Identification of Inhibitory Effect on *Streptococcus mutans* by Oleanolic Acid

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Among endogenous oral microflora, *Streptococcus mutans* plays a critical role in dental plaque formation, which mainly contributes to the development of caries and periodontal disease. Phytochemicals are plant-derived chemical compounds that have been studied as beneficial nutrients to human health. The purpose of this study was to determine the effects of phytochemicals against *S. mutans*. Among them, oleanolic acid (OA) and 5-(hydroxymethyl)-2-furfural (HF) from Thomson seedless raisins were tested for anti-microbial effects against various clinically important bacteria. OA inhibited the growth of Gram-positive bacteria, but not Gram-negative bacteria. However, HF did not display any antibacterial effect against any of the strains tested. OA also exhibited inhibitory effects in surface adherence and biofilm formation of *S. mutans*. The results suggest that OA can be utilized as a potential anti-plaque and anti-caries agent by controlling the physiological characteristics of *S. mutans* on teeth.

Key words : *Streptococcus mutans*, phytochemicals, oleanolic acid, biofilm

Introduction

Streptococcus mutans is the principal causative agent of dental caries, in which lactic acids are produced by bacterial fermentation of dietary carbohydrates, resulting in the demineralization of the tooth enamel and further dental caries (Fig. 1). It produces glucosyltransferases (GTFs) that catalyze the transfer of glucosyl groups from one compound to another, resulting in the formation of glucan which provides binding site for the adherence of the bacteria to tooth surfaces and is a major contributor of persistant biofilm formation [1,17].

Phytochemicals, aromatic compound produced by most fruits and vegetables, include phenols, alkaloids, and terpenes. Among them, oleanolic acid, an important pentacyclic triterpenoid, has been considered as a key pharmaceutical substance. This compound and its derivatives have been known to exhibit a variety of biological activities, including anti-inflammatory [5], anti-HIV [9], anti-angiogenesis [18], anti-mutagenic [2], and gastroprotective and ulcer-healing [16] activities. In addition, oleanolic acid was suggested to display an antibacterial or bactericidal effect against different bacteria including *Mycobacterium tuberculosis* [8], *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA) [6].

Recently, researchers showed that highly-concentrated cranberry polyphenol extract (at the concentration of 500 μg /ml) inhibited the growth of *S. mutans* [22]. This finding brought to launch dental products containing the cranberry extract as an agent for preventing dental caries in the U.S. More recently, Thomson seedless raisins were tested for the possible use as a beneficial food that prevents oral diseases [25]. Five phytochemicals extracted from the raisins included oleanolic acid, oleanolic aldehyde, linoleic acid, linolenic acid, betulin, betulinic acid, and 5-(hydroxymethyl)-2-furfural [25].

Therefore, the aim of the present study was to investigate the effect of the phytochemicals found in the raisins on *S. mutans* and further to emphasize the development to a potential therapeutic agent which controls the growth of various oral pathogens.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains used in this study are listed as follows: Gram-negative bacteria (*Acinetobacter baumannii* ATCC19606, *Burkholderia thailandensis* E264, *Escherichia coli* DH5α, *Klebsiella*

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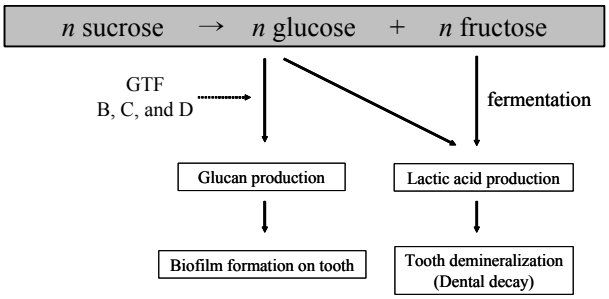

Fig. 1. Mechanism of generation of dental plaque and decay by *S. mutans*. *S. mutans* metabolizes sucrose to two monosaccharides, glucose and fructose. Glucans are synthesized from glucoses by glucosyltransferases (GTFs) encoded by the *gtfB*, *gtfC* and *gtfD* genes, resulting in facilitating bacterial adherence to the tooth surface, followed by dental biofilm formation. In addition, lactic acid is produced by the fermentation process of the fructose, thus lowering pH of oral environment, which causes the tooth demineralization and decay.

pneumoniae ATCC25306 and *Pseudomonas aeruginosa* PA14) and Gram-positive bacteria (*Enterococcus faecalis* ATCC35038, *Enterococcus faecium* ATCC19434, *Listeria monocytogenes* ATCC15313, *Streptococcus mutans* ATCC25175 and *Streptococcus sanguinis* CUG). The strains were routinely maintained on brain heart infusion medium (BHI, Difco). Stock solutions of oleanolic acid (OA, Sigma) and 5-(hydroxymethyl)-2-furfural (HF, Sigma) were freshly prepared at 4.567 mg/ml (10^{-2} mol/l) in 100% dimethyl sulfoxide. These solutions were diluted in BHI medium to 1,024 μg/ml for further experiments.

Determination of minimum inhibitory concentrations (MICs) of phytochemicals against various clinically important bacteria

Two-fold serial dilutions of OA and HF were prepared in fresh BHI medium in 96-well plates. An equal volume of bacterial inoculum of 5×10^7 CFU was added to each well on the 96-well plate containing 100 μl of the serially-diluted drugs. After incubation for 24 hr at 37°C, the MICs were obtained by observing the optical density at 600 nm by spectrophotometer.

Biofilm assay

Biofilm formation was assessed by using the slightly modified protocol of Loo *et al* [11]. BHI medium containing 1% sucrose was used for biofilm assay. Attached cells were stained using 1% crystal violet (v/v) for 15 min followed by rinsing the wells three times with 200 μl of H_2O and solublizing the stained cells in 95% ethanol. Biofilm formation was quantified by measuring the optical density at 575 nm by spectrophotometer.

Adherence assay

The bacteria were grown at an angle of 30° in a glass tube containing 10 ml of BHI medium supplemented with 1% sucrose in the presence of various concentrations of OA. After growth, unattached cells were removed and attached cells were collected by adding 0.5 M of sodium hydroxide. The adherence ability was quantified by measuring the optical density at 600 nm by spectrophotometer [7].

Statistical Analysis

Optical density data were analyzed by the general linear model of SAS® version 9.2 (SAS Institute, Cary, NC, USA), and all mean comparisons were performed with the Tukey's method at alpha=0.05.

Results and Discussion

OA inhibits the growth of Gram-positive bacteria

The results of MIC values against various bacteria were shown in Table 1. OA exhibited antimicrobial effects against all Gram-positive bacteria tested at low concentration of the compound, but not Gram-negative bacteria. However, HF did not display any antibacterial activity against all strains tested in this study. Horiuch research group suggested that the compounds are not active on Gram-negative bacterium, including *E. coli*, *P. aeruginosa* and *Serratia marcescens*, due to the existence of the outer membrane which is equipped

Table 1. MICs of OA and HF against several Gram-negative and Gram-positive bacteria

Strains	MIC (mg/ml)	
	OA	HF
Gram-negative bacterium		
Acinetobacter baumannii ATCC19606	>512	512
Burkholderia thailandensis E264	>512	512
Escherichia coli DH5a	>512	>512
Klebsiella pneumoniae ATCC25306	>512	512
Pseudomonas aeruginosa PA14	>512	512
Gram-positive bacterium		
Enterococcus faecalis ATCC35038	16	>512
Enterococcus faecium ATCC19434	2	>512
Listeria monocytogenes ATCC15313	4	>512
Streptococcus mutans ATCC25175	2	>512
Streptococcus sanguinis CUG	2	512

with several multidrug efflux pumps [6]. Of the Gram-positive strains tested in this study, *E. faecalis* and *E. faecium* are commonly isolated from human infections and resistant to many antimicrobial agents in many cases [19]. *Listeria monocytogenes* is a clinically important human pathogen causing listeriosis with a high fatality rate [21] and has been reported as an antimicrobial-resistant strain [14]. Therefore, the discovery of a novel drug will be indispensable to treat patients suffering with the infection of the drug-resistant enterococcal species and *L. monocytogenes*.

OA significantly suppressed the growth of two common endogenous oral pathogens, *Streptococcus mutans* and *Streptococcus sanguinis*, suggesting the possibility of the development to a potential therapeutic substance originated from plants to prevent serious dental caries. The results suggest that OA can be utilized as a valuable medical drug to eliminate the proliferation of clinically important human pathogens, particularly Gram-positive bacteria.

OA inhibits surface adherence and biofilm formation of *S. mutans*

To determine if OA hinders the biofilm formation of *S. mutans* on surface, cells were grown on the 96-well polystyrene plate containing BHI medium supplemented with 1% sucrose in the presence of various amounts of OA. In this experiment, sucrose was added to the medium for providing enough substrate of GTFs, which synthesizes glucan polymers, key factors in the development of a biofilm [1]. OA solutions ranging from 0 to 256 μg/ml were prepared by routine two-fold serial dilution method. As shown in Fig. 2, OA inhibited biofilm formation in a dose-dependent manner, that was completely repressed at the concentration of 4 μg/ml of OA.

The level of bacterial adherence to glass surface was examined in the presence of OA if the compound affects *in vitro* adherence of *S. mutans* to surface. The effects of different concentrations of OA on adherence of *S. mutans* to glass tubes are shown in Fig. 3. OA significantly reduced the bacterial adherence to the glass surface at the concentration of 8 μg/ml by 80%. Adherence ability to glass surface was slightly higher than the one to polystyrene plates in the adherence assay. The initial adhesion between bacteria and the surface substrate is governed by the physical properties of the bacteria and the surface material. Charged bacteria adhere in more amounts to hydrophilic glass surface than to hydrophobic polystyrene surface. In addition, adhesion

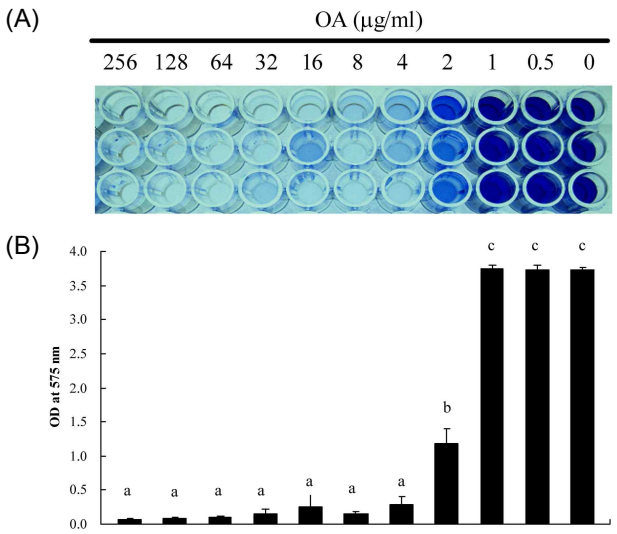

Fig. 2. Inhibitory effect on biofilm formation of *S. mutans* by OA. A 5×10^7 CFU amount of *S. mutans* cells was added to each well on the 96-well plate containing different amounts of OA ranging from 0 to 256 μg/ml. The degree of biofilm formation was performed by staining the cells with crystal violet (A) and measuring the optical density at 575 nm (B). [abc]: means with different letters are different ($p<0.05$)

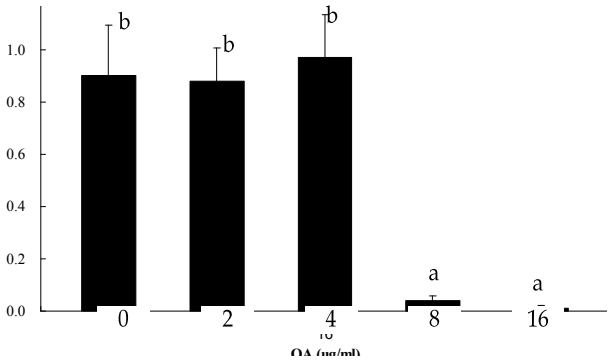

Fig. 3. Anti-adherence activity of OA against *S. mutans*. The bacteria cells were grown at an angle of 30° in a glass tube in presence of different concentrations of OA ranging from 0 to 16 μg/ml. Attached cells to the tube were solublized by adding sodium hydroxide, followed by measuring the optical density of the cells at 600 nm. [ab]: means with different letters are different ($p<0.05$)

increases with increasing contact angle [15,20].

Taken together, the data suggest that OA, quite common in nature, is capable of suppressing *S. mutans* associated with dental caries or periodontal diseases by hindering the growth of bacterial cells, the dental plaque formation or the bacterial adherence on the tooth surface.

Previously, it has been reported that OA inhibited peptidoglycan metabolism in *L. monocytogenes*, resulting in the im-

paired proliferation of the bacterial cells [10]. Therefore, it is necessary to examine if same mechanism applies to the case of *S. mutans* by OA addition.

The GTFs produced by *S. mutans* are responsible for synthesizing glucan polymers, which plays a critical role in biofilm formation and cell adhesion [1,17]. Among three GTFs, GtfB, GtfC and GtfD, the amount of GtfB and GtfC controls biofilm formation [12]. The expression of the *gtfBC* operon encoding two glucosyltransferase enzymes is negatively governed by a global regulator, CovR [4]. Besides glucan polymers, glucan-binding proteins (GbpA, GbpB, GbpC, and GbpD) synthesized by *S. mutans* have been found to influence biofilm formation or adhesion [3].

S. mutans also produces autoinducer 2 (AI-2) by which virulence characteristics of the pathogen are regulated. The synthesis of the molecule is known to be carried out by the LuxS enzyme [24]. The loss of the LuxS enzyme was shown to inhibit biofilm growth in *S. mutans* [13,23].

Based on the findings explained above, it also will be necessary to determine what molecular mechanism(s) is/are described for inhibitory effect of OA on biofilm formation and/or cell adhesion to surface of *S. mutans*.

In conclusion, this work represents considerable possibility that OA, found in natural foods, is able to be utilized as an alternative for prevention and medical treatment of dental diseases. However, further experiments are required to understand how OA works on extirpating *S. mutans* on tooth.

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References

1. Aoki, H., T. Shiroza, M. Hayakawa, S. Sato, and H. K. Kuramitsu. 1986. Cloning of a *Streptococcus mutans* glucosyltransferase gene coding for insoluble glucan synthesis. *Infect Immun.* **3**, 587-594.
2. Aparecida Resende, F., C. A. de Andrade Barcala, M. C. da Silva Faria, F. H. Kato, W. R. Cunha, and D. C. Tavares. 2006. Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice. *Life Sci.* **79**, 1268-1273.
3. Banas, J. A. and M. M. Vickerman. 2003. Glucan-binding proteins of the oral streptococci. *Crit. Rev. Oral Biol. Med.* **14**, 89-99.
4. Biswas, S. and I. Biswas. 2006. Regulation of the glucosyltransferase (*gtfBC*) operon by CovR in *Streptococcus mutans*. *J. Bacteriol.* **188**, 988-998.
5. Giner-Larza, E. M., S. Máñez, M. C. Recio, R. M. Giner, J. M. Prieto, M. Cerdá-Nicolás, and J. L. Ríos. 2001. Oleanonic acid, a 3-oxotriterpene from *Pistacia*, inhibits leukotriene synthesis and has anti-inflammatory activity. *Eur. J. Pharmacol.* **428**, 137-143.
6. Horiuchi, K., S. Shiota, T. Hatano, T. Yoshida, T. Kuroda, and T. Tsuchiya. 2007. Antimicrobial activity of oleanolic acid from *Salvia officinalis* and related compounds on vancomycin-resistant enterococci (VRE). *Biol. Pharm. Bull.* **30**, 1147-1149.
7. Islam, B., S. N. Khan, I. Haque, M. Alam, M. Mushfiq, and A. U. Khan. 2008. Novel anti-adherence activity of mulberry leaves: inhibition of *Streptococcus mutans* biofilm by 1-deoxynojirimycin isolated from *Morus alba*. *J. Antimicrob. Chemother.* **62**, 751-757.
8. Jiménez-Arellanes, A., M. Meckes, J. Torres, and J. Luna-Herrera. 2007. Antimycobacterial triterpenoids from *Lantana hispida* (*Verbenaceae*). *J. Ethnopharmacol.* **111**, 202-205.
9. Kashiwada, Y., H. K. Wang, T. Nagao, S. Kitanaka, I. Yasuda, T. Fujioka, T. Yamagishi, L. M. Cosentino, M. Kozuka, H. Okabe, Y. Ikeshiro, C. Q. Hu, E. Yeh, and K. H. Lee. 1998. Anti-AIDS agents. 30. Anti-HIV activity of oleanolic acid, pomolic acid, and structurally related triterpenoids. *J. Nat. Prod.* **61**, 1090-1095.
10. Kurek, A., A. M. Grudniak, M. Szwed, A. Klicka, L. Samluk, K. I. Wolska, W. Janiszowska, and M. Popowska. 2009. Oleanolic acid and ursolic acid affect peptidoglycan metabolism in *Listeria monocytogenes*. *Antonie Van Leeuwenhoek* in press.
11. Loo, C. Y., D. A. Corliss, and N. Ganeshkumar. 2000. *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. *J. Bacteriol.* **182**, 1374-1382.
12. Mattos-Graner, R. O., M. H. Napimoga, K. Fukushima, M. J. Duncan, and D. J. Smith. 2004. Comparative analysis of Gtf isozyme production and diversity in isolates of *Streptococcus mutans* with different biofilm growth phenotypes. *J. Clin. Microbiol.* **42**, 4586-4592.
13. Merritt, J., F. Qi, S. D. Goodman, M. H. Anderson, and W. Shi. 2003. Mutation of luxS affects biofilm formation in Streptococcus mutans. *Infect. Immun.* **71**, 1972-1979.
14. Poroś-Głuchowska, J. and Z. Markiewicz. 2003. Antimicrobial resistance of *Listeria monocytogenes*. *Acta Microbiol. Pol.* **52**, 113-129.
15. Rutter, P. R. and A. Abbott. 1978. A study of the interaction between oral Streptococci and hard surfaces. *J. Gen. Microbiol.* **105**, 219-226.

16. Sánchez, M., C. Theoduloz, G. Schmeda-Hirschmann, I. Razmilic, T. Yáñez, and J. A. Rodríguez. 2006. Gastroprotective and ulcer-healing activity of oleanolic acid derivatives: *in vitro-in vivo* relationships. *Life Sci.* **79**, 1349-1356.
17. Schilling, K., M. and W. H. Bowen. 1992. Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. *Infect. Immun.* **60**, 284-295.
18. Sohn, K. H., H. Y. Lee, H. Y. Chung, H. S. Young, S. Y. Yi, and K. W. Kim. 1995. Anti-angiogenic activity of triterpene acids. *Cancer Lett.* **94**, 213-218.
19. Takeuchi, K., H. Tomita, S. Fujimoto, M. Kudo, H. Kuwano, and Y. Ike. 2005. Drug resistance of *Enterococcus faecium* clinical isolates and the conjugative transfer of gentamicin and erythromycin resistance traits. *FEMS Microbiol. Lett.* **243**, 347-354.
20. van Loosdrecht, M. C. M., W. Norde, J. Lyklema, and A. J. B. Zehnder. 1990. Hydrophobic and electrostatic parameters in bacterial adhesion. *Aquatic Sciences* **52**, 103-114.
21. Vázquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Domínguez-Bernal, W. Goebel, B. González-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* **14**, 584-640.
22. Yamanaka-Okada, A., E. Sato, T. Kouchi, R. Kimizuka, T. Kato, and K. Okuda. 2008. Inhibitory effect of cranberry polyphenol on cariogenic bacteria. *Bull. Tokyo Dent. Coll.* **49**, 107-112.
23. Yoshida, A., T. Ansai, T. Takehara, and H. K. Kuramitsu. 2005. LuxS-based signaling affects *Streptococcus mutans* biofilm formation. *Appl. Environ. Microbiol.* **71**, 2372-2380.
24. Wen, Z. T. and R. A. Burne. 2002. Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. *Appl. Environ. Microbiol.* **68**, 1196-1203.
25. Wu, C. D. 2009. Grape products and oral health. *J. Nutr.* **139**, 1818S-1823S

초록 : Oleanolic acid(OA)의 *Streptococcus mutans*에 대한 저해효과

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구강에 상주하는 미생물 중, *Streptococcus mutans*는 충치 및 치주염의 원인인 치아플라그를 형성하는데 중요한 역할을 한다. Phytochemical은 식물에서 추출된 화학성분으로서, 사람의 건강에 유익한 영양물질로서 많은 연구들이 진행되어왔다. 본 연구는 이 phytochemical이 중요 구강미생물인 *S. mutans*에 대한 효과를 살펴보았다. 최근에 Thomson seedless raisin에서 여러 phytochemicals가 추출되었는데, 그 중 oleanolic acid (OA)와 5-(hydroxymethyl)-2-furfural (HF)의 임상적으로 중요한 여러 미생물에 대한 항균활성효과를 확인한 결과, OA가 그람음성균들에게는 항균활성효과가 나타나지 않았고, 그람양성균들에만 항균활성효과를 보였다. 그러나, HF의 경우에는 모든 균주에 대해 항균활성을 나타내지 않았다. 또한, OA는 *S. mutans* 균주의 표면부착과 생균막의 형성을 저해하기도 하였다. 따라서, 이 연구결과들은 OA가 치아에 존재하는 *S. mutans*의 생육 및 여러 생리적 특성들을 저해하므로 항플라그제나 항충치약으로서의 활용가능성을 확인할 수 있었다.