Proteases, actinidin, papain and trypsin reduce oral biofilm on the tongue in elderly subjects and in vitro

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ABSTRACT

Objective: Dental plaque is a causative factor for oral disease and a potential reservoir for respiratory infection in the elderly. Therefore, there is a critical need for the development of effective methods to remove oral biofilm. The objective of this study was to investigate the effect of proteases on oral biofilm formation and removal.

Design: The in vivo effect of actinidin, a cysteine protease, on the removal of tongue coating was assessed after orally taking a protease tablet. Effects of the proteases trypsin, papain and actinidin on Actinomyces monoculture and multispecies biofilm that was reconstructed using a plaque sample from the tongue coating were investigated using the microtiter plate method. Antimicrobial tests and limited proteolysis of fimbral shaft protein were also performed to clarify underlying mechanisms of oral biofilm removal.

Results: Tablets containing actinidin removed tongue coating in elderly subjects. Oral Actinomyces biofilm was significantly reduced by the proteases papain, actinidin and trypsin. Papain and trypsin effectively digested the major fimbral proteins, FimP and FimA, from Actinomyces. Actinidin, papain and trypsin reduced multispecies biofilm that was reconstructed in vitro. Papain and trypsin inhibited formation of multispecies biofilm in vitro.

Conclusions: This study shows that proteases reduced oral biofilm in vivo in elderly subjects and in vitro, and suggests that protease digests fimbriae and inhibits biofilm formation.

1. Introduction

The accumulation of oral plaque biofilms is thought to induce various localized and systemic infectious diseases such as periodontal disease, dental caries and infectious endocarditis (Kumar, 2013). Many types of natural or synthetic agents have been tested to prevent formation of oral biofilm (Rahmani-Badi, Sepehr, & Babaie-Naiej, 2015; Tada et al., 2016; Nakano, Shimizu, Wakabayashi, Yamauchi, & Abe, 2016; Howlin et al., 2015). The tongue dorsum especially retains a large amount of biofilm in the oral cavity as a tongue coating, which is thought to be associated with oral halitosis (Tonzetich & Ng, 1976; Tonzetich, 1977; Miyazaki, Sakao, & Katoh, 1995; Bollen & Beikler, 2012), periodontal disease (De Geest, Laleman, Teughels, Dekeyser, & Quirynen, 2000; Yaegaki & Sanada, 1992; Van Tornout, Dadamio, Coucke, & Quirynen, 2013), opportunistic infection (Peralis, de Souza Bonfim-Mendonca, Negri, Jarros, & Svidzinski, 2016), and aspiration pneumonia (Takeshita et al., 2010). Especially in edentate elderly, tongue coating has been identified as a risk indicator for aspiration pneumonia. The relative risk of developing pneumonia in the good tongue hygiene group compared with the poor tongue hygiene group was 0.12 (Abe, Ishihara, Adachi, & Okuda, 2008). Although formation of the tongue coating is a normal phenomenon that is observed even in healthy individuals, accumulation of a tongue coating is affected by various factors such as oral hygiene habitats, smoking, presence of a denture, periodontal status, and dietary habits (Van Tornout et al., 2013). It has also been reported that an increase in salivary viscosity and a decrease in salivary flow accelerates formation of the tongue coating, suggesting that the saliva also plays an important role in tongue coating accumulation (Ueno, Takeuchi, Takehara, & Kawaguchi, 2014; Suzuki et al., 2016). Although mechanical scraping is the main treatment for a tongue coating (Blom, Slot, Quirynen, & Van der Weijden, 2012; Slot, De Geest, van der Weijden, & Quirynen, 2015; Erovec Ademovski et al., 2012), scraping sometimes wounds the taste buds and induces the gag reflex (Rowley, Schuchman, Tishk, & Carlson, 1987; Christensen, 1998). Therefore, chemical and biochemical removal methods have been studied.

Oral biofilm formation starts with an initial colonizer that adheres to oral tissue and dental surfaces. This process involves multiple factors such as 36 fimbriae and fimbriae receptors on host cells, and extracellular polymeric substances (EPSs). The initial colonizer, Actinomyces...
supplemental tablets during the interval. Experimental details are shown in the between test and control treatment, and the subjects did not take any was repeated. All subjects were studied twice with a 1-week interval time was approximately 10 min, and both of the tablets were of the approximately 5 min later, they placed the second tablet there. The total Patients placed one of the tablets on their tongue dorsum, and ap- proximately 10 min, and both of the tablets were of the same type. We then reassessed the amount of tongue coating. One week later, the subjects were allocated to the alternate group and the protocol was repeated. All subjects were studied twice with a 1-week interval between test and control treatment, and the subjects did not take any tablets during the interval. Experimental details are shown in the Supplemental file (Fig. S1).

2.2. Tongue coating assessment

The tongue coating was assessed and compared using the Tongue Coating Index (TCI), according to previous studies (Shimizu, Ueda, & Sakurai, 2007). Briefly, each tongue dorsum surface was di- vided into nine equal areas from the circumvallate papillae to the tip, and the tongue coating was assessed in each of these areas as follows: 0, no coating; 1, light coating; and 2, heavy coating (Fig. S2). There was no significant difference in the TCI value among nine sections of the tongue (data not shown). The TCI was calculated based on all nine areas, and a higher TCI score indicated a greater amount of tongue coating.

2.3. Reagents

Because purified actinidin was unavailable, we used a freeze-dried extract from kiwi fruit, which was supplied as a tablet formulation and provided by Ezaki Glico. The kiwi fruit extract is thought to contain over 50% actinidin out of the total soluble protein component. The pH of the extracted supernatant (A1), which represented the equivalent activity of approximately 1.8 mg/mL papain (Fig. S4), was adjusted to 6.5 by adding a small amount of 1 M NaOH. Purified papain from carica papaya and trypsin from porcine pancreas were purchased from Wako Pure Chemical Industries (Osaka, Japan).

L-Pyroglutamylation-p-phenylalanyl-l-leucine p-nitroanilide (PFLNA) was purchased from Peptide Institute (Osaka, Japan). E-64 (a cysteine protease inhibitor) was from Merck Millipore Corporation (Darmstadt, Deutschland). All other chemicals were obtained from commercial sources and were of the highest available purity.

2.4. Bacterial strains and growth conditions

Bacteria and plasmids used in this study are listed in Table 2. Actinomyces oris and Escherichia coli were cultured at 37 °C in heart infusion broth (HIB) and Luria-Bertani (LB) broth, respectively. Unless otherwise specified, ampicillin was used at a concentration of 100 μg/mL.

2.5. Effect of protease on monospecies and multispecies biofilm

Monospecies biofilm was constructed using the A. oris strain MG-1. Strain MG-1 was grown overnight in HIB with shaking, and then diluted to a final optical density of 1.0 at 600 nm (OD600) using fresh HIB with 1% sucrose. Diluted culture (1 mL) was added to each well of a 24-well polystyrene microtitre plate (Nunc). Plates were incubated at 37 °C without shaking for 18 h. The plates were washed once with 200 μL of dH2O to remove planktonic cells. Two hundred microliters of ten-fold protease solution serial dilutions (10, 1, 10−1, 10−2, 10−3 and 10−4 113 mg/mL papain and trypsin) or A1 with or without 10 μg/mL E-64 in 0.1 M phosphate buffer (pH 6.5) with 0.5 M sucrose was added. After 10 or 30 min incubation at 37 °C without shaking, the plates were carefully washed once with 200 μL of dH2O to remove peeled biofilm and the attached protease-treated biofilms were not removed. Retained biofilm was stabilized with 200 μL of 2.5% glutaraldehyde for 5 min, stained with 250 μL of 0.5% crystal violet for 5 min, and then washed three times with 10% ethanol to remove unbound excess dye. The crystal violet was solubilized by adding 200 μL 95% ethanol and mixing using a shaker. After 5 min, 50 μL aliquot was transferred to each well of a 96-well polystyrene microtitre plate (Nunc) and the amount of residual biofilm was determined by measuring the absorbance at 590 nm.

Multispecies biofilm was constructed using samples collected from the tongue dorsum. All individuals gave written informed consent and the study protocol was approved by the Osaka Dental University Ethics Committee (approval no. 1101084). The tongue coating was incubated overnight in HIB without shaking and was resuspended at a final OD600 of 0.1 in fresh HIB with 1% sucrose. A 1-mL aliquot of the sample was added to each well of a 24-well polystyrene microtitre plate and incubated at 37 °C without shaking for 2 weeks, with 1 mL fresh media exchanged every other day. After incubation, the plates were
washed once with 200 μL of dH2O to remove planktonic cells. The bacterial flora of multispecies biofilm was evaluated using 16S rRNA gene sequencing on the Illumina MiSeq platform, and six genera (Streptococcus, Veillonella, Lactobacillus, Prevotella, Peptostreptococcus and Haemophilus) were detected more than 2% each and Actinomyces was detected at 0.4% (data not shown). Protease solutions (200 μL) at various concentration in 0.1 M phosphate buffer (pH 6.5) and 0.5 M sucrose were added and incubated at 37 °C without shaking. After 30 min of incubation, the plates were washed twice with 200 μL of dH2O to remove peeled biofilm. Bacteria retained in the residual biofilm were measured using the same method as above.

2.6. Antimicrobial assay

Strain MG-1 was grown overnight in HIB with shaking, and then pelleted by centrifugation. The cell pellet was suspended in HIB containing 1% sucrose to a final OD600 of 0.5. A 0.4-ml aliquot of the diluted culture was mixed with 50 μg/ml papain or trypsin in 0.1 M phosphate buffer (pH 6.5) containing 0.5 M sucrose, and incubated at 37 °C with shaking for 60 min. A 10 μl aliquot of the incubating solutions was temporally fractionated, serially diluted and spotted on Brucella agar plates. The number of colony forming units (CFU/mL) was determined and compared.

2.7. Overexpression and purification of FimP and FimA

A total DNA sample was extracted from an overnight culture of strain MG-1 using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer’s protocol. The 5’ expression vector pQE80L (Qiagen, Hilden Germany) was used for overproduction of the FimP and FimA proteins that were fused to the His6 tag at their N terminus. The fimP and fimA genes were PCR-amplified using the primers PFP1 plus PFP2 and PFA1 plus PFA2, respectively (Table 2). The linearized vectors of pQE80L were obtained by PCR using primers PFP3 plus PFP4 and PFA3 plus PFA4 (Table 2). The amplified fimP and fimA fragments were fused to the linearized vectors using In-Fusion HD cloning kit (TaKaRa, Tokyo, Japan) to obtain pNMP1 and pNMA1, respectively (Table 3). Escherichia coli DH5α cells harboring one of the His-tagged fusion plasmids were grown at 37 °C in LB medium containing 100 μg/ml ampicillin (Table 3). When the cell growth reached the mid-exponential phase, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a concentration of 1 mM and incubation was continued for a further 5 h. The cell growth reached the mid-exponential phase, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a concentration of 1 mM and incubation was continued for a further 5 h. The culture was diluted to a final OD600 of 0.1 using fresh HIB plus 1% sucrose. Papain and trypsin were added to final concentrations of 10, 1, 10–1, 10–2 and 10–3 mg/mL, and actinidin was also added to final concentration of 1–10–1477.0-fold serial dilutions of A1. A 1-ml aliquot of the diluted culture was added to each well of a 24-well polyurethane microtiter plate (Nunc). Plates were incubated at 37 °C without shaking for 18 h. After incubation, the plates were washed once with 200 μL of dH2O to remove planktonic cells. Bacteria retained in the residual biofilm were stabilized with 200 μL of 2.5% glutaraldehyde for 5 min, stained with 250 μL of 0.5% crystal violet for 5 min, and then washed three additional times with 10% ethanol to remove unbound excess dye. The crystal violet was solubilized by additional of 200 μL of 95% ethanol using a shaker. After 5 min, a 50-μl aliquot was transferred to each well of 96-well polyurethane microtiter plate and the amount of residual biofilm was determined by measuring the absorbance at 590 nm.

2.10. Statistical analysis

All experiments were performed in duplicate or triplicate for each condition and was repeated at least three times. All the statistical analyses were performed using the IBM SPSS statistical software package (version 21.0). The pre-specified research objective was to determine whether taking test tablets removed the tongue coating in healthy young adults and in elderly adults who required daily nursing care. The pre-specified outcome for the study was change in tongue coating after taking the test tablet compared to the change after placebo. The mean of baseline values were compared between both treatments for each age group using the Kruskal-Wallis test and compared between both age groups using the Wilcoxon signed-rank tests and paired Student’s tests. Intra-group comparisons (before and after treatment) were performed using Friedman test. Inter-group comparisons (change ratios by test vs. change ratios by placebo treatment) were performed using the Wilcoxon signed-rank test. The level for statistical significance was p < 0.05 for all analyses.

3. Results

3.1. Effect of actinidin in a tablet on tongue coating removal

To evaluate the effects of actinidin on tongue coating removal, we conducted a double-blind, crossover, placebo-controlled study involving 20 healthy young adults and 20 elderly adults receiving nursing care. There was no significant difference in the baseline value of the TCI between the test and placebo in each of the age groups (p > 0.05). The average TCI value in the young subjects was significantly lower than that in the elderly subject (p < 0.01; Table 1 and Fig. 1), which is consistent with previous reports (Ralph, 1987). Although TCI values decreased after taking the tablets in both age groups, there was a greater decrease in the TCI for subjects taking the test tablets containing kiwi fruit extract, which contains substantial amounts of the cysteine protease actinidin, compared with taking placebo in both young and elderly subjects. There was a statistically significant difference in the TCI in the elderly group but not in the young group compared to the baseline value. These results suggest that actinidin in tablet formulations facilitates the removal of the tongue coating in this experimental condition.

3.2. In vitro biofilm removal by papain, trypsin and actinidin

We further investigated the in vitro effects of proteases on oral bacterial biofilm to elucidate the mechanism of tongue coating detachment. To clarify the ability of proteases to detach biofilms, we used a mono-species biofilm formed by A. oris, which is an important initial oral biofilm colonizer, and purified two types of proteases: the cysteine protease, papain, and the well-characterized serine protease, trypsin. Concentration- and time-dependent biofilm detachment was observed...
in wells with papain added (Fig. 2). Papain reduced *Actinomyces* biofilms almost completely at 10 mg/mL with a 10-min incubation and at 1 mg/mL with a 30-min incubation. Trypsin can also reduce biofilms, but we did not observe a clear correlation in wells containing trypsin, as there was for papain. In this experimental condition, papain seems to have larger effects on removing the mono-species *Actinomyces* biofilm than trypsin. Because purified actinidin was not available, we investigated detachment of biofilms using a water-soluble fraction of kiwi fruit extract containing actinidin. After a 30-min incubation, kiwi fruit extract removed biofilm in a concentration-dependent manner, which was similar to papain. Because addition of the cysteine protease-specific inhibitor E-64 fully suppressed the effect of kiwi fruit extract, actinidin in kiwi fruit extract may be involved in removal of *Actinomyces* biofilm.

### 3.3. No antimicrobial effects of proteases on *Actinomyces*

To investigate the mechanism of *Actinomyces* biofilm detachment by proteases, we performed two different experiments. First, we...
investigated whether proteases cause potentially lethal damage to Actinomyces cells. Actinomyces cells were incubated with a final concentration of 10 mg/mL papain or trypsin for 60 min. Papain or trypsin treatments showed no decrease in the amount of viable Actinomyces cells compared to control (Fig. S3). This result suggest that papain and trypsin have no antibacterial effect on A. oris.

3.4. Limited proteolysis of purified FimP and FimA

We also investigated whether papain and trypsin digest main fimbrial subunits in Actinomyces, which are known to be essential for biofilm formation. To study the protease sensitivity of the main type 1 and 2 subunits FimP and FimA, we performed limited proteolysis of these overexpressed and purified proteins using the proteases papain and trypsin. The time-course of degradation, as monitored by SDS-PAGE, is shown in Fig. 3.

For both papain and trypsin, FimP and FimA were digested in a time-dependent manner. Although band patterns of limited proteolyzed products were different for each protease, intact FimP and intact FimA bands rapidly decreased when incubated with low enzyme concentrations (17.5 μg/mL; Fig. 3). Additionally, these limited proteolyzed products completely disappeared within 15 min when using proteases at a high concentration (0.1 mg/mL, data not shown).

3.5. Effects of proteases on an in vitro oral biofilm model

As shown above, the proteases used in this study disrupted the monospecies biofilm composed of Actinomyces. We next investigated whether these proteases can remove an oral multispecies biofilm made with plaque taken from the tongue dorsum. After application of the enzyme for 60 min, all proteases (actinidin, papain and trypsin) fully disrupted oral multispecies 256 biofilms at a high concentration, similar to the monospecies biofilm (Fig. 4). Although the actinidin’s unit was very mild compared to papain (Fig. S4), it reduced biofilms. At lower concentrations, trypsin retained some removal activity. Because actinidin we used was unpuriﬁed product, we measured this unit test.

We also examined the inhibitory effects of the proteases on biofilm formation. All proteases used in this study prevented formation of multispecies oral biofilm in a concentration-dependent manner (Fig. 5). Papain and trypsin almost completely inhibited biofilm formation at 10 mg/mL, and actinidin also sufﬁciently inhibited biofilm formation at the highest concentration. Similar experimental results were obtained using a monospecies biofilm made from Actinomyces (data not shown). These data indicate that these proteases have the ability to inhibit formation of oral biofilm.

4. Discussion

The aim of this study was to demonstrate that proteases can reduce or remove tongue coating and an in vitro oral biofilm. We found that actinidin signiﬁcantly removed tongue coating in elderly subjects, and trypsin, papain and actinidin reduced monospecies and multispecies biofilm in vitro. To date, much research has been performed on removal of the tongue coating and oral malodor to prevent various infectious diseases. Because the dorsal tongue surface possesses a large number of papillae, mechanical tongue cleaning with tongue brushes is troublesome, and chemical and biochemical methods for removal of the tongue coating are desired. Early studies showed the effectiveness of disinfectant treatment for the oral cavity, e.g. chlorhexidine (Winkel, Roldán, Van Winkelhoff, Herrera, & Sanz, 2003), cetylpyridinium chloride and zinc (Codipilly, Kaufman, & Kleinberg, 2004). Although the treatment was effective for reducing oral malodor, there was no statistically signiﬁcant difference in the TCI score. Additionally, tongue coating removal by gargling with chemical compounds is thought to be difﬁcult (Blom et al., 2012).

Oral biofilms are three-dimensional structured bacterial
creased oral malodor, and Ford (2008) demonstrated that pineapple juice removed tongue coating. Kiwi and pineapple possess a large number of cysteine proteases, actinidin and bromelain. In this study, we showed that the actinidin significantly reduced the tongue coating in elderly people. However, the effect of actinidin was not markedly greater than that of physically rubbing a tablet, and for young people, physical rubbing of a tablet may be more effective than proteases. Although actinidin in the tablet did not show remarkable effects, the TCI scores in this study were obtained using only a single intake of two tablets. Therefore, eating actinidin tablets frequently may lead to more effective reduction of the tongue coating. Moreover, because the salivary flow rate is known to be related to age (Ship, Pillemer, & Baum, 2002; Fox, van der Ven, Sonies, Weiβbach, & Baum, 1985; Osterberg, Birkhed, Johansson, & Svanberg, 1992), the difference in the concentration and retention time of proteases between the two age groups might affect the TCI results.

Carica papaya extract, which contains benzyl-isothiocyanate, papain and calpain (Adebiyi, Ganesan Adaikan, & Prasad, 2003; Nakamura et al., 2007; Da Silva et al., 2010; Julianti, Oufr, & Hamburger, 2014), was reported to have disinfectant activity (Emeruwa, 1982; Dawkins, Hewitt, Wint, Obiefuna, & Wint, 2003). Brock, Arzabe, Piñeiro, and Olivito, 1977 showed the killing effect of trypsin. Conversely, neither papain nor trypsin showed any significant antimicrobial effect under our experimental conditions. Using mouthwash with intensive antimicrobial activity may lead to the perturbation of the oral flora in the oral cavity and may increase the chance of infection by opportunistic pathogens. Therefore, our results suggest that protease treatment using the appropriate concentration and reaction time can be used as an agent in routine clinical use. Although in vitro biofilm removal required a relatively high concentration of proteases, there are synergistic effects between the flow of saliva and mechanical rubbing of tablets in the oral environment. Determination of the enzyme, concentration and reaction time requires further analysis.

In this study, we showed the inhibitory effect of the proteases on oral biofilm formation. To prevent infection, safe and natural inhibitory agents with an inhibitory effect on biofilm formation and without an antimicrobial effect have been desired and developed. Therefore, these proteases may provide a preventive effect against oral plaque formation for routine oral care in healthy and elderly people. A more detailed analysis is necessary to evaluate these proteases for prevention of oral plaque accumulation.

5. Conclusion

This study provides direct evidence that actinidin, a cysteine protease, reduces the tongue coating in elderly subjects. Oral Actinomyces biofilm and reconstructed biofilm taken from a tongue coating were significantly reduced by the proteases papain, actinidin and trypsin in
vitro. Additional results suggest that proteases digest fibrin to disrupt the biofilm structure and also inhibit biofilm formation.

Conflict of interest statement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2017.04.035.

References


