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Purification and Characterization of a Protease from *Bacteroides gingivalis* 381

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An intracellular membrane-free, trypsinlike protease was isolated from cells of *Bacteroides gingivalis* 381. The protease was extracted from the cells by ultrasonic treatment and was purified about 250-fold with a recovery of 2% by sequential procedures. The properties of the protease were as follows: (i) its optimal pH was 8.5; (ii) its activity was almost completely lost on incubation at 50°C for 15 min; (iii) its activity was inhibited by diisopropylfluorophosphate, *p*-toluenesulfonyl-L-lysine chloromethyl ketone hydrochloride, leupeptin, Mn^{2+}, Cu^{2+}, and Zn^{2+}; (iv) it hydrolyzed casein, azocasein, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), bovine serum albumin, azocoll, and gelatin, but not *N*-α-benzoyl-DL-lysine-*p*-nitroanilide or human serum immunoglobulin A; (v) its molecular weight was estimated as 45,000 by gel filtration and 50,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; and (vi) its K_m values for azocasein and BAPNA were 1.11% and 0.19 mM, respectively.

Periodontal disease is an inflammatory reaction in the gingival tissue associated with breakdown of the supporting tissue of teeth. Recently, *Bacteroides* species have been reported to be the predominant cultivable microflora in several types of periodontal diseases (16, 18, 25, 26, 28, 30–33, 38). *Bacteroides gingivalis* is the main species isolated from advanced cases of adult periodontosis and is the most important species with respect to the capacity for breakdown of tissue and host defense (15, 19, 34).

B. gingivalis has been reported to hydrolyze casein (15), fibrin (21, 24), collagen (14, 15, 22), gelatin (34), and immunoglobulin A (IgA) and IgG (10) and to produce a trypsinlike protease (12, 27, 37). Of the various *Bacteroides* species, *B. gingivalis* is the most fibrinolytic and gelatinolytic (15, 21, 24, 34), and it is the only species that produces a trypsinlike protease (12, 27). This protease may be useful for its identification and may be one of its virulence factors. Yoshimura et al. (37) reported a membrane-bound, trypsinlike protease of *B. gingivalis*, but we considered that a membrane-free protease might be more important in vivo because such an enzyme would be present in periodontal pockets in the free form after its secretion from the cells. But there is little evidence for the existence of a membrane-free trypsinlike protease in *B. gingivalis*. Therefore, we examined the localization of the trypsinlike protease in relation to the growth of *B. gingivalis* in culture and isolated a membrane-free, trypsinlike protease from strain 381 by the sequential procedures described previously (5, 6, 8, 9, 17, 20, 36). We then examined the properties of the enzyme.

MATERIALS AND METHODS

Bacterial strains. The protease-producing strains *B. gingivalis* 381 and 1312 were generously provided by H. Sagawa, Department of Microbiology, Osaka Dental College, Osaka, Japan; *Bacteroides asaccharolyticus* ATCC 25260 and *Bacteroides melaninogenicus* ATCC 15930 were obtained from the American Type Culture Collection, Rockville, Md.; *B. asaccharolyticus* GAI3051 and *B. melaninogenicus* GAI5596 were obtained from K. Ueno, Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan.

Bacterial cultivation for protease purification. Cells of *B. gingivalis* 381 in 100 ml of GAM broth (Nissui Pharmaceutical Co.) containing 0.5 μg of menadione per ml were cultivated anaerobically at 37°C for 48 h in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. The entire culture was then used to inoculate 10 liters of GAM broth, and the culture was incubated anaerobically at 37°C for 5 days (final optical density at 660 nm, 1.4) in an anaerobic box.

Enzyme assay. Proteolytic activity was determined by the method of Kunitz (11), as modified by Arvidson et al. (3), with heat-denatured casein or asocasein as substrate. The reaction mixture, containing 1 ml of 1% casein in 0.15 M phosphate buffer (pH 7.5), 1 ml of 30 mM 2-mercaptoethanol, and 0.9 ml of distilled water, was incubated for 5 min at 37°C. Then 0.1 ml of enzyme solution was added to start the reaction, and the mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 15% perchloric acid. The tubes were kept standing for 10 min at room temperature and then centrifuged, and the A_{280} of the supernatant was measured. One unit of protease activity was defined as the amount that increased the A_{280} by 0.01/min under these conditions. When azocasein was used as substrate for the qualitative examinations, the A_{430} of the supernatant was measured. In studies on the substrate specificity of the purified protease, the hydrolyses of azocoll, gelatin, human serum IgA, and *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and *N*-α-benzoyl-DL-lysine-*p*-nitroanilide (BLPNA) were measured by the methods of Boethling (5), Smith and Goodner (29), Kilian (10), and Bernard et al. (4), respectively.

Protease activities of various *Bacteroides* species. Cells of each bacterial strain were cultivated anaerobically at 37°C for 48 h on GAM agar containing 0.5 μg of menadione per ml, harvested, suspended in 2 ml of 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine, and disrupted by ultrasonic treatment (200 W for 20 min). The sonic extracts were centrifuged at 9,000 × *g* for 45 min, and the

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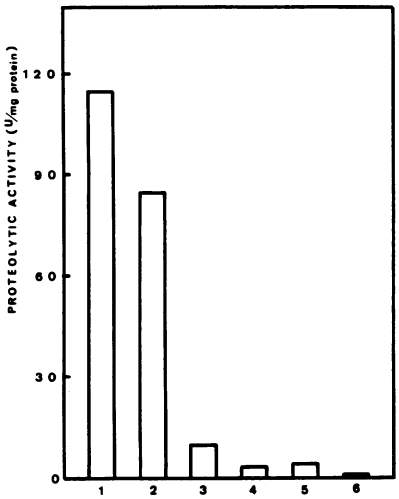

FIG. 1. Proteolytic activities in various *Bacteroides* species. The strains tested were *B. gingivalis* 381 (bar 1) and 1312 (bar 2), *B. asaccharolyticus* ATCC 25260 (bar 3) and GAI3051 (bar 4), and *B. melaninogenicus* ATCC 15930 (bar 5) and GAI5596 (bar 6).

activity of the supernatants was assayed as described above, with casein as substrate.

Growth curve of *B. gingivalis* 381 and protease activity of its cellular fractions. A volume of 4 ml of a suspension (optical density at 660 nm, 1.2) of *B. gingivalis* 381 cells was inoculated into 200 ml of GAM broth containing 0.5 μg of menadione per ml, and the culture was incubated anaerobically at 37°C for various periods. Growth was monitored by measuring the A_{660}. At appropriate times, the culture fluid and cells were separated by centrifugation. All steps for preparation of each fraction were performed at 4°C. Solid ammonium sulfate was slowly added to the culture fluid with stirring to give 70% saturation, and the mixture was centrifuged at 10,000 × *g* for 30 min. The precipitate was dissolved in 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine and dialyzed against the same buffer, and the dialyzed material was used as the extracellular fraction.

The cells were washed with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 0.15 M NaCl, suspended in 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine, and then disrupted by ultrasonic treatment (200 W for 20 min). Remaining intact cells were removed by centrifugation at 1,000 × *g* for 10 min, and the supernatant was centrifuged at 30,000 × *g* for 30 min. The resulting supernatant was used as the intracellular membrane-free fraction. The precipitate was mixed with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine and 1% Triton X-100 and centrifuged at 30,000 × *g* for 30 min. The supernatant was used as the intracellular membrane-bound fraction.

The protease activities of the extracellular and intracellular membrane-free and membrane-bound fractions were assayed as described above with azocasein (for general protease activity) or BAPNA (for trypsinlike protease activity) as substrate. One unit of enzyme activity on BAPNA was defined as the amount catalyzing the release of 1 μmol of *p*-nitroaniline in 1 min at 37°C under the conditions described above.

DEAE-cellulose column chromatography of proteases in extracellular and intracellular membrane-free and membrane-bound fractions. The extracellular fraction used for DEAE-cellulose (Whatman DE-32) column chromatography was prepared from a 5-day culture, and the intracellular membrane-free and membrane-bound fractions were from 2-day cultures. Each fraction was applied to a DEAE-cellulose column (1.3 by 10 cm) previously equilibrated with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine. Proteins were first eluted with 40 ml of equilibration buffer and then with 200 ml of a linear gradient of 0 to 0.4 M NaCl in the same buffer at a flow rate of 20 ml/h. For chromatography of the intracellular membrane-bound fraction, 1% Triton X-100 was added to the equilibration and elution buffers. The protease activity on azocasein or BAPNA was assayed as described above.

Protein determination. Protein concentration was determined by the method of Lowry et al. (13) or Ross and Schatz (23) with bovine serum albumin as a standard.

Purification of the protease. All steps in purification of the protease were performed at 4°C unless otherwise specified. In all column chromatographies, 5-ml fractions were collected, and their A_{280} and proteolytic activity were measured.

(i) Preparation of crude extract and S-30 and S-100 fractions. Cells (24 g) from a 10-liter culture of *B. gingivalis* 381 were harvested by continuous centrifugation at 8,000 × *g*, washed twice with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 0.15 M NaCl, and suspended in 120 ml of 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine. The cells were then disrupted by ultrasonic treatment (200 W for 20 min) and centrifuged at 9,000 × *g* for 45 min. The supernatant was fractionated by differential centrifugation at 30,000 × *g* for 30 min and 100,000 × *g* for 2 h. The supernatants were used as the crude extract and S-30 and S-100 fractions, respectively.

(ii) Ammonium sulfate fractionation. To 143 ml of the S-100 fraction, solid ammonium sulfate was added slowly with stirring to give 70% saturation. The suspension was allowed to stand for 15 min and was then centrifuged at 10,000 × *g* for 30 min. The brown precipitate was dissolved in 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine and dialyzed overnight against the same buffer.

(iii) DEAE-cellulose column chromatography. The dialyzed material was applied to a DEAE-cellulose (Whatman DE-32) column (1.7 by 30 cm) previously equilibrated with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine. The proteins were then eluted with 120 ml of the same buffer followed by 500 ml of a linear gradient of 0 to 0.7 M NaCl in the same buffer at a flow rate of 15 ml/h. The main protease activity was found in fractions 50 to 80. These fractions were pooled, concentrated by precipitation with ammonium sulfate, and dialyzed overnight against 1 mM potassium phosphate buffer (pH 6.8) containing 5 mM 2-mercaptoethanol.

(iv) Hydroxyapatite column chromatography. The dialyzed material was applied to a hydroxyapatite column (1.8 by 14 cm) previously equilibrated with 1 mM potassium phosphate buffer (pH 6.8) containing 5 mM 2-mercaptoethanol. The column was washed with 70 ml of the same buffer, and proteins were eluted with a 300-ml linear gradient of 0.001 to 0.4 M potassium phosphate buffer (pH 6.8) containing 5 mM 2-mercaptoethanol at a flow rate of 25 ml/h. The active fractions (37 to 55) were combined, concentrated by precipitation with ammonium sulfate, and dialyzed overnight against 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine.

(v) Cellulofine GCL 2000 column chromatography. The dialyzed material was applied to a column (2 by 80 cm) of

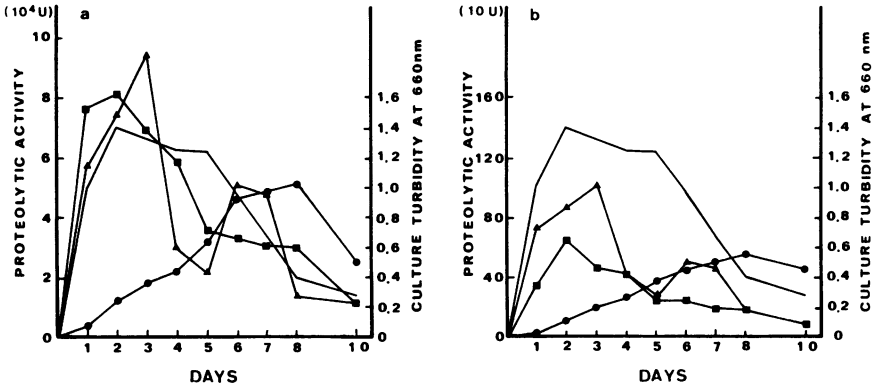

FIG. 2. Changes in turbidity of *B. gingivalis* 381 during cultivation and protease activities of three cell fractions on BAPNA (a) and azocasein (b). The turbidity of the culture was measured at 660 nm. The cells were collected on the indicated days of culture and fractionated into extracellular, intracellular membrane-free, and intracellular membrane-bound fractions. The protease activities of the three fractions were assayed with BAPNA or azocasein as substrate. Symbols: ——, culture turbidity; ●, extracellular fraction; ▲, intracellular membrane-free fraction; ■, intracellular membrane-bound fraction.

Cellulofine GCL 2000 previously equilibrated with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine, and proteins were eluted with the same buffer at a flow rate of 30 ml/h. The active fractions (32 to 38) were combined.

(vi) Second DEAE-cellulose column chromatography. The material with protease activity from the first Cellulofine GCL 2000 column was applied to a second DEAE-cellulose column (1.3 by 11 cm), and proteins were eluted with a 150-ml linear gradient of 0 to 0.4 M NaCl in 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine at a flow rate of 20 ml/h. The active fractions (19 to 24) were combined and dialyzed against 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine.

(vii) Third DEAE-cellulose column chromatography. The dialyzed material was applied to a third DEAE-cellulose column (1.3 by 8 cm), and the column was eluted with 50 ml of the dialyzing buffer followed by a 300-ml linear gradient of 0 to 0.3 M NaCl in the same buffer at a flow rate of 20 ml/h. The active fractions (25 to 30) were combined and concentrated to 4 ml by positive-pressure ultrafiltration through an Amicon PM 10 membrane.

(viii) Second Cellulofine GCL 2000 column chromatography. The concentrated material was applied to a second Cellulofine GCL 2000 column (2 by 80 cm), and proteins were eluted as in the first Cellulofine column chromatography. The active fractions (32 to 36) were combined to give the final preparation.

Effect of pH on protease activity. The reaction mixture for determination of the protease activity at different pH values consisted of 1 ml of 0.3% azocasein in distilled water, 1 ml of an appropriate buffer (0.15 M citrate-phosphate buffer for pH 4.0 to 7.5; 0.15 M Tris hydrochloride buffer for pH 7.0 to 9.0; and 0.15 M diethanolamine hydrochloride buffer for pH 8.0 to 10.0), 0.9 ml of 30 mM 2-mercaptoethanol, and 0.1 ml of enzyme preparation.

Effects of group-specific reagents, EDTA, and metal ions on enzyme activity. The effects of various compounds on enzyme activity were tested at the following final concentrations: diisopropylfluorophosphate (DFP), 10 and 0.5 mM; *p*-toluenesulfonyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 2.5 and 0.5 mM; *p*-chloromercuribenzoic acid (PCMB), 0.5 and 0.1 mM; *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK), 5 and 1 mM; iodoacetate, 5 and 0.5 mM; leupeptin, chymostatin, elastatinal, soybean trypsin inhibitor, and egg trypsin inhibitor, 50 and 5 μg/ml; and phenylacetate, L-cysteine, and EDTA, 10 mM. Various metal ions were tested at 10 mM. The activities of reaction mixtures containing these reagents were assayed as described above.

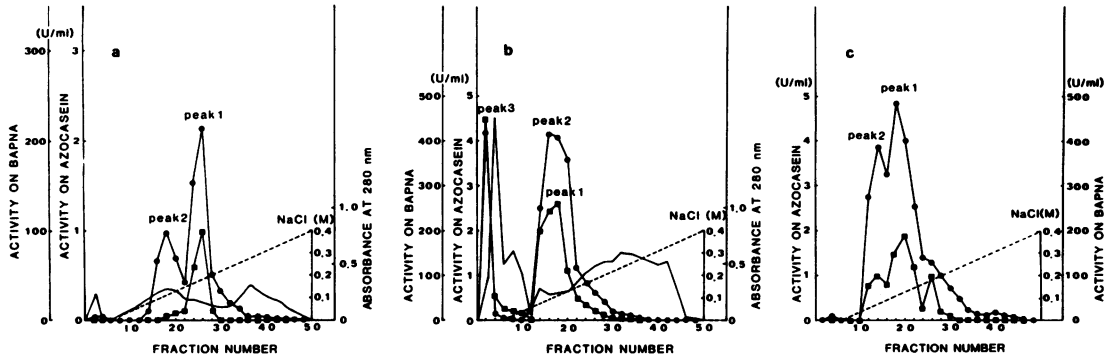

FIG. 3. DEAE-cellulose column chromatography of extracellular (a), intracellular membrane-free (b), and intracellular membrane-bound (c) fractions. Each fraction was applied to a DE-32 column (1.3 by 10 cm) equilibrated with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine. The column was washed with 40 ml of the equilibration buffer. Proteins were then eluted with 200 ml of a linear gradient of 0 to 0.4 M NaCl in the same buffer. For chromatography of the intracellular membrane-bound fraction, 1% Triton X-100 was added to the equilibration and elution buffer. Symbols: ●, activity on BAPNA; ■, activity on azocasein; ——, A_{280}; – – –, NaCl concentration (M).

TABLE 1. Purification of a protease from *B. gingivalis* 381

Fraction	Vol (ml)	Amt of protein (mg)	Activity (U)	Sp act (U/ml)	Purification (fold)	Yield (%)
S-9	150	1,100	11,500	10	1.0	100
S-30	145	980	9,850	10	1.0	85
S-100	143	810	9,310	11	1.1	81
Ammonium sulfate precipitation	43	363	7,970	22	2.2	69
DEAE-cellulose (DE-32)	13	83	7,870	95	9.5	68
Hydroxyapatite	6.5	34	4,130	133	13.3	36
Cellulofine GCL 2000	34	9	1,570	174	17.4	14
Second DEAE-cellulose	30	2	1,290	646	64.6	11
Third DEAE-cellulose	28	0.4	970	2,430	242	8
Second Cellulofine GCL 2000	25	0.1	250	2,500	250	2

Determination of molecular weight. The molecular weight of the protease was estimated by gel filtration on a Cellulofine GCL 2000 column (2 by 80 cm) by the method of Andrews (1) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Weber and Osborn (35).

(i) Gel filtration. The standard proteins used were bovine serum albumin, ovalbumin, chymotrypsinogen A, and RNase A. The elution buffer was 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine. The elution volumes of the standard proteins were determined from the A_{280}, and that of the protease was determined by measuring its activity.

(ii) SDS-PAGE. The partially purified protease fraction obtained by the first Cellulofine gel filtration was subjected to 5% PAGE in the absence or presence of 0.1 mM TLCK at 5 mA per tube at 4°C for 2 h by the method of Davis (7). After electrophoresis in the absence of TLCK and SDS, the gel was dyed by reaction with BAPNA solution (pH 8.5) for 15 min at 37°C; the portion with the protease-active fraction stained yellow. After electrophoresis in the absence and presence of TLCK, gels were stained with Coomassie brilliant blue R-250. The parts corresponding in position to the protease-active fraction found in parallel nonstained gels with and without TLCK were cut out, and the proteins were extracted with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine and 0.1 mM TLCK at 4°C for 3 h. The extracted proteins were mixed with 1/10 volume of 10% SDS, heated in boiling water for 5 min, and applied to 5% polyacrylamide gels containing 0.1% SDS. SDS-PAGE was performed by the method of Weber and Osborn (35). After electrophoresis, the gels were stained with Coomassie brilliant blue. The standard proteins used were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor.

Chemicals. EDTA, L-cysteine, perchloric acid, ammonium sulfate, and hydroxyapatite were purchased from Wako Pure Chemical Industries, Osaka, Japan. Bovine serum albumin, azocasein, BAPNA, BLPNA, soybean and egg trypsin inhibitors, and azocoll were from Sigma Chemical Co., St. Louis, Mo. DFP, TLCK, TPCK, iodoacetate, PCMB, phenylacetate, menadione, and Cellulofine GCL 2000 were from Nakarai Chemical Co., Kyoto, Japan. Casein and 2-mercaptoethanol were from E. Merck Japan, Tokyo. Molecular weight standards for gel filtration and electrophoresis were from Pharmacia Fine Chemical Japan Co., Tokyo. Gelatin, human serum IgA, and DEAE-cellulose (DE-32) were obtained from Difco Laboratories, Detroit, Mich., Cooper Biomedical, Inc., West Chester, Pa., and Whatman Biochemicals, Maidstone, Kent, England, respectively.

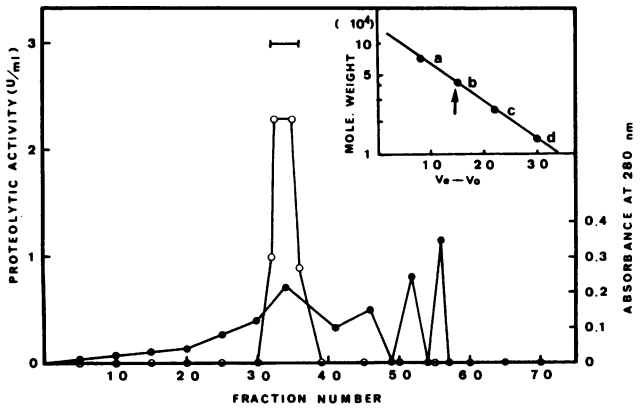

FIG. 4. Second Cellulofine GCL 2000 chromatography of a protease from *B. gingivalis* 381. The concentrated material of the pooled fractions from the third DEAE-cellulose column chromatography was applied to a Cellulofine GCL 2000 column (2.0 by 80 cm) equilibrated with 0.05 M Tris hydrochloride buffer (pH 7.5) containing 5 mM L-cysteine. Proteins were eluted with 350 ml of the equilibration buffer at a flow rate of 30 ml/h. Symbols: ○, activity; ●, A_{280}. The active fractions are indicated by the horizontal bar. The molecular weight was determined by the method of Andrews (1). The standard proteins used were bovine serum albumin (a) ovalbumin (b), chymotrypsinogen A (c), and RNase A (d). The arrow denotes the membrane-free, trypsinlike protease.

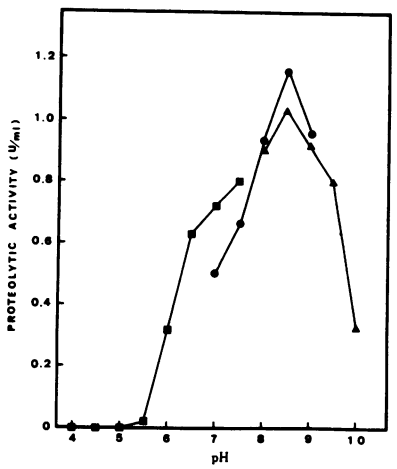

FIG. 5. Effect of pH on protease activity. The assay system was adjusted to various pH values with the following buffers: ■, 0.15 M citrate-phosphate, pH 4.0 to 7.5; ●, 0.15 M Tris hydrochloride, pH 7.0 to 9.0; ▲, 0.15 M diethanolamine hydrochloride, pH 8.0 to 10.0.

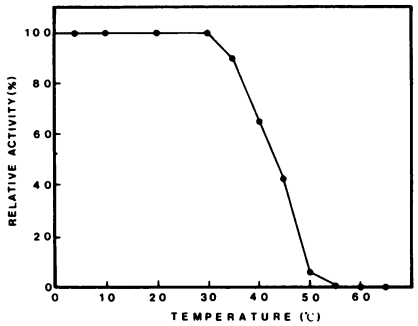

FIG. 6. Heat stability of protease from *B. gingivalis* 381. Portions of the purified protease in 0.05 M Tris hydrochloride buffer (pH 8.5) containing 5 mM L-cysteine were incubated for 15 min at the indicated temperatures, and then their activity was assayed at 37°C for 15 min.

Chymostatin, leupeptin, and elastatinal were obtained from Peptide Institute Inc., Osaka, Japan.

RESULTS

Proteolytic activities of various *Bacteroides* species. The proteolytic activities of *B. gingivalis* 381 and 1312 were much higher than those of *B. asaccharolyticus* ATCC 25260 and GAI3051 and *B. melaninogenicus* ATCC 15930 and GAI5596 (Fig. 1). Since *B. gingivalis* 381 had the highest activity, the protease was purified from this strain.

Growth curve of *B. gingivalis* 381 and protease activity of cellular fractions. *B. gingivalis* 381 proliferated for 2 days, maintained a constant turbidity for 3 days, and then lysed rapidly (Fig. 2). The proteolytic activities of the three fractions on BAPNA and azocasein showed similar patterns. The protease activities on both BAPNA and azocasein of the extracellular fractions increased linearly until day 8, those of the intracellular membrane-free fractions showed a major peak on day 3 and a minor peak on day 6, and those of the intracellular membrane-bound fractions showed patterns similar to those of bacterial growth.

DEAE-cellulose column chromatography of protease in extracellular and intracellular membrane-free and membrane-bound fractions. On DEAE-cellulose column chromatography of each of the three cellular fractions, two protease peaks were eluted with an NaCl gradient (Fig. 3). Peak 1 of all three fractions was active on both BAPNA and azocasein, whereas peak 2 had activity on BAPNA but little activity on azocasein. The peak 3 fraction shown in Fig. 3b, which was not adsorbed to cellulose, disappeared during DEAE-cellulose column chromatography in the presence of Triton X-100 with the appearance of peaks 1 and 2 (Fig. 3c), suggesting that it contained protease bound to small membrane fragments. No peak such as peak 3 was detected in the extracellular fraction. The intracellular membrane-bound fraction was not adsorbed to DEAE-cellulose in the absence of Triton X-100. These results suggest that the proteases in the culture fluid were membrane free.

TABLE 2. Effects of group-specific reagents on protease activity[a]

Reagent	Final concn (mM)	Activity (%)
2-Mercaptoethanol	10	100
L-Cysteine	10	90
DFP	10	25
	0.5	86
TLCK	2.5	19
	0.5	19
PCMB	0.5	80
	0.1	80
TPCK	5	81
	1	88
Iodoacetate	5	84
	0.5	100
Phenylacetate	10	86
None		58

[a] Protease activity was determined in the presence of the test reagents at the indicated concentrations. The standard assay mixture contained 10 mmol (final concentration) of 2-mercaptoethanol, except with L-cysteine.

TABLE 3. Effects of various inhibitors on protease activity[a]

Reagent	Final concn (μg/ml)	Activity (%)
Leupeptin	50	0
	5	10
Soybean trypsin inhibitor	50	90
	5	100
Egg trypsin inhibitor	50	35
	5	100
Chymostatin	50	48
	5	90
Elastatinal	50	90
	5	99
None		100

[a] Protease activity was determined in the presence of the test compounds at the indicated concentrations. The standard assay mixture contained 10 mmol (final concentration) of 2-mercaptoethanol.

Purification of a protease. Cells from a 5-day culture were used as starting material, as described in Materials and Methods. Since a smaller bacterial inoculum was used, this 5-day culture corresponded to the 2- or 3-day culture shown in Fig. 2.

The specific activity of the protease obtained after sequential purification procedures was 250 times that of the crude extract, and the recovery of activity was 2% (Table 1). The material used as the final purified preparation is indicated by the horizontal bar in Fig. 4.

Effect of pH on protease activity. The optimal pH of the protease was 8.5 with azocasein as substrate. The activity decreased rapidly above pH 9.5 and below pH 6.5, and almost no activity was found below pH 5.0 (Fig. 5).

Heat stability. The purified preparation was incubated at various temperatures for 15 min. At 4°C it retained full activity, but at temperatures above 35°C, its activity decreased with an increase in temperature; after incubation at 30°C, the activity was the same as that at 4°C, but after incubation at 35 and 40°C, the activities were 90 and 70%,

TABLE 4. Effects of EDTA and metal ions on protease activity[a]

Addition	Activity (%)
None	100
Na^+	90
Ca^{2+}	80
Mn^{2+}	16
Cu^{2+}	8
Mg^{2+}	70
Zn^{2+}	17
EDTA	63

[a] Protease activity was determined in the presence of metal ions or EDTA at a final concentration of 10 mM. The standard assay mixture contained 10 mmol (final concentration) of 2-mercaptoethanol.

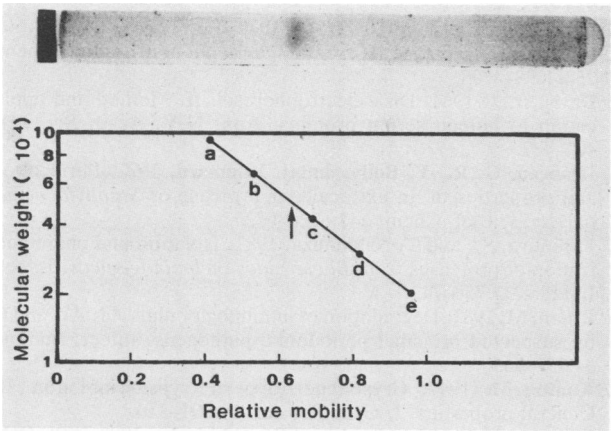

FIG. 7. SDS-PAGE of a protease from *B. gingivalis* 381. The molecular weight of the protease extracted from the gel after PAGE without SDS in the presence of 0.1 mM TLCK was determined by SDS-PAGE by the method of Weber and Osborn (35). The standard proteins used were phosphorylase *b* (a), bovine serum albumin (b), ovalbumin (c), carbonic anhydrase (d), and trypsin inhibitor (e). The arrow denotes the position of the membrane-free, trypsinlike protease.

respectively, of that at 4°C. Scarcely any activity was found after incubation at 50°C (Fig. 6).

Effects of various reagents on protease activity. At both concentrations tested, TLCK and leupeptin inhibited the protease activity 81% and more than 90%, respectively (Tables 2 and 3). PCMB, TPCK, iodoacetate, soybean trypsin inhibitor, and elastatinal did not significantly affect the activity of the protease at the two concentrations tested (less than 20% inhibition). DFP, egg trypsin inhibitor, and chymostatin inhibited the protease activity at the high concentration tested but not at the low concentration. L-Cysteine (10 mM) and 2-mercaptoethanol (10 mM) increased the activity of the protease (Table 2).

Effects of EDTA and metal ions on protease activity. Mn^{2+}, Cu^{2+}, and Zn^{2+} at 10 mM inhibited the protease activity 84, 92, and 83%, respectively. The other metal ions tested did not significantly affect the activity of the protease. EDTA at 10 mM caused 37% inhibition of the protease activity (Table 4).

Substrate specificity. The purified protease hydrolyzed casein, azocasein, bovine serum albumin, azocoll, gelatin, and BAPNA but did not hydrolyze human serum IgA or BLPNA (data not shown).

Molecular weight determination. (i) Gel filtration. The molecular weight of the protease was determined to be about 45,000 by Cellulofine GCL 2000 gel filtration (Fig. 4).

(ii) SDS-PAGE. On PAGE without SDS, the protease gave a single protein band which stained with Coomassie brilliant blue. Partial degradation of the protease after PAGE in the presence of TLCK was avoided by mixing the part of the gel with protease activity with extraction buffer containing TLCK. On SDS-PAGE, the protease extracted in this way gave a single band, and its molecular weight was estimated to be 50,000 by comparison of its mobility with those of marker proteins (Fig. 7).

Kinetics. The rates of hydrolysis of azocasein and BAPNA determined as functions of substrate concentration showed that the reactions followed Michaelis-Menten kinetics. From the double-reciprocal plots of the reaction velocity versus the concentration of these substrates (Fig. 8), the K_m values were determined as 1.11% for azocasein and 0.19 mM for BAPNA.

DISCUSSION

The finding of differences in proteolytic activities in various *Bacteroides* species (Fig. 1) is consistent with reports (12, 15, 21, 24, 27, 34) that a trypsinlike protease is produced only by *B. gingivalis* and that *B. gingivalis* is the most proteolytic *Bacteroides* species.

The protease from *B. gingivalis* 381 was purified by sequential procedures. The specific activity of the final preparation was 250 times that of the crude extract. The purified protease seemed to correspond to the peak 1 protease shown in Fig. 3b, judging from the NaCl concentration at which it was eluted from DEAE-cellulose and from the fact that it hydrolyzed both BAPNA and azocasein. The molecular weight of the protease (about 50,000 as determined by SDS-PAGE) was less than that of an IgA1 protease (17) and proteases I and II (9) from *B. melaninogenicus* and a fibrinolysin from *Treponema denticola* (21) (molecular weights, 62,000, 420,000, 73,000, and 1,000,000, respectively). The K_m values of this protease (1.11% for azocasein and 0.19 mM for BAPNA) were less than that of protease I (1.39%) from *B. melaninogenicus* (9) and more than that of protease II (0.91%) from *B. melaninogenicus* (9) and a trypsinlike, membrane-bound protease (0.01 mM) from *B. gingivalis* (37).

This protease seemed to be secreted into the culture fluid, judging from the following findings: (i) the protease in the extracellular fraction was membrane-free protease, not membrane-bound protease; (ii) the protease activity in the extracellular fraction increase as a function of time before bacterial lysis (Fig. 2); (iii) peaks 1 and 2 were observed in both intracellular membrane-free and extracellular fractions (Fig. 3); and (iv) a halo was observed when cells were grown on agar containing 1% casein or 1 mM BAPNA (data not shown). However, the identity of the peak 1 protease of the intracellular membrane-free fraction with the peak 1 protease of the extracellular fraction requires investigation.

The optimal pH of the protease (pH 8.5) was higher than that of an IgA1 protease (17) and proteases I and II (9) from *B. melaninogenicus* and *T. denticola* fibrinolysin (21) and close to that of a fibrinolytic enzyme from *B. asaccharolyticus* ATCC 25260 (20), protease II from *Staphylococcus aureus* (2), and trypsin. This indicates that the *B. gingivalis* 381 protease is an alkaline protease. The pH of the saliva is about 6 to 8, and this protease was active in this pH range.

Microbial alkaline proteases are generally serine proteases and are inhibited by DFP. The purified protease was inhibited significantly (more than 50%) by DFP (10 mM), TLCK

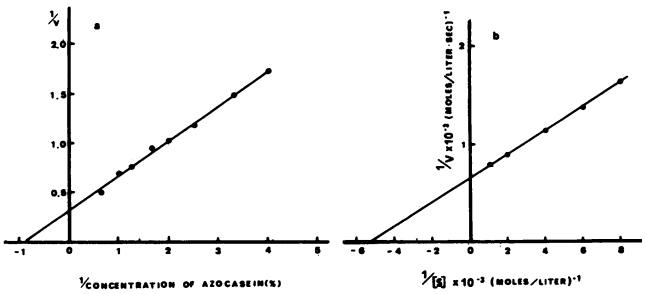

FIG. 8. Lineweaver-Burk plots of *B. gingivalis* 381 protease activity on azocasein (a) and BAPNA (b).

(2.5 and 0.5 mM), egg trypsin inhibitor (50 μg/ml), and leupeptin (50 and 5 μg/ml). This spectrum of inhibition is the same as that of trypsin and similar to that of a fibrinolytic enzyme from *B. asaccharolyticus* ATCC 25260 (20). These results indicate that the purified protease is a serine protease and trypsinlike protease, whereas proteases I and II from *B. melaninogenicus* are not serine proteases (9). Of the inhibitors tested, DFP and egg trypsin inhibitor did not affect the activity of the protease at low concentrations. A similar phenomenon was observed by Yoshimura et al. (37) for inhibition of chymostatin.

When the protease was purified without addition of L-cysteine or 2-mercaptoethanol to the buffer, its activity decreased with time. Its stability was increased by the addition of 5 mmol of L-cysteine or 2-mercaptoethanol to the buffer, like the stabilities of IgA1 protease (17), protease II from *S. aureus* (2), and a trypsinlike, membrane-bound protease (37). The addition of 10 mmol of L-cysteine or 2-mercaptoethanol to the reaction mixture increased the activity of the protease. However, PCMB did not significantly affect its activity, although it affected the activity of the membrane-bound protease reported by Yoshimura et al. (37). The fact that the protease of *B. gingivalis* 381 required reducing conditions for activity indicates the suitability of the enzyme for functioning in the gingival crevices.

The proteolytic activity of the protease was inhibited by Zn^{2+}, Mn^{2+}, and Cu^{2+}. In this, it resembled protease II from *S. aureus* (2). The activity of the protease was inhibited by EDTA (37% inhibition). This suggests that it could be a collagenase or metal protease. However, collagenases are generally activated by Ca^{2+} and Zn^{2+} and inhibited by L-cysteine, and metal proteases are activated by Mn^{2+} and Zn^{2+}; however, this protease was significantly inhibited by these metal ions and activated by L-cysteine, indicating that it is not a collagenase or metal protease. On the other hand, the trypsinlike, membrane-bound protease of Yoshimura et al. (37) was significantly inhibited by EDTA (more than 95% inhibition) and required Mg^{2+} for recovery of activity, whereas our protease was slightly inhibited (30%) by Mg^{2+}.

The activity of the protease was lost on heating at 50°C for 15 min. In contrast, the fibrinolytic enzymes from *T. denticola* (21) and *B. asaccharolyticus* ATCC 25260 (20) are heat stable. The slight instability of the protease at body temperature (37°C) was probably due to autodigestion; in fact, at a higher concentration, 65% of the activity of the purified protease was lost during incubation for 15 min at 37°C (data not shown). However, although we do not know the exact concentration of the protease in the periodontal pocket, we consider that the residual activity in this location at 37°C (80% after 15 min under our conditions) may be sufficient to have an effect on the tissue.

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