COMPARISON OF THE ACTIVITY OF FOUR CHITOSAN DERIVATIVES IN REDUCING INITIAL ADHERENCE OF ORAL BACTERIA ONTO TOOTH SURFACES

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Received 9 October, 2001/Accepted for Publication 30 November, 2001

Abstract

We examined the effects of four kinds of chitosan derivatives on initial adherence of oral bacteria onto human anterior teeth surfaces. The buccal surfaces of anterior teeth were used as the experimental surfaces. They were divided into five rectangle areas with outer dimensions of about 2 mm x 4 mm. After applying two ml of a sample solution onto the tooth surfaces, an examiner wiped each rectangle area with a sterilized plastic swab one, three and six hours later. Then we measured bacterial counts in sterilized swabs with mitis salivarius agar.

We found that the order of magnitude of the inhibitory effect on the adherence of oral bacteria was low molecular chitosan > phosphorylated chitosan > amorphous chitosan > carboxymethyl chitosan. The solution containing 0.5% low molecular chitosan depressed the bacterial adherence to the same extent as a 50 ppm chlorhexidine digluconate solution for three hours, and 0.1% phosphorylated chitosan also exhibited an inhibitory effect in bacterial adherence for one hour. Amorphous chitosan had a moderate inhibitory effect, but no clear inhibitory activity was found with 0.1% carboxymethyl chitosan. These results suggest that low molecular chitosan and phosphorylated chitosan have the potential to effectively inhibit the initial adherence of oral bacteria onto human tooth surfaces.

Key words: Low molecular chitosan—Phosphorylated chitosan—Initial adherence—Oral bacteria—Tooth surface

INTRODUCTION

The process of bacterial adherence to the tooth surface involves a variety of physico-chemical interactions. The nature of this process seemed to include electrostatic\textsuperscript{10}, hydrophobic\textsuperscript{10} and lectin-like\textsuperscript{2} interactions as binding mechanisms. After the initial adherence, oral bacteria are ready to proceed to aggregations and production of the extracellular poly-
saccharides, which may lead from thin biofilms to mature plaque accumulations.

The inhibition of the initial adherence of cariogenic bacteria onto the tooth surface could be a useful strategy for preventing their colonization and reducing of the progression of dental caries. Therefore, many researchers have attempted to develop chemical plaque control agents in the last twenty years.

Chitosan is a poly-aminosugar which possesses free amino residues. It has been reported that derivatives of these poly-aminosugars have several interesting characteristics caused by their polycationic nature.

Our previous study demonstrated that low molecular weight chitosan binds to both hydroxyapatite beads and oral bacterial cell surfaces and that some water-soluble chitin derivatives inhibit the adsorption of S. sobrinus, S. sanguis, and S. mitis to saliva-coated hydroxyapatite beads. Moreover, we showed a cariostatic effect of low molecular weight chitosan on experimental rats infected with S. cricetus.

In this study, we developed a method to determine the number of bacteria adsorbed onto the teeth surface in situ and examined the effect of four chitosan derivatives on the adherence of the oral bacteria to human tooth surfaces.

MATERIALS AND METHODS

1. Preparation for chitosan derivatives

Four kinds of chitosan derivatives were prepared from commercially available chitosan (Kyowa Yushi Kogyo Co., Tokyo). Low molecular chitosan (LMCS, mw: 5 kDa, degree of deacetylation (DA); 0.75) was prepared by the method of depolymerization of chitosan with nitrous acid. Phosphorylated chitosan (PPPS, mw; 20 kDa, DA; 0.70) was prepared by the reaction of chitin with phosphorus pentoxide. Carboxymethyl chitosan (PCMS, mw; 70 kDa, DA; 0.70) were prepared by the reaction of alkali-chitin with monochloroacetic acid. Amorphous chitosan (PDAS, mw; 200 kDa, DA; 0.75) was prepared by the partial N-acetylation of chitosan. The molecular weight was determined by the GPC method with N-acetyl-d-glucosamine oligomer and pullulan standards. Measurement of the degree of deacetylation was carried out by the colloid titration method and the MBTH method.

2. Subject and study design

A 29-year-old male was selected as the subject because he was healthy, not taking any medication, and had good oral hygiene, including (i) no signs of periodontitis, (ii) no extensive dental restorations and (iii) not systematic antibiotic treatment before or during the experimental period.

The subject was asked to maintain his normal dietary habits during the study. He participated in ten separate experiments, and a wash-out period of three days preceded each of the experimental periods.

Experimental procedures are diagramed in Fig. 1. We selected five areas with the outer dimensions of approximately 2 mm x 4 mm for measurement of bacterial counts (Fig. 2). At the beginning of the each experimental cycle, the buccal surfaces of his anterior teeth were cleaned extensively with a silica abrasive. After
washing the surfaces with a ten ml of sterilized saline, we applied two ml of sample solution to the tooth surfaces in thirty seconds with a plastic syringe. The subject was asked not to rinse his mouth with water just after application of the solution. One, three, and six hours later, the examiner washed the surfaces with ten ml of sterilized saline, then wiped any adsorbed bacteria off each measurement area with a sterilized small plastic swab (i.d.: 2 mm, length: 6 mm) using ten strokes and constant pressure. Only one examiner performed this operation during the study to minimize experimental variation. We immediately put each swab into a test tube containing two ml of sterilized saline and glass beads (i.d.: 1.5 mm); then the tubes were mixed extensively by a vortex mixer for thirty seconds. Microbial counts were obtained by spreading 0.1 ml of the appropriate dilutions on duplicate plates of mitis salivarius agar (Difco, Detroit, MI, USA) using sterile bent plastic rods. The agar cultures were incubated anaerobically in a chamber with an atmosphere of 80% N₂, 10% H₂ and 10% CO₂ at 37 for 78 hours.

3. Test solutions

The following formulations were used. A): a solution containing 10% glycerin, 10% ethanol, 0.05% sodium saccharin, 0.48% nonionic surfactant (polyoxyethylene hydrogenated castor oil), 0.16% peppermint flavor, and either 0.5% or 0.1% of a chitosan derivative. C): a solution of 50 ppm chlorhexidine digluconate (a positive control). Each test solution was adjusted to 100 w/w% by adding deionized water.

4. Statistical analysis

The data were analyzed with ANOVA. The factors analyzed were the different kinds of chitosan derivative, the different concentrations of chitosan derivative and the different time points at which the measurement of bacterial counts were performed. The differences of the average of chitosan derivatives were analyzed with the least significant difference (student l.s.d) test. Statistical significance was accepted at the 5% probability level.

RESULTS

The changes in bacterial counts on the tooth surfaces after exposure to a 50 ppm chlorhexidine (CHX) solution and a blank solution are shown in Fig.3. After applying the blank solution, we found that microbial counts on the tooth surfaces was increased with elapsing time. The chlorhexidine solution (a positive control) kept the adherence of oral streptococci depressed for as long as
6 hours. At twenty four hours, no difference was found in bacterial counts on the tooth surfaces between the two solutions. From this preliminary test, we decided to measure microbial counts for six hours to compare the inhibiting abilities among the solutions of chitosan derivatives.

After applying the chitosan solutions, we found the number of oral streptococci on the tooth surfaces were increased with elapsing time \( (p < 0.01) \). The inhibitory effects of chitosan derivatives on adherence of oral streptococci depended on the concentration of samples \( (p < 0.05) \) and kind of chitosan derivative \( (p < 0.05) \). The order of inhibiting ability was LMCS > PPPS > PDAS > PCMS, and significant differences \( (p < 0.05) \) were found between LMCS v.s. PCMS and PPPS v.s. PCMS (Table 1).

A solution containing 0.5% LMCS showed a relatively stronger inhibition of adherence of oral streptococci to tooth surfaces for as long as three hours (Fig. 4). A solution containing PPPS \( (0.1, 0.5\%) \) also had an inhibitory effect for one hour (Fig. 4, 5). A solution containing 0.5% PDAS had an inhibitory effect for one hour of the same magnitude as 0.5% PPPS (Fig. 5). The solutions containing 0.1% and 0.5% PCMS had the least inhibitory effects on bacterial adherence to tooth surfaces among the four chitosan derivatives.

**DISCUSSION**

We designed a novel method for detecting the initial bacterial adherence on the tooth surfaces by using a small piece of a sterilized swab. A preliminary trial showed that a positive control of 50ppm chlorhexidine digluconate inhibited oral bacterial adherence for six hours in comparison with a blank solution. This result indicated that the method could
be beneficial for estimating influences of test samples on the initial adherence of oral bacterial under in situ conditions.

Chitosan is an all-natural product derived from the polysaccharide chitin. The difference between cellulose and chitosan is that the 2-hydroxy group of cellulose is replaced with amino group. This positively charged group might give chitosan the ability to chemically bond with negatively charged bacterial surfaces and tooth pellicle.

Our results indicated that the order of magnitude of inhibitory effect of chitosan derivatives on the initial adherence of oral streptococci was LMCS>H11022PPPS>PDAS>PCMS (Fig. 4, 5. Table 1). We previously demonstrated that LMCS had a binding affinity with both Streptococcus sobrinus 6715 and saliva treated hydroxyapatite beads. The results obtained in this study with LMCS was supported in a part by findings of Tarsi et al. They demonstrated that low-molecular-weight chitosan had a selective action against S. mutans adhesive properties and reduced the adsorption to saliva-treated hydroxyapatite beads. They then hypothesized that chitosan mechanism of anti-adherence activity seems likely to involve (i) bacterial surface modifications, (ii) alterations in the expression levels of bacterial surface ligands, and (iii) chitosan adsorption to host surfaces. Although it is not proper to make direct comparisons with other experiments because of different molecular weights and deacetylation of chitosan preparations, these results are consistent with our findings in which LMCS, binding both bacterial cell and saliva-treated hydroxyapatite beads, showed an inhibitory effect of adherence of oral bacteria on the tooth surfaces. In general, LMCS seems to discourage bacterial colonization on the tooth surface because of its binding activity toward the both bacterial cell and tooth surface. However, there remains the possibility that there is an optimal specification of molecular weight and deacetylation for LMCS to inhibit bacterial adherence. The mechanism behind this phenomenon is not fully understood and seems to be beyond the scope of this study. Further research on the mechanism of the anti-adherence activity of LMCS on oral bacteria is needed.

We also previously showed that LMCS can aggregate oral bacteria. Liljemark et al. reported that aggregating oral bacteria may reduce their adherence to tooth surfaces. The polycationic nature of chitosan might reduce the initial bacterial adherence onto the teeth surfaces, at least in part, by generating bacterial aggregation.

On the other hand, PDAS was relatively less effective in inhibiting the adherence of oral bacteria than LMCS. LMCS and PDAS have the same degree of deacetylation (0.75), but their molecular weights are 5 kDa and 200 kDa, respectively. The results obtained by Bough et al. indicated that the magnitude of aggregation depends not only on the degree of deacetylation but also on the molecular weight of the chitosan polymer. The effect of the degree of deacetylation and the molecular weight in the inhibition of bacterial adherence should be studied and further evaluated.

The results obtained in this study also demonstrated that PPPS suppressed the initial bacterial adherence to the tooth surface. Our previous study showed that phosphorylated chitosan strongly bound hydroxyapatite beads and inhibited adsorption of oral streptococci to saliva-coated hydroxyapatite beads. It is suggested by Olsson et al. that inorganic phosphate binds with high affinity to hydroxyapatite and might be effective as a cleansing agent, by displacing proteins and other organic substances from the enamel surfaces. These findings suggest that the inhibitory effect of phosphorylated chitosan on the adherence of oral streptococci is partially due to reduction of the interaction between the bacterial cell surface and tooth surface. Phosphorylated chitosan contains both phosphate groups and amino residues in the same glucosamine units. These amphoteric units might interfere with the binding of a variety of plaque bacteria, which may adsorb to the enamel surface by electrostatic interactions. Therefore, the postulated mechanism of the action of PPPS might include of formation of polyelectrolyte complexes on the tooth surface, which permit
less interaction between oral bacteria and tooth enamel.

PCMS, in contrast, has the least inhibiting ability on bacterial adherence. The reason for the inactivity of PCMS is still unclear, but PCMS has an isoelectric point near neutrality and might tend to be precipitated by even a slight pH change during the test period (from pH 7.7 to 7.3). Although there were no insoluble particles in the test solutions at the beginning of the test period, the components in the test solution might influence the solubility of PCMS during the test period. We therefore hypothesize that the inactivation of PCMS is partially due to formation of invisible and insoluble minute particles derived from interactions between PCMS and ingredients such as a nonionic surfactant or flavor. A test product containing PCMS should be carefully prepared before any judgment concerning its clinical effect is made.

Chitosan derivatives showed no bacteriostatic effect on oral streptococci in our previous studies (unpublished data). The mechanism behind the inhibition of adherence of oral streptococci to the tooth surface by chitin derivatives is not yet properly understood. The clinical use of chitin derivatives should be further studied, including a detailed evaluation of their electrostatic interactions with bacterial cell walls and tooth surfaces.

In conclusion, our findings suggest that chitosan derivatives such as LMCS and PPPS could relatively reduce the adherence of oral streptococci to the tooth surfaces and might minimize bacterial colonization.

REFERENCES

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