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Original Article

EFFECT OF MOLECULAR MASS AND DEGREE OF DEACETYLATION OF CHITOSAN ON ADSORPTION OF *STREPTOCOCCUS SOBRINUS* 6715 TO SALIVA TREATED HYDROXYAPATITE

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Abstract

We evaluated the influence of molecular mass and degree of deacetylation of chitosan on the adsorption of *Streptococcus sobrinus* 6715 to saliva-treated hydroxyapatite (S-HA) by measuring the optical density of the bacterial cell suspensions released from saliva-treated hydroxyapatite. Twenty-five chitosan samples with different molecular masses (0.8–6 kDa) and degrees of deacetylation (10–95%) were prepared for the study. We found that the inhibition of adsorption of *S. sobrinus* 6715 to S-HA correlated positively with the molecular mass of chitosan ($R=0.876$) and that the optimal degree of deacetylation was 50–60% for maximum inhibition of bacterial binding to S-HA. We also examined the effect of chitosan on zeta potentials of the oral bacteria and their surface hydrophobicities. It was observed that chitosan reduced the magnitude of the zeta potential and surface hydrophobicities of the oral bacteria. Thus, the results demonstrated that chitosan with a molecular mass of 5–6 kDa and a degree of deacetylation of 50–60% might have the potential to act as an effective anti-plaque agent because of its polycationic properties.

Key words: Chitosan—Molecular mass—Degree of deacetylation—Adsorption—Hydroxyapatite

INTRODUCTION

The development of dental caries is dependent on many factors including plaque accumulation, exposure to fermentable carbohydrate and salivary buffering capacity. Bacterial plaque on the tooth surfaces has been

believed to be a major etiologic factor in the development of dental caries. Specific types of microorganisms are associated with plaque as it develops and matures. Reduction in the accumulation of dental plaque has therefore been the objective of many *in vitro* and clinical investigations^{2,4,14,19}.

The ability of cariogenic organisms to become attached to tooth surfaces is commonly acknowledged to be a first step in the process of dental caries. The nature of this attachment appears to be complex and is ascribed, at least in part, to electrostatic and hydrophobic interactions^{11,16,18}. The inhibition of the adsorption of the cariogenic bacteria to the enamel would be a promising approach for preventing their colonization and the progression of the dental caries. This goal may be accomplished, to some extent, by either ionic or nonionic compounds such as alkyl phosphates¹⁷, poly-L glutamic acid¹², phytate⁷, sulfolane²⁷, and nonionic propoxylated surfactants⁸ which modify the hydroxyapatite surface, reducing oral bacterial adsorption.

Chitosan is a natural polysaccharide derived from chitin by N-deacetylation. The glucosamine backbone produced by the N-deacetylation process gives chitosan a polycationic character. Because of these positive charges, such derivatives have been receiving much attention as potential candidates for use as a stimulant for regenerating oral soft tissues¹⁵ and some blood coagulant factors⁶.

Our previous study demonstrated that five chitin derivatives reduced the adsorption of oral streptococci onto saliva-treated hydroxyapatite and detached these bacteria from hydroxyapatite beads²⁰. We also showed that water-soluble chitosan (pKa: 6.5) was suitable as a food additive to buffer the oral pH to a high enough level to prevent the deleterious action of organic acids on the tooth surfaces²². Moreover, it was found that water-soluble chitosan possesses a cariostatic effect on caries increment in rats²¹. Recently Tarsi *et al.*²⁴ also indicated that low molecular mass chitosan had an inhibitory effect on oral streptococci in a range of sub-minimal inhibitory concentrations.

The aim of this study was to investigate the optimum molecular mass and degree of deacetylation of chitosan for the inhibition of adsorption of *Streptococcus sobrinus* 6715 to S-HA and also to assess the effect of chitosan on cell surface zeta-potential and hydropho-

bicity of the oral streptococci.

MATERIALS AND METHODS

1. Preparation of chitosan

The various types of chitosan (n = 25) were prepared by the depolymerizing of commercially available chitosan (Fronac NA-500: Kyowa Yushi Kogyo Co., Japan) with sodium nitrite by changing concentrations of the depolymerization reagent and reaction times following the methods of Hirano⁵. The molecular mass of each chitosan preparation was determined by the gel permeation chromatography method with N-acetyl-D-glucosamine oligomer and pullulan standards²². We measured the degree of deacetylation of chitosan using a colloid titration method¹⁰ and the MBTH (3-methyl-2-benzothiazolone hydrazone hydrochloride) method²⁵. The molecular mass and degrees of deacetylation of chitosan used in this study were in the ranges of 0.8–6 kDa and 10–95%, respectively.

2. Bacterial strain and culture conditions

S. sobrinus 6715 and *Streptococcus mitis* 9811 were cultured aerobically overnight in Brain Heart Infusion broth (DIFCO Laboratories, Detroit, MI, USA) and Tryptocase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA), respectively. The cells were harvested by centrifugation (10,000 × g, 15 min 4°C), washed three times in buffered KCl³ (0.05 M KCl, 1 mM KH₂PO₄, 1 mM CaCl₂, 0.1 mM MgCl₂, pH 6.0), and resuspended in buffered KCl to produce a suspension containing about 5 × 10⁸ CFU/ml, corresponding to an optical density of 1.5 at 660 nm.

3. Preparation of saliva-treated hydroxyapatite

Paraffin-stimulated whole saliva from one donor was collected in a container chilled over ice. The saliva was heated at 60°C for 30 min to inactivate degradative enzymes and clarified by centrifugation. Twenty milligrams of hydroxyapatite beads (Central Glass Co., Ltd. Tokyo, Japan) with a surface area of

4–6 m²/g were placed in polypropylene microtest tubes and allowed to equilibrate in 1 ml of buffered KCl for 18 hours. The supernatant solution was removed, and the beads were then incubated with 1 ml of the clarified saliva for 2 hours at room temperature. They were washed three times with buffered KCl before use.

4. Adsorption and desorption assay

Inhibition studies of *S. sobrinus* 6715 binding to S-HA were carried out using various chitosans. A mixture of a suspension of 1 ml of bacterial cells and 1 ml of test solution (250 ppm) was incubated with 20 mg of S-HA beads for 20 min. The S-HA beads in the control were incubated with buffered KCl solution. After incubation, the supernatant was removed and washed twice with buffered KCl. Then 2 ml of 0.5 N HCl was added to solubilize the S-HA beads. The number of bacterial cells that had attached to the S-HA beads was determined by turbidity at 660 nm. The percent inhibition was calculated by the following formula: $100 \times \{(\text{cells adsorbed to S-HA treated with control} - \text{cells adsorbed to S-HA treated with chitosan}) / (\text{cells adsorbed to S-HA treated with control})\}$. All assays were done in triplicate and expressed as mean values.

The ability of chitosan to desorb cells from S-HA was determined by the following procedure. An aliquot of 20 mg S-HA beads was incubated with 2 ml of bacterial cell suspension for 30 min. These beads were washed twice with buffered KCl and then incubated with 2 ml of chitosan solution (2,500 ppm) for 30 min. The S-HA beads in the control were incubated with buffered KCl. After incubation, the supernatant was removed and washed twice with buffered KCl. Then 2 ml of 0.5 N HCl were added to solubilize the S-HA beads. The number of bacterial cells which had attached to the S-HA beads was determined by turbidity at 660 nm, and each percentage of desorption was calculated. All assays were done in triplicate and expressed as mean values.

5. Measurement of zeta potential

The zeta potentials of *S. sobrinus* 6715 and *S. mitis* 9811 were measured by using a microelectrophoresis apparatus (Laser Zee, Model 501, Pen Ken Co., New York, USA)⁹⁾. Before electrophoresis, the bacteria were suspended to an appropriate density (10⁸ CFU/ml) and mixed with various amounts of chitosan concentrations (25, 250, 2,500, and 5,000 ppm). These suspensions were then poured into a glass cell that had been thoroughly washed and rinsed several times in distilled water. All assays were done 10 times and expressed as mean values.

6. Measurement of hydrophobicity

The measurement of hydrophobicity was based on the adherence of bacterial cells to hexadecane³⁶⁾. A 1.2 ml aliquot of washed cell suspension (optical density of 1.0 at 660 nm) was mixed with 0.2 ml of hexadecane for 60 sec and stand at room temperature for 20 min. The lower aqueous phase was then removed, and the OD₆₆₀ of the suspension was measured with a spectrophotometer. The results were expressed as the percentage of the initial OD₆₆₀. All assays were done in triplicate and expressed as mean values.

RESULTS

1. Adsorption inhibiting assay

The influence of chitosan molecular mass and degree of deacetylation on the adsorption of *Streptococcus sobrinus* 6715 to S-HA is shown in Figs. 1 and 2. A linear relationship ($R=0.876$) was observed between the molecular mass and the % inhibition of adsorption of bacterial cells to S-HA (Fig. 1). More than 70% of the adsorption of bacterial cells to S-HA was suppressed by the chitosan with molecular mass of 5–6 kDa. The optimum degree of deacetylation was about 60% for maximum inhibition of adsorption to S-HA. More than 90% of the adsorption of *S. sobrinus* 6715 to S-HA was inhibited by the chitosan with degree of deacetylation around 60% (Fig. 2).

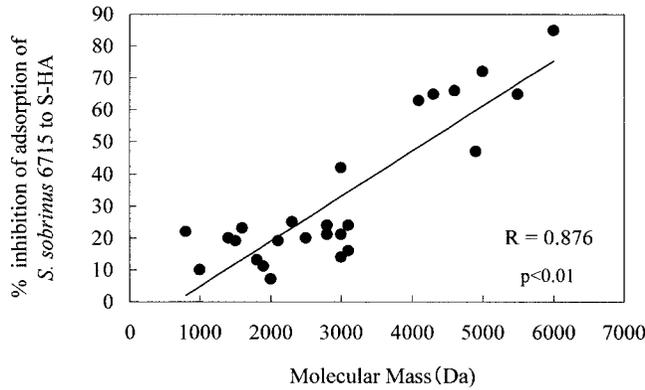


Fig. 1 Effect of the molecular mass of chitosan on inhibition of the adsorption of *Streptococcus sobrinus* 6715 to S-HA

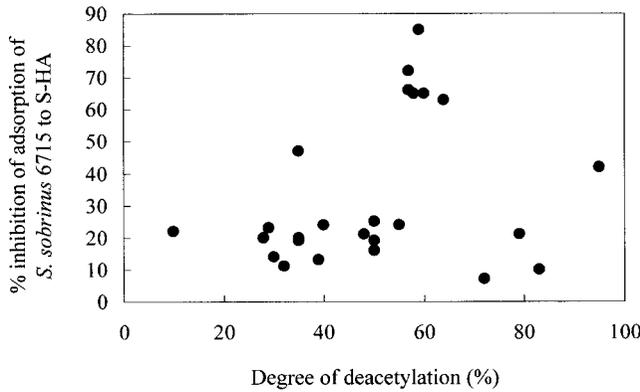


Fig. 2 Effect of the degree of deacetylation of chitosan on the inhibition of adsorption of *Streptococcus sobrinus* 6715 to S-HA

2. Desorption assay

The influence of molecular mass and degree of deacetylation on the desorption of cells previously adsorbed to S-HA is shown in Figs. 3 and 4. There was a relatively low correlation ($R=0.351$) between the degree of molecular mass of chitosan and % desorption of previously attached cells from S-HA (Fig. 3). However, the chitosan with the degree of deacetylation of 50-60% showed the strongest desorption activity among the samples tested (Fig. 4). It desorbed more than 70% of preadsorbed bacterial cells from S-HA.

3. Change of the zeta potentials and cell surface hydrophobicity of two kinds of bacteria

The results indicated that increasing concentrations of chitosan in bacterial suspensions gave a successive decrease of the zeta potentials in a range of negative values (Fig. 5). The bacterial hydrophobicities decreased with increasing chitosan concentrations in bacterial suspensions (Fig. 6).

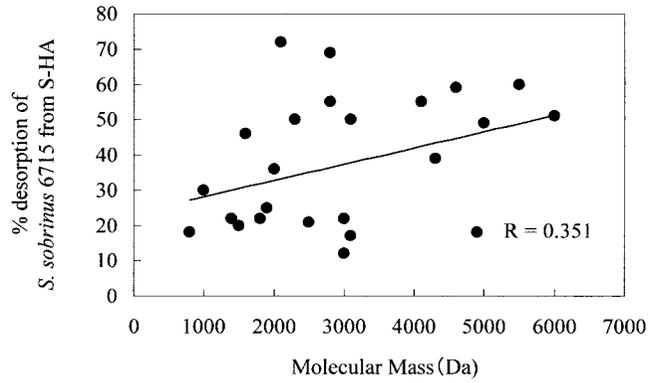


Fig. 3 Effect of the molecular mass of chitosan on the desorption of *Streptococcus sobrinus* 6715 from S-HA

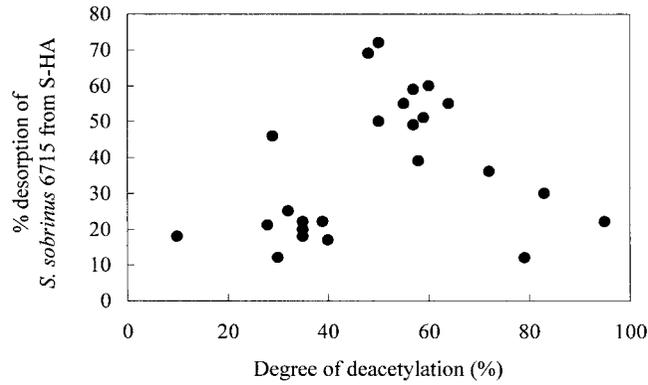


Fig. 4 Effect of the degree of deacetylation of chitosan on the desorption of *Streptococcus sobrinus* 6715 from S-HA

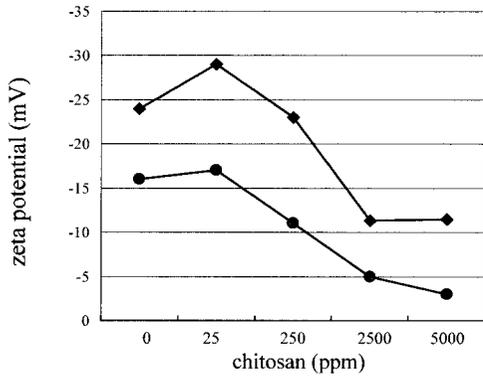


Fig. 5 Effect of chitosan on the zeta potential of *Streptococcus sobrinus* 6715 and *Streptococcus mitis* 9811
 ●: *S. sobrinus* 6715, ◆: *S. mitis* 9811

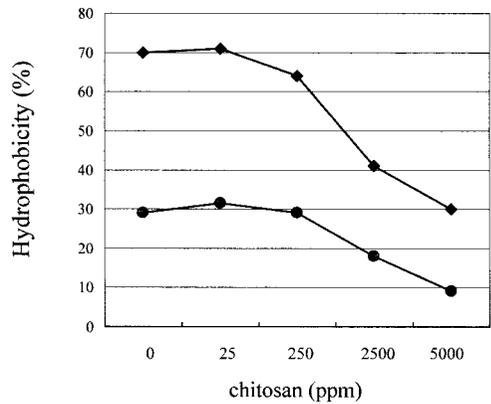


Fig. 6 Effect of chitosan on the hydrophobicity of *Streptococcus sobrinus* 6715 and *Streptococcus mitis* 9811
 ●: *S. sobrinus* 6715, ◆: *S. mitis* 9811

DISCUSSION

Our previous *in vitro* study²⁰⁾ showed that chitosan moderately inhibited the adsorption of *Streptococcus sobrinus* 6715 to S-HA. Tarsi *et al.*²⁴⁾ also 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 assumed that the mechanism of anti-adherence activity seems to involve (1) bacterial surface modifications, (2) alterations in the expression levels of bacterial surface ligands, and (3) chitosan adsorption to host surfaces to change hydroxyapatite ionic properties.

In the study, we attempted to clarify the influence of average molecular mass and degree of deacetylation of chitosan on the binding ability of *S. sobrinus* 6715 to S-HA. The present study clearly demonstrated that chitosan having a molecular mass of 5–6 kDa is effective in inhibiting of adsorption of *S. sobrinus* 6715 onto S-HA and that a degree of deacetylation of 50–60% produces a maximal inhibitory effect on both the adsorption and desorption of *S. sobrinus* 6715 in S-HA binding assay.

Bough *et al.*¹⁾ reported that chitosan with a higher molecular mass was a more effective coagulating agent for activated sludge than the chitosan with the lowest molecular mass. Thus, higher values for molecular mass were predictive of greater effectiveness for coagulation of activated sludge suspensions. On the basis of mutans streptococci surface charge and characteristics of chitosan, we can say that chitosan interaction with mutans streptococci cells is electrostatic: polymer chains attach themselves to the negatively charged bacterial cell surface by means of their positively charged groups. If these chains are of sufficient length to bind more than one cell, bridges are formed between bacterial cells. Then, as the bridging becomes effective, flocs are formed¹⁾, and the mutans streptococci cannot adsorb onto S-HA.

We also found that chitosan with a degree of deacetylation of 50–60 % produced a maxi-

mal inhibitory effect on both the adsorption and desorption of *S. sobrinus* 6715 in S-HA binding assays. The mechanism behind this finding is not fully understood. Shibasaki *et al.*²²⁾ reported that chitosan with a molecular mass of 3 kDa and a degree of deacetylation of 50% was most effective in inhibition plaque pH fall following direct application of 5% glucose solution, contrary to their assumption. McNee *et al.*¹³⁾ also reported that the diffusion of electrolytes into dental plaque was inhibited by electrostatic interactions between the ions and the charged components of the plaque. Thus the electric properties, like the degree of deacetylation, could also be considerable factors in the interaction with dental plaque. If chitosans possess relatively low degrees of deacetylation, they don't have enough ability to bind both bacterial surface and S-HA by polycationic action. On the other hand, when they have a relatively high degree of deacetylation, chitosans form linearly chained structures due to intramolecular electrostatic repulsion. Therefore, both inhibition of the adsorption and the desorption of *S. sobrinus* 6715 in S-HA might be retarded by less entrapment or aggregation of bacteria because of the steric hindrance.

We also revealed that the zeta potential for bacterial cell surfaces increased with increasing chitosan concentrations in oral bacterial suspensions. This finding is similar to the colloidal study that examined the influence of a cationic detergent on the stability of bacterial suspensions²³⁾. These findings strongly suggested that, because the chitosan used in this study carried positive charges, it functioned as a polycationic agent by adsorbing onto cell surfaces and by bridging together into aggregates to prevent adsorption of bacteria onto S-HA.

We also showed that increasing concentrations of chitosan induced a successive decrease in cell hydrophobicity. It is generally thought that surface hydrophobicity is related to adherence ability¹⁶⁾; therefore, the inhibiting effects of chitosan may depend, at least partially, on alterations of bacterial cell surface hydrophobic expression.

In conclusion, chitosan with a molecular mass of 5–6 kDa and a degree of deacetylation of 50–60% might have the potential to be an effective anti plaque agent.

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