이학석사 학위 논문

The role of enolase as a virulence factor of *Tannerella forsythia*

Tannerella forsythia 의 병독력 인자로서 enolase 의 역할

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치의과학과 면역 및 분자미생물 치의학 전공

이 준 영

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지도교수 : 최 봉 규

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이준영의 석사 학위논문을 인준함

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위 원 장 이 승 복 (인)

부 위 원 장 최 봉 규 (인)

위 원 최 영 님 (인)

ABSTRACT

The role of enolase as a virulence factor of *Tannerella forsythia*

Jun Young Lee

Department of Dental Science, Major of Immunology and Molecular Microbiology in Dentistry, Graduate School, Seoul National University

(Directed by professor Bong-Kyu Choi, Ph.D.)

Objectives

Periodontal disease is a chronic inflammatory disease in the periodontium caused by multi-species oral bacteria. The progression of the disease leads to destruction of periodontal tissues and alveolar bone loss. *Tannerella forsythia* is considered to be a periodontopathogen because it has been detected more frequently in periodontitis patients than in healthy subjects. In spite of the association of *T. forsythia* with periodontal disease, its virulence factors have not been fully studied. Although enolase presents mainly

in the cytosol of many organisms, it is a multiple-functional "moonlighting protein" that exists in distinct locations. Some bacterial enolase has been reported to be transported to extracellular space. A secreted protein of bacteria is tightly correlated with host cell infection and might be an excellent candidate of virulence factors. In addition, recent research has demonstrated that some enolases function as a human plasminogen receptor. The purpose of this study was to elucidate the pathogenic potential of *T. forsythia* enolase.

Methods

The viability of *T. forsythia* was examined by measuring the growth curve and live/dead staining. To identify the secreted proteins, the bacteria were cultured in new oral spirochete (NOS) medium and the culture supernatants were obtained by centrifugation. The culture supernatants were filtered using a membrane filter with pore size of 0.22 ㎛. The collected culture supernatants were then concentrated by using a 3 kDa-Centricon and subjected to SDS-PAGE to determine the secreted proteins. The secreted proteins of *T. forsythia* were analyzed by MALDI-TOF. Recombinant *T. forsythia* enolase was expressed in *Escherichia coli*, recombinant proteins were purified and endotoxin decontamination was verified. To examine whether *T. forsythia* enolase is exposed on the bacterial surface, immunoblotting and flow cytometry analysis were performed using bacterial enolase antibody. The binding ability of *T. forsythia* enolase to plasminogen was analyzed, as well as the activating ability of the enolase-bound plasminogen. Fibronectin degradation by *T.*

forsythia enolase-activated plasmin was analyzed using immunoblotting. To find out the effect of *T. forsythia* enolase on the proinflammatory responses in THP-1, the cells were treated with *T. forsythia* enolase for 24 h. The expression of IL-1β, IL-6, IL-8 and TNF-α was determined by real-time RT-PCR and ELISA.

Results

The secreted proteins were identified in the culture supernatants of a *T. forsythia* 24 h culture which was in the exponential phase. Enolase was identified as one of the secreted proteins. *T. forsythia* enolase was not only expressed on the bacterial surface but also secreted out of the bacteria. *T. forsythia* enolase bound to human plasminogen, and a plasminogen activator activated the enolase-bound plasminogen to plasmin. *T. forsythia* enolase-activated plasmin degraded fibronectin secreted from human gingival fibroblasts. *T. forsythia* enolase significantly induced IL-1β, IL-6, IL-8 and TNF-α in THP-1 cells at the gene and protein level.

Conclusion

T. forsythia enolase has a pathogenic potential to host by plasminogen binding and activation as well as induction of proinflammatory cytokines. These results suggest that *T. forsythia* enolase might induce tissue destruction and inflammatory response which could exaggerate inflammation, a characteristic of periodontitis.

Key words: Periodontitis, *Tannerella forsythia*, Enolase, Pasminogen activation, Proinflammatory cytokines

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CONTENTS

Ⅰ. INTRODUCTION

Periodontal disease is an inflammatory disease in the tissues surrounding the teeth. It is closely associated with the accumulation of periodontopathic bacteria such as *Tannerella forsythia, Porphyromonas gingivalis* and *Treponema denticola*. The chronic and progressive bacterial infection in periodontium leads to tissue damage and alveolar bone destruction by degrading the extracellular matrix such as fibronectin, dissociating fibrin clots or by promoting the production of inflammatory mediators including interleukin-1β, interleukin-6, interleukin-8 and tumor necrosis factor -α.

T. forsythia is a Gram-negative anaerobe with a fusiform rod shape. The bacteria is a member of the *Cytophaga-Bacteriodes* family and was previously referred to as *Bacteroides forsythus* by Tanner *et al* [1]. *T. forsythia* is considered a periodontal pathogen because it has been detected more frequently in periodontitis patients than in healthy individuals [2]. In spite of the evidence of its role in the pathogenesis of periodontal disease, it is still under-studied due to the difficulty in cultivating this pathogen. Although a few putative virulence factors including BspA, S-layer and PrtH have been identified, the virulence factors of *T. forsythia* are not fully understood [3].

Enolase (molecular weight of 46 kDa) is found in all living organisms and its sequence has been highly conserved throughout evolution. For example, *T. forsythia* enolase has 59% homology and 71% similarity in its sequence with *Fusobacterium nucleatum* enolase. It also shows 52% identity and 68% similarity with human enolase.

Enolase presents mainly in the cytosol. However, it is called a "moonlighting protein" that has multiple functions and exists in distinct locations [4, 5]. Some bacterial proteins without a signal peptide have been found to be secreted [6]. This phenomenon is termed *unconventional secretion*. Enolase is also transported to extracellular space despite the lack of a signal peptide. Because secreted proteins are tightly correlated with host cell infection, they might be a potential virulence factor. However, the mechanism of non-classical secretion protein is still unknown. A classical function of enolase involved in glycolysis and gluconeogenesis is catalyzing the dehydration of 2-phosphoglycerate to phosphoenolpyruvate. Recently, enolase has been reported to function as a plasminogen receptor, playing a role in the invasiveness and virulence of bacteria [7].

The interaction of enolase with host plasminogen and its subsequent activation to plasmin represents a mechanism to enhance the bacterial virulence by degrading fibrin and extracellular matrix (ECM) components [8]. Furthermore, lysine residues play an

important role in plasminogen binding, which is significantly inhibited by a lysine analog. Plasminogen is a glycoprotein (molecular weight of 92 kDa) which circulates in the blood and is mainly synthesized in the liver. The plasminogen is also expressed in broad extrahepatic locations which include the adrenal glands, kidneys, brain, testis, heart, lungs, uterus, spleen, thymus and gut [9]. Plasminogen is the zymogen of plasmin, and its conversion to plasmin is activated by tissue-type (tPA) and urokinase-type (uPA) plasminogen activators. The plasminogen activation system plays an essential role in fibrinolysis, ECM degradation, tissue remodeling, inflammation and cell migration. It is closely associated with the development of periodontal disease due to its ability to enhance bacterial invasion and regeneration of periodontal tissue [10].

The aim of this study was to elucidate the pathogenic potential of *T. forsythia* enolase. The surface exposure of enolase on *T. forsythia* was demonstrated using anti-enolase antibodies. The enolase bound to human plasminogen, which was activated to plasmin in the presence of uPA. In addition, the enolase induced the expression of proinflammatory cytokines in THP-1 cells.

Ⅱ. Material and methods

1) Bacteria strains and growth conditions

Tannerella forsythia (ATCC 43037) was cultured in new oral spirochete (NOS) broth (ATCC medium 1494) supplemented with vitamin K (0.02 ㎍/ml) and N-acetylmuramic acid (0.01 ㎍/ml). The bacteria were incubated under an anaerobic atmosphere (5% H_2, 10% CO_2, and 85% N_2) at 37℃ for 1 day.

2) Bacterial viability

T. forsythia was inoculated at OD_{600}=0.1 and cultured for 1 to 84 h. One milliliter of the bacteria was harvested by centrifugation at 10,000 x g for 10 min. The supernatants were removed and the bacteria were resuspended in phosphate-buffered saline (PBS). The pellet was collected by centrifugation and incubated with 1.5 ㎕ of propidium iodide and SYTO9 (Live/Dead-BacLight bacterial viability kit, Invitrogen, Grand Island, NY, USA) in PBS (1 ml) at room temperature in the dark for 20 min. The bacteria were washed twice with PBS and resuspended in 200 ㎕ of PBS. To separate a single bacteria, bacteria were sonicated by a VC130 Ultrasonic processor (Sonics & Materials Inc., Danbury, CT, USA). The bacteria were trapped between a slide and a coverslip with mounting oil. Viability of *T. forsythia* was observed using a confocal laser scanning microscope (Olympus FV300, Tokyo, Japan) at a magnification of

1,000x.

3) Preparation of culture supernatants

T. forsythia was cultured in NOS broth without serum components for 1 day. Twenty-one milliliters of culture supernatants (3.6×10^8 CFU/ml) was collected by centrifugation (High speed centrifuge, VISION SCIENTIFIC, Daejeon, Korea) at 7,000 x g for 30 min at 4℃. The culture supernatants were filtered using 0.22 ㎛-pore size membrane. Subsequently, it was concentrated approximately 28 fold by a Centricon 3 kDa exclusion filter (Millipore, Bedford, MA, USA).

4) MALDI-TOF identification of secreted proteins of *T. forsythia*

Electrophoresis of the concentrated culture supernatants of *T. forsythia* was performed using 10% SDS-polyacrylamide gel. The gel was stained in Coomassie blue (Bio-Rad Laboratories, Richmond, CA, USA) for 10 min and destained in the destaining buffer (methanol 10%, acetic acid 10% and distilled water 80%). To identify the visualized bands by peptide mass fingerprinting, the bands were excised, digested using trypsin and analyzed by MALDI-TOF (Ettan MALDI-TOF/Pro system, Amersham Biosciences, Bucks, UK). Amino acid sequences were searched using the MASCOT.

5) Purification of *T. forsythia* enolase

The nucleotide sequence of *T. forsythia* enolase was identified in

NCBI. The *T. forsythia* enolase gene was amplified from the genomic DNA by PCR. The sequences of the primers used for PCR were 5′-AAC TGA GCT CAT GAG AAT AGA ACA GAT T - 3′ (SacI-tagged) and 5′ - AAC TCT GCA GTT ATT TCA CTT TTT TAT ACC - 3′ (PstI-tagged). The PCR products of *T. forsythia* enolase were cloned into a TA cloning vector. The plasmid DNA was isolated and digested with the restriction enzyme (SacI and PstI). The fragments of genes were gel-purified using a Power Gel extraction Kit (Dyne Bio, Suongnam, Korea) and ligated into the predigested pQE30 expression vector using T4 DNA ligase. The expression of the recombinant *T. forsythia* enolase was induced with isopropyl-β-D-thiogalactopyranoside (IPTG). The proteins separated through a polyacrylamide gel were detected with Coomassie blue staining. The recombinant enolase was purified under the native conditions using nickel-nitrotriacetic acid agarose (Ni-NTA agarose, Qiagen, Valencia, CA, USA). The enolase was confirmed by SDS-PAGE gel and Coomassie blue staining.

6) Endotoxin removal

Endotoxin in the recombinant protein was removed by polymyxin B (Detoxi-Gel Endotoxin Removing Columns, Thermo, Rockford, USA). Endotoxin decontamination of the recombinant enolase was verified using the NF-kB reporter cell line. CHO/CD14/TLR4 cells were cultured to 70% confluency in Hams-F12 medium (Gibco, Invitrogen,

Grand Island, NY, USA) supplemented with 2% FBS (fetal bovine serum, Hyclone Laboratories, Logan, UT, USA). The cells were seeded at 1 x 10^5 cells/500 ㎕ in 24-well culture plates (Corning, Corning, NY, USA) and were reacted with recombinant *T. forsythia* enolase (10 ㎍/㎖) or LPS (1 ㎍/㎖) for 16 h. The cells were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD25 (Becon Dickinson, San Diego, CA, USA). The expression of CD25 was analyzed using flow cytometry (BD FACSCalibur, BD) and CellQuest acquisition analysis software (BD). In addition, the endotoxin activity of the recombinant enolase was measured by Limulus amoebocyte lysate assay using a LAL Endochrome Kit (Charles River Endosafe, Wilmington, MA, USA) according to the manufacturer's protocol.

7) Affinity purification of antibodies

In the present study, enolase-specific antibodies purified from *Fusobacterium nucleatum*-injected rabbit serum were used, due to the high homology of *F. nucleatum* enolase and *T. forsythia* enolase (59% identity and 71% similarity). One hundred micrograms of recombinant *T. forsythia* enolase was applied onto a neutral nylon membrane (Hybond-N, GE Healthcare Amersham, Slough, Berkshire, UK), and the membrane was blocked with 3% BSA (Bovine serum albumin, Bovogen, Australia) for 1 h at room temperature. Then, the membrane was incubated with *Fusobacterium nucleatum*-injected

rabbit serum for 5 h at room temperature and washed with PBS. Enolase-specific antibodies were eluted with 0.2 M glycine (pH 2.8, Duchefa Biochemio, Haarlem, Netherland) and 1 mM EGTA (Boehringer Mannheim GmbH, Mannheim, Germany). The eluted antibodies were immediately neutralized with 0.1 volume of 1 M Tris (Duchefa Biochemio, pH 8.5), and 0.1 volume of 10x PBS was added. Sodium azide was added to a final concentration of 0.02%. Finally, the pH of the collected antibodies was tested using pH paper.

8) Immunodot blotting

A 3 microliter volume of the concentrated *T. forsythia* culture supernatants (1.15 x 10^8 bacteria), *T. forsythia* lysates (1.5 x 10^8 bacteria), medium of bacteria (negative control) and THP-1 lysates (8 x 10^4 cells) were applied onto the neutral nylon membrane and dried. The membrane was blocked with 5% skim milk (Difco™ skim milk, BD, Sparks, MD, USA) in PBST (0.1% Tween20) for 1 h at room temperature and washed in PBST three times. The membrane was incubated with the affinity-purified enolase antibody (0.05 ㎍/㎖) in 5% BSA in PBST overnight at 4℃ and washed in PBST three times. The membrane was then incubated with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG, R&D Systems™, Minneapolis, MN, USA) for 1 h at room temperature. After washing with PBST, the membrane was developed with TMB (3,3′,5,5′-Tetramethylbenzidine Liquid Substrate System for

Membranes, Sigma-Aldrich, Louis, MO, USA).

9) Immunoblotting

The *T. forsythia* lysates (1.5 x 10^8 bacteria), medium of bacteria (negative control), recombinant *T. forsythia* enolase (100 ng) and THP-1 lysates (8 x 10^4 cells) were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon p, Millipore). The membrane was blocked with 5% skim milk in PBST (0.1% Tween20) overnight and reacted with the primary antibody (0.05 ㎍/㎖, bacterial enolase antibody) overnight at 4℃. Then, the membrane was incubated with a secondary antibody for 2 h. After washing with PBST, the immunoreactive bands were detected with a standard ECL reaction (Amersham/Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions.

10) Immunofluorescence assay

One-day-old cultures of *T. forsythia* were harvested by centrifugation at 10,000 x g and blocked in a mixture of 5% BSA and 5% goat serum (Gibco) for 1 h at 37℃. After washing in PBS, the bacteria were incubated with 0.03 mg/ml of the purified enolase antibody or rabbit anti-non neuronal enolase antibody (Abcam, Cambridge, MA, USA) in a blocking buffer for 2 h at 37℃ and washed in PBS three times. The bacteria were then incubated with Alexa Fluor 555-conjugated anti-rabbit-IgG (1/200, Thermo Fisher

Scientific Inc.) in 5% BSA and washed with PBS three times. The bacteria were fixed in 2% paraformaldehyde for 10 min at room temperature, followed by washing with PBS and sterilized distilled water. The location of enolase was observed by confocal scanning laser microscopy (Carl Zeiss LSM 700, Oberkochen, Germany).

11) Flow cytometric analysis

T. forsythia was harvested by centrifugation at 10,000 x g. After removing the culture supernatants, the bacteria were blocked in a mixture of 1% BSA and 2% goat serum in PBS for 90 min at room temperature. The bacteria were incubated with 0.01 mg/ml the purified enolase antibody or rabbit anti-non neuronal enolase antibody (Abcam) in blocking buffer for 1 h at 37℃, followed by washing with PBS three times. The bacteria were incubated with Alexa Fluor 555-conjugated anti-rabbit-IgG (1/50) in blocking buffer for 40 min. The surface-exposed enolase was quantitated by flow cytometry (BD).

12) Plasminogen binding assay

Ninety six-well plates (SPL Life sciences, Pocheon, Korea) were coated with 100 ㎕ of 0-5 ㎍/ml recombinant *T. forsythia* enolase in PBS overnight at 4℃. After washing with PBST (0.1% Tween20) three times, the immobilized enolase was blocked with 3% BSA in PBST for 1 h at room temperature. Subsequently, the blocked

enolase was washed in PBST three times, followed by incubating with 100 ㎕ of 10 ㎍/ml human plasminogen (Merk KGaA, Darmstadt, Germany) in the presence or absence of 30 mM lysine analog (6-Aminohexanoic acid, SigmaUltra, Sigma-aldrich) for 2 h at 37℃. After washing with PBST, the samples were incubated with biotin-labeled goat anti-plasminogen antibody (1:1000 dilution, Abcam) in PBS for 2 h at 37℃. The plates were washed with PBST three times and incubated with streptavidin-HRP (1:200 dilution, R&D Systems) for 2 h at room temperature. The plates were washed with PBST and incubated with TMB solution (Sigma) for 20 min at room temperature, followed by adding 50 ㎕ of stop solution (2 N H_2SO_4). The binding of *T. forsythia* enolase to human plasminogen was measured spectrophotometrically at 450 nm.

13) Plasminogen activation assay

Ninety six-well plates were coated with 100 ㎕ of 10 ㎍/ml recombinant *T. forsythia* enolase or BSA in PBS overnight at 4℃, followed by washing in PBST(0.1% Tween20) three times. The immobilized enolase was blocked with 3% BSA (in PBST) for 2 h at room temperature and washed in PBST three times. Then, 100 ㎕ of 20 ㎍/ml plasminogen was added, incubated for 2 h at 37℃ and washed in PBST three times. Subsequently, 100 ㎕ human of uPA (urokinase, Chemicon, Temecula, CA, USA) was added to adjust the final concentration of uPA 300 ng/ml and incubated for 2 h at 37℃.

Fifty micrograms of 0.3 mM substrate (S-2251, Chromogenix, Lexington, MA, USA) in buffer (64 mM Tris-HCl, 350 mM NaCl, 0.15% TritonX-100, pH 7.5) was added and incubated overnight at 37℃. Plasminogen activation was quantitated spectrophotometrically at 405 nm.

14) Fibronectin degradation assay

To analyze fibronectin degradation, human gingival fibroblasts (HGFs) were cultured in DMEM supplemented with 10% FBS, 100 ㎍ streptomycin, and 100 U of penicillin per ml (Gibco). Fibronectins secreted from cultured HGFs were used. Ninety six-well plates were coated with 100 ㎕ of 10 ㎍/ml recombinant *T. forsythia* enolase or BSA in PBS overnight at 4℃, followed by washing in PBST(0.1% Tween20) three times. The immobilized enolase was blocked with 3% BSA (in PBST) for 1 h at room temperature and washed in PBST three times. Then, 100 ㎕ of 20 ㎍/ml plasminogen was added and incubated for 2 h at 37℃. After washing with PBS, 70 ㎕ of human uPA (300 ng/ml) was added and incubated for 2 h at 37℃. Finally, the plates were incubated with 30 ㎕ of culture supernatants of HGFs (2.5×10^5 cell/ml) for 36 h at 37℃. The incubated culture supernatants were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 5% skim milk in PBST overnight and reacted with mouse anti-fibronectin antibody (1:5000, Sigma) overnight. Then, the membrane was incubated with

a secondary antibody (R&D Systems™) for 2 h. After washing with PBST, the immunoreactive bands were detected with a standard ECL reaction according to the manufacturer's instructions.

15) Cell culture and treatment

THP-1 cells (ATCC TIB-202), known as human monocytic cell line, were cultured in RPMI 1640 supplemented with 10% FBS, 100 ㎍ streptomycin, and 100 U of penicillin per ml (Gibco). The cells were maintained in a humidified 5% CO_2 atmosphere at 37℃. THP-1 cells (2×10^5 cells/ml in 24-well plates) were stimulated with recombinant *T. forsythia* enolase for 24 h.

16) Real time RT- PCR

The cells were harvested by centrifugation at 6,000 x g for 5 min. RNA was isolated using the Easy-BLUE total extraction kit (iNtRON Biotechnology, Sungnam, Korea), and cDNA was synthesized from 1 ㎍ of RNA using a Maxime RT PreMix kit (Promega) according to the manufacturer's protocols. The cDNA (1 ㎕) was mixed with 10 ㎕ of SYBR Premix Ex Taq (Takara Bio Inc., Tokyo, Japan) and 4 pM of primer pairs (Table 1) in a 20 ㎕ reaction volume, followed by PCR for 40 cycles of the following protocol: a denaturation step at 95℃ for 15 sec, an annealing step at 60℃ for 15 sec, and an extension step at 72℃ for 33 sec in an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR

products were subjected to melting curve analysis to verify the presence of a single amplification product. PCR without reverse transcription was performed as a negative control. The housekeeping gene encoding glyceraldehyde dehydrogenase (GAPDH) was used as a reference for normalization of the gene expression levels.

17) Enzyme-linked immunosorbent assay (ELISA)

The level of IL-1β, IL-6, IL-8 and TNF-α in the culture supernatants of THP-1 cells treated with recombinant *T. forsythia* enolase were determined by ELISA kits (R&D SystemsTM).

18) Statistical analysis

Statistically significant differences among experiments were analyzed with an unpaired, one-tailed Student's t-test. Data are shown as the mean ± SD. A p value of <0.05 was considered statistically significant.

Table 1. Primers used for real time RT-PCR analysis

Genes	Primer sequences
Interleukin-1 β	5′-GCCAATCTTCATTGCTCAAGTGTC-3′ 5′-TTGCTGTAGTGGTGGTCGGA-3′
Interleukin-6	5′-GATTCAATGAGGAGACTTGCCTGG-3′ 5′-GCAGGAACTGGATCAGGACTTT-3′
Interleukin-8	5′-CTGTGTGAAGGTGCAGTTTTGC-3′ 5′-AACTTCTCCACAACCCTCTGC-3′
Tumor necrosis factor- α	5′-CCTGCTGCACTTTGG AGTGA-3′ 5′-CTCAGCTTG AGGGTTTGCTACA-3′

Ⅲ. RESULTS

1) Determination of bacterial viability

Prior to identification of secreted proteins from *T. forsythia*, the bacterial viability at various incubation times was determined to select the time point for collecting bacterial culture medium to avoid the release of proteins by bacterial death. To determine the time for reaching mid log-phase, *T. forsythia* (OD_{600nm}=0.1) was inoculated, and the optical density at 600 nm was measured for 84 h. As shown Figure 1A, the log-phase of *T. forsythia* was initiated at 12 h and sustained until 36 h. The stationary phase was started at from 40 h. To further confirm the bacterial viability, *T. forsythia* was incubated for various time periods (0, 12, 24, 36, or 72 h) and the bacterial viability was determined using a Live/Dead-BacLight bacterial viability kit (Figure 1B). As expected, the bacterial death was not observed during the log-phase (12 and 24 h), whereas bacterial death was detected in the stationary phases (36 and 72 h).

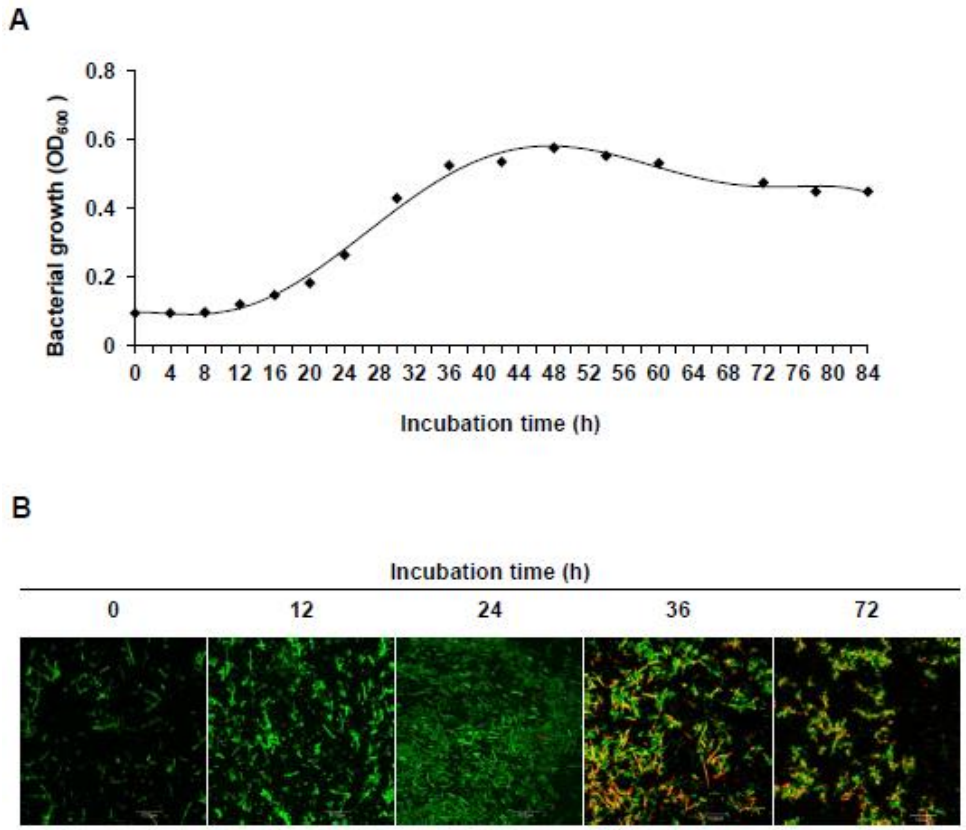

Figure 1. The growth curve of *T. forsythia* and bacterial viability.

To determinate bacterial viability according to the growth phase, *T. forsythia* was inoculated at OD_{600nm}=0.1. The optical density of the culture was measured at 600 nm for 84 h (A). The cultured *T. forsythia* was stained using a Live/Dead-BacLight bacterial viability kit and observed using a confocal laser scanning microscope (B). Original magnification x1,000

2) Identification of enolase in culture supernatants of *T. forsythia*

To identify the proteins that are secreted or released from *T. forsythia* during bacterial growth, the culture supernatants at 24 h were collected and concentrated using a Centricon 3 kDa exclusion filter. After separating the concentrated supernatants with 12% SDS-PAGE, the proteins were visualized on the gel by staining with Coomassie blue (Figure 2A). Among various bands on the gel, three bands around at 60 kDa, 45 kDa and 40 kDa were selected and subjected to the peptide identification using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The mass spectrometry analysis showed that 60 kDa, 45 kDa and 40 kDa proteins were matched with phosphoenolpyruvate carboxykinase, enolase, and phosphoserine aminotransferase, respectively (Figure 2 and Table 2). Bacterial enolase has been reported not only to be secreted from bacteria but also to be surface-exposed and associated with bacterial pathogenesis by interacting with plasminogen resulting in degradation of the extracellular matrix and fibrin [11, 12], *T. forsythia* enolase was thus chosen for further experiments. To confirm the existence of enolase in the culture supernatants, analysis of the secreted proteins was repeated under identical conditions as described above. In further investigation, the band of 46 kDa *T. forsythia* protein was identified as enolase, tetratricopeptide repeat protein and phosphoglycerate kinase (Figure 3 and Table 3).

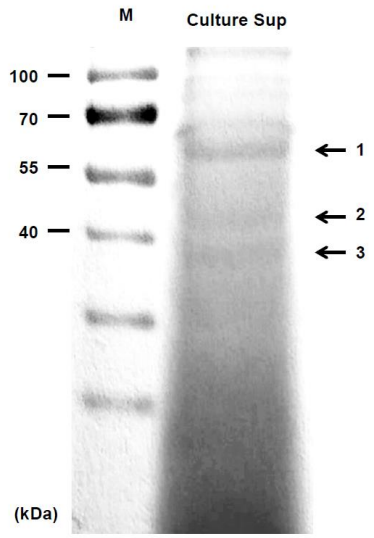

Figure 2. Identification of secreted proteins from culture supernatants of *T. forsythia.* After culturing *T. forsythia* for 24 h, the culture supernatants were collected and concentrated with a Centricon filter (MWCO: 3 Kda). The culture supernatants were subjected to SDS-PAGE, followed by visualization using Coomassie blue staining. The protein bands (arrow) were obtained from the gel, and the bands were excised, digested trypsin and analyzed by MALDI-TOF. Amino acid sequences were searched using MASOT.

Table 2. Identification of secreted proteins from culture supernatant of *T. forsythia* using MALDI-TOF

	Accession number	Protein ID	mass (kDa)	Peptide Sequence
1	gi|117620524	phosphoenolpyruvate carboxykinase [Aeromonas hydrophila subsp. hydrophila ATCC 7966]	60	AIIDAILDGSIEK
2	gi|333382777	enolase [Dysgonomonas gadei ATCC BAA-286]	46	GLSTAVGDEGGFAPALNGTEDALDSIL KAIK
3	gi|319955675	phosphoserine aminotransferase [Cellulophaga algicola DSM 14237]	40	FFGEVVEVASSK

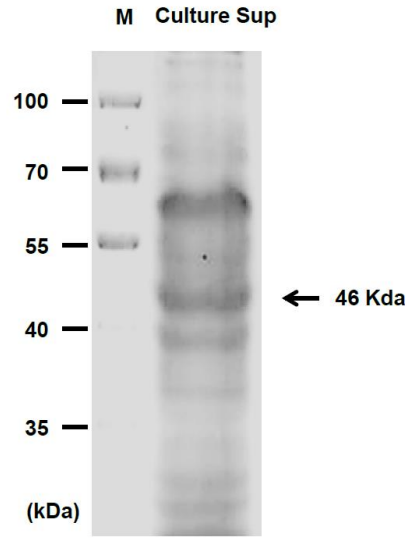

Figure 3. Identification of enolase from culture supernatants of *T. forsythia.* To identify the presence of the enolase in culture supernatants, *T. forsythia* was cultured at 37℃ for 24 h. And the culture supernatants were concentrated using a Centricon 3 kDa exclusion filter. After separating via SDS-PAGE (12% polyacryl amide), the proteins on the gel were stained with Coomassie blue. The expected size of enolase is around 46 kDa (Arrowhead). The bands at 46 kDa were analyzed by MALDI-TOF.

Table 3. Identification of 46 kDa protein using MALDI-TOF

Accession number	Protein ID	Mass (Kda)	Peptide Sequence
gi|375254021	Enolase [Tannerella forsythia ATCC 43037]	46	FVQDFVTVSSIFDSQIK
gi|375256526	tetratricopeptide repeat protein [Tannerella forsythia ATCC 43037]	52	GLSTAVGDEGGFAPA LNGTEDALDS IIQAIK
gi|375255755	phosphoglycerate kinase [Tannerella forsythia ATCC 43037]	45	FGLADGVSYVSTGGGALLEAIEGK

3) Purification of recombinant *T. forsythia* enolase

To prepare recombinant *T. forsythia* enolase, an expression vector called PQE-30 that habored the *T. forsythia* enolase gene was introduced into *E. coli*. After confirming the IPTG-induced expression of *T. forsythia* enolase (46 kDa) in *E. coli* using Coomassie blue staining (Figure 4A), the *T. forsythia* enolase was purified by affinity chromatography using Ni-NTA agarose (Figure 4B). Subsequently, the purified protein was subjected to the polymyxin B agarose column chromatography to remove endotoxin contamination. The decontamination of endotoxin in the purified recombinant *T. forsythia* was confirmed using NF-κB reporter cells (Figure 5). CHO/CD14/TLR4 cells express CD25 upon TLR4-dependent NF-κB activation. The recombinant *T. forsythia* enolase did not cause an increase in CD25 expression in the cells, whereas LPS significantly increased the expression of CD25. The endotoxin activity of the recombinant enolase was 0.04 endotoxin unit/μg of the protein. This activity was about 1/112,500 of *E. coli* LPS of the same amount, when measured by Limulus amoebocyte lysate assay. These results indicated that endotoxin was successfully removed in the recombinant enolase.

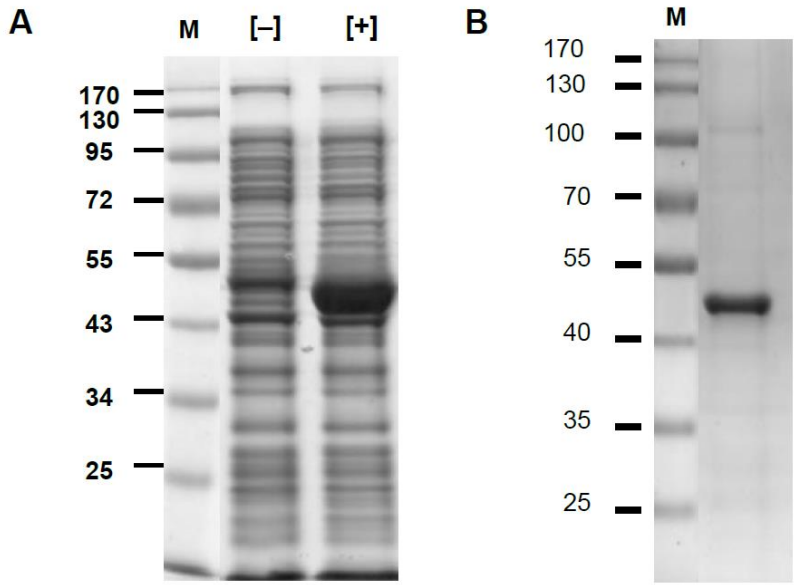

Figure 4. Preparation of recombinant *T. forsythia* enolase. (A) The *T. forsythia* enolase gene was cloned in *E. coli*, and the expression of histidine-tagged recombinant protein was analyzed by SDS-PAGE after induction with IPTG. [–], noninduced *E. coli* cell lysate; [+], IPTG-induced *E. coli* cell lysate. The position of protein size markers (M) are indicated. (B) Purified recombinant enolase was confirmed by SDS-PAGE.

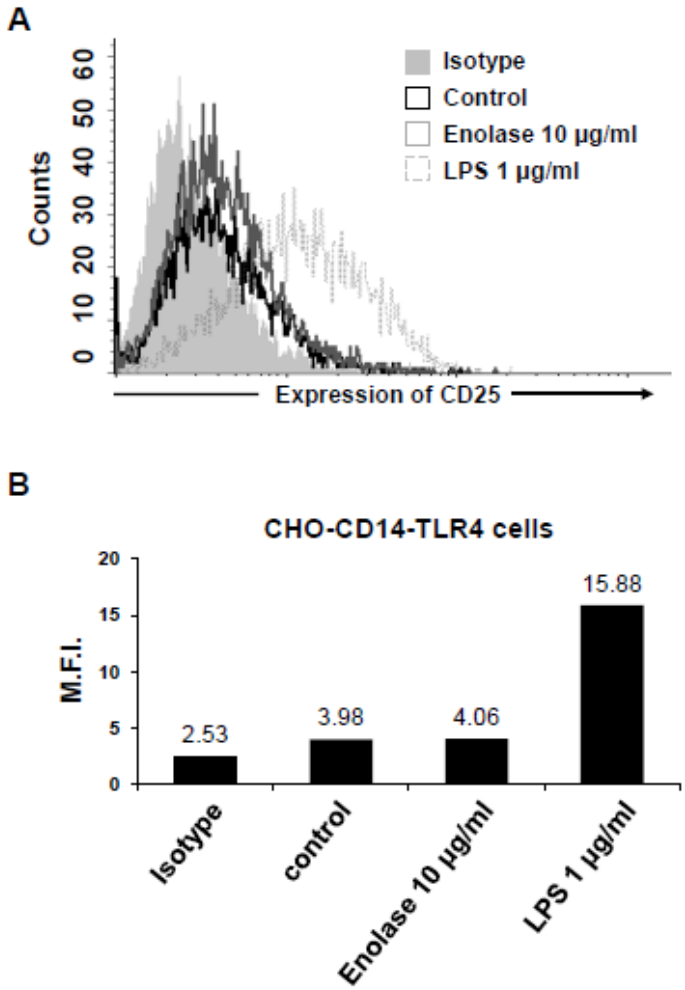

Figure 5. Verification of endotoxin decontamination of recombinant enolase protein using NF-κB reporter cell line. After removal of endotoxin from the recombinant enolase, CHO/CD14/TLR4 cells (1 x 10^5 cells/500 ㎕) in 24-well plates were treated with recombinant enolase (10 ㎍/ml) or *E. coli* LPS (1 ㎍/ml) in the presence of 2% FBS for 16 h. The cells were stained with FITC-labeled anti-human CD25 antibody for 30 min at 4℃. The CD25 expression was analyzed by flow cytometry. LPS-treated cells were used as a positive control.

4) Extracellular location of *T. forsythia* enolase

Immunodot blotting was performed to verify the existence of *T. forsythia* enolase in culture supernatants using the purified enolase antibody. Like positive controls such as recombinant enolase and THP-1 lysate, *T. forsythia* enolase was detected in the culture supernatant as well as the whole lysates of *T. forsythia* in its log phase(Figure 6), confirming that *T. forsythia* enolase can be secreted during bacterial growth. As it has been reported that several bacterial species express the enolase on their surface, confocal microscopy and flow cytometry analysis using anti-enolase antibodies were performed to detect expression of *T. forsythia* enolase on the surface. Interestingly, the *T. forsythia* enolase was detected on the bacterial surface (Figure 7 and 8).

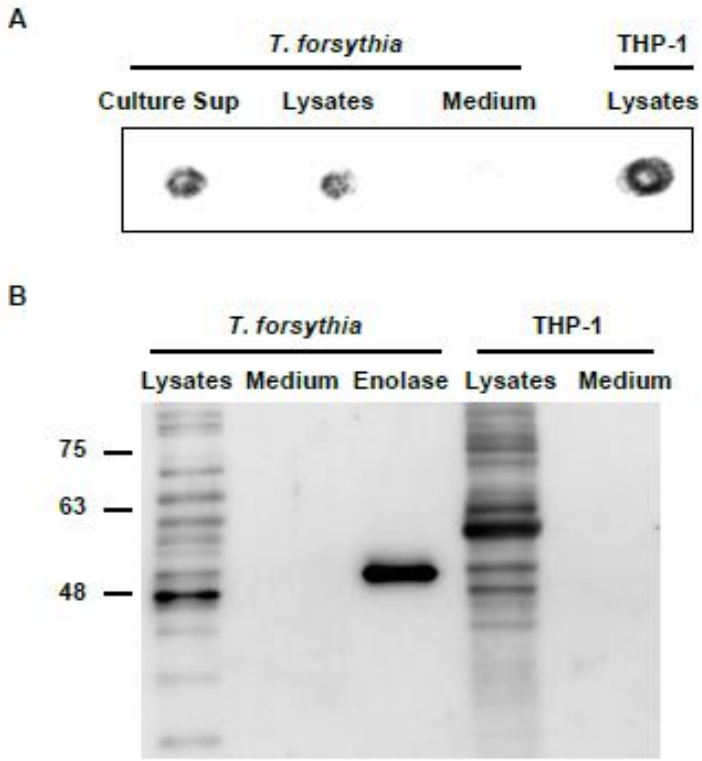

Figure 6. Reactivity of *T. forsythia* culture supernatants with anti-bacterial enolase antibody. The cross-reactivity of the *T. forsythia* culture supernatants with the anti-bacterial enolase antibody was examined by using an immunodot blotting. *T. forsythia* culture supernatants, *T. forsythia* lysates and THP-1 lysates were applied onto a nitrocellulose membrane and reacted with affinity-purified enolase antibody. As a negative control, medium were included. The detection was performed using HRP-labeled anti-rabbit IgG and TMB solution (A). Similarly, *T. forsythia* whole cell lysates, recombinant enolase and THP-1 lysates were subjected to immunoblotting with affinity-purified enolase antibody (B).

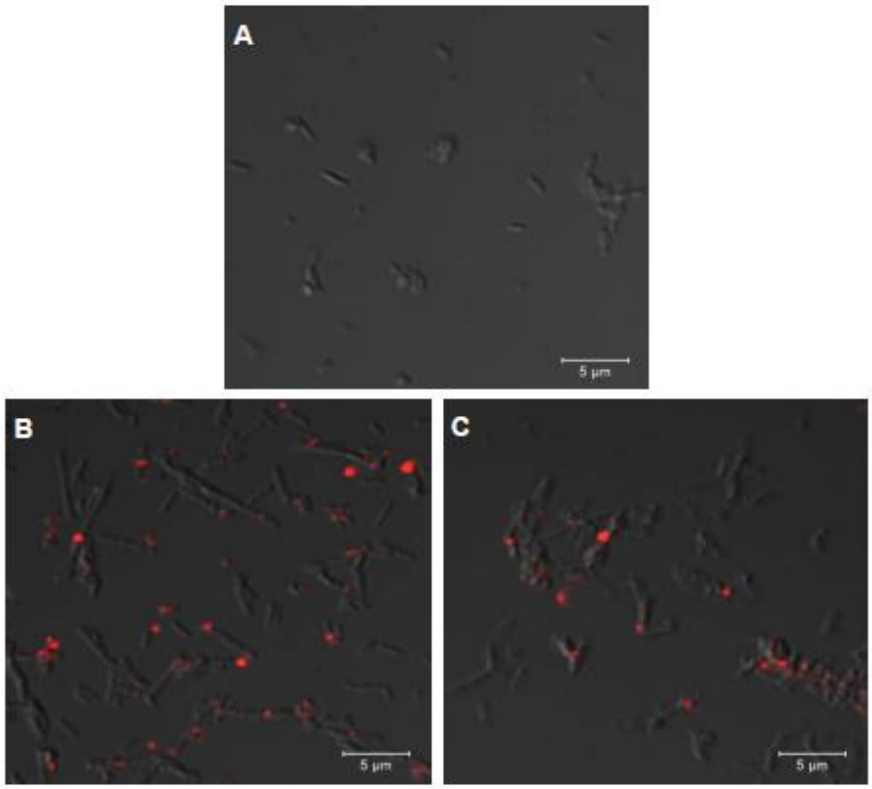

Figure 7. The immunofluorescence assay of *T. forsythia* enolase on the bacterial surface. The ability of the intact bacteria to react with the enolase antibody was assessed using indirect immunofluorescence. Cultured *T. forsythia* was reacted with an isotype control (A), affinity-purified enolase antibody (B), or anti-human enolase antibody (C), followed by reacting with Cy3-labeled anti-rabbit IgG. The surface location of enolase was observed by a confocal microscope. An isotype-matched antibody (rabbit-IgG) was used as a negative control. Original magnification x2,000

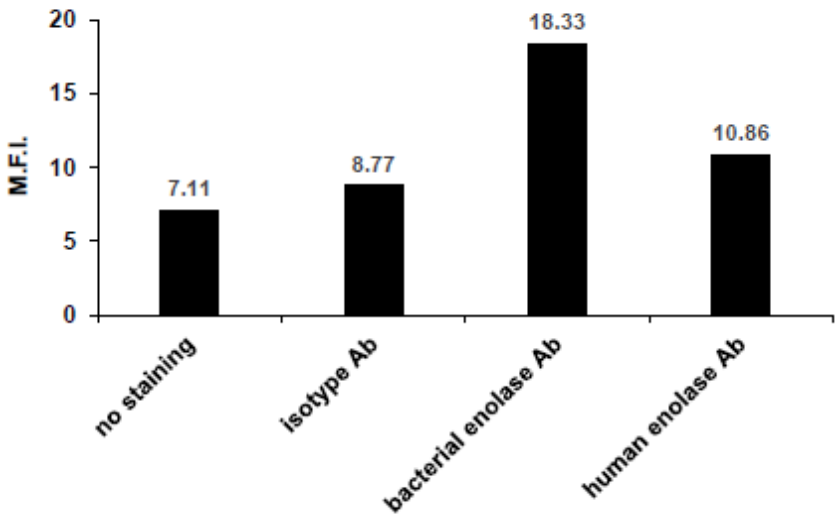

Figure 8. The flow cytometric analysis of *T. forsythia* enolase on the bacterial surface. *T. forsythia* was reacted with an isotype control, anti-bactreial enolase antibody (affinity-purified enolase antibody), or anti-human enolase antibody, followed by reacting with Cy3-labeled anti-rabbit IgG. The surface location of enolase was analyzed by flow cytometry. An isotype-matched antibody (rabbit-IgG) was used as a negative control.

5) Binding of *T. forsythia* enolase to human plasminogen and activation of *T. forsythia* enolase-bound plasminogen

Previous studies have reported that several bacterial enolases can interact with plasminogen. Furthermore, lysine residues of enolases play an important role in plasminogen binding, which is significantly inhibited by a lysine analog [13-15]. To examine whether *T. forsythia* enolase binds plasminogen, various amounts of *T. forsythia* enolase were incubated with plasminogen. *T. forsythia* enolase bound to human plasminogen in a dose-dependent manner, and this interaction was inhibited in the presence of the lysine analog (Figure 9A). To examine whether the *T. forsythia* enolase-bound plasminogen had an ability to convert plasminogen to plasmin, a plasminogen activation assay was performed after incubating plasminogen in the enolase-coated plate. Plasminogen that bound to *T. forsythia* enolase was activated to plasmin by the plasminogen activator, thereby degrading the plasmin-specific substrate. Plasminogen activation was not observed in the negative control group using BSA (Figure 9B).

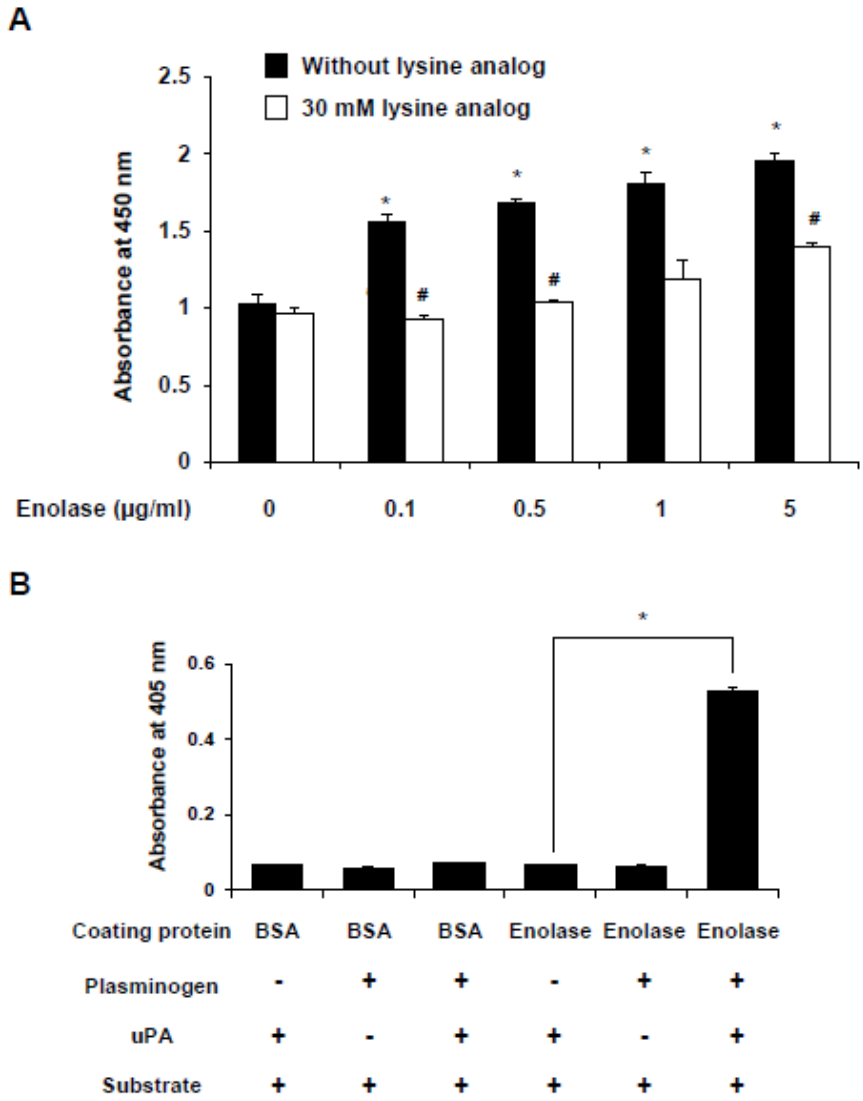

Figure 9. Binding of *T. forsythia* enolase to human plasminogen, and its converting activity to plasmin. (A) Binding of human plasminogen to immobilized enolase (0 to 5 ㎍/ml) was analyzed by ELISA. Human plasminogen (10 ㎍/ml) was incubated in the presence and absence of a lysine analog. Plasminogen bound to enolase was detected with

a biotin-labeled anti-plasminogen antibody and Streptavidin-HRP, followed by developing with TMB. The absorbance was measured at 450 nm. $^{*}p<0.05$, Statistical significance compared to nontreated control, $^{\#}p<0.05$, Statistical significance compared to enolase-treated group without the lysine analog. The experiments were performed three times in triplicates and the representative data are shown. (B) Enolase-coated plates (10 ㎍/ml) were incubated with human plasminogen (20 ㎍/ml), plasminogen activator (4 ng/ml), and plasmin-specific chromogenic substrate. The absorbance was measured at 405. $^{*}p<0.05$, Statistical significance compared to control group without plasminogen. The experiments were performed three times in triplicates and the representative data are shown.

6) Degradation of fibronectin by enolase-bound plasmin activity

The interaction of host plasminogen with enolase and its subsequent activation to plasmin can lead to degradation of ECM components [16, 17]. As one of the major ECM components, fibronectin is secreted from HGF cells [18, 19]. To investigate whether *T. forsythia* enolase affects fibronectin in HGF culture supernatants, HGF culture supernatants were incubated with enolase-bound and uPA-activated plasminogen. Unlike negative controls such as BSA, the activated plasminogen that was bound to *T. forsythia* enolase degraded fibronectin. HGF culture supernatants incubated with plasmin were used a positive control.

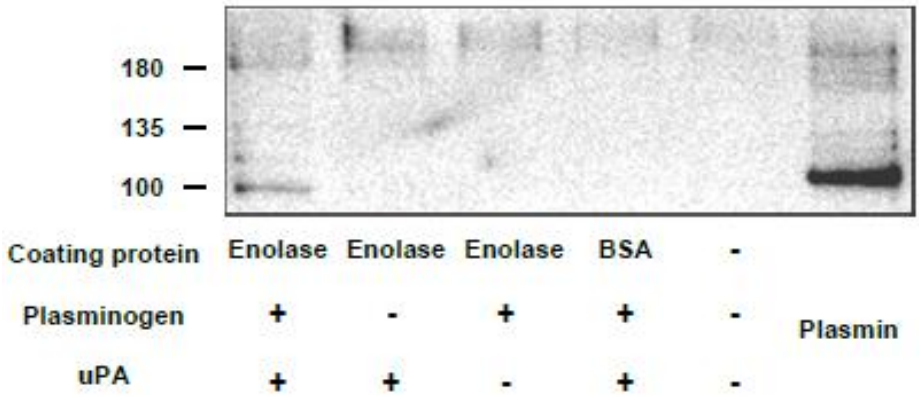

Figure 10. Degradation of fibronectin by uPA activated *T. forsythia* enolase-bound plasminogen. Enolase-coated plates (10 ㎍/ml) were incubated with human plasminogen (20 ㎍/ml), plasminogen activator (uPA, 300 ng/ml), and HGF culture supernatants. The reaction mixtures were subjected in SDS-PAGE and transferred to PVDF membrane. The membrane was reacted with anti-fibronectin antibody and HRP-labeled secondary antibody followed by ECL reaction.

8) Proinflammatory responses to *T. forsythia* enolase

To test whether *T. forsythia* enolase is involved in the pathogenesis of *T. forsythia* infection, the expression of proinflammatory cytokines was examined. THP-1 monocytes were stimulated with recombinant *T. forsythia* enolase for 6 or 24 h. The enolase significantly up-regulated mRNA expression of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α in a dose-dependent manner (Figure 11A). In addition, the protein levels of the cytokines were significantly increased by *T. forsythia* enolase in a dose-dependent manner (Figure 11B).

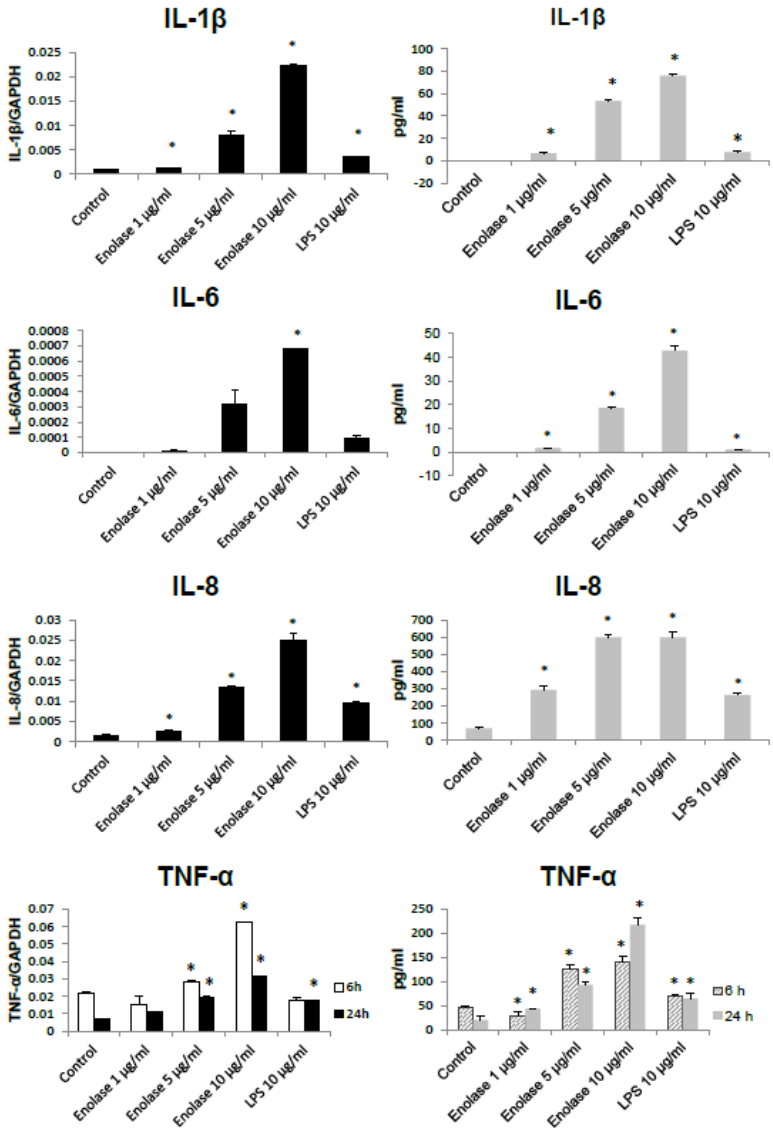

Figure 11. Upregulation of proinflammatory cytokines by *T. forsythia* enolase in THP-1 cells. THP-1 cells (2 x 10^5 cells/ml) were stimulated with recombinant *T. forsythia* enolase for 6 or 24 h (A). RNA was isolated from the cells and subjected to real-time RT-PCR. (B) The conditioned medium were used for ELISA. * $p<0.05$, Statistical significance compared to control.

Ⅳ. DISCUSSION

Although *T. forsythia* is a major periodontopathogen which is closely related to the progression of chronic periodontitis, its pathogenic mechanisms are not fully understood due to the difficulty of the bacterial cultivation. In this study, *T. forsythia* enolase was identified as a novel secreted protein regarding the degradation of the extracellular matrix and the induction of proinflammatory mediators in host cells.

T. forsythia enolase is capable of binding to human plasminogen. The results of the present study correspond well with earlier studies on the enolases of the human pathogens *Streptococcus pneumonia* and *Bacillus anthracis* [20, 21]. Enolases of *Bifidobacterium* and *Lactobacillus* species, which are considered commensal bacteria, can also bind to human plasminogen [22, 23]. However, pathogenic bacterial enolases show a slightly higher affinity for human plasminogen than commensal bacterial enolases [22]. The enolase-plasminogen binding activity might represent a high benefit for bacterial colonization and ECM degradation. The enolase-bound plasminogen activates plasmin, which can degrade ECM proteins such as laminin, fibronectin, and connective tissue proteins such as elastin and collagen [16, 17].

T. forsythia enolase may be involved in the interaction with host

cells due to its presence on the bacterial surface and its secretion into outside. However, there have been no reports on the potential role of enolase to induce proinflammatory responses. In the present study, *T. forsythia* enolase significantly enhanced the production of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α. This increase was not due to endotoxin contamination of *T. forsythia* enolase. Another moonlighting protein (proteins of multiple function like enolase), GroEL has also been reported to increase IL-8 secretion in macrophages [22]. The results suggest that *T. forsythia* enolase affects host inflammation. However, the mechanisms of proinflammatory responses induced by *T. forsythia* enolase are still undiscovered.

Several proteins were detected in the culture supernatants of *T. forsythia*. The proteins were identified as phosphoenolpyruvate carboxykinase, phosphoserine aminotransferase, tetratricopeptide repeat protein, phosphoglycerate kinase and enolase. With the exception of tetratricopeptide repeat protein, they are all glycolytic enzymes. Bacterial tetratricopeptide repeat motif has been demonstrated to serve an essential for function of a chaperone [24, 25]. The result of bacterial viability indicates that detection of these proteins in the culture supernatants was not caused by the release of cytosolic components after bacterial death. These findings imply that the identified proteins are secreted. Thus, the identified proteins might be potential candidates for virulence factors due to secreted

proteins being tightly correlated with host infection [26].

Among those proteins identified in culture supernatants, enolase was also exposed on the bacterial surface in spite of lacking a signal peptide for the secretion or for an anchor domain to expose on the surface. It is known that other bacterial components such as GAPDH and GroEL are also secreted although they do not possess a secretion signal sequence, nor do they have a domain for an anchor [27]. This phenomenon is termed unconventional secretion [6]. Although automodification of amino acid sequence and stress condition would be possible mechanisms [28, 29], further studies are needed to explain the exact mechanism of secretion.

It is important to identify the virulence factors of bacteria in order to understand the pathogenesis in bacterial infection. In this study, *T. forsythia* enolase was identified as a novel secreted protein involved in tissue degradation and inflammation. This study may provide some insight into the role of *T. forsythia* enolase as a virulence factor and a potential target for understanding periodontal disease. Further research on the *in vivo* role of periodontopathogen enolases, such as those of *T. forsythia*, would clarify the association of enolase in periodontitis.

Ⅴ. CONCLUSION

T. forsythia enolase can be an important virulence factor which contributes to periodontitis pathogenesis with plasminogen-activating and proinflammatory cytokine-stimulating abilities, resulting in tissue damage.

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국문초록

Tannerella forsythia 의 병독력 인자로서 enolase 의 역할

이 준 영

서울대학교 대학원

치의과학과 면역 및 분자미생물 치의학 전공

지도교수: 최 봉 규

1. 연구목적

치주질환은 다양한 세균의 집합체에 의해 발병하는 구강 내 염증질환으로 잘 알려져 있다. *Tannerella forsythia* 는 치주병원성과 관련이 높은 치주유발세균 중 하나로 밝혀져 있으며, 배양의 어려움으로 인해 그 병독력 인자가 많이 밝혀져 있지 않다. 또한, 세포 내 존재하는 단백질이 세포 밖에서 존재하여 새로운 기능을 한다는 '문라이팅 단백질' 연구가 최근에 시행 된 바가 있다. Enolase 도 문라이팅 단백질의 하나로,

세포 밖으로 분비된다고 밝혀진 사례가 있다. 이러한 분비되는 물질은 독성인자로써 훌륭한 후보물질로 생각되어진다. 따라서 본 논문에서는 *T. forsythia* 의 enolase 가 병독력 인자로서의 가능성을 제시하기 위한 연구를 하였다.

2. 연구방법

T. forsythia 의 생장곡선 측정과 현미경 관측을 통해 세균의 활성도가 높은 조건을 확인하였다. 이 생장조건에서 세균을 배양하여 주사 여과기 (0.2 ㎛ pore size) 로 여과 후 YM-3 3-kDa Centricon으로 배양액을 농축하였다. 농축된 배양액을 SDS-PAGE로 분리한 후 단백질 밴드를 선택, 펩타이드 분석 (MALDI-TOF) 을 수행하여 배양액으로 분비된 단백질을 동정하였다. 동정된 단백질 중 하나인 enolase를 유전자 재조합 기술을 이용하여 *Esherichia coli* 에서 발현 시켜 추출 하였다. 정제된 재조합 단백질은 내독소의 오염을 제거 한 뒤 실험에 사용하였다. 세포 밖으로 분비되는 enolse를 확인하기 위하여 면역 블롯 (immunoblot, immuno dotblot)을 수행하였다. 또한, 세포표면의 enolase 존재를 유세포분석과 공초점현미경을 통하여 분석하였다. 그리고 재조합 단백질을 이용하여 사람의 플라스미노겐과 결합하는지, 그 결합이 플라스민으로 활성화되는지, 그리고 활성화된 플라스민이 파이브로넥틴을 분해 할 수 있는지 확인하였다. 그리고 enolase가 THP-1 세포에서의 염증성 사이토카인 생성에 기여하는 바를 실시간 중합효소 연쇄반응과 효소결합면역흡착검사로 확인하였다.

3. 연구결과

*T. forsythia*는 24시간 배양에서 세균의 생활력이 가장 높았다. 이

생장조건에서 분비된 단백질 중 하나로 enolase를 확인하였다. 그리고 enolase는 실제로 분비되고, 또한 세포표면에 존재 하는 것을 확인하였다. 또한, 재조합 단백질로 만든 enolase가 사람의 플라스미노겐과 결합하고, 그 결합이 플라스민으로 활성화되는 것을 확인하였다. 이 활성화된 플라스민은 HGF의 배양액에 존재하는 파이브로넥틴을 분해시킬 수 있었다. 그리고 enolase에 의해서 사람단핵구세포인 THP-1 에서 IL-1β, IL-6, IL-8, TNF-α 같은 염증 성 사이토카인 발현이 증가되었다.

4. 결 론

T. forsythia enolase는 치주 병인기전에서 플라스미노겐을 활성화 시키고 염증성 사이토카인을 유도함으로써, 조직을 손상시킬 수 있는 주요한 병독력 인자가 될 수 있을 것이다.

주요어 : 치주염, *Tannerella forsythia*, Enolase, 플라스미노겐 활성화, 염증 성 사이토카인

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