World Journal of Advanced Research and Reviews, 2020, 08(03), 154–161

World Journal of Advanced Research and Reviews

e-ISSN: 2581-9615, Cross Ref DOI: 10.30574/wjarr

Journal homepage: https://www.wjarr.com

(RESEARCH ARTICLE)

Identification of MMP-like protein from *Streptococcus mitis*

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Publication history**:** Received on 04 December 2020; revised on 11 December 2020; accepted on 13 December 2020

Article DOI: https://doi.org/10.30574/wjarr.2020.8.3.0473

Abstract

Streptococcus mitis is a normal commensal of the human oral cavity and oropharynx. This microorganism is an opportunistic pathogen in immune compromised hosts and a cause of invasive diseases such as infective endocarditis. We isolated a matrix metalloprotease (MMP)-like protein of *S. mitis*. The gene for the MMP-like protein was found on the genome sequence database of *S. mitis* . The gene encodes a protein consisting of 240 amino acid residues with a conserved zinc-binding motif (HEXXHXXGXXH) among the matrix metallopeptidase family. The gene was PCR-amplified from *S. mitis* ATCC 49456 and cloned for construction of the recombinant protein. The recombinant MMP-like protein with a GST-tag was purified and the enzymatic activity was assessed. The recombinant protein showed approximately half the level of MMP-like activity as compared to human recombinant MMP-8. The MMP-like protein of *S. mitis* may be involved in the pathogenesis of infection to the dentin with collagen fibers and systemic invasive diseases.

Keywords: *Streptococcus mitis*; Matrix metalloprotease; Collagen; Zinc-binding motif

1. Introduction

Matrix metalloproteases (MMPs) are a family of proteolytic enzymes that play important roles in defending hosts against infections. They are required for leukocyte recruitment, cytokine and chemokine processing, and remodeling of the extracellular matrix. However, excess MMP activity destroys infected and inflamed tissues, and sometimes leads to host morbidity or mortality and bacterial dissemination and persistence. MMPs are classified by specificity to the substrates, which include collagenases (MMP-1, -8, -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -13) and elastases (MMP-7 and -12) [1]. Bacterial collagenases are reported to be involved in the degradation of the extracellular matrices of animal cells [2]. These enzymes are important virulence factors in a variety of pathogenic bacteria. The MMP-like activities of *Enterococcus faecalis* may contribute to deterioration of the structural integrity of the dentin [3]. In addition to direct pathogenic roles, MMP-like proteins of bacteria are reported to activate human MMPs by processing pro-MMPs and leading the host immune response toward tissue destruction [1].

Streptococcus mitis, a Gram positive viridans streptococcus, is a normal commensal in the human oral cavity. *S. mitis* is a pathogen of human dental caries [4, 5], including root caries [6]. This microorganism is reported to be the dominant bacterial species in infected root canals [7]. The pathogenicity of *S. mitis* is relatively low and rarely causes systemic diseases. However, *S. mitis* is reported to be an emerging pathogen of bloodstream infections in immune-compromised hosts such as neutropenic patients or patients undergoing cytotoxic anti-cancer chemotherapy [8], and to cause a variety of invasive diseases such as endocarditis [9].

As described above, *S. mitis* has two characteristic aspects, as a normal commensal microbiota and as a pathogen of invasive diseases and bloodstream infections. Immunoglobulin A1 protease [10] and bacteriophage lysin [11] have been

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suggested to be virulence factors of *S. mitis* . However, these molecules have not been shown to play a direct role in the pathogenesis of *S. mitis* infections. There is also a series of candidate virulence genes on the genome database of *S. mitis.* Since *S. mitis* is the closest resembling species of the major human pathogen *Streptococcus pneumoniae* [12], virulence factors homologous with those of *S. pneumoniae* have been identified on the genome [9]. In spite of the plentiful information on the genome, little is known about the virulence factors and their roles in its infection. Among the candidate virulence factors on the genome database, we selected the MMP-like protein in the current study. We identified MMP (MMP-1, -8 and -13) homologues on the genome database of *S. mitis* and examined its enzymatic activity

2. Material and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *S. mitis* ATCC 49456, *Streptococcus salivarius* ATCC 9758, *Rothia mucilaginosa* DY-18 and *E. faecalis* ATCC 19433. Each *S. mitis*, *S. salivarius* and *R. mucilaginosa* was cultured anaerobically in tryptic soy broth (Becton Dickinson and Company, NJ, USA) supplemented with 0.5% yeast extract (Becton Dickinson and Company). *E. faecalis* was cultivated in brain heart infusion broth (Merck Japan, Tokyo, Japan). The bacterial cells were collected at the early plateau phase by centrifugation and suspended in assay buffer supplied in SensoLyte® 520 Generic MMP Activity Kit *Fluorimetric* (AnaSpec, San Jose, CA, USA). The bacterial cells in the assay buffer were disrupted by sonication as described previously [13] and subjected to the MMP activity assay.

2.2. Database search

A blast homology search was performed on the *Streptococcus* genome database of the National Center for Biotechnology Information (NCBI) and the human oral microbiome database using the amino acid sequence of human MMP-1, -8 and -13 as queries. Nucleotide and amino acid sequences of the MMP-like proteins were obtained from the NCBI database and used for the sequence alignments and cloning of the gene. Sequence alignment was performed using Genetyx ver. 8 (Genetyx Co., Ltd., Tokyo, Japan).

2.3. PCR and Cloning of the gene for MMP-like protein from *S. mitis* .

Primers for polymerase chain reaction (PCR) were designed to amplify the whole length of the open reading frame (ORF) for the MMP-like protein of *S. mitis*. The nucleotide sequence of the primers containing restriction sites (*Xho*I site in forward primer and *Not*I site in reverse primer) were as follows: forward primer 5'-AATATCTCGAGATGCGCTGGATTTTTTGT-3' and reverse primer 5'-CTAGTGCGGCCGCTTACTCACTAGTCTCAT-3'. Five extra nucleotides with a random sequence were added to the 5'-end of each primer for digestion of the PCR product with the restriction enzymes. PCR was performed in a volume of 25 µl, using TaKaRa Taq™ HS Perfect Mix (Takara, Shiga, Japan) with 20 pmol of forward and reverse primers and approximately 200 ng of genomic DNA of *S. mitis* . The thermal cycling program was 35 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. The amplicon was digested with *Xho*I and *Not*I, and the digested DNA fragment was cloned into the corresponding site of the pEU-E01-GST-PS-MCS-N2 (Cell-free Sciences, Ehime, Japan) in frame with the gene encoding N-terminal glutathione S-transferase (GST).

2.4. Synthesis and purification of the recombinant MMP-like protein

Synthesis and purification of the recombinant MMP-like protein were performed by Cell-free Sciences by wheat germ cell-free translation system (Cell-free Sciences). Briefly, *in vitro* transcription and translation were performed using the CFS-TRI-1240G kit (Cell-free Sciences) and the constructed expression vector described above. Transcription was carried out at 37°C for 6 h in the transcription buffer supplied in the kit with the recombinant expression vector (100 ng/µl), SP6 RNA polymerase (1 unit/µl), RNase inhibitor (1 unit/µl) and 2.5 mM NTPs. Translation was carried out using 5.5 ml of SUB-AMIX (proprietary buffer containing all 20 amino acids) and 250 µl of the transcription products, 1 µl of creatine kinase (20 mg/ml) and 250 µl of WEPRO1240G supplied with the kit for 16 h at 17°C. Glutathione sepharose 4B gel (GE Healthcare Bio-Sciences, Rydalmere, NSW, Australia) was used for purification of the synthesized GST-tagged recombinant proteins. Sample fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Gelatin zymography

Gelatin zymography was performed for recombinant MMP-like protein of *S. mitis* using a Gelatin-zymography Kit (Cosmo Bio type) (Cosmo Bio Co., Ltd., Tokyo, Japan). Approximately 200 ng of the recombinant protein was subjected to SDS-PAGE using a gel containing 1% gelatin as the substrate. After the electrophoresis, the gel was incubated in a 2.5% Triton X-100 solution at 37°C for 1 h, then washed in 50 mM Tris-HCl buffer (pH 7.5) supplied in the kit. For the

enzyme reaction, the gel was immersed in a buffer containing 200 mM NaCl, 10mM $CaCl_2$ and 0.005% Brij-35 for 25 h. After the reaction, the gel was stained with Coomassie Brilliant Blue and destained in a solution with 30% methanol and 10% acetic acid.

2.6. MMP-like activity

The MMP-like activities of the recombinant protein and whole cell sonication samples of *S. mitis* , *S. salivarius*, *R. mucilaginosa* and *E. faecalis* were assessed using SensoLyte® 520 Generic MMP Activity Kit *Fluorimetric* (AnaSpec) according to the manufacturer's instructions. Briefly, 50 µl of the recombinant protein (80 µg/ml), MMP-8 (80 µg/ml) (recombinant human MMP-8 Life Laboratory Co., Yamagata, Japan) and each of the sonicated bacterial cells (1 mg/ml) suspended in assay buffer were mixed with 50 µl of MMP substrate solution provided in the kit. The enzyme reaction was carried out on a 96-well plate (BRAND GMBH + CO KG, Wertheim, Germany) and the fluorescence intensity was measured at 490 nm/520 nm and recorded every 5 min for 70 min using SpectraMax M5 (Molecular Devices Co., San Jose, CA, USA). The fluorescence intensity was normalized to assay buffer with the substrate and shown in relative fluorescence units (RFU). To estimate the amounts of enzymatic reaction products, the fluorescence intensities of serial diluted 5-FAM-Pro-Leu-OH (2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 and 0 µM) were measured, and a standard curve was constructed. The amounts of digested products in each reaction at the end point (70 min) were calculated from the standard curve. Each sample was measured in triplicate.

3. Results

3.1. Database search

A gene encoding MMP-like protein was identified on the genome database of *S. mitis* through the blast homology search. The ORF consisted of 699 bp (233 amino acids) and its molecular weight estimated from the deduced amino acid sequence was approximately 26.6 kDa. A few differences were seen in the sequences among the strains (data not shown). The sequence data of *S. mitis* NCTC 12261 (NZ_CP028414.1) was obtained from the NCBI genome database and was used for primer design and cloning.

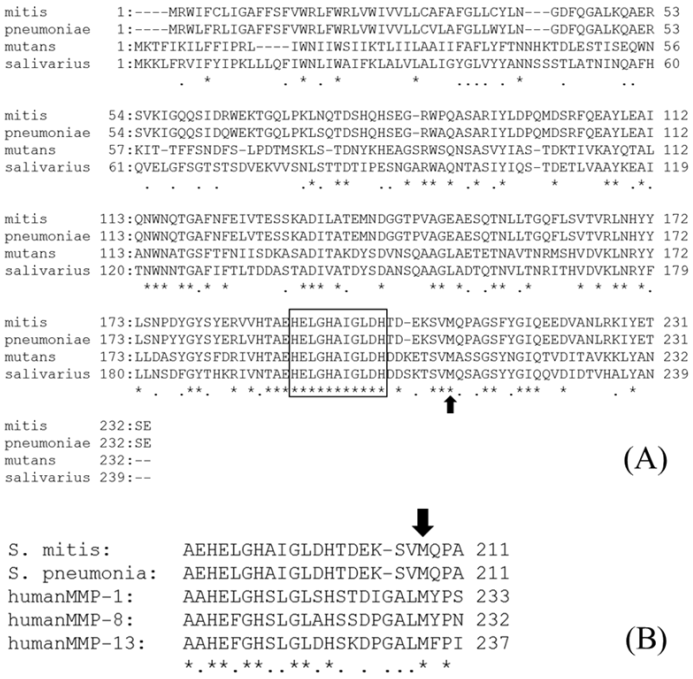

Figure1 Alignment of amino acid sequence of MMP-like protein of *S. mitis* with the corresponding sequences of other *Streptococcus* species (*S. pneumoniae*, *S. mutans* and *S. salivarius*) (A). The 3-histidine zinc-binding motif (HEXXHXXGXXH) is indicated by a box, and the methionine turn is indicated by an arrow. Asterisks indicate the conserved amino acid sequence among the homologues. The zinc-binding motif of *S. mitis* MMP-like protein is compared with those of *S. pneumoniae*, human MMP-1, -8 and -13 (B). The methionine turn near the motif is indicated by an arrow.

Homologous molecules of the MMP-like protein were widely conserved among the *Streptococcus* species. Amino acid sequences of the MMP-like protein of *S. mitis* NCTC 12261, *S. salivarius* NCTC8618 (NZ_LR134274.1), *S. mutans* NCTC10499 (NZ_LS483349.1) and *S. pneumoniae* NCTC7465 (NZ_LN831051.1) were aligned, as shown in Fig. 1A. The MMP-like protein of *S. mitis* showed the highest sequence identity with that of *S. pneumoniae* (95.7%). The sequence identities with *S. mutans* and *S. salivarius* were 36.1% and 41.6%, respectively. The conserved residues in the 3-histidine zinc-binding motif (HEXXHXXGXXH) among the matrix metallopeptidases (family M10A) were compared to those of human MMP-1, -8, -13 and *S. pneumoniae* MMP-like protein, as shown in Fig. 1B. A conserved methionine turn following the active site was also identified in the MMP-like protein of *S. mitis*.

3.2. Molecular cloning of the gene encoding MMP-like protein

The PCR amplified an approximately 700 bp DNA fragment by using genomic DNA of *S. mitis* ATCC 49456 as the template, and the fragment was cloned into the expression vector pEU-E01-GST-PS-MCS-N2 at *Xho*I-*Not*I site. The nucleotide sequence of the insert DNA was analyzed using the SP6 primer and was confirmed to be identical to the gene encoding MMP-like protein of the NCTC 12261 strain on the genome database (data not shown).

3.3. Construction of the recombinant protein

The recombinant protein of *S. mitis* MMP-like protein was successfully expressed in the wheat germ cell-free translation system (Cell-free Sciences). The recombinant protein with the GST-tag was purified by affinity chromatography, and the protein profiles of the elution sample of the chromatography are shown in Fig. 2. The purified protein with the GST tag showed the expected molecular mass of approximately 53 kDa. Approximately 29-kDa protein and multiple ladder-like bans were also seen on the gel.

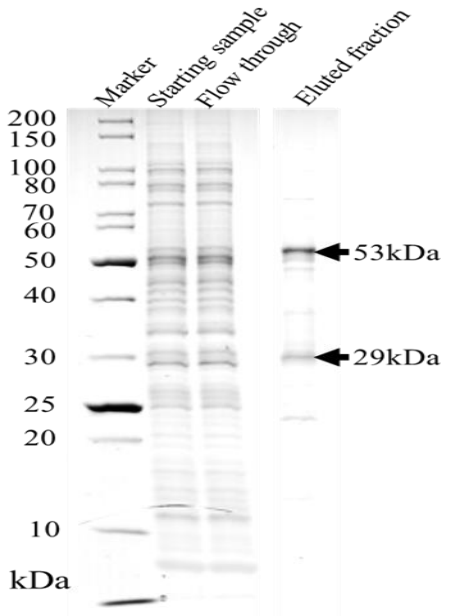

Figure 2 Protein profiles of the recombinant protein during purification. Pooled fractions of the starting sample flowing through the sample from the glutathione sepharose 4B gel and the eluted sample were analyzed by SDS-PAGE. Approximately 500 ng of purified protein (eluted sample) was loaded on the gel.

3.4. MMP-like activity and gelatin zymography

The recombinant protein and sonicated *S. mitis* cells both demonstrated generic MMP-like activity. Representative kinetics curves of generic MMP-like activity of the recombinant protein are shown with that of the activated human MMP-8 in Fig. 3A. The curves reached a plateau after reacting for 70 min. At the end-point, the fluorescence intensity resulting from the generic MMP-like activity of the recombinant protein was 2.2 times less than that of the human MMP-8. The kinetics curves of generic MMP-like activities of the sonicated bacterial cells are shown in Fig. 3B. The kinetics curves are similar among the species, and *R. mucilaginosa* demonstrated the highest MMP-like activity, with statistical significance at the end point (Mann-Whitney U test, $P < 0.05$).

At the end point (70 min), the number of moles of digested substrates was calculated from the fluorescence intensities in each reaction, as shown in Fig. 4. The generic MMP-like activity (mean ± SD) of the recombinant MMP-like protein of *S. mitis* , recombinant human MMP-8, sonicated *S. mitis* , sonicated *S. salivarius*, sonicated *R. mucilaginosa* and sonicated *E. faecalis* was 16.65 ± 3.22, 37.45 ± 5.16, 17.84 ± 2.41, 20.7 ± 3.37, 27.7 ± 5.59 and 19.14 ± 4.44 (μM/mg), respectively.

In gelatin zymography, the recombinant protein showed no gelatinase activity in the gel (data not shown).

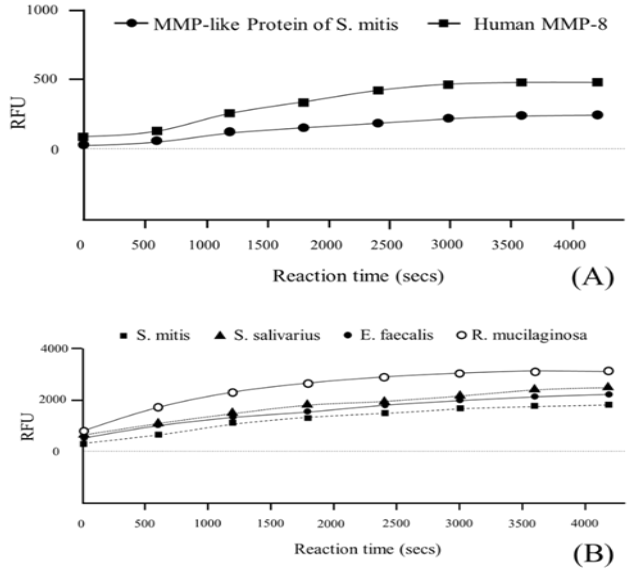

Figure 3 Kinetics of generic MMP-like activity of the recombinant MMP-like protein of *S. mitis* (A) and sonicated cells of *S. mitis* , S. salivarius, E. faecalis and R. mucilaginosa (B). RFU denotes the fluorescence intensity resulting from the degradation of the substrates and is shown after normalization with the negative control. Recombinant human MMP-8 was used as a positive control.

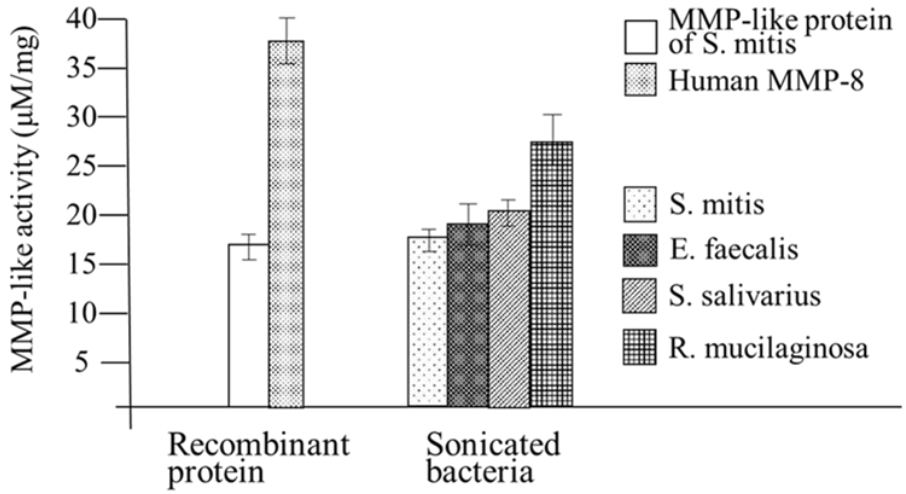

Figure 4 Generic MMP-like activity of the recombinant proteins and sonicated bacterial cells at the end point of the reaction (70 min). The number of moles of the digested substrates was calculated in each reaction and is shown as the enzymatic activity. Each sample was measured twice in triplicate, and the data are shown as mean ± SD. Significant differences (*) are seen between the recombinant human MMP-8 and the recombinant MMP-like protein of *S. mitis* . Significant differences are also seen between the sonicated R. mucilaginosa and each of the other bacterial species (Mann-Whitney U test, $P < 0.05$).

4. Discussion

S. mitis is a very interesting bacterium, walking the line between a normal commensal and a pathogen [9]. However, very few virulence factors have been identified and characterized. Bacterial proteases are major virulence factors in various infectious diseases [14, 15]. They promote the adhesion of bacteria to host tissues and invasion into the tight junction barrier of epithelial cells. Bacterial proteases inhibit the activation and transmigration of various immune cells to evade opsono-phagocytosis and intracellular bacterial killing. They also block the pathways of complement activation. *S. mitis* is closely related to dentin infectious diseases, such as root caries and endodontic infections. Since type 1 collagen is the major protein in dentin, MMP-like protein was suggested to be a candidate virulence factor of *S. mitis* . Collagen-binding protein (Cnm) of *S. mutans,* a potent pathogen of dental caries, has been suggested to be a virulence factor in dental caries and systemic diseases such as endocarditis and IgA nephropathy [16]. We hypothesized that MMP, as a collagen-associated molecule, is a potential virulence factor for both dentin infectious diseases and systemic diseases. As the first step of the investigation, MMP-like protein was identified in *S. mitis* .

Amino acid sequences of MMP-1, -8 and -13 with enzymatic activity for type 1 collagen were used as queries in a blast homology search. The MMP-like protein was identified on the genome of *S. mitis* , and was widely conserved among *Streptococcus* species. The blast search of the human oral microbiome database revealed that homologous proteins were also conserved in *Lactobacillus* species (data not shown). *Streptococcus* and *Lactobacillus* species are both pathogens of dental caries, and therefore, the MMP-like protein may be associated with the pathogenesis of dental caries. Among the *Streptococcus* species, the MMP-like protein of *S. mitis* showed a higher sequence identity with the homologue of *S. pneumoniae* than those of *S. mutans* or *S. salivarius*. The sequence identity was 95.7% for *S. pneumoniae*, and 36.1% and 41.6% for *S. mutans* and *S. salivarius*, respectively. It has been suggested that *S. mitis* evolved from a major human pathogen *S. pneumonia* [17]. Although a series of virulence factors have been lost through evolution, some are still conserved among the *S. mitis* strains. Comparative genome analysis suggests that zinc-metalloproteases are conserved virulence factors [12]. The MMP-like protein of *S. mitis* may show similar enzymatic properties to that of *S. pneumoniae,* and the enzymatic properties may be different from those of *S. mutans* and *S. salivarius*. Further elucidation is required for the characterization of each enzyme.

Alignment of the amino acid sequence of the MMP-like protein identified a 3-histidine zinc-binding motif (HEXXHXXGXXH) on the sequence of *S. mitis* . In addition, a methionine turn, which contributes to enzyme structural integrity [18], was also identified following the active site. These structural characteristics strongly suggest that the MMP-like protein of *S. mitis* might show similar enzymatic properties to those of the MMP family (family M10A).

To investigate the enzymatic properties of the MMP-like protein, the gene was cloned, and the recombinant protein was constructed. The MMP-like protein was designed to fuse to GST and was purified by affinity chromatography. Since the molecular weight of GST was approximately 26 kDa, the purified recombinant fusion protein was expected to have a molecular mass of 53 kDa. The protein profile of the purified recombinant protein included the 53-kDa protein as the most intensive band. This result suggests that the recombinant protein was successfully expressed by the wheat germ cell-free translation system and was purified through affinity chromatography. In addition to the major protein band at the expected size, multiple ladder-like protein bands were seen on the gel. Karim *et al.* reported the construction of the recombinant MMP-like protein of *Tannerella forsythia* and its enzymatic properties [19]. The autoprocessing of the GST-tagged recombinant protein of *T. forsythia* has been reported. Multiple bands other than 53-kDa protein were probably the products of autoprocessing.

As the first step in characterizing the MMP-like protein of *S. mitis* , the generic MMP activity was assessed. The recombinant MMP-like protein demonstrated approximately half the level of MMP-like activity as compared to that of human recombinant MMP-8. The result may suggest a similar enzymatic reaction between the human MMP and the MMP-like protein of *S. mitis* and an ability to degrade human tissue. It has been reported that the recombinant MMP-like protein of *T. forsythia* shows enzymatic activity under a GST-tagged condition, and shows higher activity after autoprocessing [19]. Removing the GST tag may enhance the MMP-like activity of the recombinant protein of *S. mitis* . On the other hand, the MMP-like protein of *S. mitis* did not demonstrate gelatinase activity. Human MMP-1 and -13 are collagenase enzymes and also have the ability to degrade gelatin slowly [20]. The MMP-like protein of *S. mitis* may have different properties in this regard. The substrate information of the generic MMP activity kit used in this study is not open, and the substrate specificity of the *S. mitis* MMP-like protein requires further elucidation.

Each of the sonic extracts of the bacterial cells used in this study demonstrated relatively strong MMP-like activity. The MMP-like activities of intact cells of *E. faecalis* and *Micrococcus luteus* are reported to be at least 50 times more than those reported for dentinal and salivary MMPs [3]. The sonic extracts of *S. mitis* demonstrated a similar level of MMP-like activity as compared to *E. faecalis*. Bacterial MMP-like proteins and other proteases, especially those in dental caries

associated bacteria, may contribute more significantly to dentin degradation than human MMPs in dentin or saliva. Among the bacterial species tested in this study, *R. mucilaginosa* showed the highest MMP-like activity. The microorganism has been reported as a pathogen of persistent periapical periodontitis [21], and as an emerging opportunistic pathogen of serious systemic infections [22]. MMP-like proteins in *R. mucilaginosa* may play significant roles in the infection.

Streptococcus, *Enterococcus* and *Rothia* species associated with dentin infectious diseases possess MMP-like activity. They also have an aspect of opportunistic pathogens that cause systemic invasive diseases and blood stream infections in compromised hosts. MMP-like proteins in these microorganisms are potent virulence factors in diseases. Characterization of the MMP-like proteins will facilitate studies elucidating the pathogenesis of both dentin and systemic infectious diseases.

5. Conclusion

We isolated MMP-like protein from *S. mitis* and constructed a recombinant protein, which demonstrated generic MMP-like activity. The MMP-like protein seems to play pathogenic roles in the degradation of the host tissue.

Compliance with ethical standards

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research (Grant number: 18K09613) from the Japan Society for the Promotion of Science.

Disclosure of conflict of interest

The authors have no conflicts of interest to declare

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