Inhibition of *Streptococcus mutans* Biofilm by LL-37

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Abstract

Streptococcus mutans is a primary agent of human dental caries. One of its important virulence properties is the ability to mediate the adherence to tooth surfaces. LL-37 (cationic antimicrobial peptide) acts as a component of innate immunity, and is the only member of the cathelicidin family found in humans. In this study, we investigated the antimicrobial activity of LL-37 against S. mutans. LL-37 inhibitedthe biofilm formation while did not the growth. LL-37 can interact with streptococcal lipoteichoic acid (LTA). This finding suggests that LL-37 interacts with streptococcal LTAto inhibit biofilm formation.

KEYWORDS:*Streptococcus mutans*, LL-37, biofilm, streptococcal LTA

Streptococcus mutans, Gram-positive bacteria, is a primary agent of human dental caries. One of the most well documented characteristics of the virulence of *S. mutans* is its ability to produce glucosyltransferases, which synthesize intracellular and extracellular polysaccharides (EPS). The EPS, especially water-insoluble glucans, mediate the adherence of *S. mutans* to tooth surfaces, contributing to the formation of dental plaque biofilms [10]. Thus, microbial biofilms anchored to oral surfaces and protected by a self-produced polymeric matrix.The lipoteichoic acid (LTA) besides peptidoglycan is an important component of the cell surface of Gram-positive bacteria [12]. Biofilm formations can resistance against antibiotics and oxygen, resulting in failure the prevention or treatment on oral diseases.

Cationic antimicrobial peptides (AMPs) represent the first line of defense against many invading pathogens [9]. The only member of the cathelidicin family found to date in humans is hCAP18/LL-37, an 18 kDa peptide [1] with 37 amino acid residues starting with two leucines, hence being named LL-37 [9]. hCAP18/LL-37 has appeared to express in many tissues and body fluids like saliva, gingiva, sweat, amniotic fluids, seminal plasma and more tissues [3]. It is reported that the intravital concentration of hCAP18/LL-37 is about 86 µg/mL in seminal plasma from healthy donors [8], from less than 1.2 to more than 80 µg/mL in nasal secretion which is unstimulated and nonpurulent [7], respectively.

It has been reported that there are no correlation between dental caries of salivary LL-37 in children[13].The cariogenic species *S. mutans*, *Streptococcus sobrinus*, *Lactobacillus paracasei* and *Actinomyces viscosus* were resistant to LL-37in growth inhibition test and bactericidal test [2].

Despite many researchers evaluating the efficacy of antimicrobial peptides, there are few reports about LL-37 against biofilm, including *S. mutans* biofilm. It is essential to clarify whether antimicrobial peptide is able to inhibit biofilm formation on research to develop new drug.

The aim of this study is to examine anti-biofilm formation activity of LL-37 against *S. mutans*. We examined electron microscopic observations of *S. mutans* biofilm was formed by bacteria culture was treating with LL-37. We also measured whether binding between streptococcal LTA and LL-37 occurs or not by quartz crystal microbalance (QCM) binding assay.

We used ten strains of *S. mutans* that were isolated from children in their saliva as described by Hirose et al.[4]. The bacteria were grown in Brain Heart Infusion (BHI) broth (Merck KGaA, Darmstadt, Germany) for 24 hours at 37℃.

LL-37 (Sequence: LLGDF FRKSK EKIGK EFKRI VQRIK DFLRN LVPRT ES) was synthesized as described previously by the solid-phase method by Isogai et al.[6].

The pre-culture cells were diluted to make an optical density (OD) at 660 nm of 0.5 (JASCO Corporation, Tokyo, Japan) by diluting the cells with BHI broth. The bacteria were diluted further to a final concentration of 1×10^4 cells/mL with BHI broth, and then 1 mL of the cell suspension and 1 mL of two-fold serial dilutions of LL-37 solution were mixed together, of which final concentrations were 20, 10, 5, 2.5, and 1.25 µg/mL. Each mixture of bacteria and LL-37 solution was incubated at 37℃. Controls were prepared by mixing 1 mL of cells, 0.9 mL of BHI broth and 0.1 mL of Hanks' Balanced Salt Solution (HBSS, pH 7.4; Gibco, Grand Island, NY, USA). Cells were incubated with LL-37 for 24 hours to measure minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC was defined as the lowest concentration of LL-37 that inhibited the visible growth of LL-37 after overnight incubation. The MBC is defined as the lowest concentration of LL-37 that killed 99.9% of the initial inoculum in a given time using a plate count of viable cells.

The effects of LL-37 on biofilm formation were measured by using the Minimum Biofilm Eradication Concentration-High Throughput Plate (MBECTM-HTP, Innovotech, Inc, Edmonton, AB, Canada). The bacteria were diluted to a final concentration of 107 CFU/mL. A hundred microliter of the cells dilution was mixed with 100 µl of LL-37. The MBECTM-HTP was covered with lid equipped with 96 pegs, surface of which are covered with hydroxyapatite, in which each peg was dipped in cells suspension. Thus biofilm could be formed on the surface of pegs. Controls were a mixture of 100 µl each of the cells and 100 µl of BHI broth. All measurements were done in triplicate. The microtiter plate was incubated at 37℃ with shaking for 12, 24 and 36 hours. After different periods of incubation, we broke off pegs and disrupted biofilms from the surface of the pegs in the 200 µl of physiological saline solution (PSS) with a sonicator, and 20

µl of the cell suspension was removed on BHI agar plates, and plates incubated for 48 h at 37℃, and then colonies were counted.

Pegs were removed from the plate, on which biofilms were formed, and were rinsed in 0.9 % saline for 1 minute to remove planktonic culture. The samples were fixed with 2.5 % glutaraldehyde (Kanto Chemical co., Inc. Tokyo, Japan) in 0.1 M cacodylic acid (Wako Pure Chemical Industries, Ltd. Osaka, Japan) at 4℃ for 16 hours. The pegs were washed in 0.1 M cacodylic acid and with distilled water for approximately 10 minutes each. After that 70% ethanol was applied for 15-20 minutes and then air dried for a minimum of 24 hours. The pegs were mounted on a stage and examined by FE-SEM (SU8000; Hitachi High-Technologies Corporation, Tokyo, Japan).

A quartz crystal microbalance (QCM) is highly sensitive mass measuring devices because of changes in their resonant frequency upon a weight increase on their surface. The use of QCM transducers offers an in situ sensitive detection of hybridization events, without the need for optical or redox indicators 14). We used QCM (Single-Q, AS ONE Co., Ltd. Japan), which has automatic injection mechanism, mixer, sensor crystal oscillator, and low capacity reaction vessel. Substances have attached to sensor crystal oscillator that is defined as the vibration frequency changes for 200 Hz. Five hundred µl of HBSS was added into low capacity reaction vessel and mixed with 6000 rotation per minute (rpm) to stabilize at 37℃. Afterwards, 5 µl of LL-37 (1 mg/mL) was injected into the low capacity reaction vessel to stabilize. Subsequently, 5 µl of block reagent that does not react with substances injected to fix the gap of sensor which was not bound by LL-37. Then, 5 µl of LTA (1 mg/mL) that was isolated from *Streptococcus sanguis* as described by Isogai et al. [13)] was injected to measure the frequency change.

We used the function program contained in Microsoft Excel (Microsoft Corporation) to conduct F-test and t-test of our results (bacterial effect and estimation of bacterial cell). The type of function used to analyze is shown with each result. After statistical analysis, P-values of less than 0.05 were considered statistically significant.

The growths of *S. mutans* were compared added different concentrations of LL-37, culture with untreated controls, and found they had the similar growths. LL-37 has not inhibited the growth of *S. mutans* as shown in Fig. 1.

The inhibitory effect of LL-37 on biofilm formation was measured by counting colonies, which was recovered from biofilm formed on the surface of pegs. The CFU obtained from biofilm, which had been formed in the presence of LL-37, was less than that formed in control culture (Fig. 2, t-test, $P < 0.05$), indicating that LL-37 exhibited an inhibitory effect on *S. mutans* biofilm formation.In the observation of FE-SEM,*S. mutans* biofilm was inhibited by the treatment of LL-37 (Fig.3).

When 5 µl of LL-37 injection, the vibration frequency decreased by 750 Hz, and then 5 µl of streptococcal LTA was injected that the vibration frequency reduced by 470 Hzin QCM (Fig. 4). Similar results were obtained in triplicate. This result reveals that LL-37 and streptococcal LTA had intermolecular interactions. A major class of these cell surface glycopolymers is the teichoic acid, which is phosphate rich molecular found in a wide range of Gram-positive bacteria, pathogens [11)]. In the intermolecular interaction

with streptococcal LTA test, the result revealed that LL-37 and streptococcal LTA have intermolecular interaction. We consider that LL-37 interacts with streptococcal LTA to inhibit biofilm formation on the tooth surface.

In this study, we found that *S. mutans* strains from children with active caries showed greater resistance to LL-37. LL-37 had not inhibited/killed the growth of *S. mutans*. This findings is in agreement with a recent study that found no growth inhibition and no killing activities of LL-37 to *S. mutans*[2)]. It may be considered a potential virulence factor for this species, *S. mutans* strains that are more resistant to these peptides may have an ecological advantage to preferentially colonize within dental plaque and increase the risk of dental caries. In the biofilm formation experiment, the colonies obtained from biofilm, which had been formed in the presence of LL-37, were less than that formed in control culture. Although LL-37 does not inhibit/kill the growth, while does interact with something of cell to inhibit biofilm formation of *S. mutans*.

In conclusion,we found that (1) LL-37 has not inhibited the growth of *S. mutans*, (2) LL-37 can inhibit biofilm formation, (3) LL-37 and streptococcal LTA have intermolecular interaction.

Acknowledgments

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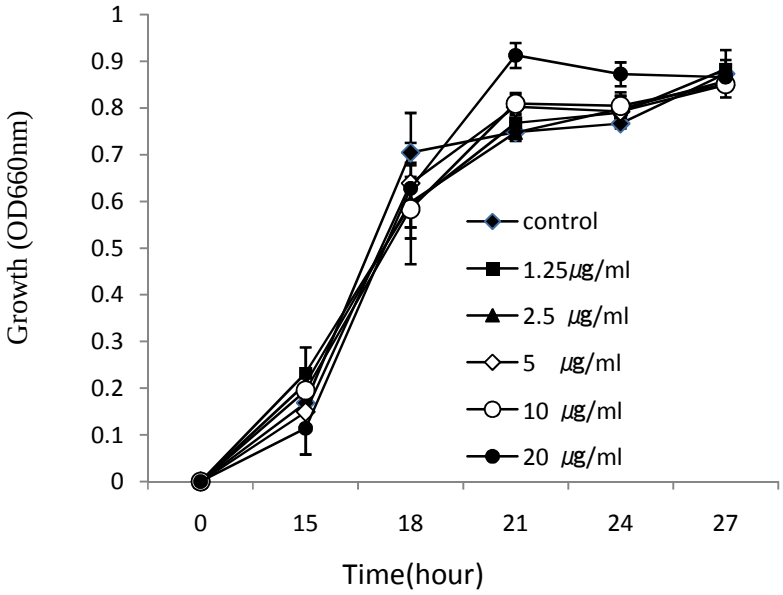


Fig. 1. Effect of LL-37on growth of*S. mutans*

The bacteria cultures that added different concentrations of peptide LL-37 were incubate at 37℃,andmeasured at 660 nm of optical densityvalue for every 3 hours after 15 hours incubation.Standard deviations indicated by error bars were calculated from 3 independent experiments.

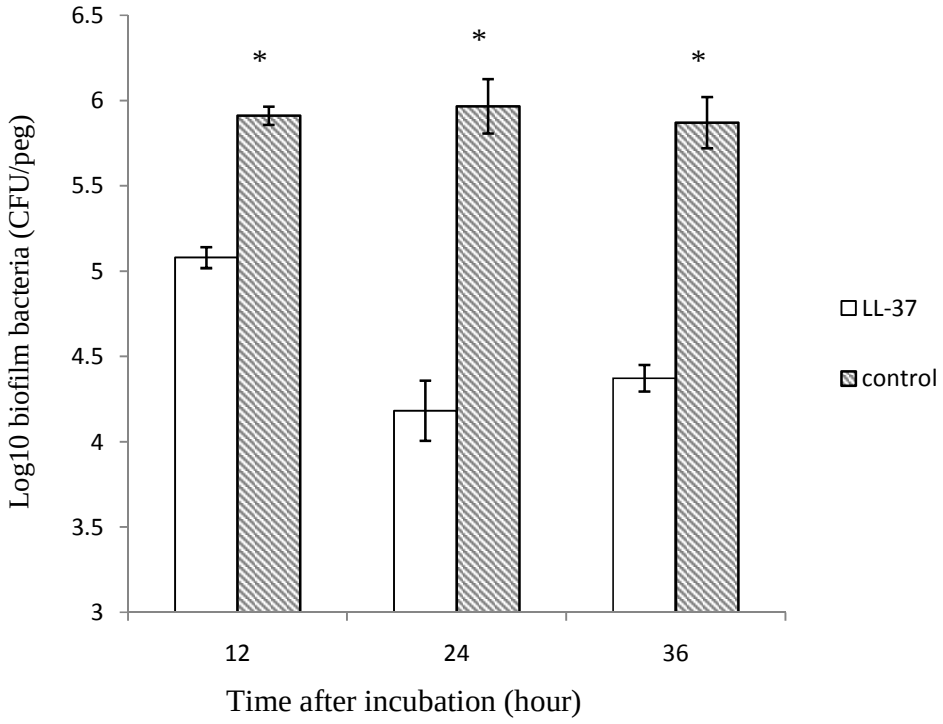


Fig. 2. Inhibitory effect of LL-37 on biofilm formation.

The pegs inserted into 107 CFU/mL planktonic cultures that added LL-37 (80 µg/mL) to incubate for 12, 24, and 36 hours, respectively. Standard deviations were calculated from 3 independent experiments.

: Significantly different from the untreated controls ($P < 0.05$).

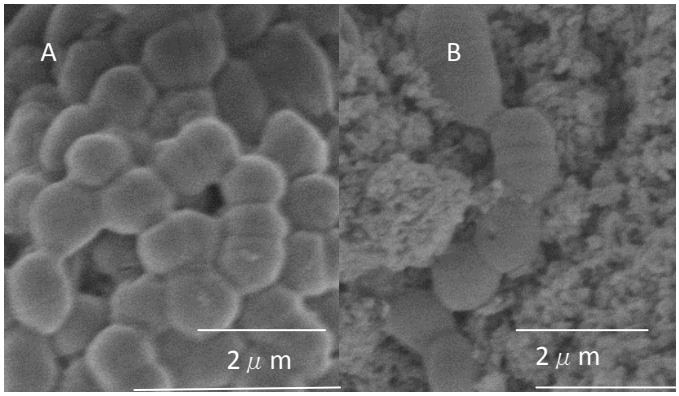
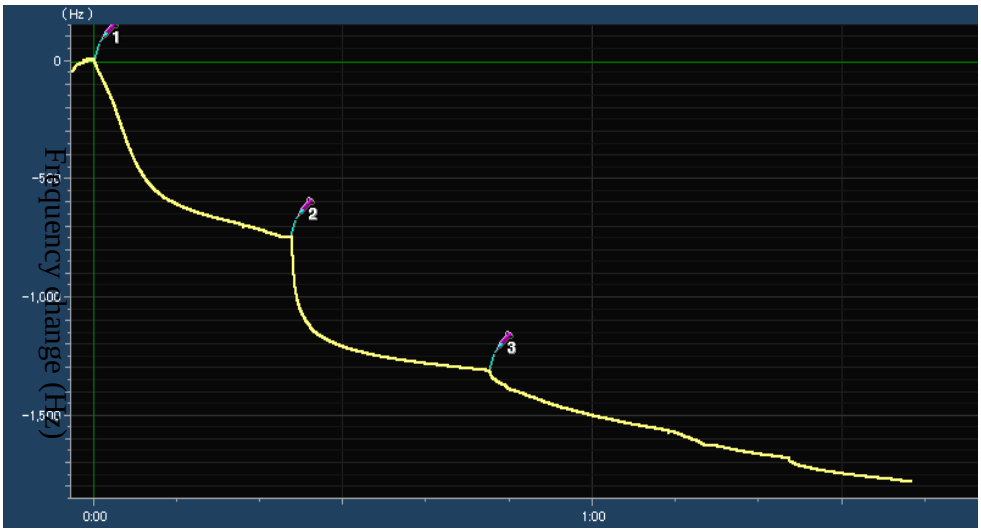


Fig. 3. Field emission-scanning electron microscope of biofilm.

A:Untreated control of biofilm on MBECTM-HTP peg. B:LL-37 treated *S. mutans*. The biofilm on MBECTM-HTP peg isinhibited by the treatmentwith 80 µg/mL of LL-37 for 24 hours.



Time (minute)

Fig. 4. Intermolecular interaction between LL-37 and streptococcal LTA in QCM.

1: LL-37 (5 µl of 1mg/mL solution); 2:block reagent (5 µl);3: of streptococcal LTA (5 µlof 1mg/mL solution).

The numbers in the figure represent number of auto-injections.