Heat stability. Full activity was retained when elastase was incubated at 30°C for 10 min, but half the activity was lost by heating at 53°C, and the enzyme was completely inactivated at 60°C.

Optimum pH. Function of pH on the enzyme activity was tested with various buffers. Maximum activity was found at pH 7.5 to 8.0. No activity was observed below pH 5.0.

Effects of group-specific and divalent ions on enzyme activity. Elastolytic activity in the presence of various reagents is summarized in Table 2. Elastase was completely inhibited by 0.1 mM diisopropylfluorophosphate and phenylmethylsulfonyl fluoride. Inhibition by tosylphenylalanine chloromethyl ketone was also strong. However, neither significant inhibition nor activation was observed with other reagents, except for inhibition by Zn^{2+}.

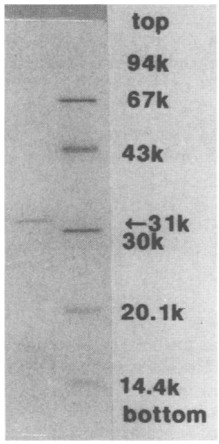

FIG. 2. SDS-PAGE of the purified elastase (lane A) and standard proteins (lane B).

TABLE 2. Effects of inhibitors and metal ions on elastase

Reagent[a]	Concn (mM)	Enzyme activity (%)
Control		100
DFP	0.1	0
PMSF	0.1	0
N-Ethylmaleimide	1.0	102
Iodoacetate	1.0	113
TLCK	0.5	86
TPCK	0.5	6
EDTA	1.0	92
EGTA	1.0	90
1,10-*O*-Phenanthroline	1.0	99
2-Mercaptoethanol	1.0	105
$CaCl_2$	1.0	113
$MgCl_2$	1.0	110
$ZnCl_2$	1.0	38

[a] DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Substrate specificity. Purified elastase most actively hydrolyzed Metsuc-Ala-Ala-Pro-Val-pNA and Glt-Ala-Ala-Pro-Leu-pNA. Suc-Ala-Pro-Ala-pNA was significantly hydrolyzed. Other synthetic substrates were not degraded or were degraded only scarcely. This enzyme was observed to hydrolyze elastin powder, but it released no ninhydrin reaction-positive materials from type I collagen which had been incubated with elastase; therefore, this enzyme has no collagenolytic activity. No hydrolysis of azocasein and azoalbumin was detected (Table 3).

DISCUSSION

Elastase has been implicated in tissue damage because of its possible hydrolytic action on connective tissue elastin. Furthermore, an important role of human leukocyte elastase was demonstrated in tissue damage (1, 38). Electron microscopic observations of the effect of human leukocyte elastase on the breakdown of gingival tissue revealed that this enzyme caused an enlargement of the intracellular spaces of the oral gingival epitherium, interruptions of basement lamina, and loss of collagen in the underlying connective tissue (6). These observations suggest that elastase is responsible for tissue damage of gingiva through hydrolysis of elastin.

TABLE 3. Substrate specificity of elastase

Substrate	Enzyme activity[a] (%)
Glt-Ala-Ala-Pro-Leu-pNA	100
Metsuc-Ala-Ala-Pro-Val-pNA	112
Suc-Ala-Ala-Ala-pNA	0
Suc-Ala-Pro-Ala-pNA	37
Suc-Ala-Ala-pNA	4
Suc-Ala-Ala-Pro-Phe-pNA	1
Azocasein	−
Azoalbumin	−
Collagen (type I)	−
Elastin	+

[a] −, negative in hydrolysis; +, positive in hydrolysis.

To date, a number of proteolytic enzymes, including collagenase (5, 16, 18, 35), trypsinlike protease (15, 20), and fibronectin-degrading enzyme (37, 41), elaborated by periodontopathogens have been considered virulence factors, and their isolation and enzymatic properties were studied.

In microbial elastases, the *Pseudomonas* enzyme has been well investigated, and it has been shown to be a virulence factor of this species (12, 13, 23, 28, 34). According to the report of Balke and Scharmann, *Pseudomonas* elastase is a serine protease and splits the bond between proline and leucine (2).

There are, however, only a few studies of elastase produced by oral bacteria. Murphy reported that 60 staphylococcal strains of 96 isolates from the human oral cavity were positive in elastin production, and a role for oral staphylococci in periodontal disease was postulated (25). Subsequently, Hartman and Murphy studied the production mechanism of elastase by oral *Staphylococcus epidermidis* (11). We detected seven elastolytic strains of *P. intermedia* in three of five patient pus samples, indicating that occurrence of elastolytic *P. intermedia* in periodontal lesions seems to be rather frequent. Nonelastolytic black colonies on the elastin-blood agar plates were found to contain the strains considered to be *P. gingivalis* on the basis of the findings that trypsin-like protease and hemagglutination of sheep erythrocytes were positive (data are not shown). No elastase production was observed in our 10 laboratory stock strains of *P. gingivalis*. These results suggest that elastase production is negative in the other, better-characterized, periodontal pathogen *P. gingivalis*.

Usually membrane-bound proteases can be solubilized with detergents; however, in the present study, elastase in the envelope could not be effectively solubilized with detergents including lauroylsarcosine, Emulgen 109P, and Triton X-100, but it was easily solubilized with guanidine-HCl, suggesting that the association mode of elastase with envelope is different from that of such proteases. At least elastase is not sensitive to inhibition by guanidine, a protein-denaturing agent.

Elastase of *P. intermedia* is a serine protease, since it was inhibited by phenylmethylsulfonyl fluoride. An elastase-like enzyme extracted from human gingival tissue was also demonstrated to be a serine protease (36). Molecular mass (31 kDa) is quite similar to that (30 kDa) of human leukocyte elastase (3) and that (32 kDa) of an *Aspergillus fumigatus* serine protease (9). Nakajima et al. reported that Metsuc-Ala-Ala-Pro-Val-pNA was an excellent substrate for human leukocyte elastase (26). Del Mar et al. showed that Glt-Ala-Ala-Pro-Leu-pNA was very actively hydrolyzed by human pancreatic elastase, as well as Suc-Ala-Ala-Pro-Met-pNA, and that the relative activity of pancreatic elastase against Suc-Ala-Ala-Pro-Leu-pNA was below 1/1,000 of this activity against Suc-Ala-Ala-Pro-Val-pNA (7). However, *P. intermedia* elastase showed about the same activity against Metsuc-Ala-Ala-Pro-Val-pNA and Suc-Ala-Ala-Pro-Leu-pNA. Pancreatic elastase could hydrolyze significantly the synthetic substrate for chymotrypsin (Suc-Ala-Ala-Pro-Phe-pNA) (7), but our elastase had no significant activity against this substrate. Suc-Ala-Ala-Ala-pNA, a traditional substrate for elastase (4), was also not hydrolyzed. The *P. intermedia* elastase could split only elastin, but it could not hydrolyze substrates for general protease, including azocasein and azoalbumin, indicating that the substrate specificity of this enzyme is rather strict.

Although chemical treatment was required to extract elastase from cell components, elastase activity could be demonstrated with whole-cell sonicates or even with washed, intact cells (data are not shown). These observations suggest that elastase has a destructive effect on the surrounding tissue of *P. intermedia*-infected areas in collaboration with other tissue-damaging enzymes such as elastase and collagenase secreted from host cells into gingival crevicular fluid (21, 40).

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