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Proteases of *Treponema denticola* Outer Sheath and Extracellular Vesicles

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Electron microscopical observations of the oral periodontopathogen *Treponema denticola* show the presence of extracellular vesicles bound to the bacterial surface or free in the surrounding medium. Extracellular vesicles from *T. denticola* ATCC 35404, 50 to 100 nm in diameter, were isolated and further characterized. Protein and proteolytic patterns of the vesicles were found to be very similar to those of isolated *T. denticola* outer sheaths. They were enriched with the major outer sheath polypeptides (molecular sizes, 113 to 234 kDa) and with outer sheath proteases of 91, 153, 173, and 228 kDa. These findings indicate that treponemal outer sheath vesicles contain the necessary adhesins and proteolytic arsenal for adherence to and damage of eucaryotic cells and mammalian matrix proteins. The major outer sheath- and vesicle-associated protease of *T. denticola* ATCC 35404 was purified and characterized. The purified enzyme had a molecular size of 91 kDa, and it dissociated into three polypeptides of 72, 38, and 35 kDa upon heating in the presence of sodium dodecyl sulfate with or without a reducing agent. The activity of the enzyme could be inhibited by diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, and phenylboronic acid. The value of the second-order rate constant of the protease inactivation by phenylmethylsulfonyl fluoride was 0.48×10^4 M^{-1} min^{-1}. Inhibition of the enzyme by phenylboronic acid was rapid (<1 min) and pH dependent. These data strongly suggest that this major surface proteolytic activity belongs to a family of serine proteases.

Electron microscopic studies of several gram-negative bacteria, including some periodontopathogens, have shown that these bacteria form vesicles (blebs) from their outer membrane which are released into the bacterial surrounding (10, 17, 34). These evaginations of the outer membrane may function as carriers of outer membrane-associated molecules (e.g., lipopolysaccharides and hydrolytic and proteolytic enzymes) and in the transport and exchange of genetic material (11, 34, 51). Vesicles were also shown to contain hemagglutinating and bacterial aggregating activities (17). The biological activities of bacterial extracellular vesicles and their possible role in the progression of periodontal disease have been reviewed extensively (21, 30).

Oral spirochetes normally observed in low numbers in subgingival plaque are found in significantly increased numbers in sites affected with periodontal disease (27, 40), acute necrotizing ulcerative gingivitis (25), and exudates from acute pericoronitis (50). Their populations are markedly decreased after treatment (24, 26).

Treponema denticola, an oral spirochete closely associated with periodontal disease (33, 45), possesses several possible virulence agents, including proteinases (28, 29, 35, 39, 46) and factors interfering with human leukocyte functions (43) and proliferation of fibroblasts (5). In addition, these spirochetes have the ability to attach to host cells and tissues (19, 36).

Treponema spp. bear an outer sheath that surrounds the endoflagella and protoplasmic cylinder of the organism (20). Several studies have noted the presence of extracellular vesicles in oral treponemes grown in culture (8, 9, 49). Treponemal vesicles were also observed by us in direct clinical samples from pericoronal exudates (50). Nevertheless, neither morphological nor biological studies on these vesicles have been reported.

We have recently characterized six different proteolytic enzymes of *T. denticola* with molecular sizes of 91, 123, 138, 151, 173, and 228 kDa (T1 to T6; respectively) (39). Proteases with molecular sizes of 91, 173, and 228 kDa were also found in the cell-free bacterial supernatant. These last enzymes were recovered in the pellet obtained by ultracentrifugation of the concentrated cell-free supernatant and partitioned into the detergent phase after Triton X-114 extraction and phase separation. Our data have pointed to their association with membrane fragments (39).

The aim of the present study was the isolation and biochemical analysis of extracellular vesicles from *T. denticola*. Purification and biochemical characterization of the major *T. denticola* outer sheath and extracellular vesicle protease are also reported.

MATERIALS AND METHODS

***T. denticola* strains and growth conditions.** *T. denticola* ATCC 35404 and ATCC 33520 were obtained from the American Type Culture Collection, Rockville, Md. A clinical strain designated GM-1 was isolated from a human periodontal pocket (43). All strains were grown in GM-1 (4) broth without glucose for 3 or 4 days in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) under an atmosphere of 85% N_2–10% H_2–5% CO_2 at 37°C. Cultures were harvested when they reached an optical density at 660 nm of 0.250, equivalent to approximately 5×10^8 *T. denticola* cells per ml. Liquid cultures were maintained by weekly transfers of a 10% inoculum to fresh GM-1 broth. Bacterial purity and motility were determined by Gram staining and phase-contrast microscopy.

Isolation of extracellular vesicles. Cells from 2 liters of culture were removed from the growth medium by centrifugation at $10,000 \times g$ for 15 min. The culture supernatant was filtered through a 0.2-μm-pore-size filter (Schleicher and Schuell, Dassel, Germany) and further concentrated 25-fold by ultrafiltration with an Amicon YM10 membrane. The concentrated supernatant was dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.8) and then centrifuged at $100,000 \times g$ for 2 h. The supernatant was discarded, and the precipitate containing the extracellular vesicles was washed twice with 50 mM Tris-HCl (pH 7.8) by centrifugation at $100,000 \times g$ for 2 h and kept at −20°C. Outer sheaths were prepared as described previously (39).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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(SDS-PAGE) was carried out by the Laemmli method (23) with a Mini Protean II cell (0.075-cm thick; Bio-Rad Laboratory, Richmond, Calif.). Gels contained 240 μg of human fibrinogen (Immuno, Heidelberg, Germany) per ml. After electrophoresis, the gels were incubated for 30 min at room temperature in Tris-buffered saline (TBS; 0.05 M Tris-HCl, 0.15 M NaCl [pH 7.8]) containing 2% Triton X-100 and then washed three times with TBS (39). Gels were incubated at 37°C for 1.30 h. Bands of activity were revealed after staining with Coomassie brilliant blue R-250. Proteolytic activity was visualized as a clear band against a blue background. Gels without the protein substrate were stained with either Coomassie brilliant blue R-250 or silver.

Samples were either dissolved at room temperature or heated at 100°C for 10 min (2:1, vol/vol) in sample buffer (192 mM Tris-HCl [pH 6.8], 30% glycerol, 9% SDS). Where stated, 15% β-mercaptoethanol was added to the sample buffer.

Molecular masses of protein bands were calculated by linear regression analysis of high- (45 to 205 kDa; Sigma), and low- (14 to 97 kDa; Bio-Rad) molecular-mass standards.

Transmission electron microscopy. *T. denticola* ATCC 35404, ATCC 33520, and GM-1 grown for 3 to 4 days in GM-1 medium were prepared for transmission electron microscopy by first mixing cells with 3% glutaraldehyde in cacodylate buffer (0.1 M; pH 7.4) for 2 h at room temperature.

The samples were sedimented, postfixed in 2% OsO_4, and rinsed in water. The samples were then stained with 2% aqueous uranyl acetate for 1 h at 4°C. Dehydration with ethyl alcohol was performed, and samples were embedded in Spurr's low-viscosity resin. Ultrathin sections were cut with an LKB ultramicrotome, picked up on copper grids, and poststained with 2% uranyl acetate (prepared in 50% methanol) and then with 0.2% lead citrate. Sections were viewed with a Philips CM 12 electron microscope at an accelerated voltage of 100 kV.

T. denticola cells or isolated vesicles were also negatively stained with 1% (wt/vol) phosphotungstic acid and observed with a Philips CM 12 electron microscope.

Scanning electron microscopy. Scanning electron microscopy was performed after processing the treponemal samples as described for transmission electron microscopy, except for the following changes. The dehydration process was accomplished through a graded series of Freon 113 in absolute ethanol rather than by critical-point drying. After triple rinsing in 100% Freon, the specimens were shaken vigorously in air, which allowed rapid evaporation of the Freon phase, leaving the samples dry. The specimens were mounted on coverslips (12-mm diameter), which were previously coated with a 0.1% aqueous solution of polylysine hydrobromide. The coverslips were then mounted on stubs, introduced into a sputter coater (Polaron ES100), and coated with gold. The specimens were examined under a Philips 505 scanning electron microscope at an accelerating voltage of 20 kV.

Purification of T1 protease. Isolated outer sheaths (39) were subjected to preparative SDS-PAGE (6.5% polyacrylamide). After electrophoresis, a thin strip from each side of the gel was cut and stained with Coomassie brilliant blue R-250 to localize the enzyme. T1 was electroeluted from the remaining gel with a Bio Trap BT 1000 electroeluter (Schleicher and Schuell) with Tris-glycine buffer (25 mM–192 mM) without SDS for 2 h at 200 V and then for 10 h at 100 V. The enzyme was stored at −20°C.

Enzymatic activity. The enzymatic activity was assayed by incubating 0.4 to 0.8 μg of enzyme per ml in TBS (pH 7.8) at 25°C with the chromogenic substrates SAAPNA (*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide), BAPNA (*N*-α-benzoyl-L-arginine-*p*-nitroanilide), L-γ-glutamyl-*p*-nitroanilide, L-proline-*p*-nitroanilide, and *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide. The change in optical density at 405 nm was recorded spectrophotometrically. The hydrolysis of FALGPA (furyacryloyl-Leu-Gly-Pro-Ala) was monitored as described previously (48). Initial velocities were calculated from reaction progress curves recorded continuously for the first 10 min of the reaction.

Degradation of the extracellular matrix proteins collagen IV from human placenta (Sigma), fibronectin from bovine plasma (Sigma), and human fibrinogen was monitored by analyzing the degradation products by SDS-PAGE (7.5% gel; Coomassie brilliant blue R-250 staining). The reaction mixtures contained 1.4 mg of protein and 0.8 to 1.6 μg of enzyme per ml of 50 mM Tris-HCl (pH 8.0). The hydrolysis was carried out for 17 h at either 28 or 37°C. Fibrinogen and fibronectin samples were neither heated nor reduced. Collagen samples were heated at 100°C for 3 min in sample buffer containing 15% β-mercaptoethanol.

Effect of inhibitors. The effect of a number of serine protease, thiol protease, carboxy protease, and metalloprotease inhibitors on the activity of T1 was investigated. Assays were performed by preincubating the enzyme (about 0.4 μg/ml) with the inhibitor in TBS (pH 7.80) for 30 min at 25°C (unless otherwise stated) before the addition of 3 mM SAAPNA. The enzymatic activity was recorded for 10 min. For those inhibitors dissolved in dimethyl sulfoxide, isopropanol, or methanol [(i.e., diisopropylfluorophosphate (DIF), phenylmethylsulfonyl fluoride (PMSF), tosylphenyl chloromethyl ketone (TPCK), *N*-*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucylagmatine (E-64), and the peptidyl diazomethyl ketones benzoyloxycarbonyl (Cbz)-Phe-Ala-CHN_2, Cbz-Phe-Phe-CHN_2, Cbz-Phe-Cys-CHN_2, and 1,10-phenanthroline], the final solvent concentration in the assay system was 5% and appropriate controls were included.

Inhibition with phenylboronic acid was performed in phosphate buffer (0.2 M) for a pH of 6 to 8.5, in acetate for a pH below 6.0, and in carbonate for a pH above 8.5. The rate of inactivation with PMSF was determined by incubating inhibitor at increasing concentrations (5 to 50 μM) with the enzyme in TBS buffer (pH 7.8) at 25°C. The rate of irreversible inhibition was monitored by withdrawing samples for assay with 3 mM SAAPNA at the intervals stated, after mixing enzyme and inhibitor. The apparent first-order rate constants for the inactivation reactions (k_{app}) were determined from the slopes of semilogarithmic plots of enzymatic activity versus time.

RESULTS

Negatively stained electron micrographs of the three *T. denticola* strains examined showed extracellular vesicles either as evaginations of the outer sheath still attached to the surface of the cells or free in the medium (Fig. 1A, strain ATCC 33520; Fig. 1B to D, strain ATCC 35404 [strain GM-1 is not shown]). The vesicle diameters ranged from 50 to 150 nm. The morphological similarity of the outer membranes surrounding both cells and vesicles (Fig. 1B) suggests that these vesicles originate by budding of the outer sheath. The spherical vesicles were observed when the treponemes were grown in GM-1 medium (Fig. 1), with or without serum, while uninoculated media were devoid of these structures (data not shown). Scanning electron micrographs (Fig. 1C) demonstrated the morphological structure and the surface pattern of *T. denticola* during the process of extracellular budding of the vesicles.

Extracellular vesicles from strain ATCC 35404 were separated from the cells by centrifugation at 8,000 × *g* for 15 min and further filtration of the supernatant through 0.2-μm-pore-size Millipore filters. This last step rendered vesicle preparations free of bacteria. The vesicles were then recovered by ultracentrifugation and further washed to eliminate contaminating growth medium proteins. Negative staining of the sedimented material revealed pure preparations of vesicle material (Fig. 1D). This methodology seemed a better choice for the preparation of vesicles from *T. denticola* cultures than ammonium sulfate precipitation of the cell-free growth media (17), in which only aggregated structures could be observed by electron microscopy (data not shown).

The availability of isolated vesicle preparations permitted the study of their protein and proteolytic contents. These patterns were found to be very similar to those of isolated outer sheaths shown by us previously (39). The vesicles were highly enriched with an oligomeric outer sheath polypeptide, with a molecular size of 113 to 234 kDa, that upon heating resolved into two major polypeptides with molecular sizes of 53 and 46 kDa (Fig. 2, OS and V). The proteolytic profiles of the isolated vesicles showed four proteolytic bands with molecular sizes of 91, 153, 173, and 228 kDa (T1, T4, T5, and T6, respectively). These proteases were also observed in isolated outer sheath preparations (Fig. 3, OS and V). A diffuse T1 band appeared in the zymograms (Fig. 3) as a result of the high proteolytic activity of T1 with regard to the T4, T5, and T6 proteases. Nevertheless, when the outer sheath and vesicle preparations were diluted enough to get a good resolution of T1, this protease resolved into one proteolytic band (39). Thus, *T. denticola* releases extracellular outer sheath vesicles, which possess four proteases, into the culture supernatant.

T1 is the major *T. denticola* protease associated with the bacterial outer sheath. This protease correlated with the major proteolytic activity found in extracellular vesicles as determined by the molecular weight of the enzyme from both outer sheath and vesicle preparations. T1 was purified from strain ATCC 35404 and further characterized. Purification involved the preparation of isolated outer sheaths, followed by preparative SDS-PAGE and electroelution. The outer sheaths were preferred over whole-cell sonicates as starting material for purification since they contained large quantities of protease and were free of most contaminating proteins. Homogeneous

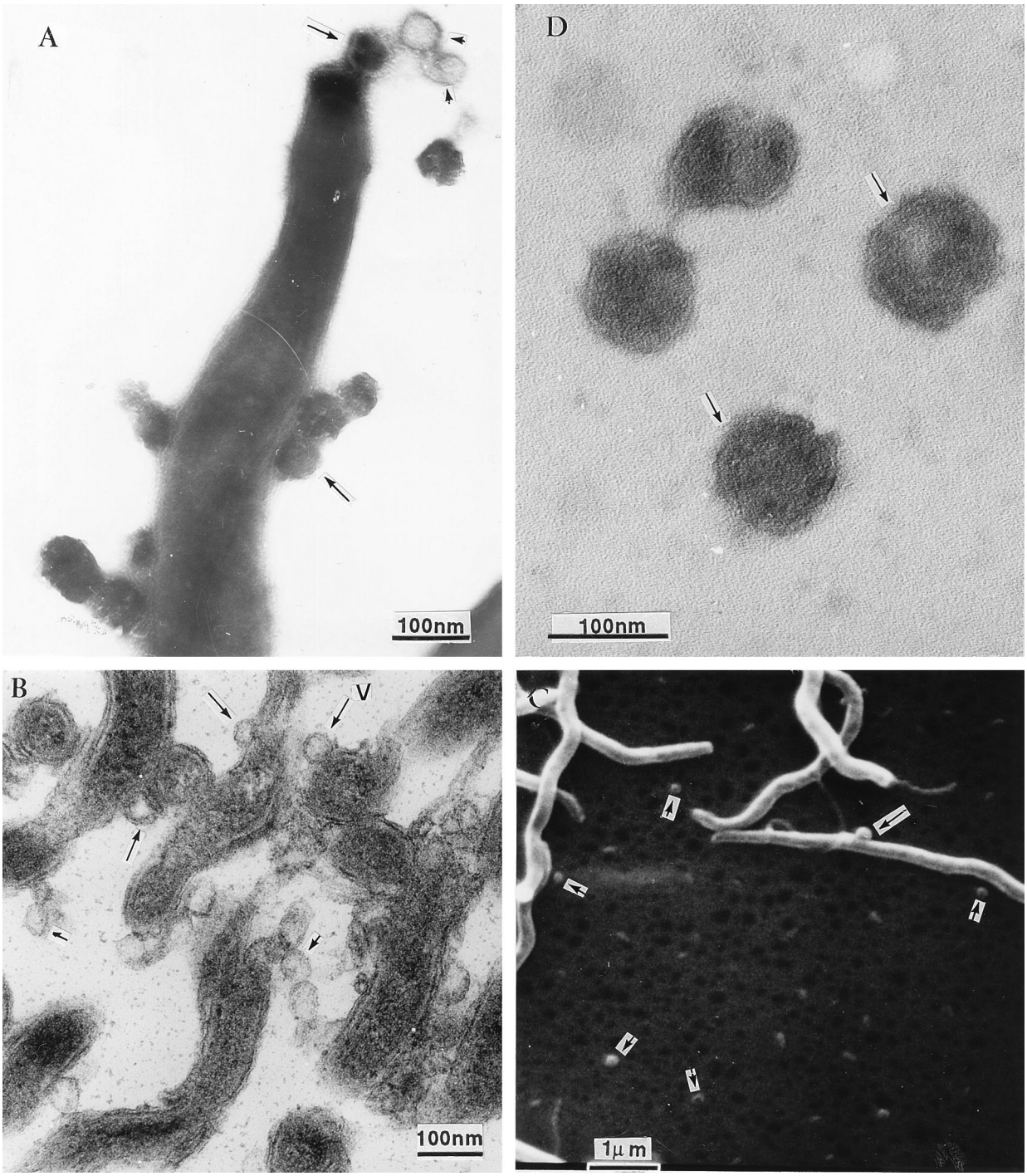

FIG. 1. Electron micrographs of *T. denticola*. (A) Negative staining of *T. denticola* ATCC 33520; (B) thin sections of *T. denticola* ATCC 35404; (C) SEMs of *T. denticola* ATCC 35404 (note the membranous vesicles bound to the surface of the cells [long arrows] or free in the extracellular medium [short arrows]); (D) negative staining of an isolated vesicle preparation of *T. denticola* ATCC 35404.

T1 preparations were obtained after the last purification (80 μg of purified enzyme per liter of treponemal cultures). A zymogram of the purified T1 is shown in Fig. 3 (lane T1). The specific activity of the purified enzyme was on the order of 100 to 150 U/mg, a 100-fold purification from total treponemal cells (1.2 U/mg) and a 15-fold purification from outer sheath preparations (8 U/mg). The specific activities of T1 purified from outer sheath and vesicle preparations were very similar. Nevertheless, 1.6-fold more membranes per liter of treponemal cultures were obtained from outer sheath than from vesicle preparations. Therefore, T1 purification from outer sheaths was pursued.

SDS-PAGE of the purified T1 is shown in Fig. 4. The molecular size of the native enzyme is 91 kDa. Upon heating, T1

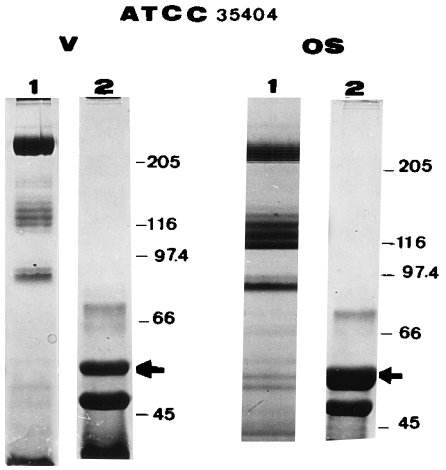

FIG. 2. *T. denticola* ATCC 35404 protein profiles. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. V, extracellular vesicle proteins; OS, outer sheath proteins. Lanes: 1, unheated samples; 2, samples heated at 100°C for 10 min as described in Materials and Methods. The arrows indicate the 53-kDa band calculated by linear regression of molecular mass standards. Numbers to the right of the gels are molecular sizes in kilodaltons.

dissociated into three polypeptides with molecular sizes of 72, 38, and 35 kDa (Fig. 4, lanes 1 and 2). The same polypeptide pattern was obtained when the enzyme was boiled in the presence or absence of a reducing agent (data not shown). Inhibition of the enzyme by PMSF, prior to boiling and SDS-PAGE, to prevent autodigestion of T1, failed to change this electrophoretic pattern (Fig. 4, lane 3). We therefore conclude that the three bands obtained after boiling were possibly due to the dissociation of strong noncovalent bonds between the polypeptides rather than to autodigestion.

A number of synthetic compounds were used to determine the substrate specificity of T1. The purified enzyme had a preference for phenylalanine in the P-1 position in synthetic substrates, readily hydrolyzing the chromogenic substrate

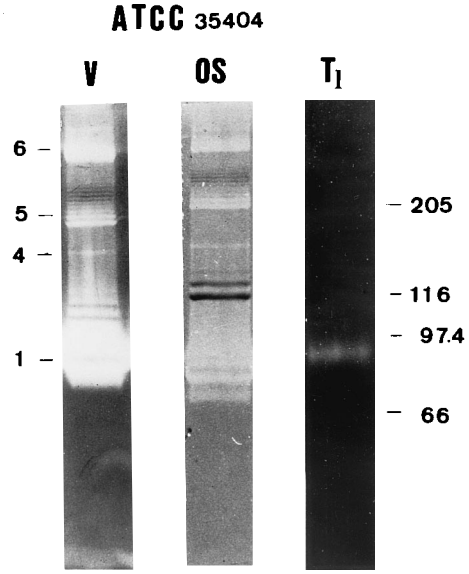

FIG. 3. *T. denticola* ATCC 35404 protease profiles on fibrinogen-containing polyacrylamide gels. V, extracellular vesicle proteases; OS, outer sheath proteases. T1, purified T1. Numbers to the right are molecular sizes in kilodaltons.

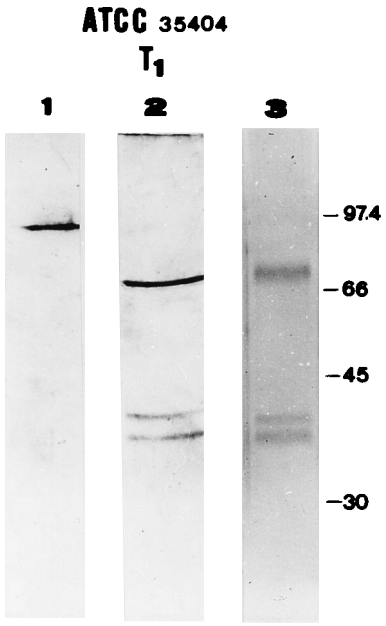

FIG. 4. Silver-stained SDS-polyacrylamide gel of the purified T1 protease (T_1) from *T. denticola* ATCC 35404. Lanes: 1, unheated sample; 2, sample heated at 100°C for 5 min; 3, sample heated at 100°C for 5 min after 10 min of preincubation of T1 with 1 mM PMSF. Numbers to the right are molecular sizes in kilodaltons.

SAAPNA. The K_m of T1 for this substrate was found to be 0.65 mM (determined by double reciprocal plots), and the optimal pH was found to be 7.8 to 8.0. BAPNA, FALGPA, L-γ-glutamyl-*p*-nitroanilide, L-proline-*p*-nitroanilide, and *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide, which do not contain phenylalanine, were not hydrolyzed by T1.

The ability of the purified T1 to degrade extracellular matrix proteins was evaluated. The protease cleaved fibronectin into small degradation products that were not detected on the gels (Fig. 5, lanes 1). Fibrinogen was hydrolyzed to polypeptides with molecular sizes below 56 kDa (Fig. 5, lanes 2). Degradation of the α chains of collagen IV was observed at 28 and 37°C (Fig. 5, lanes 3 and 4). The increased hydrolysis of collagen at 37°C was probably due to the enhanced thermal denaturation

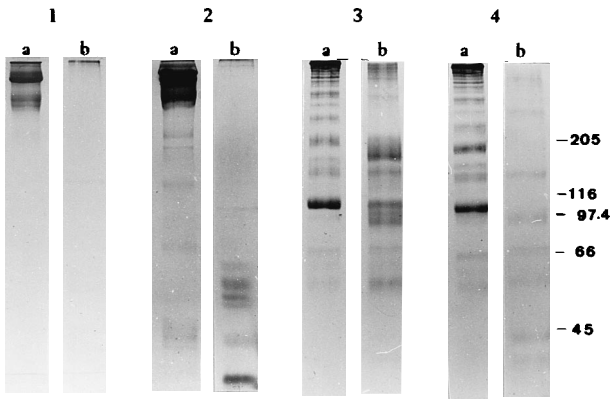

FIG. 5. Degradation of extracellular matrix proteins by purified T1 performed as described in Materials and Methods. Lanes: 1, fibronectin; 2, fibrinogen; 3, collagen IV incubated at 28°C; 4, collagen IV incubated at 37°C; a, proteins incubated without enzyme; b, proteins incubated with enzyme. Numbers to the right are molecular sizes in kilodaltons.

TABLE 1. Effect of inhibitors on T1 activity

Type of inhibitor	Agent	Concn	Remaining activity (%)
Serine protease	DIF	2 mM	4
	PMSF	0.005 mM	50
	PMSF	0.010 mM	27
	PMSF	0.050 mM	0
	TPCK	0.5 mM	50
	TLCK[a]	0.5 mM	100
	Phenylboronic acid	0.1 mM	75
	Phenylboronic acid	1 mM	43
	Phenylboronic acid	5 mM	5
	Chymostatin	30 μg/ml	78
	Chymostatin	150 μg/ml	50
Thiol protease	Dithiothreitol	5.0 mM	100
	Cysteine	5.0 mM	100
	Cysteine	25.0 mM	100
	β-Mercaptoethanol	5.0 mM	100
	E-64	1.5 mM	100
	Cbz-Phe-Ala-CHN_2	0.1 mM	100
	Cbz-Phe-Cys-CHN_2	0.1 mM	100
	Cbz-Phe-Phe-CHN_2	0.1 mM	100
	p-Chloromercuri-benzoate	0.05 mM	0
	$HgCl_2$	0.05 mM	0
Metalloprotease	$MgCl_2$	1.0 mM	100
	$CaCl_2$	1.0 mM	100
	$ZnCl_2$	1.0 mM	34
	1,10-Phenanthroline	5 mM	100
Carboxyl protease	Pepstatin	30 μg/ml	100
	Pepstatin	150 μg/ml	100

[a] TLCK, *N*α-*p*-tosyl-L-lysine chloromethyl ketone.

of the molecule at this temperature. At 28°C, collagen I from rat tail was not susceptible to degradation by the protease (data not shown).

The effect of various proteinase inhibitors on the purified T1 was examined (Table 1). All specific serine proteinase inhibitors, i.e., DIF, PMSF, and phenylboronic acid, strongly inhibited T1 activity. The time course of T1 inactivation by PMSF is shown in Fig. 6A. The secondary plot of $1/k_{app}$ versus 1/[PMSF] produced a straight line with a slope of 0.9 (Fig. 6B). These data clearly indicate that inactivation of T1 by PMSF is time and concentration dependent. The second-order rate constant (k_{app}/[inhibitor]) was calculated to be 0.48×10^4 M^{-1} min^{-1}. Inhibition by phenylboronic acid was rapid (<1 min) and pH dependent. Maximal inhibition was obtained at pH 8.0 to 8.5 (data not shown), similar to the rapid pH-dependent inhibition of chymotrypsin and subtilisin (37).

Chymostatin, a chymotrypsin inhibitor of microbial origin, was also found to inhibit T1 (Table 1).

T1 was not activated by the sulfhydryl group reagents L-cysteine, dithiothreitol, or β-mercaptoethanol and was not inhibited by the thiol-specific reagent E-64 or the peptidyl diazomethyl ketones Cbz-Phe-Cys-CHN_2, Cbz-Phe-Ala-CHN_2, and Cbz-Phe-Phe-CHN_2 (Table 1). This last compound did not inhibit T1 activity although it satisfied its specificity.

The enzyme activity was unaffected by Ca and Mg, while it was strongly inhibited by Zn and Hg. Neither 1,10-phenanthroline nor pepstatin had any effect on the activity of the enzyme.

DISCUSSION

This report presents morphological and biochemical evidence for the formation and release of extracellular outer

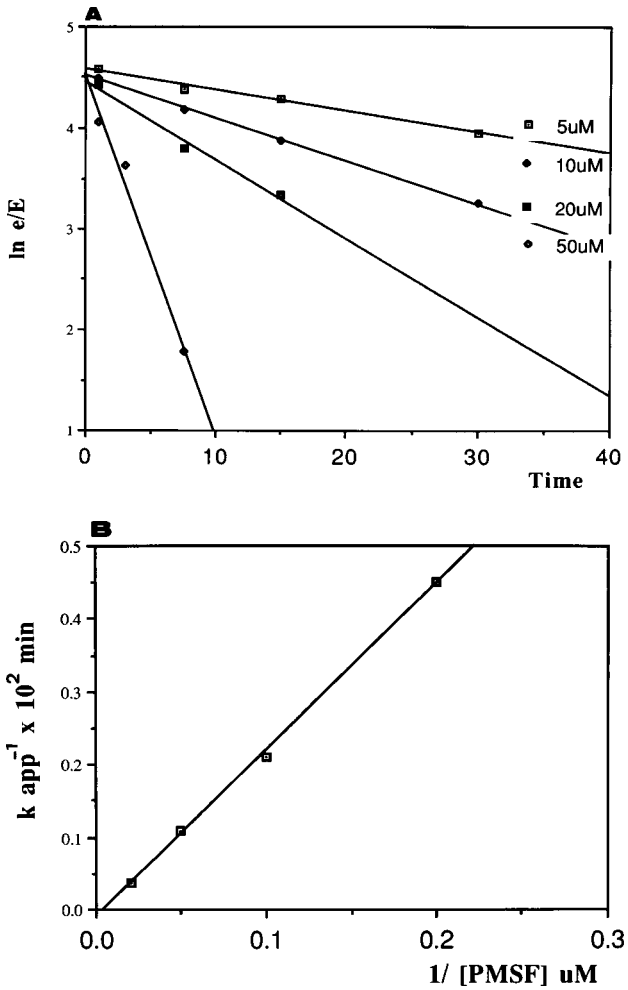

FIG. 6. (A) Progressive development of inhibition produced by reaction of T1 with four different PMSF concentrations. e/E, percentage of remaining activity at the stated time intervals. The values of the apparent rate constants of inactivation, k_{app} (per minute), were calculated from the slopes of semilogarithmic plots of enzymatic activity versus time. (B) Dependence of k_{app} upon the concentration of PMSF plotted as reciprocals.

sheath vesicles by the oral spirochete *T. denticola*. The polypeptide composition of these extracellular vesicles is very similar to the one found in treponemal outer sheaths. They contain a major outer sheath oligomeric polypeptide (molecular size, 113 to 243 kDa) and four outer sheath proteases with molecular sizes of 91, 153, 173, and 228 kDa. Previous studies of *T. denticola* isolated outer sheath demonstrated that this membrane shows a regular hexagonal array containing an oligomeric polypeptide (9, 31, 49). This polypeptide resolved into one major protein upon heating (9, 49). The resulting monomer varied in its molecular size among the different *T. denticola* strains (62 to 64 kDa for E-21 and GM-1 strains; 53 kDa for strains ATCC 35404, ATCC 33405, and ATCC 33520) (9, 19, 31, 39, 49). The 53-kDa polypeptide can be observed clearly in the protein profiles of outer sheath and vesicle preparations of *T. denticola* ATCC 35404 (Fig. 2). This 53-kDa surface protein has been shown to bind mammalian matrix proteins and to mimic a porin in vitro (12, 19). The 64-kDa outer membrane protein present in the GM-1 strain was found to adhere to eucaryotic cells (49).

We have shown previously that *T. denticola* outer sheaths

contain proteases with molecular sizes of 91, 153, 173, and 228 kDa which are strongly inhibited by PMSF, $ZnCl_2$, and mercurial compounds (39). T1 and T5 were found to hydrolyze the three proteic substrates tested, i.e., fibrinogen, collagen IV, and albumin. T6 degraded fibrinogen and collagen IV, while T4 had only fibrinolytic activity (39). Therefore, the polypeptide composition of the outer sheath and vesicles provides the spirochetes with adhesins and proteolytic enzymes capable of adhering and degrading periodontal tissues.

It has been reported recently that exposure of human gingival fibroblasts to *T. denticola* induces actin rearrangements and cell detachment from the substratum with concomitant fibronectin degradation (2, 13). These effects on fibroblasts, which are inhibited by PMSF, were suggested to be induced partly by a treponemal chymotrypsin-like protease. We have demonstrated the presence of several outer sheath proteases that are strongly inhibited by PMSF (39). All of these proteases together with their counterparts associated with extracellular vesicles may contribute to the damaging reactions observed in fibroblasts exposed to *T. denticola* cells.

In another recent study, scanning electron micrographs of *T. denticola* containing visible surface blebs (vesicles) were shown to adhere to hydroxyapatite beads and penetrate holes in the apatite surface (8). The high motility of spirochetes together with their capacity to release small highly proteolytic vesicles may therefore provide these microorganisms with an effective mechanism of penetration through tissues and cause tissue damage.

In this study, we have shown that the major cell surface oral treponemal protease T1 is the main protease found in the bacterial extracellular vesicles. This enzyme has been purified from *T. denticola* ATCC 35405, and its preliminary characterization has been reported (46). Because of its specificity towards phenylalanine, it has been characterized as exhibiting chymotrypsin-like activity (46). In this paper, we have demonstrated that the purified T1 protease degrades some components of connective tissue, including type IV collagen, fibrinogen, and fibronectin (Fig. 5). These findings are in agreement with previously reported data (18, 46). Under nondenaturing conditions (28°C), T1 degraded the collagen type IV α chains (Fig. 5), while those of collagen type I were resistant to hydrolysis (data not shown). The different susceptibilities of these two collagen types to T1 may reside in their structural features. Unlike the fibrillar collagen I, the type IV basement membrane collagen forms a network structure and contains interrupted triple helices that are susceptible to hydrolysis by nonspecific proteases such as pepsin and trypsin (6). These interruptions in the helical portion of the molecule, which are known to contain phenylalanine residues (22), may be responsible for the sensitivity of collagen IV to T1. Because of the possible importance of this protease in periodontal tissue destruction, we have extended the studies on the catalytic characteristics of the enzyme. The data in the present study strongly suggest that T1 is a serine protease as indicated by its inactivation by DIF, PMSF, and phenylboronic acid. The second-order rate constant of T1 inactivation by PMSF is, although smaller, of the same order of magnitude as that found for chymotrypsin (14) (0.48×10^4 M^{-1} min^{-1} versus 1.9×10^4 M^{-1} min^{-1}), suggesting similar degrees of molecular complementarity for the inhibitor and the enzymes. The rapid (<1 min) and pH-dependent inhibition of T1 by phenylboronic acid, a transition-state analog for serine proteinases, is also similar to that observed for chymotrypsin (37). This rapid and pH-dependent inhibition of chymotrypsin by phenylboronic acid is attributed to the formation of a tetrahedral enzyme-boronic acid complex. The most probable structure for this adduct contains a covalent bond between the oxygen of the catalytic serine and the inhibitor boron atom (32).

Previous reports on the T1 protease (46) demonstrated its activation by reducing agents like dithiothreitol, cysteine, and β-mercaptoethanol. Under our experimental conditions, activation of the purified enzyme could be demonstrated neither when the enzyme was purified from isolated outer sheaths nor when purification was performed from detergent phases obtained by Triton X-114 extraction of the treponemes followed by phase separation (39) (data not shown). Furthermore, qualitative analysis of zymograms showed that the addition of these reducing reagents during the proteolytic step neither enhanced the activity of the *T. denticola* proteases nor induced the appearance of new proteolytic bands in fibrinogen-containing gels (unpublished data). In this study, we show that E-64 and the peptidyl diazomethyl ketones, which are specific thiol protease inhibitors, have no effect on T1 activity (Table 1). Nevertheless, as reported previously (46), Zn and mercurial compounds are strong inhibitors of T1 activity. The inhibitory effect of Zn and mercurial compounds may indicate a critical role for free-SH groups in the active enzyme.

A protease of 30 kDa which seems to possess hemolytic activity has been cloned recently from strain ATCC 35405 by Arakawa and Kuramitsu (1). The cloned enzyme displays substrate specificity and sensitivity to inhibitors similar to that of T1. Both enzymes cleave the P1-phenylalanine position of synthetic substrates and are strongly inhibited by DIF and PMSF. However, in contrast to T1, the 30-kDa cloned protease seems to be unable to degrade a number of native proteins tested, with the exception of albumin and casein (1).

Of the anaerobic oral microorganisms capable of degrading extracellular matrix proteins, *Porphyromonas gingivalis* and *T. denticola* have been shown to possess the highest proteolytic activity (47). Both microorganisms contain a number of surface membrane proteases that are released from the cells through extracellular vesicles (17). Nevertheless, while those of *P. gingivalis* were found to be cysteine proteinases with specificity towards arginine and lysine (3, 7, 16, 38, 41), the *T. denticola* outer sheath proteases appear to belong to the serine family. The difference between the proteases from *P. gingivalis* and *T. denticola* with regard to substrate specificity and sensitivity to inhibitors may be a useful tool in differentiating between these two oral microorganisms in the progression of periodontal disease.

Oral bacteria found in subgingival plaque are predominantly anaerobic and rely on the hydrolysis of peptides and amino acids for their energy supply (15, 44). *T. denticola*, an inhabitant of the subgingival area, grows well on a Trypticase-yeast extract-based medium supplemented with serum (42). Several peptidases in these spirochetes, including a trypsin-like peptidase, a proline endopeptidase, and a FALGPA-peptidase, have been thoroughly characterized (28, 29, 35). A glutamyl-peptidase was also reported in strain ATCC 35405 (29). T1, the major outer sheath and vesicle protease described in this study, appears to complement the substrate specificities of the other *T. denticola* peptidases by hydrolyzing phenylalanine-containing proteins, including those which the organism would normally encounter in the periodontal pocket. This strong proteolytic arsenal, which is important in supplying the nutritional requirements of these spirochetes, seems to also play a role in the virulence of the organism.

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