Microbial Pathogenesis 100 (2016) 37–42

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Identification and characterization of a chymotrypsin-like serine protease from periodontal pathogen, *Tannerella forsythia*

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ARTICLE INFO

Article history:
Received 31 May 2014
Received in revised form
8 January 2016
Accepted 31 August 2016
Available online 1 September 2016

Keywords:
Tannerella forsythia
Serine protease
Periodontal disease
Type I collagen
Gelatin

ABSTRACT

Tannerella forsythia is a bacteria associated with severe periodontal disease. This study reports identification and characterization of a membrane-associated serine protease from *T. forsythia*. The protease was isolated from *T. forsythia* membrane fractions and shown to cleave both gelatin and type I collagen. The protease was able to cleave both substrates over a wide range of pH values, however optimal cleavage occurred at pH 7.5 for gelatin and 8.0 for type I collagen. The protease was also shown to cleave both gelatin and type I collagen at the average reported temperature for the gingival sulcus however it showed a lack of thermal stability with a complete loss of activity by 60 °C. When treated with protease inhibitors the enzyme's activity could only be completely inhibited by serine protease inhibitors antipain and phenylmethanesulfonyl fluoride (PMSF). Further characterization of the protease utilized serine protease synthetic peptides. The protease cleaved *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide but not N_{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) or *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide indicating that the protease is a chymotrypsin-like serine protease. Since type I collagen is a major component in the gingival tissues and periodontal ligament, identification and characterization of this enzyme provides important information regarding the role of *T. forsythia* in periodontal disease.

1. Introduction

Periodontal disease is a chronic inflammation of the periodontium that results in destruction of the connective tissue supporting teeth and bone. In addition to tooth loss, periodontal disease has also been shown to be associated with systemic diseases such as cardiovascular disease, diabetes, obesity, and rheumatoid arthritis [1–4]. *Tannerella forsythia* along with *Porphyromonas gingivalis* and *Treponema denticola* make up the "red complex" of microorganisms found in human gingival plaques [5,6] and are associated with severe periodontal disease [7]. Although *T. forsythia* plays an important role in development of periodontal disease how this bacterium interacts with host tissues and cells leading to destruction of periodontal structures has not been fully elucidated. Studies involved in understanding the role of *T. forsythia* in periodontitis have led to the identification of several potential virulence factors. They include a cell surface-associated BspA protein necessary for attachment and invasion of epithelial cells [8] and a surface glycosylated S-layer that suppresses host immunity [9]. In addition, several enzymes have been characterized and may serve as virulence factors. These include a trypsin-like protease [10], sialidases (SiaHI and NanH) [11,12], a cysteine protease (PrtH) [13], a matrix metalloprotease (karilysin) [14], and a subtilisin-like serine protease (mirolase) [15]. In addition, recent research has identified KLIKK proteases in pathogenic *T. forsythia* that are not present in normal flora *T. forsythia* implicating them as potential virulence factors [16]. The outer membrane of *T. forsythia* has been analyzed resulting in the identification of 221 proteins suggesting additional *T. forsythia* virulence factors [17].

Collagen is the primary component of periodontal connective tissue and is found in both alveolar bone and gingival connective tissue. The periodontium contains collagen types I, III, IV, V, and VI. Collagen types I, III, V and VI are fibrillar forms of collagen composed of a helical triple helix while type IV collagen contains nonhelical regions which can assemble into tetramers to form sheets. Each type of collagen has a characteristic distribution pattern in gingival tissues [18]. Type I collagen is found in gingival tissues [19] and is the predominant form of collagen in the periodontal ligament [20]. These connective tissues are necessary for the stability and functions of the periodontium and degradation of

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http://dx.doi.org/10.1016/j.micpath.2016.08.041
0882-4010/

collagen results in loss of tooth attachment and ultimately tooth loss. Degradation of type I collagen would likely result in recession of gingival tissue and decrease in tooth attachment to the alveolar bone. In this study we identified and characterized a serine protease from *T. forsythia* that degrades both gelatin and type I collagen.

2. Materials and methods

2.1. Isolation of T. forsythia protease

T. forsythia (ATCC 43037) was cultured in Hungate tubes with PY medium (ATCC 1528) containing 5% horse serum and 15 μg/mL *N*-acetylmuramic acid. Bacterial cells were incubated at 37 °C for 48 h to O.D. 600 of 0.6 then pelleted, washed with Tris-HCl pH 7.8, and resuspended in 1 mL 0.2 M Tris-HCl pH 7.8 containing 1 M sucrose, 1 mM EDTA, 1 mg/mL lysozyme. Following incubation for 20 min at room temperature 4 mL water was added and the solution was incubated at room temperature for an additional 20 min. 6 mL of 50 mM Tris-HCl pH 7.8 containing 2%Triton X-100, 10 mM $MgCl_2$, 50 μL DNase I (1 mg/mL) was added and the solution was sonicated using a sonic dismembrator set at 7 for 20 min (pulse 5 s, rest 10 s). Lysed cells were centrifuged at 5000*g* for 10 min at 4 °C to remove insoluble material. The supernatant was collected then recentrifugated 85,000*g* for 30 min at 4 °C to pellet membrane fragments. The resulting membrane containing pellet was resuspended in 1 mL 50 mM Tris-HCl, pH 7.8, 1% Triton X-100. The suspension was applied to a Sephacyl S-200 HR gel filtration column and eluted with a mobile phase consisting of 50 mM Tris-HCl, pH 7.8 with 1% Triton X-100 pumped at a rate of 1 mL/min. Fractions were collected every minute for 70 min. Each fraction was assayed for gelatinase activity.

2.2. Electrophoresis and silver staining

Fractions from Sephacyl S-200 HR gel filtration column and molecular weight markers from Promega were subjected to SDS-PAGE using 4.0–20.0% Tris-glycine gradient gels purchased from Jules Inc. Biotechnologies. The resulting gel was silver stained using Pierce Scientific silver stain solutions and procedures.

2.3. LC-MS/MS analysis of T. forsythia protease

Silver-stained proteins migrating at approximately 26 kDa and 54 kDa on 4.0–20.0% Tris-glycine gel was excised and submitted for protein sequencing by mass spectrometry (Mass Spectrometry Lab, Center for Functional Genomics, University at Albany, Rensselaer, NY). Silver-stained gel slice containing the protein of interest was subjected to alkylation of cysteines followed by in-gel tryptic digestion. The digested mixture was analyzed using a QSTAR XL (ABSCIEX, FraminghamMA) mass spectrometer equipped with the CapLC system (Waters Co. Milford, MA, USA). The amino acid sequence data generated in this study was searched against all gene and protein databases for matches using Mascot (Matrix Science, United Kingdom).

2.4. Protease enzyme assay using highly fluorescein-labeled gelatin and collagen

Highly fluorescein-labeled gelatin and collagen was purchased from Molecular Probes. Protease assays were performed at 20 °C using 1–5 nM gelatin (MW 100 kDa) or 0.1–10 nM collagen (MW 100 kDa), 30 μL enzyme, and reaction buffer (50 mM Tris-HCL, 150 mM NaCl, 5 mM $CaCl_2$, 2 mM sodium azide, pH 7.6) to 200 μL total volume. Background fluorescence was measured using gelatin or collagen in reaction buffer. Fluorescence was measured using a Perkin Elmer Luminescence Spectrometer LS50B equipped with a well plate reader. Samples were excited at 495 nm and emission recorded at 515 nm. Reported relative fluorescence was determined by subtracting the background fluorescence from fluorescence recorded from enzyme containing wells. All enzyme assays were run in triplicate.

2.5. Protease enzyme assay utilizing native collagen

Collagen type I from rat tail (Sigma-Aldrich) was solubilized in 10 mM acetic acid at a concentration of 2 μg/μL. Protease assays were performed at 20 °C and contained 10 μL of the collagen stock solution, 20 μL of assay buffer (40 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35 solution with a pH of 7.5), and 20 μL of the purified enzyme. The control samples did not contain enzyme, but had an additional 20 μL enzyme buffer (50 mM Tris-HCl pH 7.8 with 1% triton X-100). Assay mixtures were incubated at 23 °C for lengths of time ranging from 6 to 24 h. After the set incubation period, 6 μL of 10× sample buffer (0.4 M Tris HCl pH 6.8, 20% w/v SDS, 40% v/v glycerol, 0.1% w/v bromophenol blue) and 6 μL of β-mercaptoethanol were added to quench the reaction and prepare the samples for electrophoresis. Both the control and experimental samples were incubated and quenched at the same time. The SDS-PAGE assay was performed using 10% Tris-Tricine gels (Jules Biotechnologies, Inc). Quantitative analysis of collagen I degradation was accomplished by computerized optical densitometry of the Tris-Tricine gels utilizing GelDoc-It^2 imaging system (UVP Bioimaging Systems Upland, CA).

2.6. Inhibition of isolated T. forsythia collagenase

Enzyme inhibitors were purchased from Sigma Chemical. Enzyme activity was assessed in the presence of the following enzyme protease inhibitors: 100 μM antipain, 40 μM bestatin, 10 μM E-64, 1 mg/mL pepstatin, 1 mM phosphoramidon, 10 mM 1, 10-phenanthroline, and 1 mM PMSF. Background fluorescence was determined for each inhibitor by adding 3 nM gelatin or 1 nM collagen, 5 μL of inhibitor, and activation buffer to total 200 μL to each well. Experimental wells contained gelatin or collagen, 5 μL of inhibitor, 30 μL of *T. forsythia* protease, and activation buffer to a total of 200 μL. Reported relative fluorescence was determined by subtracting the background fluorescence from the experimental values. All inhibitor assays were run in triplicate.

2.7. pH and temperature assays

Highly fluorescein-labeled gelatin or type I collagen was incubated with protease in buffers ranging from pH 6.0–8.5. The following buffers were used: MES pH 6.0 and 6.5; HEPES 7.0; and Tris-HCl 7.5, 8.0, and 8.5. All buffers contained 150 mM NaCl and 5 mM $CaCl_2$. Reported relative fluorescence values shown were obtained by subtracting background fluorescence from gelatin or type I collagen alone from the fluorescence measured from incubating protease with gelatin or type I collagen. All experiments were performed in triplicate.

Protease was heated at 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, or 80 °C for 30 min then cooled to room temperature. Enzyme assays were performed as previously described. Experiments were performed in triplicate.

2.8. Cleavage of synthetic peptides

All synthetic peptides were purchased from Sigma-Aldrich. Protease cleavage of BAPNA was assessed using a modification of