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Cellular Location of a *Treponema denticola* Chymotrypsinlike Protease and Importance of the Protease in Migration through the Basement Membrane

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A number of immunological methods were used to localize a cell-associated *Treponema denticola* chymotrypsinlike protease. Indirect immunofluorescence staining, immunogold labeling, and an enzyme-linked immunosorbent assay all indicated that the protease was attached to the outside of the cell envelope. The invasive capability of *T. denticola* was evaluated by following the degradation of a reconstituted basement membrane material (Matrigel) and the release of spirochetes from the gel. Under conditions where the chymotrypsinlike activity was increased, more spirochetes migrated from the gel. Protease inhibitors strongly reduced the number of cells that moved out of the gel. The purified chymotrypsinlike protease degraded the basement membrane components type IV collagen, laminin, and fibronectin. The study suggests that the *T. denticola* chymotrypsinlike protease may play an important role in the invasion and destruction of basement membrane.

Several studies have shown that spirochetes increase in number in periodontal disease (8, 12, 16) and that their numbers decrease significantly after treatment of periodontitis (8). *Treponema denticola* appears to be the cultivable spirochete most specifically associated with affected sites (16). *T. denticola* can attach to the epithelial cells and cause morphological damage to these cells (18, 19). The ability of the spirochetes to degrade synthetic peptides and naturally occurring proteins has been demonstrated (4, 13, 14, 17, 22, 23). Trypsinlike (17) and chymotrypsinlike (23) enzymes have been partially purified, and their properties have been characterized.

Electron microscopic studies have shown that oral spirochetes invade the gingival connective tissue in some forms of periodontal disease (5, 11, 15, 20). To gain access to connective tissue, the bacteria must first overcome tissue barriers created by the epithelium and the basement membrane (1, 21). The destruction of structural and matrix components by microbial proteases may play a critical role in this invasive process. The combination of a bound protease coupled with motility would be an important pathogenic determinant for the spirochetes.

The aim of this study was to establish the location of the chymotrypsinlike protease we recently isolated from *T. denticola* (23) and to evaluate its importance in migration through a basement membrane gel.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The type strains *T. denticola* 35405 and *Treponema vincentii* 35580 were obtained from the American Type Culture Collection. The organisms were grown in liquid medium containing 12.5 mg of brain heart infusion, 10 mg of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 2.5 mg of yeast extract, 0.5 mg of sodium thioglycolate, 1 mg of cysteine, 0.25 mg of L-asparagine, 2 mg of glucose, 6 μg of thiamine pyrophosphate, and 2 mg of sodium bicarbonate per ml; 2% horse serum; and 0.2% volatile fatty acids as described earlier (2). Cultures were incubated in a Coy anaerobic chamber (N_2-H_2-CO_2, 80:10:10) at 37°C. Samples were taken at various times during the incubation to assess growth (A_{660}) and to measure the chymotrypsinlike protease activity.

Determination of chymotrypsinlike activity. The culture supernatant or the washed bacterial cells (A_{660}, 0.25) (100 μl) were incubated with 1 mM succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide in a final volume of 500 μl of 0.05 M Tris hydrochloride buffer (pH 7.2), supplemented with 2 mM dithiothreitol, for 1 h at 37°C. The change in A_{405} was recorded spectrophotometrically.

Chymotrypsinlike protease purification and antibody production. The chymotrypsinlike protease was isolated from a sonicated (five times for 45 s, 30% duty cycle, output 5; Sonifier Cell Disrupter, Branson Sonic Power Co., Danbury, Conn.) cell extract of *T. denticola* 35405 (4-day-old culture) by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (23). The purified chymotrypsinlike protease (15 μg) was injected intramuscularly into a New Zealand White rabbit with complete Freund adjuvant. Subsequent intramuscular injections, without adjuvant, were done on days 8, 14, 22, 36, and 50. The rabbit was bled via the marginal ear vein on day 57. 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).

Indirect fluorescent antibody staining and Western blotting (immunoblotting). *T. denticola* and *T. vincentii* cells from a 2-day-old culture were incubated with the anti-chymotrypsinlike protease antibodies (1:25) for 1 h at 4°C, washed in PBS, and examined by immunofluorescence with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. The *T. denticola* sonicated cell extract was electrophoresed by the

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method of Laemmli (10), and the proteins were transferred electrophoretically to nitrocellulose paper. Immunoreactivity with the chymotrypsinlike protease band was detected by the procedure described in the Bio-Rad Immun-Blot (GAR-HRP) assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Immunogold labeling. *T. denticola* cells from a 2-day-old culture were washed twice in PBS and suspended in the same buffer to an A_{660} of 1.0. A 200-μl sample of the suspension was incubated with an equal volume of the undiluted specific IgG fraction for 1 h at 4°C. Cells with bound IgG were removed by centrifugation, washed twice in PBS, and then suspended in PBS to the original volume and mixed with 25 μl of gold beads (5 nm) conjugated with goat anti-rabbit IgG (EM GAR G5; Janssen Life Sciences Products, Belgium). After incubation at 4°C overnight, the unbound secondary antiserum was removed by centrifugation. The cells were suspended in PBS (200 μl) and then 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.

Invasion assay. *T. denticola* cells from a 2-day-old culture were washed twice in 0.02 M Tris hydrochloride buffer (pH 7.5) containing 0.01 M potassium chloride and 0.2% sodium thiosulfate and suspended in the same buffer to an A_{660} of 0.3. The bacterial suspension (100 μl) was mixed with Matrigel (150 μl; Collaborative Research Inc., Lexington, Mass.), a basement membrane gel obtained from an EHS (Engelbreth-Holm-Swarm) transplantable mouse tumor, and poured into a small glass vial (1 by 3 cm). In some cases, dithiothreitol (2.5 mM), *p*-chloromercuribenzoic acid (PCMB) (5 mM), or phenylmethylsulfonyl fluoride (PMSF) (10 mM) was added to the bacterial suspension. The gel was allowed to solidify for 1 h at 37°C in an anaerobic chamber, and then 150 μl of the supplemented Tris hydrochloride buffer was placed on top of the gel. After 2 h of incubation at 37°C in anaerobic conditions, the buffer (100 μl) was removed, diluted 1:4 in carbonate coating buffer (0.05 M, pH 9.6), and sonicated for 1 min. The presence of spirochetes in the supernatant was evaluated by phase-contrast microscopy (before sonication) and by a measure of the cell-bound chymotrypsinlike enzyme as detected by an enzyme-linked immunosorbent assay (ELISA). The supernatant was analyzed for protease activity by SDS-PAGE with covalently bound bovine serum albumin (7). The invasion assay was also carried out with the purified chymotrypsinlike protease instead of the whole cells.

ELISA. The samples (purified enzyme, cellular fractions, overlying liquid phase from the invasion assay) in carbonate coating buffer (0.05 M, pH 9.6) were bound onto microdilution plates (Immulon 2; Dynatech Laboratories, Inc., Alexandria, Va.) during a 3-h incubation at 4°C. The ELISA was performed as described previously (3). Briefly, coated plates were washed with PBS containing 0.01% Tween 20, reacted with 5% bovine serum albumin (30 min), and further washed with PBS–Tween 20. Anti-protease IgG at a dilution of 1:200 or 1:1,000 in PBS–1% bovine serum albumin was added to the wells and incubated at 37°C for 2 h. This was followed by washing with PBS–Tween 20 and then incubation for 1 h at 37°C with alkaline phophatase-labeled goat anti-rabbit IgG (Helix Biotech Ltd., Vancouver, British Columbia) at a dilution of 1:4,000 in PBS–1% bovine serum albumin. After a final wash with PBS–Tween 20, the substrate *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.). In the invasion experiments, the migration units were obtained by calculating the percentage values from the highest ELISA reading.

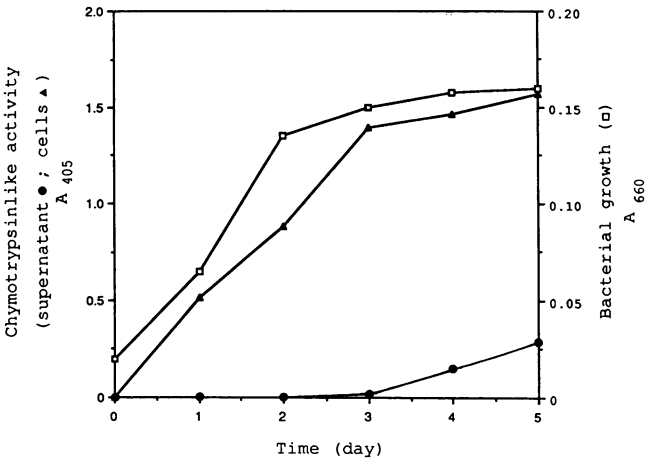

FIG. 1. Growth and chymotrypsinlike activity of *T. denticola* ATCC 35405. The bacterial cells, suspended to an A_{660} of 0.25, and the culture supernatant were assayed as described in Materials and Methods.

Degradation of basement membrane components. The degradation of Matrigel, type IV collagen, laminin, and fibronectin by the purified chymotrypsinlike protease was determined by assaying for lower-molecular-weight degradation fragments on SDS-PAGE with the buffer system of Laemmli (10). Briefly, 25 μl of the enzyme (1.5 μg) was incubated with 25 μl of the substrate (1 mg of protein per ml) and with 25 μl of 100 mM Tris hydrochloride (pH 7.2) containing 10 mM dithiothreitol for 16 h at 37°C (30°C in the case of collagen). The mixture was then boiled in the presence of SDS and 2-mercaptoethanol, and run on SDS-PAGE (12% polyacrylamide). The proteins were stained with Coomassie brilliant blue. Reference proteins were myosin (200 kilodaltons [kDa]), phospholipase *b* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and α-chymotrypsinogen (25.7 kDa).

RESULTS

The growth of *T. denticola* in the complex medium is shown in Fig. 1. Cells grow exponentially for 2 days and then enter the stationary growth phase. Cell-associated chymotrypsinlike protease activity increases during the exponential phase and during the early stationary phase. There is very little cell-free enzyme until day 4, suggesting that the enzyme is released when the cells lyse.

The chymotrypsinlike protease (0.2 mg) was purified from 600 mg of freeze-dried *T. denticola* cells. The purity of the protease was verified by silver staining and proteolytic activity detection on SDS-PAGE, which showed only a single band with an apparent molecular mass of 95 kDa. The protease showed a high specificity for the synthetic chromogenic peptide succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide, was activated by reducing agents, and was inhibited by PMSF and sulfhydryl group reagents, which was consistent with the characteristics previously reported (23).

The presence of anti-protease antibodies in the immune serum was first determined by measuring loss of succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide-degrad-

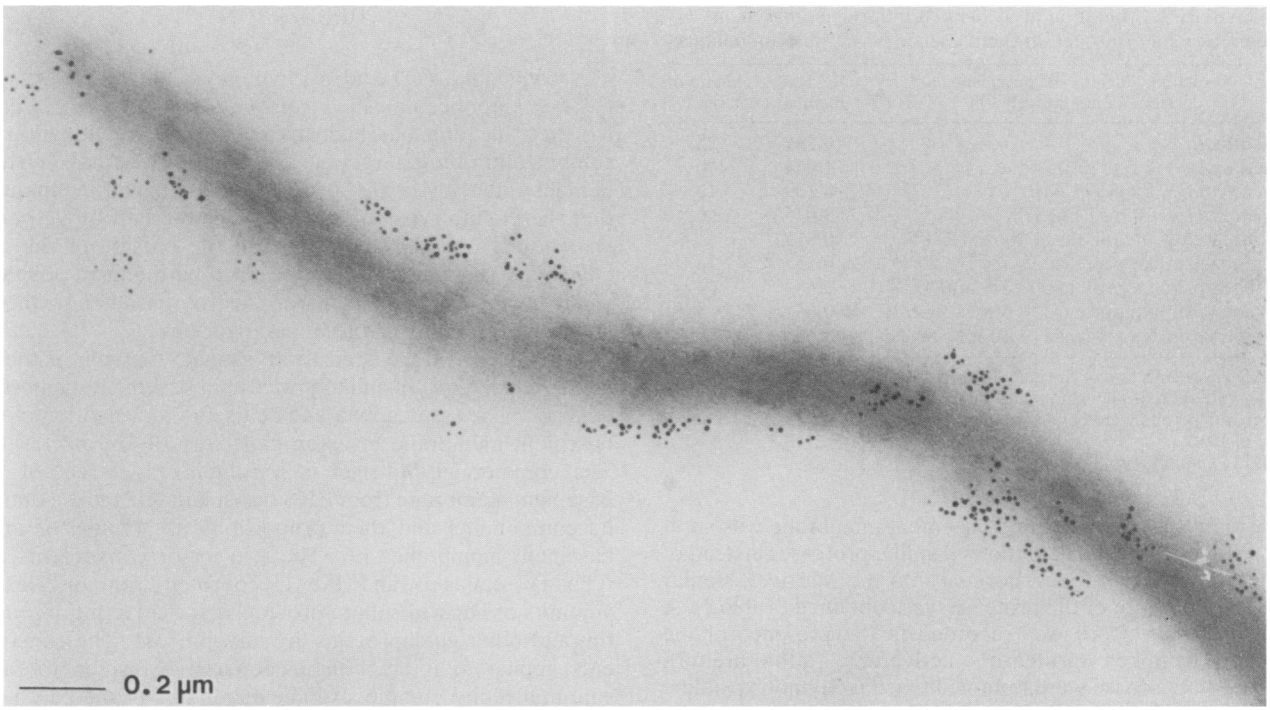

FIG. 2. Whole-mount immunoelectron microscopy of *T. denticola* 35405 cells reacted with the anti-chymotrypsinlike protease antibodies.

ing activity when purified protease (1.5 μg) was incubated for 1 h at 4°C with the immune and nonimmune sera. After centrifugation (13,000 × *g*, 10 min) to remove immune complexes, the supernatant was assayed for chymotrypsinlike activity. An A_{405} of 0.228 was obtained with the immune serum, compared with 0.928 with the control nonimmune serum. This represents a decrease of about 75% of the initial activity. An untreated sample had an A_{405} of 1.45. The immune IgG fraction appeared to be specific for the chymotrypsinlike protease as determined by Western blotting; the antibody reacted only with a 95-kDa band that represented the active form of the protease (data not shown).

T. denticola fluoresced strongly when incubated with the immune IgG fraction in the indirect immunofluorescence assay. There was no fluorescence with nonimmune serum. Incubation of *T. vincentii* cells with the immune fraction did not reveal any fluorescence in the same assay. Electron microscopic observation of *T. denticola* that had been incubated with the immune IgG fraction and gold beads conjugated to goat anti-rabbit IgG revealed that the colloidal gold beads were concentrated in specific areas on the spirochete surface (Fig. 2). Preimmune serum did not label the cells.

When the immune IgG fraction was used in an ELISA, the *T. denticola* cell extract, the high-speed pellet (80,000 × *g* for 4 h) of the same extract, and the purified chymotrypsinlike protease were the only fractions to give a significant reading (Table 1). The fact that the activity in the cell extract could be removed by ultracentrifugation suggests that the enzyme was associated with cell envelope fragments present in the preparation. The *T. vincentii* cell extract did not react with the immune IgG fraction in the ELISA.

The invasiveness capability of *T. denticola* was examined by determining the migration of the organism out of a reconstituted gel-like basement membrane material (Matrigel). After incubation, dark-field microscopic examinations of the liquid phase overlaying the gel showed numerous motile spirochetes. It was difficult to accurately determine the number of escaped spirochetes, and therefore a quantitative procedure was developed. The release of spirochetes from the gel was measured with the antibody for the cell-bound chymotrypsinlike protease by using the ELISA. The role of proteases in migration through the gel was assessed by incubation in the presence and absence of protease activators and inhibitors. The migration units were obtained by assigning the highest ELISA reading a value of 100. The *T. denticola* cells, by themselves, had a migration score of 28 (Table 2). In the presence of the reducing agent dithiothreitol (2.5 mM), which increases the proteolytic activity, the score increased to 100. Finally, when a thiol protease inhibitor (PCMB) or a serine protease inhibitor (PMSF) was included in the assay, the ability of *T. denticola* to escape from the gel was strongly reduced. The PCMB did not appear to affect the motility of *T. denticola*, whereas the PMSF reduced its motility, as verified by dark-field microscopy.

The importance of the chymotrypsinlike protease in the

TABLE 1. Reactivity of the anti-chymotrypsinlike protease IgG fraction with various cellular fractions in the ELISA

Sample[a]	ELISA reading (A_{405}) with immune IgG diluted:	
	1:200	1:1,000
T. denticola culture supernatant	0	0
T. denticola cell extract	0.35	0.20
High-speed pellet of *T. denticola* cell extract	0.29	0.22
High-speed supernatant of *T. denticola* cell extract	0.04	0
Purified chymotrypsinlike protease	0.22	0.11
T. vincentii cell extract	0.03	0.04

[a] The cell extracts were prepared by ultrasonic treatment (1 min). The high-speed supernatant and pellet were obtained by centrifugation at 80,000 × *g* for 4 h.

TABLE 2. Migration of *T. denticola* through a basement membrane gel (Matrigel) in the presence of various compounds

Fraction added to the basement membrane Matrigel[a]	ELISA reading[b]	Migration units[c] (%)
T. denticola	0.295	28
T. denticola + 2.5 mM DTT[d]	1.064	100
T. denticola + 5 mM PCMB	0.109	10
T. denticola + 10 mM PMSF	0.075	6.8
Heat-treated *T. denticola* + 10 mM DTT	0.015	1.4
Chymotrypsinlike protease	0.15	14
Chymotrypsinlike protease + 2.5 mM DTT	0.68	64
Chymotrypsinlike protease + 5 mM PCMB	0.05	4.7
Chymotrypsinlike protease + 10 mM PMSF	0.015	1.4

[a] Final concentration of the compound in the assay.
[b] A_{405} after 2 h of incubation with the alkaline phosphatase substrate.
[c] Migration units were obtained by assigning the highest ELISA reading a value of 100%.
[d] DTT, Dithiothreitol.

invasion of the reconstituted basement membrane was studied. When the purified chymotrypsinlike protease, instead of the living cells, was incorporated into the Matrigel, similar results for release of the protease were obtained (Table 2). A high migration score was recorded in the presence of the chymotrypsinlike protease activator (dithiothreitol), whereas low scores were obtained when a chymotrypsinlike protease inhibitor (PCMB or PMSF) was included to the system.

The presence of the active form of the chymotrypsinlike protease in the overlying liquid phase was measured in a bovine serum albumin-conjugated polyacrylamide gel electrophoresis. The 95-kDa band corresponding to the *T. denticola* chymotrypsinlike protease was detected in higher concentration when dithiothreitol was incorporated into the Matrigel with either the whole cells or the purified enzyme (data not shown).

The ability of the purified chymotrypsinlike protease to degrade the main components of the basement membrane and Matrigel is shown in Fig. 3. The enzyme degraded both glycoproteins laminin and fibronectin into fragments that were not detectable by SDS-PAGE. Some degradation of type IV collagen was observed when the incubation was carried out at 37°C. The collagen appeared to be much more resistant at 30°C (data not shown). Incubation of the protease with the Matrigel resulted in degradation of some proteins present in the preparation; no predominant fragments were produced.

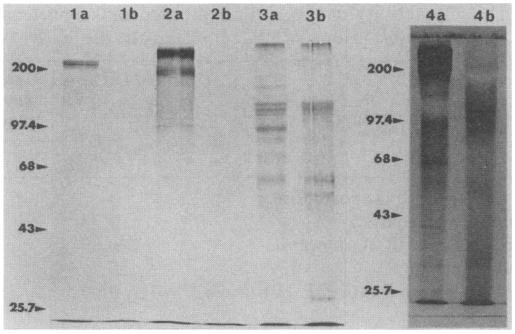

FIG. 3. Degradation of basement membrane components by the purified chymotrypsinlike protease of *T. denticola*. Lanes: 1, fibronectin; 2, laminin; 3, type IV collagen; 4, basement membrane Matrigel; a, enzyme substrate tested at time zero; b, enzyme substrate incubated for 16 h at 37°C.

DISCUSSION

A trypsinlike (17) and a chymotrypsinlike (23) enzyme have recently been purified from *T. denticola*. The evidence for their location has been based on cell fractionation or solubilization by detergents. In this study, we have clearly demonstrated, by using specific immunological techniques, that the chymotrypsinlike protease elaborated by *T. denticola* is cell bound and present on the outside of the cell envelope. The cell surface appears to be the most advantageous location for an enzyme to destroy tissue components and thereby to play a role in pathogenesis.

A model was developed to investigate the role of the *T. denticola* chymotrypsinlike protease in basement membrane invasion. The assay appears to be useful in evaluation of the basement membrane invasion capability of any bacterium. The commercial Matrigel is a solubilized extract of the basement membrane from EHS transplantable mouse tumor; its components and their proportions are typical of most basement membranes (9). The two major constituents are type IV collagen and the glycoprotein laminin; lesser amounts of heparin sulfate proteoglycan, entactin, fibronectin, and other glycoproteins are also present. The constituents appear to interact in a cooperative fashion to form supramolecular complexes. The major role of the basement membrane in the periodontium is to maintain the tissue architecture, to provide a support for normal epithelial cell function, and to serve as barrier regulating the passage of cells and particles between the tissue compartments, according to their size and charge. The bacterial invasion of the subepithelial basement membrane of gingiva is believed to contribute to the pathogenic process of periodontal disease. Recently, *Bacteroides gingivalis*, a suspected periodontal pathogen, has been shown to be able to attach to a basement membrane-like matrix and degrade it (25). Furthermore, a strong basement membrane collagen-degrading enzyme activity has been recently demonstrated in extracts from *B. gingivalis* and *T. denticola* (24).

We observed that *T. denticola* could invade the basement membrane Matrigel and that the chymotrypsinlike protease seems to be important in the invasion capability of this bacteria. The protease can degrade the major components of the basement membrane under physiological conditions. Furthermore, under conditions where the enzymatic activity was increased, the spirochetes were more easily released from the basement membrane gel. In contrast, the presence of protease inhibitors strongly reduced their release. This study suggests that the invasion of connective tissues by *T. denticola* is due to an active process involving the chymotrypsinlike protease instead of a bacterial translocation within the tissues. However, the data obtained do not exclude the possibility of participation of other *T. denticola* hydrolytic enzymes in the invasion process. By destroying immunoglobulins and plasma proteinase inhibitors (23), the chymotrypsinlike protease may also help the organism to escape the host defense system. In the first stage of invasion, *T. denticola* could adhere to the epithelial cells and cause their damage and detachment (18, 19). The presence of microulcerations (6) in the epithelium could also allow the spirochetes to reach the basement membrane. In the second stage, the spirochetes could migrate to the basement membrane, degrade its components, and then migrate further to the connective tissue.

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