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Isolation and Characterization of a Protease from *Bacteroides gingivalis*

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A protease was purified from *Bacteroides gingivalis* ATCC 33277 culture fluid by sequential procedures including ammonium sulfate precipitation, ion-exchange chromatography, and isoelectric focusing. The enzyme was active against benzoyl-L-arginine-*p*-nitroanilide, carbobenzoxy-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide, azoalbumin, azocasein, azocoll, and *p*-tosyl-L-arginine methyl ester. The molecular weight of the enzyme was about 300,000 as determined by gel filtration. Its isoelectric point was 5.0. The maximum activity was found at pH 7.5, and the optimum temperature for activity was between 40 and 45°C. The apparent K_m value for benzoyl-L-arginine-*p*-nitroanilide was 2 mM. The enzyme was inhibited by sulfhydryl group-blocking reagents, tosyl-L-lysine chloromethyl ketone, and EDTA. Soybean trypsin inhibitor and diisopropylfluorophosphate were not inhibitory.

Although it is clear that periodontal disease is initiated by bacteria, relatively few species may be involved as the causative agents. It has been suggested that many of the gram-negative isolates from periodontitis lesions, including the black-pigmented *Bacteroides* group and particularly *Bacteroides gingivalis*, are strongly associated with the etiology and pathogenesis of periodontal disease (16, 26, 27). One criterion for the identification of *B. gingivalis* from other species of *Bacteroides* is production of a trypsin-like protease from this organism (11). It has been suggested that this enzyme is an important pathogenic factor of *B. gingivalis* in periodontal disease (12). Recently, Yoshimura et al. (34) partially purified this enzyme from the envelope fraction of *B. gingivalis*. We also have found proteolytic activity in the culture fluid of a strain of *B. gingivalis*. In this report we describe the purification and characterization of this enzyme.

MATERIALS AND METHODS

Bacterial strain and cultivation methods. *B. gingivalis* ATCC 33277 was used as the producer strain of protease. The strain was grown in a general anaerobic medium broth (Nissui Seiyaku Co., Tokyo, Japan) supplemented with hemin (5 mg/liter) and menadione (0.5 mg/liter) at 37°C in an anaerobic glove box filled with a mixture of gases (N_2-H_2-CO_2; 85:10:5) for 6 days. The composition of the general anaerobic medium broth was as follows, in grams per liter: peptone, 10.0; soybean peptone, 3.0; proteose peptone, 10.0; digested serum powder, 13.0; yeast extract, 5.0; beef extract, 2.2; liver extract, 1.2; glucose, 3.0; dibasic potassium phosphate, 2.5; NaCl, 3.0; soluble starch, 5.0; L-cystein, 0.3; and thioglycolate, 0.3. The pH of the medium was 7.2.

Assay of enzyme activity. (i) Hydrolysis of Bz-L-Arg-pNA and other *p*-nitroanilide derivatives of amino acids or peptide. Routine assay of protease activity was carried out with a synthetic chromogenic substrate, benzoyl-L-arginine-*p*-nitroanilide (Bz-L-Arg-pNA), by the method of Erlanger et al. (7). The reaction mixture consisted of 2.5 ml of 1 mM Bz-L-Arg-pNA in 0.05 M Tris maleate buffer (pH 7.5), 0.4 ml of 0.05 M Tris maleate buffer (pH 7.5), and 0.1 ml of enzyme. After incubation of the reaction mixture at 37°C for 30 min, 0.5 ml of 5 N acetic acid was added to the reaction mixture to stop the reaction, and the released *p*-nitroaniline was determined by measuring the A_{410}. One unit of activity was defined as the liberation of 1 μmol of *p*-nitroaniline per min under these conditions.

(ii) Hydrolysis of azoalbumin and azocasein. Proteolytic activity against azoalbumin and azocasein was determined by a modification of the method described by Leighton et al. (13). The reaction mixture, containing 0.2 ml of 5% azocasein or azoalbumin dissolved in 0.05 M Tris maleate buffer (pH 7.5), 0.7 ml of Tris maleate buffer (pH 7.5), and 0.1 ml of enzyme solution, was incubated at 37°C for 30 min. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid. The precipitate was removed by centrifugation, and 0.2 ml of 1.8 N NaOH was added to 0.8 ml of the supernatant solution containing the diazotized trichloroacetic acid-soluble peptides. The A_{420} was measured, and 1 U of activity was defined as the increase of the A_{420} by 1.0 per min.

(iii) Hydrolysis of azocoll. Hydrolysis of azocoll was examined in centrifuge tubes. Each tube contained 2 mg of azocoll, 0.9 ml of 0.05 M Tris maleate buffer (pH 7.5), and 0.1 ml of enzyme solution. After incubation at 37°C for 30 min, the tubes were immediately chilled in an ice water bath. Subsequently, the tubes were centrifuged for 5 min at 1,000 × *g* at 4°C, and the A_{520} of the supernatant solution was measured. The activity was defined as the increase of the A_{520} by 1.0 per min.

(iv) Collagenase activity. Hydrolysis of insoluble collagen was tested by the method described by Loesche et al. (15) with some modifications. Bovine Achilles tendon collagen (5 mg; Sigma Chemical Co., St. Louis, Mo.) was incubated with 1 ml of the purified enzyme preparation in 0.05 M Tris maleate buffer (pH 7.5) at 37°C with shaking for 8 h. After incubation the reaction mixtures were filtered through a filter membrane (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and the filtrates were subjected to the ninhydrin reaction (18) to detect the hydrolytic products. In addition, collagenase activity was assessed by using a synthetic peptide substrate for collagenase, carbenzoyl-glycyl-L-prolyl-L-leucyl-glycyl-L-proline (Protein Institute, Inc., Osaka, Japan), as described by Nagai et al. (19). The ninhydrin

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reaction was employed to determine the hydrolytic products by the enzyme from this substrate.

(v) **Esterase activity.** Esterolytic activity was assayed by using *p*-tosyl-L-arginine methyl ester by the method described by Rick (24).

Protein determination. Protein was estimated by the method described by Lowry et al. (17). For samples containing Triton X-100, the procedure was carried out with sodium dodecyl sulfate (SDS) (25).

Determination of optimum pH. Proteolytic activity was assayed at different pH values by using appropriate buffers. Samples of the purified protease preparation (0.1 ml) in 0.05 M Tris maleate buffer (pH 7.5) were added to assay mixtures containing 2.5 ml of Bz-L-Arg-pNA solution in 0.1 M of the different test buffers and 0.4 ml of 0.1M Tris maleate buffer. Acetate buffer (pH 4.5, 5.0, 5.5, 6.0), phosphate buffer (pH 6.5, 7.0, and 7.5), and Tris hydrochloride buffer (pH 8.0, 8.5, and 9.0) were used.

Effect of various reagents on the enzyme. The effects of inhibitors and metal ions on the enzyme activity were tested at pH 7.5. The purified enzyme preparation was preincubated with each reagent at 25°C for 30 min. Subsequently, the substrate solution was added to start the proteolytic reaction. The activity was expressed as the percentage of the value for the control experiment, which did not include the test reagents.

Polyacrylamide gel electrophoresis. To examine the purity of the sample, polyacrylamide gel electrophoresis was carried out by the method described by Davis (5). The concentration of the gel was 6%. Protein bands were stained with Coomassie brilliant blue G-250. Simultaneously, an unstained gel was cut into 1-mm-thickness pieces, and each piece was incubated in 1 ml of 1 mM Bz-L-Arg-pNA in 0.2 M Tris maleate buffer (pH 7.5) to determine the location of enzyme activity in the gel.

Determination of molecular weight. The molecular weight was determined by gel filtration on a Sephadex G-200 column, as described by Andrews (2). The marker proteins were catalase (M_r, 232,000), aldolase (M_r, 158,000), bovine serum albumin (M_r, 67,000), chymotrypsinogen A (M_r, 25,000), and RNase A (M_r, 13,700).

Preparation of cell extract. The cells of *B. gingivalis* ATCC 33277 that were harvested from 2,000 ml of culture (13 g [wet weight]) were suspended in 0.05 M Tris hydrochloride buffer (pH 7.5), and they were subjected to ultrasonic treatment at 9 KHz for 10 min at 4°C. Triton X-100 was added at 1% to the sonicated material, followed by stirring for 2 h at 4°C. Finally, this sample was centrifuged at 100,000 × *g* for 20 min, and a clear cell extract was obtained.

Isoelectric focusing. Isoelectric focusing was carried out at about 4°C, with a 110-ml capacity column (LKB Instruments, Inc., Rockville, Md.) with 1% (vol/vol) ampholine (pH 3.5 to 10.0), by the method described by Vesterberg et al. (33). Focusing was done for 30 h under constant voltage (400 V). Fractions of 2 ml were collected, and the pH of each fraction was measured. The assay for proteolytic activity was carried out by using 0.1-ml portions of each fraction by the method described above.

Purification. Cell-free culture fluid (2,000 ml) was prepared by centrifugation at 10,000 × *g* for 10 min. Ammonium sulfate was added to this sample at 30% saturation, and the mixture was stirred for an additional 1 h. The precipitate was collected by centrifugation at 15,000 × *g* for 20 min. This precipitate was suspended in 50 ml of 0.05 M Tris hydrochloride buffer (pH 8.1) containing 0.01 M 2-mercaptoethanol (TM) and centrifuged at 100,000 × *g* for 20 min. The precipitate was dissolved in 40 ml of TM containing 1% Triton X-100 (TMT). A slight amount of insoluble material was removed by centrifugation at 100,000 × *g* for 20 min. The supernatant (TMT-soluble fraction) containing the enzyme activity was applied to a column (1.5 by 45 cm) of DEAE-cellulose, which had been equilibrated earlier with TMT. After the column was washed with 500 ml of TMT, the column was eluted with a linear concentration gradient of NaCl, which was generated by mixing 250 ml of TMT containing 0.4 M NaCl into an equal volume of TMT. The flow rate was 25 ml/h, and 5-ml fractions were collected. One major and one minor peak of the proteolytic activity emerged from the column at NaCl concentrations of about 0.1 and 0.2 M, respectively (Fig. 1). We undertook further purification of the major fraction because the minor fraction was negligible in quantity. The active fractions indicated in Fig. 1 were collected and dialyzed against TMT, and chromatography on the DEAE-cellulose column (1.5 by 30 cm) was repeated with a linear concentration gradient of NaCl from 0 to 0.4 M (200 ml on each side). The active fractions were collected and subjected to partial removal of Triton X-100 by using Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, Calif.) by the method described by Holloway (10), followed by dialysis against 1% glycine solution containing 0.01 M 2-mercaptoethanol. When this sample was electrophoresed on an isoelectric focusing column, the enzyme activity was concentrated at the fraction collected at pH 5.0. Finally, this fraction was dialyzed against 0.05 M Tris maleate buffer (pH 7.5).

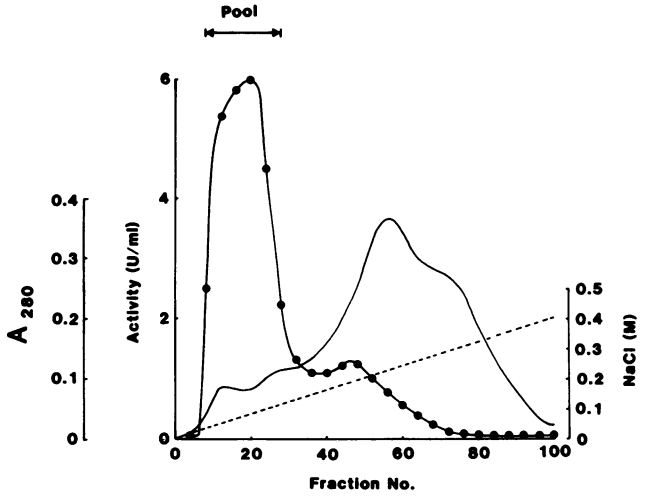

FIG. 1. First DEAE-cellulose column chromatography of protease. Symbols: ●, enzyme activity against Bz-L-Arg-pNA; —, A_{280}; ----, NaCl concentration.

RESULTS

Purification. The culture fluid (2,000 ml) that was used as the starting material for the purification procedure contained 900 U of proteolytic activity, but only 110 U was found in cell extracts from 13 g (wet weight) of the organism. About 77% of the enzyme activity in the culture fluid was recovered in the TMT-soluble fraction. The enzyme was purified 4,190 times, with a recovery of 7% (Table 1). The purified preparation was homogeneous, as judged by polyacrylamide gel electrophoresis (Fig. 2).

Molecular weight. The molecular weight, as determined by Sephadex G-200 gel filtration, was 300,000.

TABLE 1. Purification of protease

Fraction	Total activity (U)	Total protein (mg)	Sp act [a]	Yield (%)	Purification (no. of times)
Culture fluid	900	41,000	0.02	100	1
Ammonium sulfate fraction (30% saturation)	765	2,400	0.32	85	16
TMT-soluble fraction	690	400	1.73	77	87
First DEAE-cellulose fraction	250	57	4.39	28	220
Second DEAE-cellulose fraction	180	25	7.20	20	360
Isoelectric focusing	67	0.8	83.75	7	4,190

[a] Enzyme activity per milligram of protein in the fraction of each step.

Optimum temperature. When the hydrolysis of Bz-L-Arg-pNA was carried out at various temperatures, the highest activity was found at 40 to 45°C.

Optimum pH. The pH profile of the enzyme activity demonstrated that the enzyme had an optimum pH of 7.5.

K_m value. From the extrapolation of the Lineweaver-Burk plot, an apparent K_m value for Bz-L-Arg-pNA was calculated to be 2 mM (Fig. 3). At high substrate concentrations the hydrolysis rate decreased. This result could possibly be explained by a substrate inhibition phenomenon.

Substrate specificity. The activities against several substrates were compared. The enzyme preparation (0.218 U of Bz-L-Arg-pNA hydrolytic activity) exerted 0.220 U of activity for carbenzoyl-L-Phe-L-Val-L-Arg-pNA, 0.013 U for azocasein, 0.011 U for azoalbumin, 0.027 U for azocoll, and 0.050 U for *p*-tosyl-L-arginine methyl ester. L-Arg-pNa, Bz-DL-Lys-pNA, and L-Lys-pNA were not hydrolyzed. The result of the ninhydrin reaction for the filtrate of collagen that had been incubated with the enzyme was negative. From carbenzoyl-glycyl-L-prolyl-L-leucyl-glycyl-L-proline, ninhydrin-positive material was not detected after incubation with the enzyme.

Effects of inhibitors and metal ions. The possible inhibitory and activating effects of various chemicals on the protease are summarized in Table 2. The enzyme was sensitive to inhibition by *p*-chloromethyl mercuribenzoate, *N*-ethylmaleimide, and Hg^{2+}. These results indicate that the sulfhydryl group of this enzyme is very important to the enzymatic activity. Tosyl-L-lysine chloromethyl ketone also strongly inhibited the activity. The inhibitory effect of EDTA was obvious; however, the ferrous ion chelator *O*-phenanthroline had no effect. Soybean trypsin inhibitor and serine enzyme inhibitors, such as diisopropylfluoro phosphate, had substantially no effect. Considerable activation was seen in Ca^{2+} and Mg^{2+}.

Competitive inhibition by benzoyl-L-arginine. It was observed that benzoyl-L-arginine, the hydrolytic product of Bz-L-Arg-pNA, inhibited competitively the activity of the enzyme. Its K_i value was calculated to be 3.2 mM.

DISCUSSION

The protease dealt with in this study was solubilized with Triton X-100 after the culture supernatant was precipitated with a relatively low concentration of ammonium sulfate (30% saturation). The enzyme was probably liberated into the surrounding medium as the complex form, with cell debris accumulating with the progression of cell lysis.

Laughon et al. (11) demonstrated the production of a proteolytic enzyme from *B. gingivalis* strains with an API ZYM system (Analytab Products, Plainview, N.Y.) that hydrolyzed benzoyl-DL-arginine-2-naphthylamide. They referred to this enzyme as trypsin-like protease. Van Winkelhoff et al. (32) also obtained similar results. They confirmed that all the tested strains of *B. gingivalis* possessed the hydrolytic activity of benzoyl-DL-arginine-2-naphthylamide.

The enzyme presented in this report was active against not only Bz-L-Arg-pNA but also carbenzoyl-L-Phe-L-Val-L-Arg-pNA, which is a suitable substrate for trypsin, and *p*-tosyl-L-arginine methylester. These findings suggest that the enzyme is a trypsin-related protease because of its substrate specificity. General proteolytic substrates (azocasein, azoalbumin, and azocoll) were also found to be hydrolyzed

FIG. 2. Polyacrylamide gel electrophoresis of the purified protease (without SDS). Migration was from top to bottom.

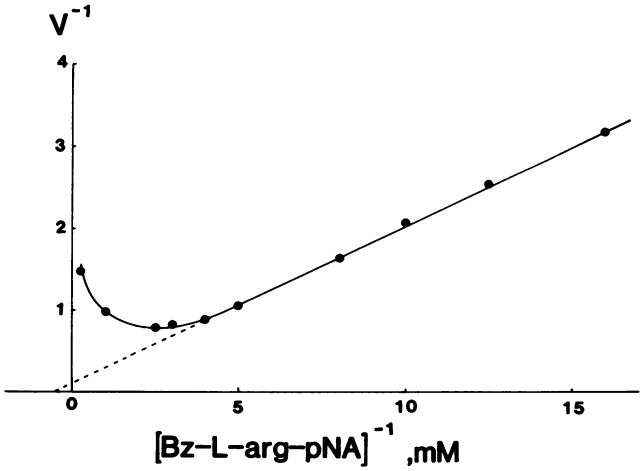

FIG. 3. Lineweaver-Burk plot of protease.

TABLE 2. Effects of inhibitors and metal ions

Reagents	Concn (mM)	Activity (%)
Control		100
p-Chloromethyl mercuribenzoate	0.01	0
N-Ethylmaleimide	1.0	0
Diisopropylfluoro phosphate	5.0	93
Tosyl-L-lysine chloromethyl ketone	0.01	0
EDTA	1.0	13
O-Phenanthroline	5.0	98
Trypsin inhibitor (soybean)	5[a]	85
Ca^{2+}	3.0	144
Mg^{2+}	3.0	141
Hg^{2+}	3.0	0

[a] 5 mg/ml.

by this enzyme. The presence of thiol-dependent collagenolytic enzyme in the culture fluid of *B. gingivalis* has been shown by Toda et al. (28). Glycylprolyl dipeptidyl aminopeptidase, which is considered to be able to cleave partially digested collagen, has been purified from *B. gingivalis* (1). Collagenase activity, however, was not demonstrated in our enzyme when examined by using collagen fibril or a synthetic peptide substrate for collagenase.

The enzyme studied here was not a serine enzyme, because no significant inhibition of the enzyme by diisopropylfluoro phosphate was observed. It was inhibited markedly by the sulfhydryl group-blocking reagents, indicating that this enzyme is a thiol enzyme. Soybean trypsin inhibitor had essentially no effect. The enzyme did not hydrolyze the lysyl bond, although it hydrolyzed the arginyl bonds and the arginyl ester. Competitive inhibition of the proteolytic activity by benzoyl-L-arginine was also reported by Bechet et al. (3) in trypsin. The K_i value in this case was 5.8 mM.

The enzymatic properties of *B. gingivalis* ATCC 33277 resembled those of the protease isolated by Yoshimura et al. (34) from another strain of *B. gingivalis*. Our K_m value for Bz-L-Arg-pNA, however, was larger than their value by 200 times, and our enzyme was much more sensitive to inhibition by tosyl-L-lysine chloromethyl ketone than was their preparation.

Nakata et al. (21) revealed that substrate inhibition of *Streptomyces* results in trypsin-like enzymes. Substrate inhibition of trypsin was demonstrated also by Lin et al. (14) when dimethylcasein was used as the substrate. Nakata and Ishii (20), however, reported opposite observations; they confirmed substrate activation in bovine pancreatic trypsin-catalyzed hydrolysis of Bz-L-Arg-pNA. Substrate inhibition of the protease from the *Bacteroides* group was also reported in *B. ruminicola* (9) and *B. amylophilus* (4).

The molecular weight was estimated to be about 300,000 by gel filtration, but by polyacrylamide gel electrophoresis with SDS and 2-mercaptoethanol, it appeared to be about 65,000 (data not shown). Although the reason for these different estimates is obscure, one likely explanation may involve the interaction between protein and Triton X-100, which results in an increase in the apparent molecular size of the protein, as discussed by Pacaud (23) in membrane-bound proteases of *Escherichia coli*. In this case the molecular weight was estimated to be 650,000 and 34,000 by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively.

Results of several studies (6, 8, 22, 30, 31) have demonstrated that the proenzyme of collagenase is activated by some factors, including proteases. Uitto and Raeste (29) have found that crevicular fluid from either healthy or inflamed gingiva contained latent collagenase that could be activated with trypsin. Based on these findings, the presence of a trypsin-like enzyme may be related to the pathogenesis of *B. gingivalis* because collagenase is considered to be an important strategem for damaging the tissue.

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