Original Article *J. Clin. Biochem. Nutr.*, **44**, 275–279, May 2009

Inhibitory Effects of Partially Decomposed Alginate on Production of Glucan and Organic Acid by *Streptococcus sobrinus* 6715

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Received 9 October, 2008; Accepted 2 December, 2008

Summary **Our previous study has already clarified that partially decomposed alginate (Alg53) by *Vibrio alginolyticus* SUN53 has a competitive inhibitory effect on sucrase. The objective of this study is to evaluate the influence of Alg53 on the production of glucan from sucrose by glucosyltransferase and acid from glucose by *Streptococcus sobrinus* 6715. Glucosyltransferase was prepared from cultural medium of *S. sobrinus* using ultrafiltration and hydroxyapatite chromatography. In order to examine the inhibitory effect of Alg53 for production of glucan by GTase, partially purified GTase, sucrose and Alg53 solution were incubated at 37°C. The influence of Alg53 on the production of acid from glucose was evaluated by a degree of pH decline during the incubation for 60 min. The original Alg53 solution markedly inhibited to 21% of the synthesis of water-insoluble glucan from sucrose and that of 10-fold diluted of Alg53 solution was 23%. However, the production of water-soluble glucan from sucrose by GTase was hardly affected by Alg53. Furthermore, Alg53 suppressed dose-dependently pH decline by organic acid converted from glucose. These results suggest that Alg53 is expected as a functional food material which prevents or reduces dental caries.**

Key Words**: partially decomposed alginate, glucan, *Streptococcus sobrinus* 6715, acid production, dental caries**

Introduction

Dental caries is induced multiple factors which are host, bacteria and substrate [*1*]. This disease is one of the most prevalent diseases all over the world. According to odontopathy investigation of actual conditions in 2005 in Japan, the prevalence of dental caries in prepuberal period tends to decrease. But, the prevalence is more than 70% in 13 years old [*2*]. This proportional is unique in diseases of digestive organs by ICD10. In addition, if they infect once dental caries, it doesn't autotherapy. Therefore, it is very important that we prevent dental caries.

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Mutans streptococci, particularly *Streptococcus mutans* and *Streptococcus sobrinus* are considered as the primary causative agents of dental caries. These bacteria produce glucosyltransferases (GTase) that synthesize water-insoluble and -soluble α-linked glucans from sucrose. And then they adhere on the tooth surface with other oral bacteria. Consequently, the adhesion of glucan brings about the formation of dental plaque. Furthermore, these bacteria in dental plaque also produce organic acids which cause the enamel demineralization. If the production of glucan by GTase can be inhibited or reduced, oral bacteria can not adhere tooth surface and dental caries is prevented. In addition, if the production of acid from sucrose by mutans streptococci is decreased, the buffer action capacity of saliva prevents the enamel demineralization.

To prevent dental caries, many studies on the anti-cariogenic effect of natural materials have been reported.

Hayacibara *et al.* found that extracts of propolis inhibited GTase and had an anticariogenic effect in rat [*3*]. Nakahara *et al.* reported that Oolong tea polyphenols inhibited GTase [*4*]. Cacao polyphenols [*5*] and apple polyphenols [*6*] also have the inhibitory effects on GTase. Acarbose and 1-deoxynojirimycin are known to inhibit sucrase, and to inhibit GTase [*7*].

Alginate, which is a copolymer of α-L-guluronate and β-D-mannuronate, is a gelling polysaccharide found in great abundance as part of the cell wall and intracellular material in the brown seaweeds [*8*]. Our previous study have clarified that partially decomposed alginate by *Vibrio alginolyticus* SUN53 (Alg53) had a competitive inhibitory effect on sucrase of rat intestinal brush border membrane vesicles [*9*]. The molecular weight was estimated approximately 1,000 by gel chromatography. Tseng *et al.* have already reported that the alginate lyase isolated from *Vibrio alginolyticus* (ATCC17749) has a specificity for poly mannuronic block [*10*], and Haug *et al.* reported that depolymerizing alginate by lyase has a product containing deoxy-uronic acid [*11*]. Therefore, we suppose that Alg53 is also penta- or hexa-mannuronic acid with deoxy-mannuronic acid as the non-reducing terminal moiety.

Acarbose and 1-deoxynojirimycin also inhibit sucrase competitively. We hypothesized that Alg53 would inhibit the synthesis of glucan by GTase, because GTase is also a sort of enzymes which is related to carbohydrate metabolism.

S. sobrinus secretes four types of GTases such as GTase-I, GTase-S_1, GTase-S_2 and GTase-S_3 [*12*]. GTase-I catalyzes the synthesis of water-insoluble glucan, which consists of principally α-1,3 linked glucan. GTase-S_1 catalyzes the synthesis of high branched water-soluble glucan, which consists of a mixture of α-1,3 linked glucan and α-1,6 glucan. GTase-S_2 and S_3 catalyze the synthesis of water-soluble glucan, which consist of principally α-1,6 linked glucan. *S. sobrinus* secretes these enzymes to extracellular. We collected the supernatant of cultural medium and partially purified GTase. This study aimed to evaluate the effects of Alg53 on synthesis of glucan by GTase and the production of acid by *S. sobrinus*.

Materials and Methods

Preparation of partially decomposed alginate by SUN53 (Alg53)

Vibrio alginolyticus SUN53 (NITE-P-14), which was isolated by Ueda S. from the sandy beach, was grown in 400 ml of culture medium〔Alginate-Na (Solgin fiber®, Average M.W.55,000), 0.5%; Yeast extract, 0.025%; Peptone, 0.05%; NaCl, 1.0%; $FePO_4$, 0.01%; SUN53, 12×10^6/ml〕with shaking culture at 25°C for 5 days. After the cultivation, the medium was centrifuged at 12,000 × *g* for 30 min at 4°C. The supernatant was added to 3 volumes of ethanol and stood for 20 h at 25°C. The treated supernatant was then centrifuged at same condition, and the collected supernatant was evaporated using an evaporator (Rotavapor R-200, Sibata Scientific Technology Ltd., Tokyo, Japan). The concentrated solution was lyophilized and the obtained powder was then dissolved in distilled water of one-tenth volumes of the cultural medium (original Alg53 solution) and stock at −20°C [*9*].

Preparation of GTase

Streptococcus sobrinus 6715, which was kindly provided by Dr. Imai S. from the National Institute of Infectious Disease (Tokyo, Japan), was grown for 24 h at 37°C in 2 L of Brain Heart Infusion (Difco, Sparks, MD). The cultural medium was centrifuged at 12,000 × *g* for 30 min at 25°C, and the protein in the supernatant was precipitated with 60% saturated $(NH_4)_2SO_4$ for 24 h at 4°C. The precipitate collected by recentrifugation was dissolved in 50 ml of 10 mM phosphate buffer (pH 6.8) and the small-sized proteins in the solution were removed using an ultrafiltration (M.W.<30,000) (Millipore Co., Bedford, MA).

Furthermore, the fluid containing crude GTase with more than 30,000 M.W. was partially purified by hydroxyapatite chromatography using a Bio-gel HTP (Bio-rad, California, Hercules, CA) column (500 mm × 20 mm) [*13–15*]. The column was first washed with 300 ml of 10 mM phosphate buffer (pH 6.8), and the enzyme was eluted with a linear gradient from 0.1 M (200 ml) to 0.6 M (200 ml) phosphate buffer (pH 6.8) (total 400 ml) containing 1 mM PMSF at a flow rate of 0.6 ml/min. 5.3 ml of effluent were collected into a test tube. GTase was eluted to around 0.5 M concentration of phosphate, and the collected fractions were used as the partially purified enzyme solution in the subsequent assays. The protein concentration of this enzyme solution was 50 μg/ml by Lowry method [*16*].

Inhibitory effect of Alg53 on glucan produced from sucrose by GTase

The substrate solution was 3% sucrose in 0.1 M phosphate buffer (pH 6.8). To measure the inhibition by Alg53 for the synthesis of glucan from sucrose, 1 ml of 3% sucrose solutions, 0.3 ml of the GTase solutions, 0.3 ml of Alg53 solution, and 1.4 ml of 0.1 M phosphate buffer were mixed and incubated at an angle of 20° for 24 h at 37°C (final concentration of sucrose; 1%). A control was used to measure the full activity of GTase that contained 0.3 ml of distilled water instead of Alg53 solution. The reaction was stopped in boiling water for 5 min. To separate of water-insoluble and water-soluble glucans, the reaction mixture was centrifuged at 2,100 × *g* for 20 min at 25°C. The precipitation containing water-insoluble glucan was washed twice with distilled water. To collect water-soluble glucan, the supernatant of reaction mixture was precipitated with 3

volumes of ethanol for 20 h at 25°C. The amount of total carbohydrate was measured by the phenol-sulfuric acid method using glucose as a standard [*17*].

Effects of Alg53 on the production of acid by S. sobrinus in vitro

To prepare the cell pack solution, *S. sobrinus* 6715 was cultured in BHI broth for 24 h at 37°C, and the cells were collected by centrifugation. After the cells were washed with Stephan's buffer (pH 7.0), they were suspended in adequate amount of the same buffer.

Next, 0.5 ml of cell pack solution, 0.5 ml of 20 mM glucose in Stephan's buffer (pH 7.0), and 0.2 ml of Alg53 original solution were mixed and incubated for 60 min at 37°C. During the incubation, the pH of the reaction medium was measured at 15-min intervals. The positive control not to produce the acid contained Stephan's buffer (pH 7.0) instead of glucose solution, and the negative control not to inhibit the production of acid contained distilled water instead of Alg53 solution. To observe the dose dependently suppressive effect of Alg53 on the production of acid, we carried out assay above using Alg53 ×2 and ×5 diluted solutions. We incubated 0.5 ml of cell pack solution, 0.5 ml of 20 mM glucose in Stephan's buffer (pH 7.0), and 0.2 ml of Alg53 original solution, ×2 or ×5 diluted solutions at 37°C, for 20 min. After incubation, we measured pH with a pH meter (pH/Ion Meter, Horiba Ltd., Kyoto, Japan).

The data was expressed the average values of duplicate assays in all experiments.

Results

Effects of Alg53 on water-insoluble and water-soluble glucan synthesis by glucosyltransferase

Water-insoluble and water-soluble glucan synthesis by GTase from *S. sobrinus* is illustrated in Fig. 1. When Alg53 was not added to the reaction mixture, water-insoluble and -soluble glucans were produced 234 μg/ml and 697 μg/ml, respectively. The original Alg53 solution and a 10-fold dilution of Alg53 solution reduced the amount of production of water-insoluble glucan to 21% (49 μg/ml) and 23% (52 μg/ml), respectively. These results have demonstrated that partially decomposed alginate by *Vibrio alginolyticus* SUN53 inhibits the synthesis of water-insoluble glucan by GTase from *S. sobrinus*. However, Alg53 hardly affected the production of water-soluble glucan by GTase.

Effects of Alg53 on the production of acid by S. sobrinus

Fig. 2 shows the results regarding the evaluation of organic acid production by *S. sobrinus* in the presence of Alg53 during 60 min of incubation. The positive control maintained the initial pH. The results indicate that Alg53 disturbs the conversion of substrate to organic acid. In contrast, the absence of Alg53 resulted in an immediate decline in pH after addition of the substrate, with the pH finally reaching 4.1. The addition of Alg53 suppressed pH decline and maintained a pH of 5.0. This suppressive effect for the production of organic acid was dependent on the concentrations of Alg53 in the reaction mixture (Fig. 3). However, only the original solution suppressed the decline of pH and maintained the upper pH than the critical pH.

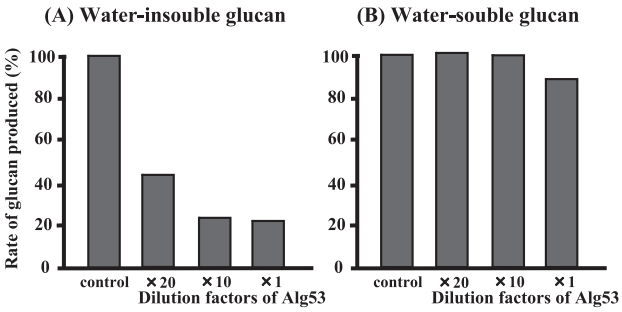

Fig. 1. Inhibitory effect of partially decomposed alginate by SUN53 on water-insoluble (A) and water-soluble glucan (B) produced by GTase. Reaction mixture [3% sucrose (final concentration 1%) in 0.1 M phosphate buffer (pH 6.8), 1 ml; GTase, 0.3 ml; 0.1 M phosphate buffer (pH 6.8), 1.4 ml; Alg53, 0.3 ml] incubated at a 20° angle for 24 h at 37°C. It was then boiled for 5 min to stop the reaction, and water-insoluble glucan was obtained for centrifugation and washing twice the precipitate with distilled water. To collect water-soluble glucan, the supernatant was precipitated with three volumes of ethanol for 20 h at 25°C. Glucan produced expressed as the relative amount (%) of glucan produced as compared to the amount produced in the absence of Alg53. The amount of total carbohydrate was measured at 490 nm by the phenol-sulfuric acid method using glucose as a standard (200 μg/ml). The data was expressed the average values of duplicate assays.

Discussion

The main finding of this study is that partially decomposed alginate (Alg53) by SUN53, which has an inhibitory effect on sucrase, also inhibits the production of glucan from sucrose by GTase. Especially, the inhibitory effect of Alg53 was remarkable for the production of water-insoluble glucan. In addition, Alg53 suppressed pH decline by the production of organic acid from glucose.

In this study, partially purified GTase from *S. sobrinus* was used for the production of glucan. *S. sobrinus* and *S. mutans* among mutans streptococci are primary oral cariogenic bacteria for human. It has been reported that *S. sobrinus* is more acidogenic and cariogenic than *S. mutans* in animals [*18*]. In addition, several epidemiological studies

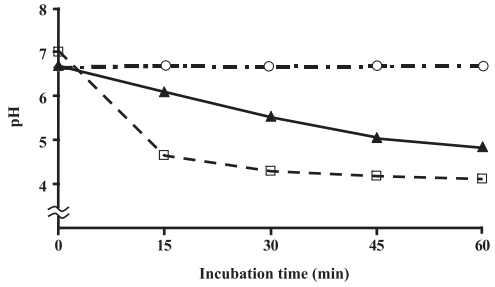

Fig. 2. Inhibitory effect of partially decomposed alginate by SUN53 on acid production from glucose by *S. sobrinus*. Open circle, positive control (no acid production); open square, negative control (no inhibition); closed triangle, with Alg53. 0.5 ml of cell pack solution, 0.5 ml of 20 mM glucose in Stephan's buffer (pH 7.0), and 0.2 ml of Alg53 were incubated at 37°C, and pH was measured at 15-min intervals for an hour with a pH meter. In the positive control (no acid production), Stephan's buffer (pH 7.0) was added instead of glucose. In the negative control (no inhibition), distilled water was added instead of Alg53. The data was expressed the average values of duplicate assays.

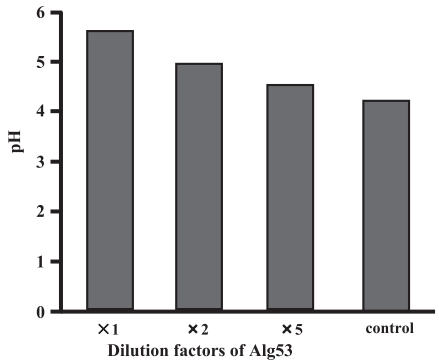

Fig. 3. Inhibitory effects by different concentration of partially decomposed alginate by SUN53 on acid production from glucose by *S. sobrinus*. 0.5 ml of cell pack solution, 0.5 ml of 20 mM glucose in Stephan's buffer (pH 7.0), and 0.2 ml of Alg53 original solution, ×2 or ×5 dilution were incubated at 37°C, for 20 min. After incubation, we measured pH with a pH meter. In the negative control (no inhibition), distilled water was added instead of Alg53. The date was expressed the average values of duplicate assays.

suggested that the presence of *S. sobrinus* is associated with high numbers of salivary mutans streptococci and with severe caries prevalence [*19–21*]. These reports indicate that *S. sobrinus* has a severe influence for dental caries.

Alg53 strongly inhibited the production of water-insoluble glucan by GTase from *S. sobrinus* in the present study, while Alg53 hardly affected for the production of water-soluble glucan by GTase. However, if we could prepare Alg53 which is more high concentration, it may provide a clear inhibitory effect on the production of water-soluble glucan, because the original Alg53 solution with highest concentration in this study slightly reduced the production of water-soluble glucan. Either way, these results suggest that Alg53 might be effective on the suppression of dental caries.

In addition, the water-soluble glucan fraction was colored brown, which was derived from the color of Alg53. It might be suggested that Alg53 cut in a reaction system of GTase and sucrose, and combine with free glucose from sucrose to synthesis original oligosaccharide or polysaccharide like panose [*22*] and isomaltose [*23*].

Phenol-sulfuric acid method determines whole carbohydrate in sample without difference between glucan and original oligosaccharide including Alg53. We therefore intend to carry out a future study regarding determination of ^{14}C transfer glucan from sucrose by GTase.

On the other hand, Alg53 suppressed pH decline by acid production. This effect was dose dependency. Oral pH declines about 4.0 immediately after the ingestion of glucose [*24*]. When oral pH declines about 5.5, enamel demineralization begins [*25*]. With addition of Alg53, pH decline suppressed that compared with control. This finding suggests that Alg53 is useful material for functional food on prevention of dental caries.

In conclusion, Alg53 had inhibitory effect on water-insoluble glucan production and suppressive effect on pH decline. But Alg53 hardly affected on water-soluble glucan production.

Our previous study demonstrated that Alg53 inhibited α-glucosidase, especially sucrase. Therefore Alg53 is expected of multiple functional food material which has effects of prevention to dental caries and diabetes.

Acknowledgements

The authors wish to thank Dr. Imai S for his kind offer of *Streptococcus sobrinus* 6715 and Kaigen Co., Ltd. (Osaka, Japan) for providing the partially hydrolyzed alginate. This study was partially supported by Siebold University of Nagasaki Fund B for Promoting Academic and Education Research.

Abbreviations

SUN53, *Vibrio alginolyticus* SUN53; Alg53, partially decomposed alginate by SUN53; GTase, glucosyltransferase; BHI, Brain Heart Infusion.

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