Surface protease of Treponema denticola hydrolyzes C3 and influences function of polymorphonuclear leukocytes

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Surface protease of *Treponema denticola* hydrolyzes C3 and influences function of polymorphonuclear leukocyte

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Abstract

*Treponema denticola* is a dominant microorganism in human periodontal lesions. One of the major virulence factors of this microorganism is its chymotrypsinlike surface protease, dentilisin. The purpose of this study was to evaluate the effect of dentilisin on human polymorphonuclear leukocytes (PMNs). We used chemiluminescence to assess production of O$_2^-$ by PMNs against *T. denticola* ATCC 35405 and dentilisin deficient mutant K1. *T. denticola* ATCC 35405 induced production of O$_2^-$, whereas dentilisin deficient K1 did not. We found that chymostatin, a protease inhibitor, strongly reduced the ability of *T. denticola* ATCC 35405 to induce production of O$_2^-$, whereas K1 was relatively unaffected. We also used Immunoblot and ELISA to evaluate the activation of complement by this microorganism in relation to PMNs. *T. denticola* ATCC 35405 hydrolyzed the α-chain of C3, producing iC3b. Furthermore, strain ATCC 35405 induced a larger release of MMP-9 from PMNs than strain K1. Dentilisin, activated PMNs *via* complement pathways, and may play a role in establishing periodontal lesions.

**Keywords:** *Treponema denticola*; Complement; Polymorphonuclear leukocyte; Protease; Dentilisin; Periodontitis
1. Introduction

Spirochetes are helically shaped motile microorganisms. They have been implicated in chronic periodontitis and acute necrotizing ulcerative gingivitis [1-4]. In the presence of disease, the percentage of spirochetes has been reported to increase to as much as 50% of the subgingival bacteria [5, 6]. One species of spirochete frequently isolated from periodontal lesions is *Treponema denticola* [7]. It has been reported that levels of *T. denticola* increased in ratio to the severity of periodontitis [8]. These findings suggest that this microorganism is a major pathogen in periodontitis. This microorganism possesses several pathogenic factors, including its major outer sheath protein and proteases [9, 10]. Dentilisin is a prolyl-phenylalanine specific surface protease and a major pathogen of *T. denticola* [11]. Dentilisin hydrolyzes host bioactive proteins [12] and is cytotoxic to periodontal ligament epithelial cells [13].

Polymorphonuclear leukocytes (PMNs) are predominant among the cells that interact with the microflora in the periodontal pocket [14, 15]. We previously reported that *T. denticola* was located primarily on the apical surface of subgingival plaque in direct contact with the periodontal pocket epithelium [16]. Various functions of PMNs, such as phagocytosis, enzyme release, and chemotaxis in response to oral bacteria, have been implicated in the progression of periodontal disease. When PMNs are stimulated by bacteria, they undergo a respiration burst characterized by increased oxygen consumption [14, 15]. In this case, superoxide free radicals (O$_2^-$), H$_2$O$_2$, and PMN enzymes can be released into the extracellular fluid. These PMN enzymes include several potentially destructive proteases, such as serine protease, elastase, cathepsin G, and also matrix metalloproteinases such as PMN collagenase (MMP-8) and PMN gelatinase B/type IV collagenase (MMP-9) [17, 18]. MMP-9 has been found to be elevated in patients with periodontitis [19]. Sela et al. [20] found that *T. denticola* suppressed the production of superoxide. Ding et al. [17, 18] showed that components of *T. denticola* such as Msp induced the production of matrix metalloproteinase by PMNs. Boehringer et al. [21] demonstrated that phagocytosed treponemes remained inside phagosomes. They found that the killing activity of spirochetes by PMNs was low, and that spirochetes affected the host defense mechanisms [21]. In this study, we evaluated the effect of dentilisin on PMN phagocytosis in order to clarify the interaction between *T. denticola* and PMNs.
2. MATERIALS AND METHODS

2.1 Growth conditions and preparation of sonic extract from T. denticola

*T. denticola* ATCC 35405 and *T. denticola* dentilisin-deficient mutant K1 [22] were maintained in TYGVS medium under anaerobic conditions as described previously [23]. *T. denticola* cells were cultured for 3 days in TYGVS and then harvested by centrifugation at 1000 x g for 20 min. They were then washed twice with phosphate buffered saline (PBS, pH 7.2) and resuspended in PBS. The cells were sonicated for 10 min on ice with a Sonicator (Branson Sonic Power Co., Danbury, CT), and the soluble fraction was collected by centrifugation at 7000 x g for 30 min. The fraction was stored at -20°C until measurement of protease activity. The protein concentration of the fraction was assayed with a DC protein assay kit (Bio-Rad, Hercules, CA).

2.2 Dentilisin activity assays

Prolyl-phenylalanine specific protease activity was measured using a synthetic substrate, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (SAAPNA, Sigma Chemical Company, St, Louis, MO), as described previously [11]. Briefly, each sonicate of *T. denticola* was added to 100 mM Tris-HCl buffer (pH 8.0) containing 1.6 mM SAAPNA. The mixture was incubated at 37°C for 15 min, and then the reaction was stopped by adding 50 µl of 20% acetic acid. Release of *p*-nitroaniline was evaluated by measuring absorbance at 405 nm.

2.3 Chemiluminescence assay for O2−

The chemiluminescence assay was performed as described previously [24]. Briefly, a total of 50 ml venous blood was collected into tubes containing heparin by venipuncture from 3 healthy donors. Written informed consent was obtained from each donor. PMNs were separated by centrifugation at 500 x g in a swing-out rotor at 20°C for 30 min using Polymorphrep (Axis-Shield PoC, Oslo, Norway). Isolated PMNs were washed with PBS and harvested following centrifugation at 400 x g for 10 min at 20°C. The PMNs were then resuspended in 4 ml of RPMI 1640 medium (Nissui, Tokyo, Japan) containing 0.5% gelatin at a concentration of 1.5 x 10⁷ cells/ml and held at room temperature.

The *T. denticola* cells grown in TYGVS medium were harvested by centrifugation at 7000 x g for 10 min and washed with PBS three times. They were then suspended at 1.8 x 10⁸ cells/ml in veronal buffer containing 0.1 % gelatin, 0.1 % glucose, 4 mM MgCl₂, and 0.6 mM CaCl₂ (GGVB⁺⁺). In the inhibitor assay, *T. denticola* was treated with 30 µg/ml protease inhibitor chymostatin (Calbiochem, San Diego, CA) at 37 °C for 30 min, after which, the cells were washed twice with PBS. The treated cells were then resuspended in GGVB⁺⁺ at 1.8 x 10⁸ /ml. C5-deficient human serum preparation (Sigma Chemical Co.) or C3-deficient
complement serum (Sigma) were used as the complement source.

Six µl of 3.3 M luminol dissolved in dimethyl sulfoxide, 150 µl of PMNs (1.5 x 10^7 cells/ml), and 784 µl of GGVB++ were mixed and preincubated at 37°C for 30 min. The mixtures, 40 µl of bacteria, 10 µl of 100 mM CaCl_2, and 10 µl of C5-deficient complement or C3-deficient complement serum were mixed (total assay volume 1 ml) and transferred into chemiluminescence test tubes. Chemiluminescence was measured with an Auto-Lumicounter Model 1422EX (Microtech-Nichion, Chiba, Japan) at 37°C for 135 min. O_2^- production was expressed as relative luminescent units (rlu).

2.4 Evaluation of C3 fragmentation

To clarify activation of C3 by dentilisin, we evaluated the level of proteolytic fragmentation of C3 with *T. denticola* ATCC 35405 and *T. denticola* K1. C3 at 0.1 µg/µl was incubated at 37°C for 17 h with sonicates of *T. denticola* at a protein concentration of 5 mg/ml in 100 mM Tris-HCl buffer at pH 8.0. C3 degradation was evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 10-20% gradient gel (Daiichi Pure Chemicals, Tokyo). The separated proteins in the gel were blotted onto PVDF membranes and immunostained with goat anti-serum to human complement C3 IgG (MP Biomedicals, Irvine, CA). The C3 antibody immobilized on the membranes was then detected with peroxidase-conjugated rabbit anti-goat IgG (Bio-Rad, Hercules, CA) as described previously [22].

2.5 Measurement of iC3b levels from C3 after treatment with *T. denticola*

Bacterial cells (1.8 x 10^8 cells/ml), 100 mM CaCl_2, and C5-deficient complement were incubated at 37°C for 30 min and 60 min. The samples were cooled on ice for one minute and then centrifuged at 1000 x g. The supernatant was stored at -80°C until used. Generation of iC3b was measured with an iC3b ELISA kit (QUIDEL Co., San Diego, USA) according to the manufacturer’s instructions. Briefly, 100 µl of diluted supernatant were added to the microassay well and incubated at room temperature for 30 min. After washing, 50 µl of iC3b antibody conjugated with peroxidase were added and incubated for 30 min. After washing, it was developed by adding 100 µl of substrate solution. Reaction was terminated with 50 µl of stop solution. Absorbance at 405 nm was evaluated with a Microplate reader (BioRad).

2.6 Induction of MMP-9 from PMN treated with *T. denticola*

Furthermore, to evaluate the activation of PMNs by dentilisin, release of MMP-9 from PMNs was investigated with an ELISA system for MMP-9 (Amersham Bioscience, Uppsala, Sweden) according to the manufacturer’s instructions. Separated PMNs were adjusted at 1.5 x 10^7 cells/ml, and bacterial cells (1.8 x 10^8 cells/ml), 100 mM CaCl_2, and C5-deficient complement were added as described above. The mixtures were incubated at 37°C for 30, 60, 90, and 120 min. After incubation, the culture supernatant was isolated. The samples obtained
were cooled on ice for 1 min and stored at -80°C until used. One hundred µl of each sample were incubated at 37°C for 1 h in the flat-bottomed microwell of the MMP-9 ELISA kit. After washing, anti-MMP-9 antibody conjugated with Horseradish peroxidase was added and incubated for 1 h. It was then washed and developed by adding substrate solution. Absorbance was measured at 450 nm with the Microplate reader (BioRad).

Activation of MMP-9 by dentilisin was evaluated with the Matrix Metalloprotease-9 Biotrak, Activity Assay System (Amersham Bioscience) according to the manufacturer’s instructions. Briefly, PMNs were incubated with *T. denticola* as described above, and after 90 min the culture supernatant was isolated. The samples obtained were cooled on ice for 1 min and stored at -80°C until used. One hundred µl of each sample were incubated at 37°C for 1 h in the provided flat-bottomed microwell. After washing, 50 µl of assay buffer were added and incubated at 37 °C for 1.5 h. After incubation, 50 µl of detection reagent were added. The plate was incubated at 37 °C for 1 h, and increase of absorbance at 450 nm from 0 h to 1 h was evaluated.

### 2.8 Statistical Analysis

Chemiluminescence activity was assessed and compared using the Mann-Whitney U test. The levels of the iC3b activity and MMP-9 were analyzed by repeated measurement One-way ANOVA followed by a Student-Newman-Keuls test.

### 3. RESULTS

#### 3.1 Enzymatic activity of protease

Dentilisin activity in the sonicates from *T. denticola* ATCC 35405 and mutant K1 were assayed with SAAPNA. *T. denticola* ATCC 35405 showed SAAPNA-hydrolyzing activity at 0.768 OD405 mg protein⁻¹ h⁻¹, while K1 only showed activity at 0.058 OD405/mg protein h.

#### 3.2 Chemiluminescence assay

Superoxide (O₂⁻) production by PMNs was evaluated with a chemiluminescence assay. Representative data for *T. denticola* ATCC 35405 and *T. denticola* K1 are shown in Fig 1. Peaks of chemiluminescence occurred at 30 min. We found that *T. denticola* ATCC 35405 induced a significantly higher production of O₂⁻ than did K1 (p<0.01). The same difference in O₂⁻ production between *T. denticola* ATCC 35405 and K1 was observed in the PMNs obtained from all three donors.

To investigate the activation of complement by dentilisin, evaluation of superoxide production from PMN by *T. denticola* was performed after treatment with protease inhibitors. Chemiluminescent activity with *T. denticola* ATCC 35405 decreased to 36.4% after treatment with chymostatin for 30 min. That with *T. denticola* K1 showed about 10% after treatment, as
shown in Table 1.

### 3.3 Analysis of C3 proteolytic activation

Hydrolysis of C3 by dentilisin was evaluated by incubation of C3 with T. denticola ATCC 35405 and K1. The results of SDS-PAGE and immunoblot after incubation are shown in Fig 2. A 110 KDa C3 α-chain band was hydrolyzed into 37 KDa and 35.5 KDa bands after incubation with T. denticola ATCC 35405; in contrast, no change was seen in this band in the C3 when incubated with mutant K1. To determine whether this activation was directly caused by the splitting of C3 into C3b, a chemiluminescence assay was performed using C3-deficient serum. As shown in Fig. 3, activation of PMNs by T. denticola ATCC 35405 was at almost the same level as that with T. denticola K1.

To determine activation of C3 by dentilisin, we evaluated production of iC3b using an iC3b ELISA kit. The results are shown in Fig 4. We found that generation of iC3b was significantly higher with T. denticola ATCC 35405 than with mutant K1 (p<0.01). To confirm the involvement of dentilisin, the effect of a protease inhibitor was investigated. In the presence of the protease inhibitor, chymostatin, production of iC3b was reduced from 12.20 ± 2.21 to 1.78 ± 1.04 at 30 min and from 6.27 ± 2.70 to 1.65 ± 1.49 at 60 min. A t-test showed that these reductions were statistically significant. (p<0.01).

### 3.4 Release of PMN-proteinases during phagocytosis

To evaluate the activation of PMNs by T. denticola, we measured release of MMP-9. As shown Fig 5 the culture supernatant of PMNs incubated with T. denticola contained significantly higher MMP-9 than that incubated with mutant K1 from 30 min to 120 min (p<0.01). The activity of MMP-9 after exposure to T. denticola was also investigated. As shown in Fig. 6, activation of MMP-9 by exposure to T. denticola was confirmed, and this activity was significantly reduced by chymostatin.

### 4. Discussion

The present study demonstrated that T. denticola dentilisin is capable of activating complement. Significant differences were found in the production of superoxide and release of MMP-9 between T. denticola ATCC 35405 and K1. Treatment with protease inhibitor chymostatin induced a reduction in production of \( \text{O}_2^\cdot \), iC3b production and active MMP production. These results implicate dentilisin in the activation of PMNs via activation of complement. They suggest that enhancement of chemiluminescence was caused by activation of complement.

C3b is a key factor in opsonization [15]. Phagocytes bear receptors on their surfaces that are specifically reactive with C3b and contribute to enhanced opsonization of foreign particles.
Our immunoblot analysis demonstrated that *T. denticola* ATCC 35405 hydrolyzed the α-chain of C3, and that K1 did not. C3a and C3b are generated by degradation of C3 and by activation of complement [14]. We confirmed that iC3b was produced from C3 by wild type *T. denticola*. iC3b has the ability to bind complement receptor type 3 (CR3) [26]. CR3 is expressed on the plasma membrane of mammalian PMNs, most mononuclear phagocytes and natural killer cells, and contributes to opsonization. These results indicate that *T. denticola* dentilisin hydrolyzes C3 and activates PMNs via a complement pathway, producing the C3-derived iC3b.

In the present study, chemiluminescence and complement analysis were performed in the absence of any specific antibody. Specific antibodies have a major impact on the susceptibility of bacteria to phagocytosis, and enhance opsonization. In our preliminary results, enhancement of opsonization was also low in mutant K1 compared with wild type strain in the presence of antibody. Our results show that dentilisin generated iC3b, and that this generation was significantly reduced by chymostatin. Shenkein et al. [27] suggested C3 activation by accumulation of C3 component on the surface of *T. denticola* strains TD2 and FM. The results in the present study agree with that report. It has been reported that the lipoproteins of *T. denticola* enhanced chemiluminescence [28] and that PMNs were activated by the Msp of *T. denticola* [17, 18, 21]. Our results indicate that, in addition to with other stimulants such as Msp, this microorganism activates PMNs with dentilisin via activation of complement.

In the present study, PMNs were activated by dentilisin. It has been reported that *T. denticola* adheres to PMNs and is ingested into phagosomes [18, 21]. Observations indicate that phagocytosed treponemes remain inside the phagosome, even after 60 min [21]. It has been suggested that the humoral immune response to *T. denticola* is not capable of resolving *T. denticola* infection [29], and it has also been shown that mouse antibody against *T. denticola* only slowed initial *T. denticola* replication and did not alter the maximum growth in vitro. In our preliminary results, growth of *T. denticola* ATCC 35405 over a two-day period after treatment with PMNs and complement was almost the same as that when not treated with PMNs or complement (data not shown). It is possible that, while *T. denticola* activates PMNs, killing of the microorganism is attenuated.

Dentilisin activated the production of reactive oxygen and released MMP-9 from PMNs. Released MMP-9 possessed activity, and this release was significantly reduced by chymostatin. Uncontrolled releases of reactive oxygen radicals and proteinases from neutrophils are known to cause cell injury [14, 15, 30, 31]. Following stimulation by bacterial antigens, PMNs produce O$_2^-$ via the metabolic pathway of the ‘respiratory burst’ catalyzed by NADPH oxidase during phagocytosis. O$_2^-$ is released into the phagosomal space and also into the extracellular
environment [30]. It has been suggested that reactive oxygens such as $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ play a role in the activation of osteoclasts, in addition to their bactericidal function [32]. It has also been demonstrated that reactive oxygen molecules are capable of activating latent PMN collagenase in gingival crevices [33, 34] and damaging connective tissues as part of the pathology of periodontal disease. It has also been reported that reactive oxygen degrades components of the extracellular matrix [30]. Furthermore, it has been demonstrated that MMP-2 and MMP-9 are capable of damaging connective tissues as part of the pathology of periodontal disease [35]. Our previous results have indicated that the protease mutant forms significantly smaller lesions in mice than does the wild type [24]. Further study is needed to elucidate the role of the activation of PMNs by dentilisin in colonization by $T. \text{denticola}$ and inflammation of periodontal tissue.

Taken together, these results indicate that the activation of production of reactive oxygen and MMP-9 by $T. \text{denticola}$ dentilisin may be involved in the process of progressive breakdown of periodontal tissues. The present findings of this study support a new virulence profile for $T. \text{denticola}$.

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References


Figure legend

Figure 1. Chemiluminescence responses of human PMNs. PMNs (1.5 x 10^7 cells/ml) were exposed to 1.8 x 10^8 cells/ml of freshly harvested *T. denticola* ATCC 35405 and dentilisin deficient K1 in presence of C5-deficient serum.

Figure 2. Immunoblot analysis of C3. C3 (0.1 µg/µl) after incubation with *T. denticola* ATCC 35405 and dentilisin deficient K1 using anti-human complement C3 serum. Lane 1: *T. denticola* ATCC 35405; lane 2: mutant K1; lane 3: control (complement alone). Arrowheads indicate high-molecular-mass oligomeric protein and α-chain and β-chain of C3 band.

Figure 3. Chemiluminescence responses of human PMNs using C3b-deficient serum. PMNs (1.5 x 10^7 cells/ml) were exposed to 1.8 x 10^8 cells/ml of freshly harvested *T. denticola* ATCC 35405 and dentilisin deficient K1 in presence of C3-deficient serum.

Figure 4. Generation of iC3b from C3 by *T. denticola*. C5-deficient complement and 1.8 x 10^8 cells/ml *T. denticola* after incubation at 37°C for 30 min and 60 min. iC3b concentrations are shown as mean (pg) ± standard deviation. One-way ANOVA for repeated measurements was used for inter-group comparisons. A Student-Newman-Keuls test was used for multiple comparisons. (*p<0.01)

Fig. 5. Induction of MMP-9 release by *T. denticola*. PMNs were incubated with 1.8 x 10^8 cells/ml of *T. denticola* in the presence of C5-deficient complement at 37°C for 30 min, 60 min, 90 min and 120 min. MMP-9 concentrations of culture supernatants are shown as mean (pg) ± standard deviation. One-way ANOVA for repeated measurements was used for inter-group comparisons. A Student-Newman-Keuls test was used for multiple comparisons. (*p<0.01)

Fig. 6. Induction of active MMP-9 release by *T. denticola*. PMNs were incubated with 1.8 x 10^8 cells/ml of *T. denticola* in the presence of C5-deficient complement at 37°C for 90 min. Amounts of active MMP-9 concentrations of culture supernatants are shown as mean (pg) ± standard deviation. One-way ANOVA for repeated measurements was used for inter-group comparisons. A Student-Newman-Keuls test was used for multiple comparisons. (*p<0.01)
Table 1. Effect of protease inhibitor on superoxide production induced by *T. denticola*

<table>
<thead>
<tr>
<th>Strain</th>
<th>% inhibition²</th>
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<tbody>
<tr>
<td><em>T. denticola</em> ATCC 35405 + chymostatin</td>
<td>36.4 ± 16.5</td>
</tr>
<tr>
<td>Mutant K1 + chymostatin</td>
<td>10.0 ± 5.61</td>
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</tbody>
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Inhibition of superoxide production evaluated by chemiluminescence assay of *T. denticola* (1.8 x 10⁸ cells/ml) treated with chymostatin (30 μg/ml). ² Mean ± standard error of three experiments done in duplicate samples.
Figure 1
Figure 2

![Image showing a gel with molecular weight markers: 175 KDa, 83 KDa, 62 KDa, 47.5 KDa, 32.5 KDa. The gel has three lanes (1, 2, 3) with some bands indicated by arrows.](image-url)
Figure 3

- ATCC 35405 with C3 deficient-serum
- Dentilisin-deficient K1 with C3-deficient serum
Figure 4

iC3b µg/ml

30 min 60 min

35405 Dentilisin-deficient K1 Control

**P < 0.01
Figure 5

![Graph showing MMP-9 levels over time for ATCC 35405, Dentilisin-deficient K1, and Control groups. The graph includes error bars indicating standard deviation. Asterisks (*) indicate statistically significant differences (* P < 0.05).]
Fig. 6

Active MMP-9 (ng/ml)

ATCC35405  ATCC35405 + Chymostatin  Control

* P < 0.05,
** P < 0.01