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Characterization of Fibrinolytic Activities of *Treponema denticola*

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Several fibrinolytic activities of *Treponema denticola*, an oral spirochete associated with gingivitis and periodontal disease, were identified and characterized following phase partitioning with the nonionic detergent Triton X-114. The apparent molecular masses of the proteases ranged from 91 to 228 kDa when analyzed in sodium dodecyl sulfate-polyacrylamide gels containing fibrinogen as the protease substrate. A qualitative analysis of zymograms showed that the proteases were highly enriched in the detergent phase, although the 91-, 173-, and 228-kDa proteases were also found in the aqueous phase. Zymograms of crude outer sheaths prepared by repeated freezing-thawing revealed that the proteases may be associated with this subcellular compartment. The proteases displayed substrate specificity towards fibrinogen, were susceptible to sulfhydryl group reagents, and had a pH optimum between 7 and 8. The similarities in their sensitivity to inhibitors, temperature stability, pH optimum, and laddered protein profiles suggest that these hydrolytic enzymes may be part of a family of oligomeric proteases that may play an important role in the invasiveness of and tissue damage caused by the spirochete.

Spirochetes are helically shaped, motile bacteria found in a variety of ecological niches as free-living or host-associated species (3). Oral spirochetes are normally observed in low numbers in subgingival plaque, in direct contact with the pocket epithelium. Their numbers increase drastically in both acute necrotizing ulcerative gingivitis (ANUG) (20, 26) and various types of periodontal disease (17, 19, 29). In ANUG, spirochetes compose 30% of the microscopic count and appear to invade the epithelium and the underlying connective tissue (18). Spirochetal invasion of gingival tissues has also been suggested for periodontitis (31).

A number of proteolytic activities that might be important in the process of the destruction of gingival tissues were described for various strains of *Treponema denticola*, a spirochete commonly found in the subgingival flora. Some treponemal enzymes are capable of degrading synthetic peptides with specificities for arginine (22, 28) or proline (22, 23). These enzymes generally appear to have low activities against native proteins (28). A 100-kDa treponemal protease isolated from several treponemal strains was shown to hydrolyze the synthetic polypeptide phenylazobenzyl-oxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (24). Collagen and collagen-derived substrates were also reported to be hydrolyzed by this protease purified from a treponemal clinical isolate (24). *T. denticola* also produces a cell surface chymotrypsin-like protease capable of degrading major protein components of the basement membrane (collagen IV, laminin, and fibronectin) and of directly damaging epithelial cells (10, 37, 38). These microbial proteases may exert their action by direct proteolysis of periodontal tissues and by the activation of mammalian proteases (34).

Morphologically, *Treponema* spp. bear an outer sheath that surrounds the endoflagella, cytoplasmatic membrane, and protoplasmatic cylinder of the organism (14). Analysis of the outer sheath protein profiles of *T. denticola* ATCC 35404, GM-1, and ATCC 33520 was recently reported (5, 11, 40). The major outer sheath proteins appear to be dissociated forms of high-molecular-mass oligomeric units ranging from 116 to 200 kDa. A group of 97-, 95-, and 70-kDa polypeptides has also been observed. Upon heating, these oligomeric polypeptides resolve as one major outer sheath protein of 53 to 64 kDa for various *T. denticola* strains (5, 11, 25, 40). This protein is the major antigen recognized by rabbit antisera to *T. denticola* and human sera from patients with ANUG and localized juvenile periodontitis (39). It has also been suggested to be involved in the adhesion of oral treponemes to eukaryotic cells (40) and mammalian matrix proteins (11). Nevertheless, up to now, no comprehensive study has been performed on outer sheath surface enzymatic activities in these microorganisms and their possible involvement in the progression of periodontal disease. The aim of the present study was to identify and characterize proteolytic activities found on the cell surface of *T. denticola*.

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 (33). All strains were grown in GM-1 (1) broth (without glucose) for 3 to 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 dark-field microscopy.

Extraction and phase separation of treponemal proteases with Triton X-114. A total of 5×10^9 late-log-phase bacteria were harvested by centrifugation at 9,000 × *g* for 20 min. The supernatant was filtered through a 0.20-μm-pore-size filter (Schleicher & Schuell, Dassel, Germany) and further concentrated four times by ultrafiltration through a 5,000-molecular-weight-cutoff membrane (concentrated supernatant). The concentrated supernatant was further centrifuged at 100,000 × *g* for 3 h, and both pellet and supernatant were analyzed for proteolytic activity. The cells were washed three times with phosphate-buffered saline and further extracted with the detergent Triton X-114 (Sigma) as described by Etges et al. (8).

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Five hundred microliters of 2% (vol/vol) precondensed Triton X-114 (unless stated otherwise) (2) in TBS (100 mM Tris, 150 mM NaCl [pH 7.4]) was used for each 5×10^9 cells (detergent/protein ratio, approximately 20:1). After overnight extraction at 4°C, the insoluble material was precipitated by centrifugation at 30,000 × *g* for 45 min at the same temperature. The supernatant was applied to a 6% sucrose cushion, incubated for 10 min at 37°C, and centrifuged at 13,000 × *g* for 10 min at room temperature to sediment the detergent aggregates through the cushion. The aqueous and detergent phases were recovered below and above the cushion, respectively. Alternatively, sucrose cushions were omitted, and extensive washings of the detergent and aqueous phases were performed as described by Radolf et al. (30). In brief, after removal of the insoluble material by centrifugation, the supernatant was warmed for 10 min at 37°C and then centrifuged at 13,000 × *g* for 10 min. The separated detergent and aqueous phases were washed four times as follows. The detergent phase was diluted to 500 μl in the original buffer at 0°C, rewarmed, and centrifuged as described above. The aqueous phase was cleansed by the repeated addition of fresh 12% Triton X-114 to a final concentration of 2% and phase separated as described above. Similar proteolytic patterns were obtained with the two procedures. Treponemes were also extracted with 0.02, 0.1, 1, or 2% Triton X-114 for 20 min at 4°C. Insoluble material was removed by centrifugation as described above. Supernatants containing Triton X-114 at concentrations of less than 1% were brought to a detergent concentration of 2% by the addition of appropriate volumes of a 12% Triton X-114 stock solution in TBS before phase separation.

Preparation of the outer sheath. The outer sheath was prepared essentially by the method described by Masuda and Kawata (25) and modified by Weinberg and Holt (40). In brief, cells harvested from 0.5 liter of culture medium were washed once with saline at 4°C. The pellet was resuspended in 6 ml of 0.05 M Tris-HCl buffer (pH 7.2) containing 0.02 M $MgCl_2$ and DNase I from bovine pancreas (Sigma) (4 U/ml). No protease inhibitors were added. The cells were subjected to 40 freeze-thaw cycles. The disrupted cell suspension was centrifuged at 8,000 × *g* for 10 min to remove unbroken cells and debris. The supernatant was centrifuged again at 25,000 × *g* for 30 min to obtain a crude outer sheath preparation. The pellet obtained after centrifugation was washed twice with Tris-HCl (0.05 M; pH 7.2). This crude outer sheath preparation was used for the detection of proteolytic activity.

A similar preparation was further suspended in 6 ml of 0.1 M sodium acetate buffer (pH 3.0) and mixed for 2 h to remove contaminating flagella (25). The preparation was centrifuged at 25,000 × *g* for 30 min at 4°C. The outer sheath was immediately neutralized to pH 7.2 and submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the detection of proteolytic activity.

Electrophoresis. SDS-PAGE was carried out by the Laemmli method (16) with a Mini Protean II cell (0.075-cm thick; Bio-Rad Laboratories, Richmond, Calif.). Gels (7.5% polyacrylamide) contained 240 μg of human fibrinogen or bovine albumin per ml. Collagen-containing gels were prepared as follows: 1.25 mg of collagen type IV (human placenta; Sigma) was dissolved in 200 μl of 0.2% acetic acid and added to 4.8 ml of running gel mixture (16). Prior to electrophoresis, detergent-phase samples were diluted 15-fold in TBS, whereas aqueous-phase samples were not diluted. Ten microliters of detergent phase and 20 μl of aqueous phase were resuspended in sample buffer (4% [wt/vol] SDS, 10% glycerol, 0.18 M Tris-HCl [pH 6.8]) and neither heated nor reduced.

After electrophoresis, the gels were incubated for 30 min in TBS containing 2% Triton X-100 and then washed three times with TBS (9). Gels were incubated at 37°C for 1.30 h. Bands of activity were revealed after staining with Coomassie blue. Proteolytic activity was visualized as a clear band against a blue background. Gels (0.15-cm thick; 7.5% acrylamide) without the protein substrate were silver stained (35).

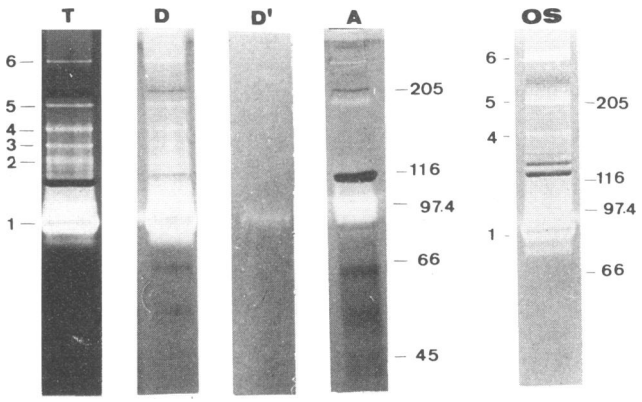

FIG. 1. *T. denticola* ATCC 35404 protease profiles on fibrinogen-containing polyacrylamide gels. T (total), 5×10^7 whole solubilized *T. denticola* cells (5 μg of protein); D, detergent-phase proteases (extract diluted 15-fold); D′, detergent-phase proteases (extract diluted 1,200-fold); A, aqueous-phase proteases (undiluted); OS, outer sheath proteases (4 μg of protein). Molecular mass markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine albumin (66 kDa), and egg albumin (45 kDa).

Molecular masses of protein bands were calculated by linear regression analysis of high (45 to 205 kDa; Sigma)-molecular-mass standards.

Effect of inhibitors. The effect of protease inhibitors was determined by incubating the gels with TBS containing the compounds of interest. These were *p*-chloromercuribenzoic acid (PCMB), phenylmethylsulfonyl fluoride (PMSF), EDTA, $ZnCl_2$, $MgCl_2$, $CaCl_2$, $HgCl_2$, and *N* α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK).

Effect of pH. The effect of pH on proteolytic activity was evaluated by comparing the intensities of the bands visualized by incubation of the gels with TBS (pH 7.5) and those obtained in 200 mM sodium citrate (pHs 4.00, 5.00, and 6.00) or 200 mM sodium carbonate (pHs 9.00, 10.00 and 11.00) buffer.

Effect of temperature. The heat stability of the proteases was determined by incubating the samples at 50, 60, and 70°C for 30 min before electrophoresis.

RESULTS

Protease profiles. The protease activities of proteins extracted with Triton X-114 from *T. denticola* ATCC 35404 were analyzed by SDS-PAGE with gels containing fibrinogen as a substrate. The protease activity of whole cells (Fig. 1, T) was associated with six different bands with apparent molecular masses of 91, 123, 138, 151, 173, and 228 kDa (T_1 to T_6, respectively). Treponemes were extracted with 2% Triton X-114, and the solubilized material was phase partitioned. To obtain a good resolution of proteolytic bands on zymograms to permit a comparison of activities in both detergent and aqueous phases, the detergent extract had to be diluted because of the high concentrations of the proteases in this phase (more than a threefold-higher concentration in the detergent phase than in the aqueous phase). The proteolytic profiles of the detergent and aqueous phases are shown in Fig. 1 D and A. All

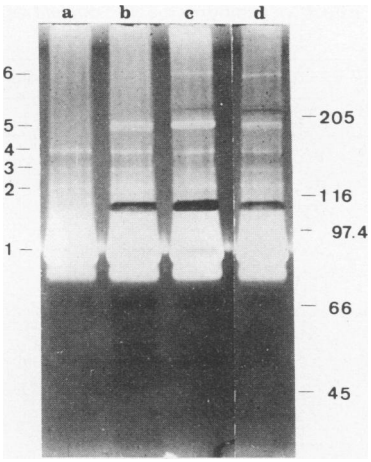

FIG. 2. Detection of proteolytic activities in the detergent phase of *T. denticola* ATCC 35404 cells extracted with 0.02, 0.1, 1, and 2% Triton X-114 (lanes a through d, respectively). The detergent extracts were diluted 1/15 for the 1 and 2% Triton X-114 phases and 1/3 for the 0.02 and 0.1% Triton X-114 phases. Numbers at right are in kilodaltons.

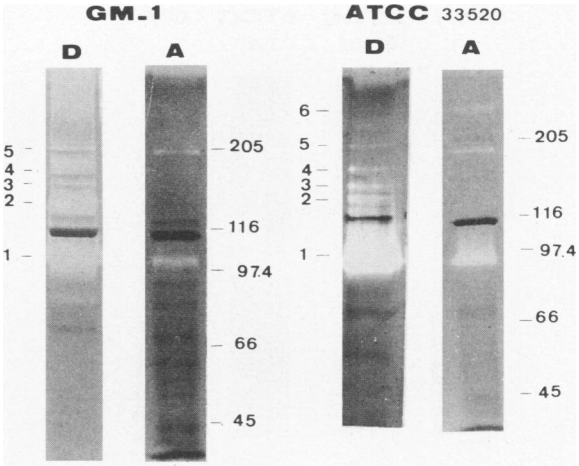

FIG. 3. *T. denticola* GM-1 and ATCC 33520 protease profiles on fibrinogen-containing polyacrylamide gels. D, detergent-phase proteases; A, aqueous-phase proteases. Numbers at right are in kilodaltons.

the proteases were present in the detergent phase (Fig. 1, D). A qualitative analysis of zymograms showed that T_2, T_3, and T_4 were visualized only in this phase; the other proteases, T_1, T_5, and T_6 were unequally distributed between the detergent and aqueous phases. Although they were markedly enriched in the detergent phase, some activity remained in the aqueous phase even after repeated washings. Despite dilution of the detergent phase, the T_1 protease appeared as a very diffuse band. When the detergent phase was diluted enough to obtain a good resolution of T_1, this protease was resolved into one proteolytic band (Fig. 1, D′).

The Triton X-114 concentration necessary to solubilize the proteases was investigated. Strain ATCC 35404 was extracted for 20 min at 4°C with increasing Triton X-114 concentrations (0.02 to 2%), and then phase separation was done. The proteolytic profiles of the detergent phase are shown in Fig. 2. The complete proteolytic pattern was detected in the detergent phase only when the Triton X-114 concentration was increased to 1%. The T_1 to T_4 proteases were extracted by 0.02% Triton X-114. Triton X-114 at 0.1% solubilized T_5 as well; higher concentrations of Triton X-114 were needed to solubilize T_6.

Minor differences in the proteolytic profile were observed for strain ATCC 33520, for which T_6 was visualized as a weak proteolytic band in the aqueous phase (Fig. 3). For the GM-1 strain, five proteolytic bands were constantly solubilized during extraction. The T_6 protease was found in the remaining Triton X-114-insoluble material and in some preparations of the detergent phase (data not shown). The proteases of this clinical isolate had a slower electrophoretic mobility than those of the reference strain.

The preferential partitioning of proteases T_1 to T_6 into the detergent phase indicates that they may be membrane-associated polypeptides. To assess the subcellular location of these proteases, the outer sheaths were isolated from the treponemes (25). Proteolytic analysis of crude outer sheaths prepared by repeated freezing-thawing (before acidification) revealed the presence of four proteolytic bands: T_1, T_4, T_5, and T_6 (Fig. 1, OS). Further treatment with acid left the outer sheath preparations with only residual T_1 activity (data not shown). The protein profiles of these outer sheaths obtained after SDS-PAGE and silver staining were examined (Fig. 4 OS, lane 1). The unheated outer sheath preparations separated in SDS-PAGE as laddered polypeptides in the molecular mass range of 113 to 234 kDa. In addition, polypeptides were resolved at 95 and 91 kDa. Heating the outer sheath preparations for 5 min at 100°C resulted in the modification of the laddered polypeptides to major outer sheath proteins of 53 and 46 kDa (Fig. 4, OS, lane 2). These two polypeptides were the predominant proteins reported to be present in the outer sheaths of *T. denticola* ATCC 35404 and ATCC 33520 (5, 11, 39).

The heat-modifiable high-molecular-mass oligomers were also the major proteins found in the Triton X-114 phase (Fig. 4, D, lanes 1 and 2). Nevertheless, polypeptides from the high-molecular-mass oligomeric units seemed to be differently enriched in the detergent and outer sheath fractions. Heating

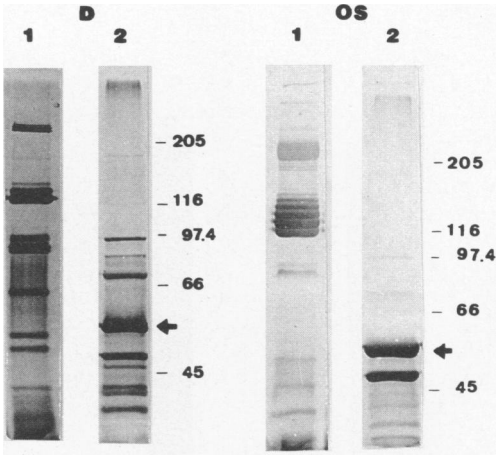

FIG. 4. Effect of heat on *T. denticola* ATCC 35404 protein profiles. D, detergent-phase proteins; OS, outer sheath proteins. Lanes: 1, unheated samples; 2, samples heated at 100°C for 5 min (15 μg of protein). The arrows indicate the 53-kDa band calculated by linear regression of the molecular mass standards. Numbers at right are in kilodaltons.

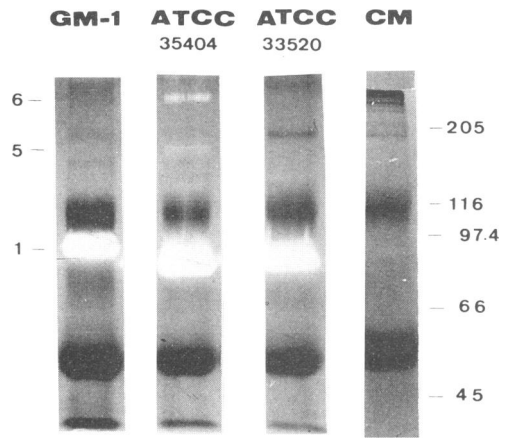

FIG. 5. Supernatant protease profiles of *T. denticola* GM-1, ATCC 35404, and ATCC 33520. CM, culture medium. Numbers at right are in kilodaltons.

the outer sheath or detergent fractions at 70°C for 5 min was adequate to cause dissociation of the laddered polypeptides (data not shown) (11, 40).

To evaluate the possibility that the proteases are released into the extracellular medium, the proteolytic pattern of the cell-free growth medium was also analyzed. For all three strains, the main protease found in the culture medium was T_1, similar to the main proteolytic activity associated with the cells (Fig. 5). The culture supernatant of strain ATCC 35404 contained two additional weak bands, related by their apparent molecular masses to the T_5 and T_6 cell-associated proteases. The concentrated cell-free supernatant was ultracentrifuged at 100,000 × *g* for 3 h (7). Zymograms of the resulting fractions showed that the proteases were removed from the supernatant and appeared in the pellet. These proteases were partitioned preferentially into the detergent phase after Triton X-114 extraction and phase separation (data not shown).

The substrate specificity of the proteases was evaluated by replacing fibrinogen in the gels with albumin or collagen IV. Figure 6 shows the proteolytic pattern of the detergent phase of *T. denticola* ATCC 35404 in SDS-PAGE done with either

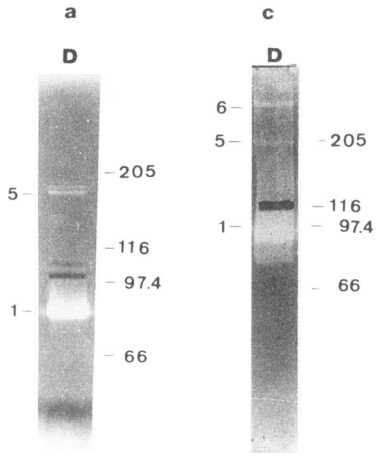

FIG. 6. Detection of *T. denticola* ATCC 35404 proteolytic activities with albumin- (lane a) and collagen (lane c)-containing polyacrylamide gels. D, Detergent phase. Numbers at right are in kilodaltons.

TABLE 1. Effect[a] of inhibitors on *T. denticola* ATCC 35404 proteolytic activities

Inhibitor	Concn (mM)	Inhibition of the following proteolytic band:					
		T_1	T_2	T_3	T_4	T_5	T_6
PCMB	0.01	+	+	+	+	+	+
	0.05	+	++	++	++	+	+
	0.1	++	+++	+++	+++	+++	+++
PMSF	1	+	+	+	+	+	+
	2	+	++	++	++	+	+
	4	+	++	++	++	+	+
EDTA	10	−	−	−	−	−	−
TLCK	2	−	−	−	−	−	−
$ZnCl_2$	0.1	+	++	++	++	+	+
	1	++	+++	+++	+++	++	++
$MgCl_2$	10	−	−	−	−	−	−
$CaCl_2$	10	−	−	−	−	−	−
$HgCl_2$	0.01	+	+	+	+	+	+
	0.05	+	++	++	++	+	+
	0.1	++	+++	+++	+++	++	++

[a] The concentrations shown are the minimum concentrations required for partial inhibition (+), strong inhibition (++), complete inhibition (+++), or no inhibition (−) of fibrinogen hydrolysis by the proteases.

albumin or collagen IV. Albumin was less degraded than fibrinogen by the *T. denticola* proteases (Fig. 6). Under the same proteolytic conditions as those used for fibrinogen, weaker T_1 and T_5 proteolytic bands could be detected with this substrate. Collagen-containing gels showed three proteolytic bands, corresponding to T_1, T_5, and T_6 (Fig. 6).

Inhibitors. Table 1 shows the effects of different inhibitory compounds on *T. denticola* proteolytic activities. All the enzyme activities were found to be sulfhydryl dependent, since they were inhibited to various extents by the sulfhydryl-blocking reagents PCMB and $HgCl_2$. $ZnCl_2$ caused partial to complete inhibition of the proteases. The enzymes were also inhibited by the serine protease inhibitor PMSF. However, no effect on the activities of the proteases was observed with EDTA, TLCK, $CaCl_2$, or $MgCl_2$.

Effect of temperature and pH. The proteolytic activities of the bands were not affected by preincubation at 50 or 60°C. Preincubation at 70°C inactivated bands T_2, T_3, T_4, T_5, and T_6, leaving only residual T_1 activity.

The optimal pH for protease activities was 7 to 8. At pH 5, bands T_2, T_3, and T_4 were not visualized. At pH 4, only residual T_1 activity could be detected. Increasing the pH from 8 to 10 decreased the activities of all the bands, although residual activities were still seen for all the proteases at pH 11 (data not shown).

DISCUSSION

The present report describes the identification and preliminary characterization of six proteolytic activities of two reference strains (ATCC 35404 and ATCC 35520) and one clinical isolate (GM-1) of *T. denticola* by SDS-PAGE done with the protease substrate. The position of the bands relative to that of the molecular mass standards was identical in every preparation. Despite the fact that these values did not necessarily

reflect the molecular masses of the monomeric forms of the enzymes, they were a constant parameter for the native forms of the enzymes.

Proteolytic activity in *T. denticola* ATCC 35405 was previously described by Uitto et al. (37). The chymotrypsin-like protease probably corresponds to T_1, as assessed by its molecular mass and sensitivity to inhibitors. These authors found T_1 to be the sole proteolytic band in albumin SDS-PAGE. The appearance of additional proteolytic bands in our zymograms may have been due to differences in the preparation and/or polyacrylamide concentrations of the gels (37). The T_1 protease was shown to be located outside the cell envelope (10). We also demonstrated the presence of T_1 in crude outer sheath preparations, in addition to T_4, T_5, and T_6. The absence of T_2 and T_3 activities from outer sheaths prepared by repeated freezing-thawing may have been due to their inactivation by this procedure. This assumption was sustained by the fact that T_2 and T_3 were still found in 8,000 $\times$ *g* supernatants of bacteria after 20 freeze-thaw cycles but were absent from treponemes devoid of outer sheaths obtained after 40 freeze-thaw cycles (unpublished data).

To further characterize the proteases, we solubilized the treponemal proteins with the nonionic detergent Triton X-114 and then carried out temperature-induced phase separation. The solution separated into two phases, a Triton X-114 (detergent) phase, containing amphipilic proteins, and an aqueous phase, containing hydrophilic proteins (2). Generally, membrane-associated proteins with an amphiphilic nature are recovered in their native form in the detergent phase. The proteolytic patterns obtained in this report showed a differential partitioning of the proteases into the aqueous and detergent phases of the extraction. T_2, T_3, and T_4 were solely observed in the detergent phase. T_1, T_5, and T_6 were highly enriched in the detergent phase, but some activity was still observed in the aqueous phase, even after repeated washes. A possible explanation could be an incomplete phase separation that led to a low level of contamination of the aqueous phase with detergent-phase proteins. On the other hand, there have been reports that integral membrane proteins with substantially ionic and hydrophilic amino acid sequences or highly glycosylated regions show some degree of or complete partitioning into the aqueous phase (4, 21).

While all three *T. denticola* strains studied released the T_1 protease into the growth medium, the T_5 and T_6 proteases of strain ATCC 35404 were also found in the culture supernatant (Fig. 5). The presence of the proteases in the pellet obtained by ultracentrifugation of the concentrated cell-free culture medium, together with their preferential partitioning into the detergent phase after Triton X-114 extraction and phase separation, suggests that these extracellular proteases are associated with membrane fragments and that they retain the amphiphilicity of their cell-associated counterparts. Thus, the presence of these proteases in the extracellular medium seems to be due to a process of "blebbing" of the outer sheath, which is known to occur in treponemes (5), rather than the release of soluble proteases.

Localization of almost any protein of a spirochete in a particular cellular membrane or compartment is very difficult because of the fragility of the outer sheath. Using a multiple freeze-thaw technique, Masuda and Kawata obtained enriched outer sheath preparations for *T. denticola* (25). In our study, the protein profiles of outer sheaths isolated by this procedure agreed with previously reported data (40).

Surface proteins of *T. denticola* have also been solubilized with a range of nonionic (0.1 to 0.2%) (5, 11) and anionic (11) detergents. We also found that the Triton X-114 phase was enriched with the heat-modifiable major outer sheath polypeptide (Fig. 4, D). While Triton X-114 solubilization of outer sheaths has an advantage over the freeze-thaw technique, in which all the proteases remain in their native active form, the latter methodology seems to permit a much clearer protein profile analysis of outer sheaths than the use of detergents (40). It is interesting to note that under conditions similar to those described in this paper (Fig. 2), treatment of *T. pallidum* with Triton X-114 appeared to selectively solubilize the bacterial outer membrane (6, 30). Still, as reviewed recently by Norris et al., definite information about the outer membrane of *T. pallidum* cannot be obtained from detergent treatment studies because of the possibility that proteins from other structures are nonspecifically released (27).

In our study, Triton X-114 extraction of *T. denticola* appeared to solubilize some of the oligomeric units of the high-molecular-mass outer sheath polypeptides (Fig. 4). Selective release with mild detergents, such as Triton X-114, is partly due to the insolubility of large, continuous membrane structures stabilized by protein-protein interactions (12). On the other hand, while low Triton X-114 concentrations (0.02 to 0.1%) were able to solubilize T_1 to T_5, solubilization of T_6 required higher detergent/membrane ratios. The increase in detergent concentration required for the sequential release of integral proteins from biological membranes has been widely reported (12, 15, 36). Our data suggest that once the outer sheath is isolated by the freeze-thaw technique, SDS solubilization of the outer sheath permits a more complete and representative analysis of its polypeptides than Triton X-114 extraction. On the other hand, if one assumes that the proteases are located in the outer sheath, an increase in the Triton X-114 concentration may be required for their sequential release. This assumption may explain the higher detergent concentrations needed to solubilize T_6. Alternatively, the need for higher detergent concentrations to solubilize T_6 may point to its possible location in a different subcellular compartment. Thus, the presence of the proteases in crude outer sheath preparations, together with their preferential partitioning into the Triton X-114 phase, suggests their association with the outer sheath. However, as periplasmic proteins may be released by the two methodologies, these proteases may also be derived from the periplasm. Therefore, although the data presented in this report suggest the association of the proteases with the outer sheath, further experimental data are needed to verify the final location of these proteases and their possible relationship to the major outer sheath protein.

The proteases described in the present study (T_1 to T_6) were susceptible to inhibition by mercurial agents, suggesting that sulfhydryl groups are involved in their activities. PMSF, a reagent that has been used to probe active serine residues in enzymes, caused partial to strong inhibition of the proteases. Neither the chelator EDTA nor TLCK, a reagent known to inhibit trypsin-like enzymes, had any effect on the proteases.

The present communication describes the presence of high-molecular-mass proteolytic activities in *T. denticola*. The similarities in their substrate specificity, sensitivity to inhibitors, temperature stability, pH optimum, and laddered protein profiles suggest that these hydrolytic enzymes may be part of a family of oligomeric proteases. These proteases may play an important role in the nutrition of the microorganism and in its virulence for the host. Human isolates of *T. denticola* grow well on Trypticase-yeast extract-based media supplemented with serum (32), suggesting the importance of bacterial extra- and intracellular proteases for the metabolic requirements of the cells. Concomitantly, these proteases may contribute to the invasion and destruction of the gingival tissues through the

proteolytic activation of inflammatory activators (13), direct proteolysis of gingival tissues (10), or activation of mammalian proteases (34).

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