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Original

Inhibition of bacterial adherence to saliva-coated through plant lectins

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Abstract: In the present study, we evaluated the ability of lectin from *Talisia esculenta* (TEL) and a protein from *Labramia bojeri* seeds (Labramin) to inhibit adherence of microorganisms and exert antimicrobial effects. The minimum inhibitory and bactericidal concentrations of these proteins were determined using 5 species of bacteria: *Streptococcus mutans* UA159, *Streptococcus sobrinus* 6715, *Streptococcus sanguinis* ATCC10556, *Streptococcus mitis* ATCC903 and *Streptococcus oralis* PB182. In addition, an adherence assay was performed using these 5 bacterial species and sterile polystyrene microtiter plates coated with human saliva. Filtered protein solutions (6.25 to 100 μg/ml) were added to saliva-coated plates, and the plates were then incubated for 1 h at 37°C. After incubation, the plates were washed, and a bacterial suspension (10^6 CFU/ml) was then transferred to each plate, followed by incubation at 37°C for 1 h (10% CO_2). Adherence of bacteria to the acquired pellicle was visualized by staining with crystal violet, and absorbance was measured using a plate reader at 575 nm. Neither Labramin nor TEL, at any of the concentrations used, inhibited growth of any of the microorganisms. However, Labramin inhibited adherence of *S. mutans* and *S. sobrinus*. The present results indicate that Labramin is potentially useful as a biofilm-inhibiting drug. (J. Oral Sci. 49, 141-145, 2007)

Keywords: streptococci; plant lectins; adherence; dental biofilm.

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Introduction

Dental surfaces exposed to the oral environment are almost instantaneously covered by a proteinaceous film, which is known as the acquired enamel pellicle. This protein film is formed by selective adsorption of salivary proteins, peptides and other organic molecules (1). The initial process of bacterial adhesion to the acquired enamel pellicle comprises specific interaction between bacterial species present in the oral cavity and the acquired enamel pellicle (2).

Dental caries are caused by colonization and accumulation of oral microorganisms, and adherence of these microorganisms is the first step in colonization (3,4). Oral organisms attach to salivary components of the acquired enamel pellicle and, through growth and interaction between species, form a biofilm community (5). Consequently, bacterial recognition of salivary receptors on the tooth surface represents an important early step in the pathogenesis of oral disease.

In vitro findings indicate that there are differences between saliva-coated and uncoated hydroxyapatite in terms of the types of bacteria that adhere to them (1). This suggests that the nature of the pellicle somehow determines the nature of the initial stages of bacterial attachment to the tooth surface. Most bacteria grow into populations by adhering to solid surfaces and ultimately forming mixed-culture biofilms. Actinomyces and streptococci predominate in biofilms collected within the first hours of their formation (6).

Because of their role in adhesion and agglutination, lectins are considered to be important in both symbiotic and pathogenic interactions between some microorganisms and hosts (7). In bacteria, surface carbohydrates such as peptidoglycan, teichoic acids and lipopolysaccharides are potential lectin-reactive sites. The ability of lectins to complex with microbial glycoconjugates has made it possible to use lectins as probes to investigate cell surface structures and functions. One of the important uses of lectins in microbiology is the direct aggregation of suspended microorganisms (8). Lectins are the only plant proteins capable of recognizing and binding glyco-conjugates present on the surface of microorganisms such as bacteria and fungi, and thereby inhibiting their motility and multiplication (9,10). A previous study indicated that plant lectins could be effectively used to inhibit oral streptococci from adhering to the acquired pellicle in vitro (11).

The purpose of the present study was to examine the effects of *Talisia esculenta lectin* (TEL) and a protein extracted from *Labramia bojeri* seeds with lectin-like properties (Labramin) on biofilm formation *in vitro*, and to determine whether TEL and Labramin have antibacterial properties, by performing minimal inhibitory and bactericidal concentration assays using these 2 proteins.

Materials and Methods

Microorganisms

Exponentially growing cells of 5 oral streptococcal species (*Streptococcus mutans* UA159, *Streptococcus sobrinus* 6715, *Streptococcus sanguinis* ATCC 10556, *Streptococcus mitis* ATCC 903, and *Streptococcus oralis* PB 182) were centrifuged at 10,000 rpm for 5 min, washed twice with phosphate-buffered saline (PBS, pH 6.8), and then re-suspended. Each cell suspension was than spectrophotometrically (Genesys 10UV, Rochester, USA) adjusted to obtain a final concentration of 10^6 CFU/ml.

Isolation of TEL and Labramin

TEL was prepared as previously described by Freire et al. (12), and Labramin was obtained as described by Macedo et al. (13). Each one was purified by a combination of affinity and ion-exchange chromatography. The purity level of each protein was evaluated by performing PAGE-SDS using the method of Laemmli (14).

Collection of whole saliva

Written informed consent was obtained from all individuals who participated, and the experimental procedures were approved by the Institutional Ethics Committee of the Faculty of Dentistry of Piracicaba, State University of Campinas. Saliva was collected under masticatory stimulation from individuals who had refrained from eating and tooth brushing for 2 h. The saliva was mixed, clarified by centrifugation (14,000 rpm, 4°C, 20 min), and then immediately used.

Minimum inhibitory and bactericidal concentrations (MIC and MBC)

The antimicrobial activity of the 2 proteins was determined by performing the conventional broth macrodilution test (15). Aliquots (50 µl) of previously adjusted suspensions (10^5 CFU/ml) of each microorganism were added to screw-capped sterile tubes containing 5 ml of BHI and serial two-fold dilutions of 1 of the 2 proteins (400, 200, 100, 50, 25 and 0 µg/ml). The positive controls were tubes containing the standardized inoculum and broth. The negative controls were tubes containing broth plus dilutions of the proteins.

Before and after incubation (10% CO_2, 37°C, 18 h), the optical density (OD at 660 nm) of each tube was determined using a spectrophotometer. The MIC was defined as the lowest concentration that completely suppressed growth; i.e., that resulted in an OD that was identical to that of the negative controls. The MBC was defined as the lowest concentration that killed 99.9% of the inoculum. The MBC was determined by spreading a 25-µl sample of each tube in a petri dish containing 9 ml of BHI agar (Difco Co., Detroit, MI, USA), and then incubating the dish at 37°C for 18 h in an atmosphere containing 10% CO_2.

Adherence of bacteria to the surface of microtiter plates

Aliquots (100 µl) of clarified saliva were transferred to sterile polystyrene U-bottom microtiter plates (Dynatech Lab, Chantilly, VA, USA), which were then incubated aerobically for 2 h at 37°C. The plates were washed with PBS twice, and 100-µl aliquots of previously filtered (pore size, 0.2 µm) solutions of the proteins (6.25, 12.5, 25, 50 and 100 µg/ml) were then added to the plates, which were then incubated for 1 h at 37°C. The plates were then washed with PBS, and a 100-µl aliquot of a bacterial

suspension (10^6 CFU/ml) was transferred to each plate, which was then incubated at 37°C for 1 h in an atmosphere of 10% CO_2.

The adherence of bacteria to the acquired pellicle was visualized and quantified by staining with crystal violet (11). Crystal violet absorbance was measured using a plate reader at 575 nm (Versa Max Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). The assay was performed in triplicate for all bacterial species and both proteins. The negative controls consisted of the acquired pellicle, which was derived from saliva, plus a protein solution. The positive controls consisted of the acquired pellicle plus bacteria.

Statistical analysis

Data was statistically analyzed by submitting it to the Kruskal-Wallis test with the level of significance set at $P < 0.05$.

Results

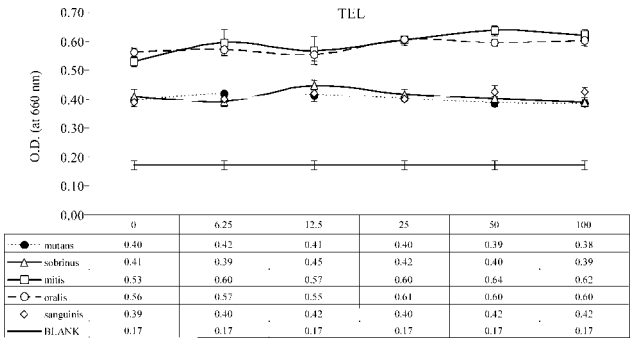
The antimicrobial activity of Labramin and TEL against the oral streptococci was determined using the broth macrodilution test, with protein concentrations of 0 to 400 µg/ml. Within the range of protein concentrations assayed, neither Labramin nor TEL killed or inhibited growth of any of the microorganisms, regardless of the concentration.

The inhibitory effects of the 2 proteins on adherence are shown in Fig. 1 (TEL) and Fig. 2 (Labramin). To determine the response trend and the concentration that most effectively inhibited adherence, various concentrations of both proteins were tested. Labramin at a concentration of 100 µg/ml had a significant inhibitory effect against adherence of the cariogenic streptococci *S. mutans* and *S. sobrinus* ($P < 0.05$). No concentration of Labramin efficiently inhibited adherence of *S. mitis*, *S. oralis* or *S. sanguinis*. TEL at 100 and 50 µg/ml caused an increase in biofilm formation by *S. mitis*. TEL did not inhibit adherence of the other 4 streptococcal species.

Discussion

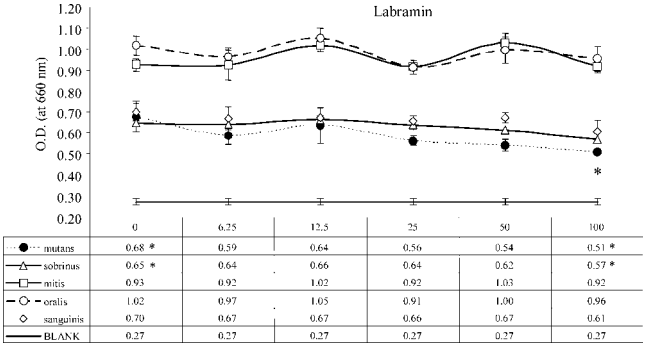
Long-term survival of bacteria in the oral cavity requires that the organisms adhere to a tissue surface or colonize a suitable niche in the complex multi-species biofilm that exists on human oral tissues. However, the molecular mechanisms used by many oral microorganisms to recognize and distinguish appropriate environmental niches in the oral biofilm are still not well understood (16). Microbial colonization of the human tooth surface is initiated by attachment of oral streptococci to salivary components of the acquired pellicle, followed by growth of these bacteria together with neighboring members of the developing biofilm community. One of the many molecular mechanisms involved in adherence of bacteria and development of mixed-species oral biofilms is specific lectin-carbohydrate interaction between bacteria, such as the interaction involved in lactose-sensitive coaggregations of actinomyces and streptococci (17).

Anti-adhesion therapy consists of inhibition or complete blocking of lectin-mediated adhesion (18). It is thought that reducing the mass of mutans streptococci in dental biofilm could lower the incidence of dental caries. The use of anti-adhesion agents that disengage mutans streptococci from dental biofilms or interfere with their adhesion without affecting their viability may prove to be clinically useful as a means of preventing selective pressure and overgrowth of resistant bacteria (19). Thus, there is a need for studies of substances, such as lectins, that affect



	0	6.25	12.5	25	50	100
mutans	0.40	0.42	0.41	0.40	0.39	0.38
sobrinus	0.41	0.39	0.45	0.42	0.40	0.39
mitis	0.53	0.60	0.57	0.60	0.64	0.62
oralis	0.56	0.57	0.55	0.61	0.60	0.60
sanguinis	0.39	0.40	0.42	0.40	0.42	0.42
BLANK	0.17	0.17	0.17	0.17	0.17	0.17

Fig. 1 Dose-response of TEL on adherence of *S. mutans* UA159, *S. sobrinus* 6715, *S. mitis* ATCC 903, *S. oralis* PB 182 and *S. sanguinis* ATCC 10556. Assays were performed in triplicate, and the values presented are mean OD (± SD). *: significantly different from negative control ($P < 0.05$).



	0	6.25	12.5	25	50	100
mutans	0.68 *	0.59	0.64	0.56	0.54	0.51 *
sobrinus	0.65 *	0.64	0.66	0.64	0.62	0.57 *
mitis	0.93	0.92	1.02	0.92	1.03	0.92
oralis	1.02	0.97	1.05	0.91	1.00	0.96
sanguinis	0.70	0.67	0.67	0.66	0.67	0.61
BLANK	0.27	0.27	0.27	0.27	0.27	0.27

Fig. 2 Dose-response of Labramin on adherence of *S. mutans* UA159, *S. sobrinus* 6715, *S. mitis* ATCC 903, *S. oralis* PB 182 and *S. sanguinis* ATCC 10556. Assays were performed in triplicate, and the values presented are mean OD (± SD). *: significantly different from negative control ($P < 0.05$).

adhesion of mutans streptococci but do not kill them.

Recently, 2 acid lectins were discovered in mulberry leaves, and they were found to be able to agglutinate the pathogenic bacteria *Pseudomonas syringae pv mori* (20). Other vegetable lectins, including *Griffonia simplicifolia* lectin (GS I), *Canavalia ensiformis* lectin (Con A), wheat-germ agglutinin (WGA), peanut lectin (*Arachis hypogaea*) and the lentil lectin (*Lens culinaris*), have been found to agglutinate *Pseudomonas syringae pv mori*. Other plant lectins, including soybean agglutinin (SBA) (21) and *Dolichos biflorus* (22), have been found to specifically react with group C streptococci. These lectins probably affect these bacteria by interacting with *N*-acetyl-D-galactosamine residues in their external structures. In a study by Teixeira et al. (11), several plant seed lectins that interact with glucose-mannose chains very effectively prevented adherence of streptococci, although they exhibit distinct biological activities.

Previous studies have examined the effects of TEL and Labramin on several phytobacteria strains, including *Curtobacterium flaccumfaciens pv flaccumfaciens* (Gram-positive), *Pseudomonas cichorii* (Gram-negative), *Xanthomonas campestris pv campestris* (Gram-negative), and *Xanthomonas campestris pv melonis* (Gram-negative). TEL (100 μg) inhibited growth of all 4 of these species, which exhibited an exponential pattern of growth when incubated without TEL. Inhibition of growth of *P. cichorii* by TEL mainly occurs in the initial stages of multiplication, at 3 h (76%) and 4 h (56%) after inoculation, as an early effect of blockage of bacterial motility. In contrast, TEL-induced inhibition of growth of *C. flaccumfaciens pv flaccumfaciens* (42%), *X. campestris pv campestris* (16%) and *X. melonis* (60%) occurred at 18, 32 and 12 h after inoculation, respectively. Labramin (100 μg)-induced inhibition of growth of *C. flaccumfaciens pv flaccumfaciens* (20%) and *X. campestris pv melonis* (23%) occured at 14 and 18 h after inoculation, respectively (23). These findings are rather different from the results of the present study, in which Labramin effectively inhibited bacterial growth but TEL did not. This apparent discrepancy is probably due to differences between the species of bacteria used.

In the present study, Labramin inhibited biofilm formation by cariogenic streptococci, but did not inhibit biofilm formation by the other streptococci tested. Interestingly, neither Labramin nor TEL exhibited antimicrobial activity against the bacteria tested. Lee et al. (24) examined interactions between lectins of various specificities and several species of oral streptococci. They observed that certain lectins only interacted with certain species, indicating that lectins can be used to specifically target certain microorganisms for aggregation.

Unique ligands on the surfaces of microorganisms appear to interact specifically with pellicle components via diverse mechanisms, including cell-cell and substrate-cell interactions (25,26). In the case of several oral bacterial species, this interaction may involve lectin-like components of the organism, which bind to saccharide receptors in the salivary glycoproteins that make up the pellicle (27,28). Approximately 40 to 50% of the amino acids in TEL and its isoforms are hydrophobic, and TEL contains 3 specific carbohydrate linkages (D-mannose, D-glucose and *N*-acetylglucosamine). Labramin contains 2 specific carbohydrate linkages: D-mannose and D-glucose.

The present results are consistent with previous findings that indicate that lectins would be useful for anti-adhesion therapy. The present results elucidate a novel aspect of plant lectins: their effects on biofilm formation. The present study was conducted using single-species cultures. Further studies using multi-species cultures will further elucidate the effectiveness of Labramin and TEL as anti-biofilm agents.

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