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Isolation of a Membrane-Associated *Bacteroides gingivalis* Glycylprolyl Protease

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A low-molecular-weight proteolytic enzyme was purified 47-fold from outer membranes of *Bacteroides gingivalis* ATCC 33277 by preparative polyacrylamide gel electrophoresis. The enzyme was present in all *B. gingivalis* strains tested but was not found in other species of black-pigmented *Bacteroides*. The molecular weight, determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, was 19,500 when the enzyme was heated to 100°C in SDS before electrophoresis and 29,000 when it was mixed with SDS but not heated. The optimum pH, with azocasein as the substrate, was between 6.0 and 6.5. The activity was inhibited by phenylmethylsulfonyl fluoride, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, Hg^{2+}, and various reducing agents. The enzyme was active against azocasein, azocoll, proline-rich protein from saliva, and the synthetic peptide glycyl-L-proline–*p*-nitroanilide. The enzyme did not degrade acid-soluble collagen nor did it hydrolyze various arginine- and lysine-containing synthetic substrates.

Numerous microbiological studies suggest that *Bacteroides gingivalis* is an important etiologic agent (20, 28, 31) of periodontitis. This suspected periodontal pathogen has been shown to produce a number of potential virulence factors, including cell-associated and cell-free proteases (24).

Much of the work on the proteolytic activity of *B. gingivalis* has involved measuring hydrolysis of various naturally occurring proteins by whole-cell and cell-free culture supernatants (4, 5, 10, 16, 25). Unlike other organisms (21, 23), no specific role in pathogenicity has been assigned to the proteolytic activity of *B. gingivalis*. Kaufman et al. (14) have demonstrated that a cell extract of *B. melaninogenicus* (previous taxonomy) possessing collagenase activity was able to increase the experimental septicemia caused by *Fusobacterium nucleatum*. More recently, Grenier and Mayrand (12) have shown that most of the *B. gingivalis* strains, infective in pure culture, were more collagenolytic than those which failed to cause lesions in guinea pigs. Some proteases elaborated by *B. gingivalis* could be associated with the disruption of host defenses. The organism degrades immunoglobulins (16, 25), collagen (10), and complement factors C3 and C5 (25), as well as human proteinase inhibitors (4) and iron-binding proteins (5). Yoshimura et al. (29) demonstrated that a partially purified membrane-bound enzyme was active against the synthetic substrate benzoyl-L-arginine–pNA (pNA, *p*-nitroanilide). More recently, an intracellular membrane-free protease and an extracellular trypsin-like protease have been isolated and characterized by Tsutsui et al. (27) and Fujimura and Nakamura (9), respectively.

In this study, we describe the isolation and characterization of a new cell-bound protease elaborated by *B. gingivalis* 33277.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 19 strains of black-pigmented *Bacteroides* spp. were used in this investigation: *B. gingivalis* 33277, W83, W50, W12, 2D, HW11D-5, HW24D-1, RB46D-1, 22B4, 23A4, 19A4, and 17A1; *B. asaccharolyticus* BM4 and 25260; *B. endodontalis* 35406; *B. denticola* 33185; *B. levii* 29147; *B. loescheii* 15930; and *B. melaninogenicus* 25845. The strains were grown in Trypticase (1.7%; BBL Microbiology Systems)-yeast extract (0.3%) medium containing glucose (0.25%), potassium phosphate (0.25%), sodium chloride (0.5%), hemin (10 μg/ml), and vitamin K (1 μg/ml). Cultures were incubated in an anaerobic chamber (N_2-H_2-CO_2 [85:10:5]) at 37°C.

Isolation and purification of protease. (i) Outer membrane preparation. Outer membranes were prepared by the method of Boyd and McBride (3). Briefly, bacterial cells from an early-stationary-phase culture of *B. gingivalis* 33277 were washed two times with 0.15 M NaCl and suspended in 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl. Outer membranes were removed by shearing whole cells through a 26-gauge hypodermic needle followed by mixing in a Waring blender for 10 s. The mixture was centrifuged at 10,000 × *g* for 20 min to remove intact cells and debris. Outer membranes were isolated by centrifugation of the supernatant at 80,000 × *g* for 2 h. The pellet was then suspended in distilled water and lyophilized.

(ii) Preparative PAGE. Preparative polyacrylamide gel electrophoresis (PAGE) electrophoresis in a 12% resolving gel with the buffer system of Laemmli (18) was performed as in the case of an analytical gel except that spacers of 6-mm thickness were used. Each gel was loaded with 60 mg of outer membranes which had been solubilized in sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris hydrochloride, 4% SDS, 20% glycerol) at 37°C for 30 min. Electrophoresis was carried out at a constant current of 250 mA with cooling for 6 h. After the run, a narrow vertical strip was cut from one side of the gel, whereas the remaining part of the gel was stored at 4°C. The strip was assayed to detect proteolytic activity according to the method of Foltmann et al. (7). The gel strip was first treated with 2.5% Triton X-100 for 60 min to remove the SDS and then equilibrated with 0.3 M sodium acetate (pH 5.3) for 60 min. The proteases were detected by laying the strips on an agarose gel containing 1% skim milk powder as the substrate. After incubation for 3 h at 37°C, the proteolytic activity was detected as white bands

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in a clear agarose gel. The section of the gel corresponding to the proteolytic activity was removed from the remaining part of the gel and finely chopped in 10 mM Tris hydrochloride buffer (pH 7.0). The proteins were eluted overnight at 4°C with constant agitation, dialyzed at 4°C against distilled H_2O for 8 h, and lyophilized. The preparative gel electrophoretic purification procedure, with spacers of 1.5-mm thickness, was repeated with the lyophilized material. The purified protein was suspended in 50 mM Tris hydrochloride (pH 7.0) and kept at −70°C.

Determination of molecular weight. The molecular weight was determined by comparing the migration of the purified protease to that of protein standards on SDS-PAGE gels (18). The samples were solubilized by heating at 100°C for 5 min in the sample buffer containing 2% SDS and 5% 2-mercaptoethanol. After electrophoresis, the gel was stained with silver nitrate. The presence of lipopolysaccharides and glycoproteins was examined by the methods of Tsai and Frasch (26) and Zacharius et al. (30), respectively.

Determination of optimum pH. A total of 100 μl of the purified protease (5 μg/ml) was added to the assay mixtures containing 500 μl of azocasein solubilized in one of the following buffers: 0.5 M acetate buffer (pH 4.0 to 5.5), 0.5 M phosphate buffer (pH 5.5 to 8.0), 0.5 M Tris hydrochloride buffer (pH 7.0 to 9.0), or 0.5 M bicarbonate buffer (pH 9.0 to 11.0). The reaction was terminated after an incubation of 5 h at 37°C by adding 500 μl of 20% trichloroacetic acid and centrifuging at 12,000 × *g* for 5 min. The trichloroacetic acid-soluble material was mixed with 150 μl of 6 N NaOH, and the absorbance was measured at 375 nm. Separate blanks were run for each pH step. The effect of pH on the denaturation of the protease was determined by the azocasein assay. In this case, the enzyme was incubated for 3 h at room temperature in the buffers described above before incubation with the substrate at pH 7.0.

Determination of optimum temperature and heat stability. The optimum temperature for enzyme activity was determined by the azocasein assay at pH 7.0 in 50 mM Tris hydrochloride buffer carried out at 25, 30, 37, and 45°C. The temperature stability was measured as described above, except that the protease was incubated at various temperatures for 30 min before enzyme activity was measured.

Inhibition of enzyme activity. Protease activity was measured in the presence of various protease inhibitors and metal ions: EDTA, phenylmethylsulfonyl fluoride (PMSF), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), SDS, 2-mercaptoethanol, dithiothreitol, cysteine, *p*-chloromercuribenzoic acid (PCMB), pepstatin, aprotinin, $CaCl_2$, $FeCl_2$, $HgCl_2$, $MgCl_2$, and $ZnCl_2$. The enzyme was preincubated for 30 min at room temperature in the presence of the inhibitor. The activity was measured and compared with that of the control by the azocasein assay.

Measurement of peptidase activity and proteolytic activity. Peptidase activity was measured according to the method of Berdal and Olsvik (2). Briefly, 100 μl of the purified enzyme (0.5 μg) was incubated with 100 μl of a 2 mM solution of each of the chromogenic peptides and 300 μl of 75 mM Tris hydrochloride (pH 7.0) at 37°C for 5 h. The release of *p*-nitroaniline was measured by diazotization. Trichloroacetic acid (40%, 500 μl) was added to the reaction mixtures, followed by 150 μl of 0.1% sodium nitrite. The mixture was allowed to react for 5 min, 150 μl of 0.5% ammonium sulfamate was added, and the incubation was continued at room temperature for another 5 min. Finally, 150 μl of a 0.1% solution of *N*-1-naphthylethylenediamine dihydrochloride was added. Diazotization changes the absorption maxima and increases the sensitivity of the test. Semiquantitative determinations were made according to the following scale: 0, no color reaction; +/−, faint red color; and +, strong red color. The A_{545} was recorded for quantitative determinations. Proteolytic activity against azocoll and azoalbumin, as well as gelatin, was detected according to the methods described by Mayrand and McBride (19) and Kortt et al. (17), respectively. The proteolysis of proline-rich proteins (PIF-slow; kindly provided by D. I. Hay, Forsyth Dental Centre), immunoglobulin G (IgG), IgA, and acid-soluble collagen was determined by assaying for lower-molecular-weight degradation fragments on SDS-PAGE.

Detection of enzyme activity in various fractions of *B. gingivalis* 33277 and in different black-pigmented *Bacteroides* spp. Various fractions of *B. gingivalis* 33277 were prepared to detect the activity corresponding to the purified protease. These fractions from 3-day cultures were as follows. (i) Cell extract was prepared from cells broken by ultrasonic treatment (5 × 45 s, 30% duty cycle, output 5; Sonifier Cell Disrupter 350; Branson Sonic Power Co.), and cellular debris and unbroken cells were cleared from the suspension by centrifugation at 80,000 × *g* for 5 h. (ii) Vesicles were prepared according to the protocol of Grenier and Mayrand (11). (iii) Culture supernatant was obtained by centrifugation of cultures at 80,000 × *g* for 1 h.

The presence of the proteolytic activity was determined in various black-pigmented *Bacteroides* cells. Bacterial cells (20-ml culture) grown for 24 h were suspended in 500 μl of 50 mM Tris hydrochloride (pH 7.2). The cells were broken in the presence of 25 mg of glass beads (75 to 150 μm; Sigma Chemical Co., St. Louis, Mo.) by ultrasonic treatment (4 × 15 s, 30% duty cycle, output 5). Cellular debris and unbroken cells were cleared from the suspension by centrifugation (12,000 × *g* for 10 min), and the resulting supernatant was tested. The samples were solubilized in 2% SDS at 37°C for 30 min and electrophoresed on 12% SDS-PAGE. The proteases were visualized on a skim milk gel as described previously.

Chemicals. Synthetic peptides, IgA, IgG, azocoll, azocasein, azoalbumin, acid-soluble collagen, lysozyme, phospholipase C, neuraminidase, and trypsin (T-1005) were purchased from Sigma. Phospholipase A was purchased from Calbiochem-Behring, La Jolla, Calif.

RESULTS

Purification. Preliminary results in our laboratory have shown that strong proteolytic activity was associated with *B. gingivalis* 33277 cells which were harvested at early stationary phase. As the culture aged, the cell-bound activity decreased and activity in the supernatant increased. To study the proteases associated with the cells, an outer membrane fraction was prepared from an early-stationary-phase culture. The syringe shearing method yielded 8 mg (dry weight) of material per liter of culture. The SDS-PAGE pattern of the outer membrane preparation was similar to that described by Boyd and McBride (3). This profile is an indication that the method minimized whole-cell lysis and cytoplasmic membrane contamination. The outer membrane fraction was found to be highly proteolytic, as revealed by the azocasein assay. The electrophoretic zymogram obtained from an outer membrane sample which had been incubated in 2% SDS at 37°C, using skim milk as the substrate, demonstrated the presence of three closely migrating protease bands (Fig. 1). The intensity of activity of

these three bands was similar and consistent from one preparation to another. Overnight incubation did not result in the appearance of additional bands. Treatment of the outer membrane preparation with lysozyme, phospholipases A and C, neuraminidase, or trypsin at 1 mg/ml for 30 min did not affect the electrophoretic zymogram. The fastest-migrating protease was chosen for further study. The quantity of protease obtained after the second preparative PAGE was estimated to be 25 μg, according to a protein determination. This represents about 0.02% of the initial material. The purification resulted in a specific activity 47-fold greater than that of the crude material (Table 1). Electrophoresis of the unboiled purified enzyme on an SDS-polyacrylamide gel yielded a single band whose presence could be demonstrated either by silver nitrate staining or by protease activity on a skim milk gel (Fig. 1, lanes B and G). In a nondenaturing system, the purified protease migrated as a smear when revealed by silver nitrate staining and protease zymogram. Under these conditions, the outer membrane preparation showed an additional fast-migrating band.

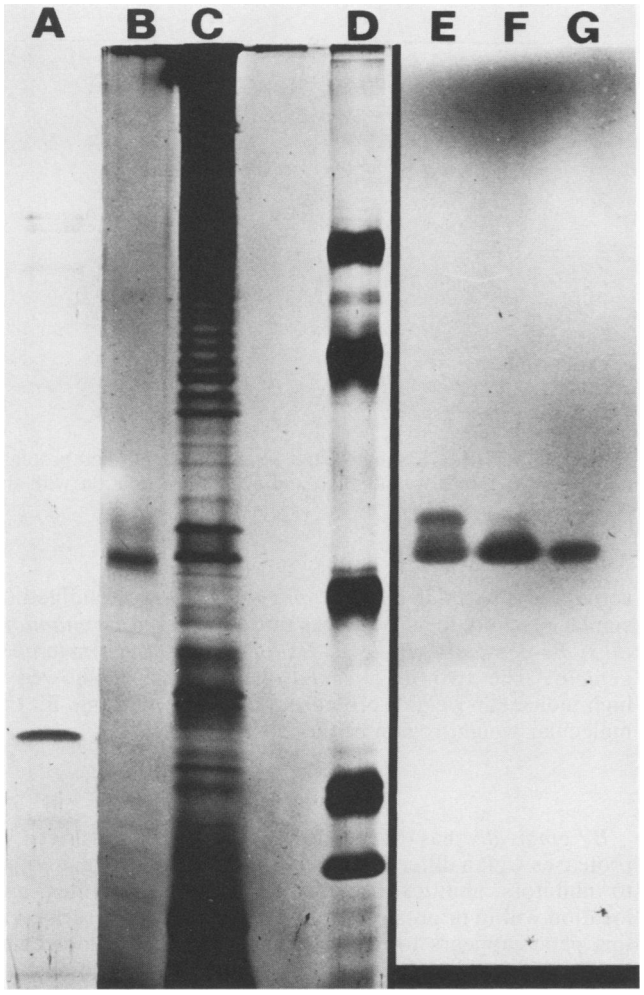

FIG. 1. SDS-PAGE of *B. gingivalis* protease preparations. Lanes A to D are stained with silver nitrate. Lanes E to G are the protease zymogram. Samples in lanes B to G were solubilized in 2% SDS at 37°C for 30 min. The sample in lane A was solubilized in 2% SDS and 5% 2-mercaptoethanol at 100°C for 5 min. Lanes: A, B, and G, purified proteases (0.25 μg); C and E, outer membrane preparations (30 μg); F, material eluted from the first preparative electrophoresis purification step; D, molecular weight markers (from top to bottom, respectively) for bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), β-lactoglobulin (18,400), and lysozyme (14,300).

TABLE 1. Purification of the membrane-associated protease from *B. gingivalis* 33277

Fraction	Protein (mg)	Total units[a]	Sp act (U/mg of protein)	Purification (fold)	Yield (%)
Outer membrane	120	168	1.4	1	100
Purified protease	0.025	1.64	65.6	47	1

[a] One unit of enzyme was arbitrarily defined as the amount of enzyme that released 0.01 μmol of *p*-nitroaniline in 30 min at 37°C from glycyl L-proline–pNA under the conditions described in Materials and Methods.

The band obtained by silver staining corresponds to a molecular weight of approximately 29,000. However, when the sample was boiled in the presence of SDS and 2-mercaptoethanol, the molecular weight was 19,500 (Fig. 1, lane A). No bands were detected when gels of the purified protease were stained for lipopolysaccharide or carbohydrate. The higher-molecular-weight bands seen in lane A are contaminants from the solubilizing solution.

Optimum pH. The cell-bound protease was quite active over the pH range of 5.5 to 7.5. The optimum pH was estimated to be between 6.0 and 6.5 (Fig. 2). The proteolytic activity was completely abolished after incubation at pH 4.0 or 10.0 for 60 min at room temperature.

Optimum temperature and heat stability. The optimum temperature, as determined by the azocasein assay, was 37°C. Preincubation of the purified protease for 30 min at temperatures below 40°C had little, if any, effect on enzyme activity. At 45°C, the enzyme activity was reduced by 60%; no activity could be recovered after preincubation for 30 min at 50°C. The enzyme remained stable for at least 3 months at

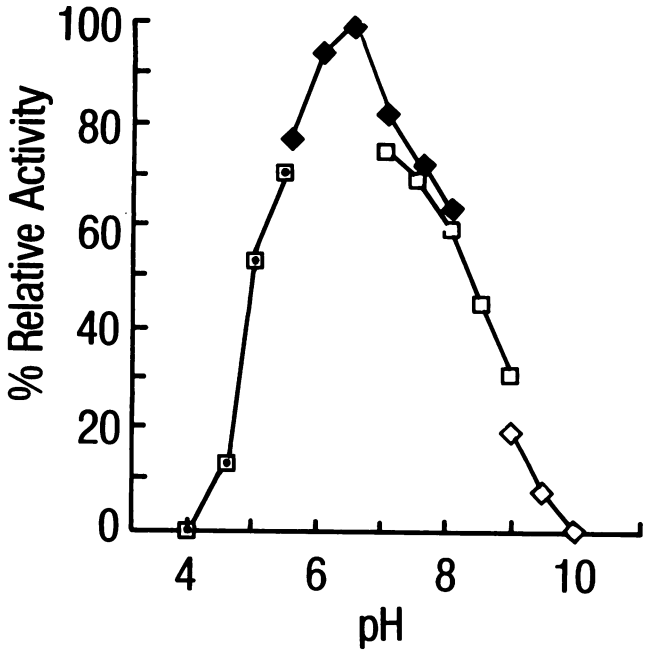

FIG. 2. Optimum pH for the hydrolysis of azocasein by the purified *B. gingivalis* protease. Symbols: ⊡, acetate buffer; ◆, phosphate buffer; □, Tris hydrochloride buffer; ◇, carbonate buffer.

−25°C; repeated freezing and thawing (five times) had no effect on the activity. A reduction of the activity was recorded when the enzyme was kept at 4°C for 24 h. Proteolytic activity was lost after storage at 37°C for 2 days. It does not appear that this loss of activity was due to self-digestion, since the 29,000-molecular-weight silver-staining band did not disappear even though enzyme activity was lost.

Inhibition assays. The effects of various enzyme inhibitors and metal ions on protease activity are shown in Table 2. Among the serine protease inhibitors tested, TLCK and, to a lesser extent, PMSF had an effect on the purified cell-bound protease. A strong inhibition was caused by exposure to reducing agents (2-mercaptoethanol, dithiothreitol, and cysteine) or by reaction with PCMB. The metal chelator EDTA had no effect on the activity, whereas the metal ions Hg^{2+} and Zn^{2+} caused a strong inhibition. There was no difference in activity when the assay was performed in an anaerobic chamber rather than in air.

Peptidase and proteolytic activities. The peptidase and proteolytic activities of the enzyme are shown in Table 3 and Fig. 3. The two azo substrates azoalbumin and azocoll, as well as gelatin and PIF-slow, were degraded by the purified enzyme. The PIF-slow was degraded to two major fragments having molecular weights of 22,500 and 15,000 and two minor fragments having molecular weights of 17,000 and 14,000. Complete hydrolysis to these four fragments occurred after 48 h of incubation with the enzyme. The protease did not hydrolyze IgG, IgA, or acid-soluble collagen. The dipeptide Gly-L-Pro–pNA was strongly hydrolyzed by the purified enzyme, whereas the tetrapeptide benzoyl-Ile-Glu-Gly-Arg–pNA was weakly degraded. Except for the weak activity on L-glutamyl–pNA, no aminopeptidase activity was detected on various L-amino acid–pNA substrates.

Purified protease activity in various fractions of *B. gingivalis* and in different black-pigmented *Bacteroides* species. In addition to being found in the outer membranes, the purified protease activity was detected in the bleb or vesicular fraction. No activity could be detected in cell extracts or in a 20-times-concentrated supernatant which had been centrifuged to remove vesicle. Weak activity was detected in a 20-times-concentrated supernatant obtained from a 7-day-old culture, probably as a result of cell lysis. The protease activity was found in all 12 *B. gingivalis* strains examined. A similar proteolytic activity was not detected in *B. endodontalis*, *B. denticola*, *B. levii*, *B. loescheii*, or *B. melaninogenicus*. The two *B. asaccharolyticus* strains showed a high-molecular-weight protease band but nothing in the molecular weight region of the 29K protease.

TABLE 2. Effect of protease inhibitors and metal ions on enzyme activity[a]

Compound	Concn (mM)	% Residual activity
None		100
EDTA	20	110
PMSF	4	40
TLCK	2	15
TPCK	2	100
SDS	20	42
2-Mercaptoethanol	20	20
Dithiothreitol	20	6
Cysteine	20	10
PCMB	4	42
Pepstatin	8 μg/ml	110
Aprotinin	8 μg/ml	109
$CaCl_2$	2	105
$FeCl_2$	2	95
$HgCl_2$	2	17
$MgCl_2$	2	110
$ZnCl_2$	2	0

[a] Enzyme activity was measured in an azocasein assay, as described in Materials and Methods.

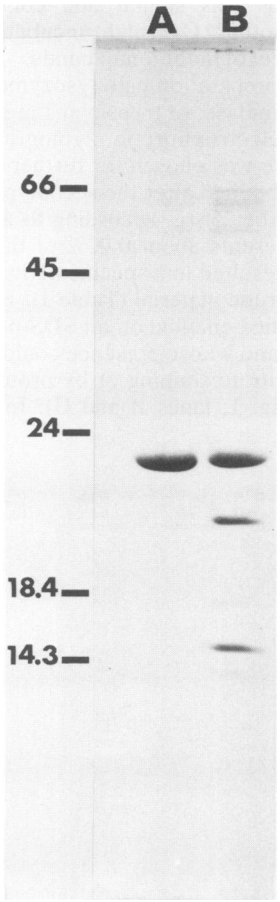

FIG. 3. SDS-PAGE showing the action of the purified protease on PIF-slow. Lanes A and B, 0 and 12 h of incubation with the protease.

DISCUSSION

B. gingivalis has been shown to possess a variety of proteases which differ in their sizes, pH optima, sensitivities to inhibitors, abilities to hydrolyze specific substrates, and location within or outside the cell. The protease described in this paper appears to be unique in terms of its molecular weight and location in an outer membrane preparation.

The purified protease has a molecular weight of 29,000 in its active form and 19,500 in the denatured state. It should be noted that the molecular weight of 29,000 was obtained in SDS under conditions where the activity was retained. Chromatography in the absence of SDS would probably indicate a much larger size. The trypsinlike proteases described by Fujimura and Nakamura (9) and Tsutsui et al. (27) were shown to have molecular weights of 65,000 and 50,000, respectively, by SDS-PAGE. The glycylprolyl dipeptidase described by Abiko et al. (1) had a molecular weight of 160,000. Proteases I and II, isolated by Fujimura and Naka-

TABLE 3. Peptidase and proteolytic activities of the purified protease

Substrate	Activity[a]
L-Alanine–pNA	0
L-Arginine–pNA	0
L-γ-Glutamyl–pNA	+/−
L-Leucine–pNA	0
L-Lysine–pNA	0
L-Methionine–pNA	0
L-Proline–pNA	0
L-Valine–pNA	0
N-α-Benzoyl-D-arginine–pNA	0
Glycyl-L-arginine–pNA	0
Glycyl-L-proline–pNA	+
L-Alanyl-L-alanyl-L-phenylalanine–pNA	0
N-Benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine–pNA	+/−
Methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine–pNA	0
Azocoll	+
Azoalbumin	+
Gelatin	+
Acid-soluble collagen	0
IgG	0
IgA	0
PIF-slow	+

[a] The determination of activity on synthetic substrates was semiquantitative according to the following scale: 0, no color reaction; +/−, faint red color; and +, strong red color. Determination of activity on the other proteins was by visualization of degradation fragments on SDS-PAGE.

mura (8), were estimated by gel filtration to have molecular weights of 420,000 and 73,000, respectively.

Unlike the other *B. gingivalis* proteases, the 29,000-molecular-weight enzyme appears to be associated with the outer membranes of the organism. The evidence supporting this view is that gentle procedures, which remove outer membranes but cause very little cell breakage, release the enzyme. Purified outer membranes possess the activity, as do vesicular structures released from growing cells. The enzyme is not present in the cytoplasmic fraction and is not secreted by actively growing cells. The possibility remains that the enzyme is periplasmic and released with the outer membrane. The precise localization of the enzyme remains to be determined. Yoshimura et al. (29) described a membrane-bound, trypsinlike enzyme but concluded on the basis of solubility in Triton X-100 that the enzyme was located on the cytoplasmic membrane. Fujimura and Nakamura (9) and Abiko et al. (1) characterized enzymes from culture supernatants obtained by centrifugation at 10,000 × *g*. These preparations would contain vesicular structures and therefore there is the possibility that they are associated with membranes.

The 29,000-molecular-weight protease described here is sensitive to TLCK and PMSF, suggesting that it is a serine protease. Interestingly, it is also sensitive to reducing agents, PCMB and Hg^{2+}, suggesting that thiol groups play a critical role in conformation or at the active site.

The enzyme actively hydrolyzed a synthetic peptide containing the glycylprolyl dipeptide and failed to hydrolyze a variety of arginine- and lysine-containing synthetic peptides. The native proline-rich protein (PIF-slow), which contains 25% proline and 21% glycine (22), was cleaved to give four large peptides. These could not be degraded further, suggesting that the enzyme was hydrolyzing specific peptide bonds. Given the high levels of glycine and proline in collagen, it was surprising to find that the enzyme would not degrade acid-soluble collagen. Possibly, the cleavage sites in collagen are not accessible when the molecule is in its native state. It could be that preliminary attack by other proteases or by a substance which separates the collagen into individual subunits would yield a substrate that could be hydrolyzed. Certainly, denatured collagen in the form of azocoll or gelatin was susceptible to hydrolysis. The enzyme described here is similar to the glycylprolyl dipeptidase characterized by Abiko et al. (1) in terms of its ability to hydrolyze glycylprolyl dipeptide and in its sensitivity to serine-reactive inhibitors, but it differs in pH optimum, in molecular weight, and in being found in the culture supernatant. Abiko et al. (1) did not mention whether their dipeptidylaminopeptidase attacked native proteins other than collagen. This comparison with the other *B. gingivalis* proteases suggests that the 29,000-molecular-weight protease is unique and is probably present in the outer membrane.

The discrepancy between the molecular weights of the SDS-heated and unheated preparations was not entirely unexpected, since it has been shown that the electrophoretic behavior of a number of outer membrane proteins varies considerably as a function of how they are prepared for electrophoresis. Porin P of *Pseudomonas aeruginosa*, which exists as a trimer when solubilized in SDS at temperatures below 60°C, has an apparent molecular weight of 94,000. The monomer, which is prepared by solubilization in SDS at 75°C, has a molecular weight of 48,000 (13; R. E. W. Hancock, personal communication). Thus, the apparent molecular weight of 29,000 observed in unboiled samples may represent a dimer with subunits having a molecular weight of 19,500. Alternatively, the molecular weight of 29,000 could be due to association with other constituents which are not seen in silver-stained gels of SDS-heated samples. It is unlikely that the material is lipopolysaccharide or carbohydrate, since the protease does not stain for either of these constituents.

Preparative gel electrophoresis of SDS-treated outer membranes coupled with detection by skim milk zymograms afforded a convenient and rapid procedure for the isolation of protease which, in its native form, is associated with the outer membranes of *B. gingivalis*. The apparent low recovery rate of the purified enzyme (1%) may be explained by the fact that the outer membranes contained other proteases which hydrolyzed the synthetic peptide glycylproline–pNA and would have contributed to the specific activity of the original preparation.

The three proteolytic bands detected in the outer membrane preparation could be three different proteases or a single protease associated with other constituents which modify its migration. Attempts to purify the higher-molecular-weight protease seen in lane E of Fig. 1 always resulted in the appearance of the 29,000-molecular-weight protein and the loss of the higher-molecular-weight enzyme, suggesting that they represent a single enzyme activity. The inability of the skim milk zymogram to detect proteases reported by others, particularly those with high molecular weights, could be attributed to the facts that (i) the assay reveals only proteases active at pH 5.3, (ii) the enzymes must be able to be reactivated upon removal of SDS, and (iii) reducing agents were not present. When another electrophoretic zymogram method was used, in which the polyacrylamide was conjugated to bovine serum albumin (15), six different higher-molecular-weight proteolytic bands were revealed when the gel was incubated in the presence of dithiothreitol (data not shown). This result indicates that *B. gingivalis* produces a variety of proteolytic

enzymes, an observation consistent with the many reports on *B. gingivalis* proteases.

The role of *B. gingivalis* proteases in the pathogenesis of periodontal disease is still unknown. However, it is reasonable to speculate that association of the protease with vesicles released by the cells would aid in the diffusion of the enzyme. The observation that *B. gingivalis* vesicles bind to serum-coated hydroxyapatite (6) or to other bacteria (11) would ensure that the protease remained within the sulcus where it would be able to exert its effect over a prolonged period of time.

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LITERATURE CITED

1. **Abiko, Y., M. Hayakawa, S. Murai, and H. Takiguchi.** 1985. Glycylprolyl dipeptidylaminopeptidase from *Bacteroides gingivalis*. J. Dent. Res. **64**:106–111.
2. **Berdal, B. P., and O. Olsvik.** 1983. *Legionella* extracellular protease activity on chromogenic peptides: a simplified procedure for biochemical enzyme identification. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **91**:89–91.
3. **Boyd, J., and B. C. McBride.** 1984. Fractionation of hemagglutinating and bacterial binding adhesins of *Bacteroides gingivalis*. Infect. Immun. **45**:403–409.
4. **Carlsson, J., B. F. Herrmann, J. F. Höfling, and G. K. Sundqvist.** 1984. Degradation of the human proteinase inhibitors alpha-1-antitrypsin and alpha-2-macroglobulin by *Bacteroides gingivalis*. Infect. Immun. **43**:644–648.
5. **Carlsson, J., J. F. Höfling, and G. K. Sundqvist.** 1984. Degradation of albumin, haemopexin, haptoglobin and transferrin, by black-pigmented *Bacteroides* species. J. Med. Microbiol. **18**: 39–46.
6. **Cimasoni, G., M. Song, and B. C. McBride.** 1987. Effect of crevicular fluid and lysosomal enzymes on the adherence of streptococci and bacteroides to hydroxyapatite. Infect. Immun. **55**:1484–1489.
7. **Foltmann, B., P. B. Szecsi, and N. I. Tarasova.** 1985. Detection of proteases by clotting of casein after gel electrophoresis. Anal. Biochem. **146**:353–360.
8. **Fujimura, S., and T. Nakamura.** 1981. Isolation and characterization of proteases from *Bacteroides melaninogenicus*. Infect. Immun. **33**:738–742.
9. **Fujimura, S., and T. Nakamura.** 1987. Isolation and characterization of a protease from *Bacteroides gingivalis*. Infect. Immun. **55**:716–720.
10. **Gibbons, R. J., and J. B. MacDonald.** 1961. Degradation of collagenous substrates by *Bacteroides melaninogenicus*. J. Bacteriol. **81**:614–621.
11. **Grenier, D., and D. Mayrand.** 1987. Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. Infect. Immun. **55**:111–117.
12. **Grenier, D., and D. Mayrand.** 1987. Selected characteristics of pathogenic and nonpathogenic strains of *Bacteroides gingivalis*. J. Clin. Microbiol. **25**:738–740.
13. **Hancock, R. E. W., K. Poole, and R. Benz.** 1982. Outer membrane protein P of *Pseudomonas aeruginosa*: regulation by phosphate deficiency and formation of small anion-specific channels in lipid bilayer membranes. J. Bacteriol. **150**:730–738.
14. **Kaufman, E. J., P. A. Mashimo, E. Hausmann, C. T. Hanks, and S. A. Ellison.** 1972. Fusobacterial infection: enhancement by cell-free extracts of *Bacteroides melaninogenicus* possessing collagenolytic activity. Arch. Oral Biol. **17**:577–580.
15. **Kelleher, P. J., and R. L. Juliano.** 1984. Detection of proteases in polyacrylamide gels containing covalently bound substrates. Anal. Biochem. **136**:470–475.
16. **Kilian, M.** 1981. Degradation of immunoglobulins A1, A2, and G by suspected principal periodontal pathogens. Infect. Immun. **34**:757–765.
17. **Kortt, A. A., J. E. Burns, and D. J. Stewart.** 1983. Detection of the extracellular proteases of *Bacteroides nodosus* in polyacrylamide gels: a rapid method of distinguishing virulent and benign ovine isolates. Res. Vet. Sci. **35**:171–174.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
19. **Mayrand, D., and B. C. McBride.** 1980. Ecological relationships of bacteria involved in a simple, mixed anaerobic infection. Infect. Immun. **27**:44–50.
20. **Moore, W. E. C., L. V. Holdeman, R. M. Smibert, D. E. Hash, J. A. Burmeister, and R. R. Ranney.** 1982. Bacteriology of severe periodontitis in young adult humans. Infect. Immun. **38**:1137–1148.
21. **Munoz, M. L., M. Rojkind, J. Calderon, M. Tanimoto, S. Arias-Negrete, and A. Martinez-Palomo.** 1984. *Entamoeba histolytica*: collagenolytic activity and virulence. J. Protozool. **31**: 468–470.
22. **Oppenheim, F. G., D. I. Hay, and C. Franzblau.** 1971. Proline-rich proteins from human parotid saliva. I. Isolation and partial characterization. Biochemistry **10**:4233–4238.
23. **Sakai, D. K.** 1985. Loss of virulence in a protease-deficient mutant of *Aeromonas salmonicida*. Infect. Immun. **48**:146–152.
24. **Slots, J., and R. J. Genco.** 1984. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival, and tissue destruction. J. Dent. Res. **63**:412–421.
25. **Sundqvist, G., J. Carlsson, B. Herrmann, and A. Tärnvik.** 1985. Degradation of human immunoglobulins G and M and complement factors C3 and C5 by black-pigmented *Bacteroides*. J. Med. Microbiol. **19**:85–94.
26. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
27. **Tsutsui, H., T. Kinouchi, Y. Wakano, and Y. Ohnishi.** 1987. Purification and characterization of a protease from *Bacteroides gingivalis* 381. Infect. Immun. **55**:420–427.
28. **White, D., and D. Mayrand.** 1981. Association of oral *Bacteroides* with gingivitis and adult periodontitis. J. Periodontal Res. **16**:259–265.
29. **Yoshimura, F., M. Nishikata, T. Suzuki, C. I. Hoover, and E. Newbrun.** 1984. Characterization of a trypsin-like protease from the bacterium *Bacteroides gingivalis* isolated from human dental plaque. Arch. Oral Biol. **29**:559–564.
30. **Zacharius, R. M., J. H. Morrison, J. J. Woodlock, and T. E. Zell.** 1969. Glycoprotein staining following electrophoresis on acrylamide gels. Anal. Biochem. **30**:148–152.
31. **Zambon, J. J., H. S. Reynolds, and J. Slots.** 1981. Black-pigmented *Bacteroides* spp. in the human oral cavity. Infect. Immun. **32**:198–203.