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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 andremoval.
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 monospecies biofilm 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 fimbrial shaft proteins 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 fimbrial 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 (Pieralisi, 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; Erovic 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

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species (Li et al., 2004; Nyvad & Fejerskov, 1987; Kilian, Larsen, Fejerskov, & Thylstrup, 1979), has two types of adhesive fimbriae that are essential for adherence to solid surfaces (Neeser, Chambaz, Del Vedovo, Prigent, & Guggenheim, 1988). Attached bacteria then develop into higher order structures with cell-to-cell communication, and they produce EPSs to accelerate the physical strength of the biofilm (Flemming & Wingender, 2010). EPSs are mostly composed of polysaccharides, DNA, lipid and proteins (Flemming & Wingender, 2010).

Agents that degrade and inhibit oral biofilm have been developed, and several types of antibiofilm agents and enzymes targeting the biofilm extracellular matrix have been reported. Many of them target polysaccharides (Singh, Parsek, Greenberg, & Welsh, 2002; Di Bonaventura et al., 2012), but recently DNA-targeting agents have been studied (Cavaliere, Ball, Turnbull, & Whitchurch, 2014; Rice et al., 2007; Fredheim et al., 2009). Additionally, the effect of proteases on removing biofilm has been studied (Gilan & Sivan, 2013; Meyle et al., 2010; Niazi et al., 2014, 2015). Trypsin, a serine protease, has been intensively studied because of its detachment effect (Niazi et al., 2014, 2015; Chaignon et al., 2007; Harris, Nigam, Sawyer, Mack, & Pritchard, 2013). Nohno, Yamaga, Kaneko, and Miyazaki, 2012 showed that tablets containing actinidin, a cysteine protease from kiwi fruit, reduces oral malodor. However, the underlying mechanisms of the proteases' oral biofilm removal effects are unclear. In this study, we assessed the reduction and antibiofilm activity of several proteases *in vivo* and *in vitro*.

2. Materials and methods

2.1. Subjects and study design

There were 20 healthy young adults (9 males, 11 females; mean age, 30.8 ± 1.69 years; age range, 18–41 years) and 20 elderly adults who were receiving daily 58 nursing care (8 males and 12 females; mean age, 71.3 ± 3.40 years; age range 63–88 years) who were enrolled into this study. Informed consent was obtained from each participant before enrolment. The study protocol was approved by the Ethics Committees of Osaka Dental University (#110821). This study was a double-blind, placebo-controlled cross-over trial with a 1-week washout period between the crossover phases. The subject flow from the initial screening to final analysis is shown in Fig. S1. All subjects were requested not to eat, drink, smoke, rinse or perform oral hygiene for at least 2 h before their appointment. In this experiment, we used two types of tablets: one with actinidin (a cysteine protease from kiwi fruits; test), and the other with no actinidin (placebo; Table S1). They were identical in size and shape, and were prepared by Ezaki Glico (Osaka, Japan). The actinidin tablets were made from freeze-dried extract of kiwi fruit was added, and they were molded without boiling during the process. After assessment of the tongue coating by taking photos of the tongue dorsum (see below) using a digital camera (EXILIM HS EX-ZR20, Casio Computer, Tokyo, Japan) at the baseline of first test phase, the subjects were randomly divided into two groups. In each crossover phase, each group was instructed to take two tablets of either test or placebo. Patients placed one of the tablets on their tongue dorsum, and approximately 5 min later, they placed the second tablet there. The total time was approximately 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 divided 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-Pyroglutamyl-L-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. 110864). 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 microtiter 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