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Surface Location of a *Bacteroides gingivalis* Glycylprolyl Protease

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Various immunological methods were used to localize a glycylprolyl protease previously isolated from *Bacteroides gingivalis* ATCC 33277. The results obtained by enzyme-linked immunosorbent assay, indirect immunofluorescence staining, and indirect immunogold labeling suggest that the glycylprolyl protease is present on the surface of the cell outer membrane and is specific to *B. gingivalis* strains. The enzyme was removed from the cell envelope by treatment of the whole cells with sodium dodecyl sulfate, Triton X-100, sodium deoxycholate, and proteinase K.

The relationship between advanced adult periodontitis and the presence of *Bacteroides gingivalis* in the affected sites is well established (15, 20, 22). This gram-negative anaerobe has been found to degrade various native proteins (3, 4, 9, 13, 18), as well as synthetic peptides (1, 8, 12, 17, 19, 21). These proteases might be involved in the dissemination of invading bacterial species, as well as in the destruction of gingival tissues. In recent years, different serine and thiol proteases have been isolated from specific fractions of *B. gingivalis*. On the basis of cell fractionation studies, these proteases have been shown to be extracellular (1, 8), membrane bound (12, 17, 21), or cytoplasmic (19).

We have recently purified and characterized a glycylprolyl protease (M_r, 29,000) from an outer membrane preparation of *B. gingivalis* (12). The protease has been found to be different from other *B. gingivalis* proteases with similar activities (1, 17). In the present study, we used immunological methods to clearly establish the location of our *B. gingivalis* glycylprolyl protease and show its specificity for this bacterial species.

MATERIALS AND METHODS

Bacterial strains and protease purification. The following strains were used in this study: *B. gingivalis* ATCC 33277, RB46D-1, and HW11D-5; *Bacteroides asaccharolyticus* ATCC 25260 and BM4; *Bacteroides endodontalis* ATCC 35406; *Bacteroides intermedius* BMH; *Bacteroides melaninogenicus* ATCC 25845; *Bacteroides loescheii* ATCC 15930; *Bacteroides denticola* ATCC 33185; and *Bacteroides levii* ATCC 29147. The bacteria were cultured anaerobically at 37°C in Trypticase (1.7%; BBL Microbiology Systems, Cockeysville, Md.)-yeast extract (0.3%) broth containing potassium phosphate (0.25%), sodium chloride (0.5%), glucose (0.1%), hemin (10 μg/ml), and vitamin K (1 μg/ml). Except when noted, experiments were done with strain ATCC 33277.

The glycylprolyl protease was isolated from an outer membrane fraction of *B. gingivalis* 33277 by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with the buffer system of Laemmli (14), as described previously (12). Briefly, the outer membranes were removed from the cells (early stationary growth phase) by shearing through a 26-gauge hypodermic needle, followed by gentle mixing in a Waring blender. The preparation was solubilized in SDS sample buffer (0.125 M Tris hydrochloride, 2% SDS, 20% glycerol) at 37°C for 30 min and then run on a 10% SDS-PAGE gel, using 6-mm-thick spacers. The protease was located by clotting of casein on a skim milk gel (7) and then eluted from the gel. The procedure was repeated twice, using 1.5-mm-thick spacers, to ensure the purity of the glycylprolyl protease.

Antibody production. The purified glycylprolyl protease was injected (10 μg) intramuscularly into a New Zealand White rabbit in the presence of complete Freund adjuvant. Subsequent intramuscular injections, without adjuvant, were done at days 8, 15, and 42. The rabbit was bled via the marginal ear vein at days 21, 29, and 53, and the antisera were pooled. The purified immunoglobulin G (IgG) fraction was prepared by passing the antiserum through a column of protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, Mo.). The sample was exhaustively washed on the column with 0.1 M borate–0.5 M NaCl buffer (pH 8.4). IgG was then eluted with 0.1 M glycine–0.5 M NaCl buffer (pH 2.5), followed by dialysis against 50 mM phosphate-buffered saline (PBS) (pH 7.2). The antilipopolysaccharide antibodies that could have been present in the antiserum were removed by adsorbing 1 ml of the IgG fraction twice with 20 mg of purified lipopolysaccharides solubilized in 2% Triton X-100. The lipopolysaccharides were prepared from *B. gingivalis* 33277 by the method of Darveau and Hancock (5).

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (14) with a 12% separating gel. The samples were solubilized in 2% SDS buffer at either 37°C (30 min) or 100°C (5 min), and the electrophoresis was carried out at a constant voltage of 200 V. The protein bands were visualized by silver nitrate staining. Molecular weight markers were phospholipase b (97,400), bovine serum albumin (BSA) (68,000), ovalbumin (45,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and cytochrome *c* (12,300). The reactivity and specificity of the antibody to the glycylprolyl protease were determined by the electroblotting technique (2). The proteins were transferred electrophoretically to nitrocellulose paper at a constant voltage of 60 V for 2 h. Immunoreactive proteins were detected by the procedure described in the Bio-Rad Immun-Blot (GAR-HRP) assay kit. The antibodies against the glycylprolyl protease were diluted 400-fold, and conjugation with peroxidase was achieved by using 3,000-fold-diluted peroxidase-conjugated goat anti-rabbit IgG.

ELISA. The bacterial suspensions (3-day-old culture; A_{660}

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= 0.5) in carbonate coating buffer (0.05 M, pH 9.6) were treated at 75°C for 1 h and allowed to attach onto microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Alexandria, Va.) by overnight incubation at 4°C. The enzyme-linked immunosorbent assay (ELISA) was performed as described previously (6). Briefly, coated plates were washed with PBS containing 0.01% Tween 20, reacted with 5% BSA (30 min), and then washed with PBS-Tween. Antibody at a dilution of 1:500 in PBS–1% BSA was added to the wells and incubated at 37°C for 2 h. This was followed by washing with PBS-Tween and then incubation for 1 h at 37°C with alkaline phosphatase-labeled goat anti-rabbit IgG (Helix Biotech Ltd., Vancouver, British Columbia, Canada) at a dilution of 1:4,000 in PBS–1% BSA. After a final wash with PBS-Tween, *p*-nitrophenylphosphate was added to each well. Color development was recorded after 2 h of incubation at 37°C by reading of the A_{405} on a Titertek Multiscan ELISA reader (Flow Laboratories, Inc., McLean, Va.).

Indirect fluorescent antibody staining. The bacterial suspensions (3-day-old culture; A_{660} = 0.5) in PBS were allowed to dry onto a glass slide at room temperature and then fixed with formaldehyde (3%) for 30 min. The antibodies to the purified protease were added at various dilutions and then detected by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. The slide was examined for fluorescence under a UV microscope.

Immunogold labeling. *B. gingivalis* cells in the early stationary phase were washed twice in PBS and resuspended in the same buffer to an A_{660} of 1.0. Two hundred microliters of the suspension was incubated with an equal volume of the antiprotease IgG fraction for 1 h. Unbound IgG was removed by centrifugation, and the cells were washed twice in PBS and then resuspended in the original volume (PBS) before being mixed with 25 μl of gold beads (5 nm) conjugated with goat anti-rabbit IgG (EM GAR G5; Janssen Life Sciences Products, Olen, Belgium). After reaction at 4°C overnight, the unbound secondary antiserum was removed by centrifugation. The cells were washed in PBS and negatively stained with 1% phosphotungstic acid. Bacteria coated with nonimmune serum were included as a control. Observations were made with a Philips EM 300 electron microscope.

Cell treatments. The bacterial suspension (A_{660} = 1.0) in PBS (pH 7.0) was treated at 37°C for 30 min in the presence of the following compounds: SDS, Triton X-100, Tween 20, *N*-lauroylsarcosine, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), and sodium deoxycholate all at 0.03%; sodium chloride and lithium chloride at 0.2 M; and EDTA at 50 mM. The cells were then washed twice in PBS and broken by sonication (2 times for 1 min each; 30% duty cycle, output 3; Sonifier cell disrupter; Branson Sonic Power Co., Danbury, Conn.). The cell extracts were solubilized in 2% SDS at 37°C and run on SDS-PAGE gels, and the glycylprolyl protease was detected by immunoblotting. The bacterial suspension (A_{660} = 1.0) in PBS was also incubated at 37°C for 1 h with trypsin or proteinase K (final concentration, 50 μg/ml). These cells were washed twice with PBS, and the glycylprolyl protease was detected by immunofluorescence. The treated cells were also broken by sonication, solubilized in 2% SDS at 37°C, and electrophoresed on SDS-PAGE gels in order to detect the enzyme by immunoblotting and clotting of casein as previously described (7).

Reagents. All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., except as specifically noted.

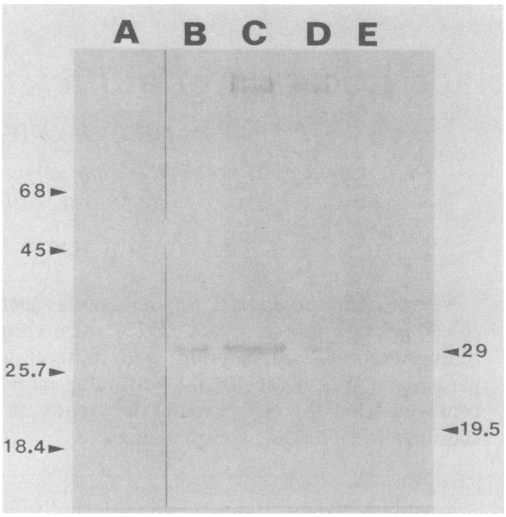

FIG. 1. Immunoblots of *B. gingivalis* 33277 fractions with anti-glycylprolyl protease antibody. Lane A, Purified glycylprolyl protease (0.6 μg incubated at 100°C in SDS buffer for 5 min); lane B, purified glycylprolyl protease (0.2 μg incubated at 37°C in SDS buffer for 30 min); lane C, vesicle preparation (1 μg incubated at 37°C in SDS buffer for 30 min); lane D, outer membrane preparation (1 μg incubated at 37°C in SDS buffer for 30 min); lane E, lipopolysaccharide fraction (5 μg incubated at 100°C in SDS buffer for 5 min). Molecular mass markers are phospholipase b (97.4 kDa), BSA (68 kDa), ovalbumin (45 kDa), α-chymotrypsinogen (25.7 kDa), and β-lactoglobulin (18.4 kDa).

RESULTS

Purification of the glycylprolyl protease yielded 0.1 mg of enzyme from 400 mg of outer membranes, as determined by a Bio-Rad protein determination microassay. The purity of the protease preparation was verified by SDS-PAGE and silver staining, which showed only a single protein band, whose molecular mass was evaluated to be 29 kilodaltons (kDa) when incubated at 37°C (30 min) in the presence of SDS and 19.5 kDa when boiled (5 min) with SDS. Inhibition of the glycylprolyl protease by 2-mercaptoethanol and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), as well as specificity for the synthetic peptide glycyl-L-proline-*p*-nitroanilide, was consistent with the properties previously reported for this enzyme (12).

Immunoblotting of the purified protease and outer membrane preparations of *B. gingivalis* 33277 resulted in staining of the 29-kDa protein band when the samples were treated with SDS at 37°C (Fig. 1, lanes B and D, respectively). The staining observed in the upper part of the resolving gel suggests that the protease may also exist as aggregates which are not dissociated by treatment at 37°C. The reactivity of the antibody with the boiled purified protease (M_r, 19.5 kDa) appeared to be very weak, since the quantity of material loaded was three times greater than that of the nonboiled sample (Fig. 1, lane A). The low reactivity of the boiled enzyme suggests that the major epitopes were modified by heating to 100°C. The absence of any reaction with *B. gingivalis* lipopolysaccharides indicates that the specific IgG fraction was free of contaminating antilipopolysaccharide antibodies (Fig. 1, lane E). When non-lipopolysaccharide-adsorbed antibodies were used for the immunodetection, some bands were visualized in both the outer membrane and the lipopolysaccharide preparations (data not shown). A vesicle fraction, obtained by ultracentrifugation (100,000 × *g*

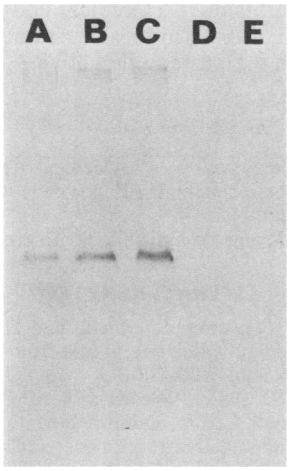

FIG. 2. Immunoblots of sonic cell extracts of different black-pigmented *Bacteroides* species, reacted with anti-glycylprolyl protease antibody. Lane A, *B. gingivalis* RB46D-1; lane B, *B. gingivalis* HW11D-5; lane C, *B. gingivalis* 33277; lane D, *B. asaccharolyticus* 25260; lane E, *B. asaccharolyticus* BM4 (2 μg of protein was loaded in each lane).

for 4 h) of the culture supernatant, also showed immunostaining of the 29-kDa band (Fig. 1, lane C). The glycylprolyl protease band was stronger in the vesicle preparation. A fivefold-concentrated vesicle-free culture supernatant of *B. gingivalis* did not show the 29-kDa band (data not shown). Crude sonic cell extracts of three strains of *B. gingivalis* (33277, RB46D-1, and HW11D-5) contained the glycylprolyl protease immunoreactive band, whereas two strains of *B. asaccharolyticus* (25260 and BM4) did not (Fig. 2).

When the antibodies were used in the ELISA, all the *B. gingivalis* strains showed a positive result (Table 1). No significant ELISA reactivity was obtained with the other black-pigmented *Bacteroides* species. A high IgG-specific titer for the glycylprolyl protease was obtained by immunofluorescence staining of the bacterial cells; the last dilution to give strong staining represented the titer endpoint. This titer was 1:400, whereas a nondiluted nonimmune control serum reacted poorly with the cells. The *B. gingivalis* cells revealed a uniform distribution of label on their cell envelopes. No fluorescence was observed when *B. asaccharolyticus* was incubated with the immune IgG fraction.

TABLE 1. Reactivity of anti-glycylprolyl protease antibody with various black-pigmented *Bacteroides* species in an ELISA

Strain[a]	ELISA reactivity (A_{405})[b]
B. gingivalis	
33277	0.41
RB46D-1	0.50
HW11D-5	0.28
B. asaccharolyticus	
25260	0
BM4	0.05
B. endodontalis 35406	0
B. intermedius BMH	0.05
B. melaninogenicus 25845	0
B. loescheii 15930	0
B. denticola 33185	0.04
B. levii 29147	0.08

[a] Bacterial suspensions were heated at 75°C for 1 h.
[b] The reading obtained with the nonimmune serum was subtracted.

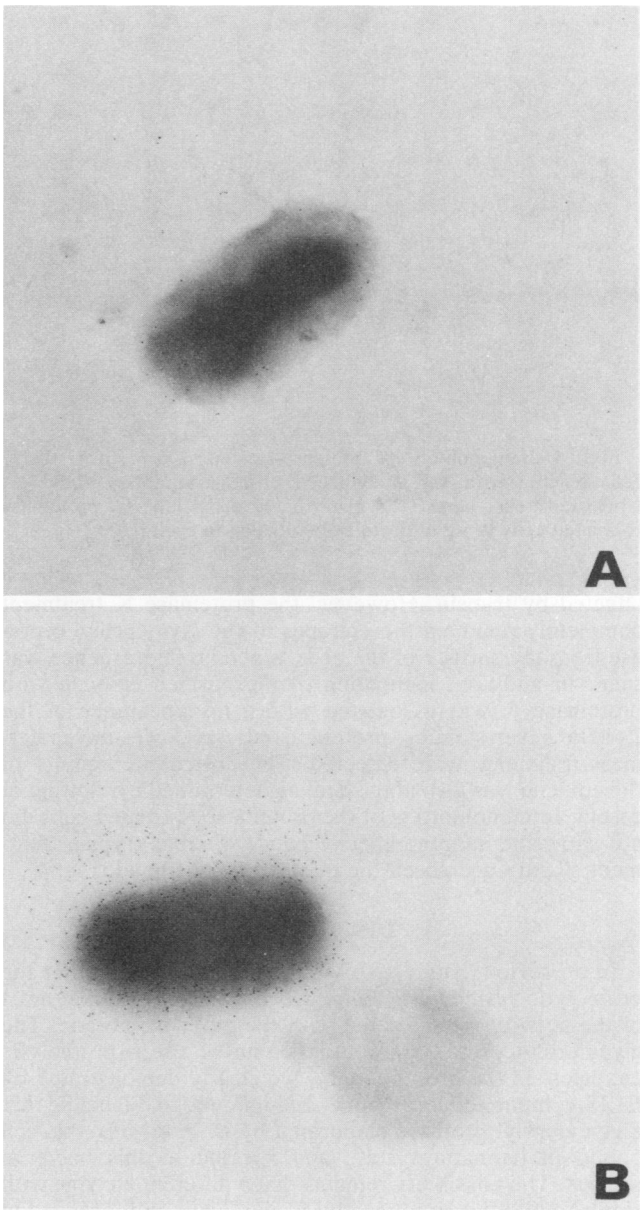

FIG. 3. Whole-mount immunoelectron microscopy of *B. gingivalis* 33277 cells reacted with nonimmune serum (A) and anti-glycylprolyl protease antibody (B). The glycylprolyl protease was detected by using a colloidal gold reagent (see the text).

Immunoelectron microscopic examination of the whole cells revealed gold beads distributed uniformly on the surface of the *B. gingivalis* cell envelope and surrounding the cell (Fig. 3). This suggests that the protease is exposed on the bacterial cell surface. There was no reaction with a nonimmune serum. The specific IgG fraction did not react with the cell surface of *B. asaccharolyticus* (data not shown).

Among the detergents used, Triton X-100, SDS, or sodium deoxycholate at a final concentration of 0.03% completely solubilized the enzyme. The protease was not released by treatment with Tween 20, *N*-lauroylsarcosine, CHAPS, sodium chloride, lithium chloride, or EDTA. Immunofluorescence detection of the glycylprolyl protease on protease-treated *B. gingivalis* cells showed that the enzyme was not

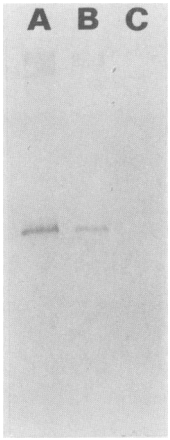

FIG. 4. Immunoblots of protease-treated *B. gingivalis* 33277 cells reacted with anti-glycylprolyl protease antibody. Lane A, Untreated cells; lane B, trypsin-treated cells; lane C, proteinase K-treated cells (5 μg of protein was loaded in each lane).

affected by trypsin. However, the proteinase K treatment completely removed the epitopes of the glycylprolyl protease from the surface of the cells, since no fluorescence was seen. In addition, incubation of the purified enzyme with proteinase K was associated with a disappearance of the 29-kDa silver-staining protein band; no lower-molecular-mass fragments were detected. The proteolytic activity of the enzyme was also abolished, as determined by clotting of casein. Immunoblotting of the proteinase K-treated cells did not show the immunoreactive band, whereas trypsin treatment slightly decreased the intensity of the band (Fig. 4).

DISCUSSION

In a previous study (10) we have demonstrated that the proteolytic system of *B. gingivalis* is complex and that most of the activity is associated with the outer membrane. The glycylprolyl protease represents one of these proteolytic enzymes. In the present study, we clearly demonstrated by ELISA, immunofluorescence, and immunogold labeling that a glycylprolyl protease elaborated by *B. gingivalis* was cell bound in its natural state and specific to this bacterial species. The possibility remains that a different enzyme with similar activity is produced by *B. gingivalis* and localized in another cellular fraction. In particular, Abiko et al. (1) have described a glycylprolyl dipeptidase with an apparent molecular weight of 160,000 which is localized in the culture supernatant of *B. gingivalis*. Furthermore, Suido et al. (17) have partially purified a cell-associated glycylprolyl peptidase which differs from ours in its sensitivity to inhibitors.

B. gingivalis is known to possess attributes which would implicate it in human periodontal disease (16). Among these virulence factors, the cell-bound and cell-free proteolytic activities can be considered to be important. Since *B. gingivalis* is dependent on peptides for growth, a major function of the proteases is to provide nutrients by degrading host proteins to peptides, which can be assimilated by the microorganisms. In addition to the nutritional role, the proteases of *B. gingivalis* could mediate tissue destruction and perturb host defense mechanisms during the pathogenic process of periodontal disease. The location of proteases in the outer membrane of *B. gingivalis* could facilitate these processes and contribute to virulence. Outer membrane fragments and vesicles, which are known to attach to oral hard surfaces (U. Singh, D. Grenier, and B. C. McBride, Oral Microbiol. Immunol., in press) and bacteria (11), would act to concentrate the proteolytic activity in specific areas.

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