Cloning, Expression and Purification of Trypsin-like Protease of the Periodontal Pathogen *Tannerella forsythia* ATCC 43037

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**ABSTRACT**

*Tannerella forsythia*, a bacterial species frequently associated with the pathogenesis of periodontal disease and its proteases are implicated as virulence factors, is known to possess trypsin-like activity. Here, we characterize a trypsin-like protease of *T. forsythia* referred to as tlp. Full length (without a signal peptide) recombinant tlp (102 kDa). The evidence suggests that the enzyme is a serine protease since it was strongly inhibited by Pefabloc, Tosyl-L-Lysyl-chloromethane hydrochloride (TLCK) and Leupeptin. The ability to degrade elastin, fibrinogen and the antimicrobial peptide LL-37 may contribute to the pathogenicity of periodontitis.

**Keywords:** Trypsin-like protease, Cloning, Periodontitis, Protein expression

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1. INTRODUCTION

A periodontal disease is a common bacteria-induced inflammatory disease in which the tooth-supporting connective tissues are progressively destroyed. It is generally divided into two types of disease: “Gingivitis” and “Periodontitis”. Gingivitis is the first sign of possible development of subsequent periodontitis and affects solely the soft epithelial and connective tissues. It thus involves inflammation of the gum surrounding the teeth, bleeding easily when pressure is applied, whereas periodontitis is the most prevalent infection-driven chronic inflammatory disease of humankind (Cobb *et al.*, 2009). Progression of the disease cause of tooth loss. More than 500 bacterial species are present in the oral cavity (Paster *et al.*, 2001), and several of these species have been implicated in periodontal disease, some of these periodontopathic microorganisms have been shown to produce multiple proteolytic enzymes such as potential virulence factors, which are thought to participate in the pathogenesis of the disease via several mechanism (Ishihara *et al.*, 1996). Among those bacteria, *Porphyromonas gingivalis, Tannerella forsythia* (formerly *Bacteroides forsythus*) and *Treponema denticola* are
referred to as the “red complex” and are considered to be the major periodontopathogens (Socransky et al., 1998). A common feature of these pathogens is production of high levels of proteolytic activity, especially with trypsin-like proteases. Before the appearance of molecular biology based diagnostic techniques, detection of a trypsin-like activity using benzoyl-arginyl-β-napthyl amide in the fluid collected from a crevice between a tooth and the gingiva (gingival crevicular fluid) was used to detect the red complex bacteria (Loesche et al., 1990; Seida et al., 1992). The latest, proteases with the trypsin-like activity from P. gingivalis and T. denticola have been characterized and their role as virulence factors is fairly well documented (Potempa and Pike, 2009, Potempa et al., 2000 and Eley and Cox, 2003). No such data are currently available concerning the nature and properties of the trypsin-like enzyme produced by T. forsythia. The aim of this study was to characterize the trypsin-like protease of T. forsythia (Los Alamos Oral Pathogens Database Accession No. TF0947).

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Restriction endonuclease BamHI and XhoII, T4 DNA ligase, and dNTP were purchased from Fermentas (Burlington, Canada). DNA polymerase and Gel Extraction Kit were obtained from Finnzyme (Massachusetts, USA) and QIAGEN (Valencia, USA), respectively. DNA clean up system and plasmid extraction kit were from A&A Biotechnology (Gdynia, Poland). Expression vector pGEX6p-1, Glutathione-Sepharose 4 Fast Flow and the 3C protease (PreScission) were purchased from Amersham Bioscience (Buckinghamshire, UK). Polyvinylidene fluoride (PVDF) membrane was from Millipore (California, United states). Fluorescent-labeled protease substrates, including FITC-Casein, DQ-gelatin and DQ-elastin were from Molecular Probes. Human fibrinogen, fibronectin, bovine serum albumin (BSA) and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Construction of recombinant plasmid

Genomic DNA of T. forsythia was extracted from strain ATCC 43037. The entire trypsin-like protease (tlp) gene (TF0947; http://www.oralgen.lanl.gov), except for the nucleotide sequence that encodes the signal peptide, was amplified by PCR by using designed forward primer, (Tlp-F) 5’-GCAGAATTCCAGATAGGCGAAGGTGGA-3’ with an EcoRI recognition site (underlined) and reverse primer (Tlp-R) 5’-CAACTCGAGTTACTTTCTGACGCCGCG-3’ with an XhoI recognition site (underlined). The PCR product was purified and cloned into the BamHI/XhoI site of pGEX-6P-1 expression vector, which provides the coding sequence for an N-terminal glutathione-S-transferase (GST). This genetic manipulation inserts five residues (Gly-Pro-Leu-Gly-Ser) before the N-terminal glutamine residue of tlp after the GST moiety is removed by cleavage with the PreScission protease cleavage. The recombinant plasmid (pGEX-6P1-tlp) was transformed into Escherichia coli strain BL21(DE3) under the control of the T7 promoter.

2.3. Recombinant tlp expression and purification

Routinely, cells freshly transformed with recombinant plasmids were grown at 37°C in 1 L LB medium to A600 = 0.5 and cooled down to 21°C for 30 min. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added into the culture (final concentration 0.3 mM), and the culture was further incubated at 21°C for 3
h to induce protein production. Cells were harvested, washed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 1.8 mM KH$_2$PO$_4$, pH 7.4) and resuspended in 50 mL of PBS. Cells were lysed by ultrasonication on ice for about 5 minutes. The cell lysates were cleared by centrifugation (12,000 × g), filtered through 0.45 μm membrane and applied onto a glutathione-Sepharose column (2 mL bed volume) equilibrated with PBS at 1 mL/min flow rate. The column was then washed with 150 mL PBS to elute the unbound proteins. The recombinant tlp was then eluted in 10 mL of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM glutathione). Alternatively, 5 mL of PBS containing 100 μL of Prescision protease stock solution (1 U/μL) was applied onto the column and incubated overnight at 4°C. Recombinant tlp was eluted with 5 mL PBS and concentrated by ultrafiltration using Vivaspin concentrator (VIVA). Final purification of tlp was accomplished by gel filtration on Superdex 75 (16/60 gel filtration column) pre-equilibrated with 50mM Tris-HCl, pH 8.0.

Individual and pooled fractions were analyzed by SDS-PAGE. The total amount of proteins recovered at each step of the purification procedure was determined by bicinchonic acid (BCA) assay using bovine serum albumin as a standard. The tag-free tlp forms were routinely purified with the yield of circa 5 mg per 1 L culture.

2.4. Gel electrophoresis

Trypsin-like protease purification was monitored by SDS-PAGE using 10% gels and the Tris-HCl/Tricine buffer system. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 10% acetic acid and destained (Schagger and von Jagow, 1987).

2.5. N-terminal sequence analysis

Trypsin-like protease was resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Protein bands were visualized by Coomassie Brilliant Blue staining, excised and analyzed by automated Edman degradation using a Procise 494HT amino acid sequencer.

2.6. Proteolytic activity assay and tlp characterization

Protease activity was detected using various fluorescent substrates including FITC-casein, DQ-gelatin and DQ-elastin. Routinely assays were performed at 37°C using 500 nM of enzyme in 100 mM Tris-HCl pH 8.0, at substrate concentrations of 25μg/mL. Released fluorescence was measured using micro-titer plate reader (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA, USA) at excitation/emission wavelengths of 485/538 nm for FITC-casein and 500/520 nm for DQ-gelatin and DQ-elastin.

Human plasma fibrinogen, fibronectin and antimicrobial peptide LL-37 were incubated with tlp at a substrate/enzyme weight ratio 100:1(fibrinogen and fibronectin) and 1000:1(LL-37) for 12 h at 37°C in 100 mM Tris-HCl pH 8.0, and samples withdrawn at specific time points were subjected to SDS-PAGE. Proteins incubated alone served as controls.

FITC-casein was used as a substrate to determine pH optimum, the enzyme thermal stability, and effect of inhibitors and divalent metal ions on tlp proteolytic activity. The pH optimum was assayed using the following buffers at 100 mM concentration: MES (pH 5.5, 6.0, and 6.5), MOPS (pH 6.5, 7.0, and 7.5), HEPES (pH 7.0, 7.5, and 8.0), Tris (pH 7.5, 8.0, and 8.5), and CAPS (pH 8.5, 9.0, and 10.0). The effect of temperature on the activity of purified tlp was performed in 100 mM Tris-
HCl, pH 8.0. The enzyme was incubated at 0, 4, 10, 18, 28, 37, 45, 55 and 70°C for 2 h then measuring the residual activity under standard conditions. To test the effect of inhibitors and metal ions on tlp activity, the enzyme was pre-incubated with inhibitors and metal ions in 100 mM Tris-HCl, pH 8.0, for 15 min at room temperature and the residual activity determined.

3. RESULTS

3.1. Cloning and sequencing of a gene encoding trypsin-like protease (TF0947)

The sequence of the entire gene excluding the signal peptide was cloned into the expression vector (pGEX-6-P1). Further, DNA sequencing of the entire construct revealed another major discrepancy in the sequence within the 3’ region of the gene. The TF0947 gene encodes a secretory protein consisting of 948 amino acid residues including the signal peptide (Genbank Accession No. GQ856797).

3.2. Trypsin-like protease expression and purification

Trypsin-like protease was overexpressed as 128 kDa GST-fusion proteins. A tag-free trypsin like protease (102 kDa) was purified to homogeneity after the N-terminal GST molecule was removed by digestion with the PreScission protease (Fig. 1). The N-terminal sequence analysis of the tag-free trypsin-like protease revealed the NH₂-Gly-Pro-Leu-Gly-Ser-Ile-Gly-Glu-Gly-Thr-Pro-Pro-Ser-sequence with first four residues derived from the vector as expected after the cleavage of the fusion protein with PreScission protease (Fig. 2). The molecular mass of the recombinant protein was determined to be 102 154 Da by MALDI-TOF mass spectroscopy in correspondence to the molecular mass of NH₂-Gly-Pro-Leu-Gly-Ser-pretlp 102 424 Da calculated based on the amino acid composition inferred from the correct DNA sequence of the TF0947 gene.

Figure 1: Expression and purification of GST-trypsin like protease. (A) E. coli cell extracts and the purified GST-free-tlp after digestion with PreScission were resolved by SDS-PAGE. Lane 1, molecular mass markers; lane 2, E. coli extract before IPTG was added; lane 3, E. coli extract after 3 h after protein expression stimulation with IPTG; lane 4, GST-free-tlp eluted from a glutathione-Sepharose (102kD); lane 5, tlp purified by gel filtration on a Superdex 75 column. (B) elution profile of the purified tlp protein from a Superdex G75 gel filtration column.

Figure 2: Trypsin-like protease sequences. (A) Nucleotide sequences with 2802 bp, small letter sequences derived from the vector. (B) Protein sequences with 923 amino acid, small letter sequences derived from the vector.
3.3. Proteolytic activity

Freshly purified trypsin-like protease (tlp) activity was measured by casein, 95.3 RFU/sec. Trypsin-like protease also cleaved gelatin and elastin but at a 2- and 3-fold lower rate, respectively, than that for casein degradation. To determine whether tlp is a potential virulence factor, we assessed its ability to degrade the human proteins fibrinogen, fibronectin, and LL-37, an antimicrobial peptide from the cathelicidin family. Trypsin-like protease efficiently degraded both fibrinogen and LL-37, and it is of note that the α-chain of fibrinogen was completely degraded within 30 min. (Figure 3). As expected for serine proteases, cleavage of both substrates was totally inhibited by pefablock, while fibronectin was resistant to cleavage tlp (data not shown).

![Figure 3: Degradation of (A) fibrinogen, (B) LL-37 by trypsin-like protease. Fibrinogen (100 μg) and LL-37 (10 μg) were incubated at 37 °C in 100 μl of 100 mM Tris-HCl, pH 8.0, at the substrate/enzyme weight ratio 100:1 (A: fibrinogen) and 1000:1 (B: LL-37). At indicated time points, aliquots (10 μl) were withdrawn from the reaction mixture, mixed with 10 μl of hot reducing SDS-PAGE sample buffer and denatured at 95°C for 5 min to stop the reaction. Samples (20 μl) were resolved by SDS-PAGE. Fibrinogen and LL-37 incubated with tlp in presence of 100 μM TLCK served as non-digested protein/peptide control.]

3.4. Inhibition profile, stability and pH optimum

Inhibitor studies showed that a variety of serine protease inhibitors, including pefabloc, TLCK and leupeptin, were strongly effective in reducing the trypsin-like protease activity (Table 1). Activity was also inhibited by Zn2+. Cysteine and metallo-protease inhibitors did not significantly affect the trypsin-like protease activity.
Table 1: Effect of inhibitors divalent cations on the proteolytic activity of trypsin-like protease

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<thead>
<tr>
<th>Inhibitors and metal ions</th>
<th>Concentration</th>
<th>Activity (%) ± SD</th>
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<tbody>
<tr>
<td>Pefabloc</td>
<td>10mM</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>TLCK</td>
<td>100μM</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>100μM</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>E-64</td>
<td>100μM</td>
<td>105 ± 4</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>10mM</td>
<td>102 ± 6</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>10mM</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>EDTA</td>
<td>10mM</td>
<td>97 ± 5</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10mM</td>
<td>108 ± 6</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>10mM</td>
<td>27 ± 3</td>
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Activity determined at 37°C in 100mM Tris-HCl, pH 8.0, using FITC-casein as a substrate was taken as 100%. Assays were run in triplicate and the mean ±SD was calculated.

The optimum incubation temperature for the activity was 37°C. More than 80% of the activity was detected following incubation at 25°C. Treatment of Tlp at 45°C for 30 min resulted in a residual activity of 15%. No activity remained after treatment at 55°C for 30 min. (Fig. 4A).

The pH optimum of tlp for the casein degradation was found at pH 8.0 but activity was dependent on buffer composition. In Tris at this pH, the tlp activity was 2-fold higher than in HEPES at the same pH (Fig. 4B). This difference in activity may reflect differences in ionic strength exerted by the buffering species themselves. Nevertheless, it is likely that in physiological conditions, the enzyme is active in the pH range from pH 6.5 to 8.5.

4. Discussion

Proteases play an essential roles in eukaryotic and prokaryotic organisms. Since proteolysis is irreversible, unregulated proteolysis can cause significant damage; therefore, it is tightly controlled in a spatial and temporal manner in all organisms.

As trypsin-like protease is elaborated by periodontopathogens such as T. denticola, P.
gingivalis and T. forsythia, this activity has been suggested as a marker for periodontal disease (Bretz et al., 1990; Grenier 1995). It is also thought that trypsin-like proteases from these bacteria may actively participate in the progression of periodontal disease. Indeed, these enzymes may play numerous roles in pathogenesis, for example, in bacterial invasion of the host tissues, in countering host defense mechanisms, and in bacterial nutrition during infections (Grenier and Mayrand, 1993; Holt and Bramanti, 1991). The present study was undertaken to determine the major characteristics of the trypsin-like protease of T. forsythia.

The evidence suggests that the enzyme is a serine protease with 102 kDa, since it was strongly inhibited by TLCK, Leupeptin and Pefabloc. The inhibition by TLCK, Leupeptin and Pefabloc indicates that the catalytic site of the enzyme involves a serine.

Trypsin-like enzymes from T. denticola and P. gingivalis have been previously characterized. The trypsin-like enzyme of T. denticola is cell-associated and has a molecular mass of approximately 69 kDa, as determined by SDSPAGE (Ohta et al., 1986). This enzyme is completely inhibited by serine protease inhibitors (TLCK and leupeptin) but is not affected by metal chelators or sulfhydryl reagents. The T. denticola enzyme hydrolyses synthetic trypsin substrates containing either an arginine or lysine bonded to pNa but does not cleave natural proteins such as casein and gelatin. The T. forsythia trypsin-like protease described in the present study appears to share numerous properties with the T. denticola enzyme. On the other hand, several trypsin-like enzymes having molecular masses ranging from 35 to 300 kDa were purified from P. gingivalis. These BAPNA-hydrolysing enzymes are activated by reducing agents, inhibited by serine and thiol protease inhibitors and are active on a variety of natural substrates (Grenier and Mayrand, 1993). Therefore, they differ considerably from the activity produced by T. forsythia.

The trypsin-like protease of T. forsythia cleaves several human substrates, the degradation of which may impact the development of periodontitis. One of these, cathelicidin LL-37, is a bactericidal peptide with strong immunomodulatory properties that seems to play a crucial role in the maintenance of homeostasis in the periodontium (Hosokawa et al., 2006; Eick et al., 2014). Levels of LL-37 are increased in patients with periodontitis compared to healthy controls (Türkoğlu et al., 2009), and the genetic lack of LL-37 is associated with severe periodontal disease (Pütsep et al., 2002). Thus, hydrolysis of LL-37 by trypsin-like protease may not only contribute to the survival of T. forsythia, which is resistant to LL-37, but also disturb the regulation of inflammation during periodontitis. Taking into account that proteases from different periodontopathogens synergistically inactivate the complement system (Jusko et al., 2012) and supply nutrients (Hajishengallis, 2014), it is tempting to speculate that trypsin like-protease allies with karilysin and gingipains, also known to degrade LL-37 (Gutner et al., 2009; Koziel et al., 2010) to synergistically eliminate the peptide from infected sites. Finally, rapid and efficient degradation of the α-chain of fibrinogen by trypsin-like protease may contribute to clotting deficiency in the periodontal pocket. Degradation of the latter two proteins may have implications in T. forsythia pathogenicity.

5. CONCLUSIONS

Trypsin-like protease is a serine protease. The ability to degrade elastin, fibrinogen and the antimicrobial peptide LL-37 may contribute to the development and progression of periodontitis.
Conflict of Interest
There is no conflict of interest.

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REFERENCES

