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Inhibitory Effects of the Phytochemicals Partially Hydrolyzed Alginate, Leaf Extracts of *Morus alba* and *Salacia* Extracts on Dental Caries

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1. Introduction

Many studies on natural materials with anticariogenic effects have been carried out. Anticariogenic materials, such as polyphenols from oolong tea (Nakahara et al, 1993) and polyphenols from cacao (Ito et al, 2003), are known to be inhibitors of glucosyltransferases (GTases). These compounds have been used in foods to prevent or reduce dental caries. Sugar alcohols and oligosaccharides, which are not utilized as the substrate of GTase, are known as alternative sweeteners to sucrose (Kawanabe et al, 1992; Makinen et al, 1995; Ooshima et al, 1992; Van Loveren, 2004).

We investigated the potentiality of inhibitory effects of some phytochemicals on dental caries, because it is very interesting that phytochemical components inhibit the activity of not only GTase but also α-glucosidase. We have clarified that some phytochemicals such as partially decomposed alginate (Alg53), extractives from the leaves of *Morus alba* (ELM) and extractives from *Salacia chinensis* (ES) have inhibitory effects on disaccharidases such as maltase, sucrase and trehalase.

Alginate is a polyuronic saccharide that is isolated from the cell walls of a number of brown seaweed species around the world, and produced as an extracellular matrix by certain bacteria (Draget et al, 2003). It has a gelling ability, stabilizing properties and high viscosity. Alginate and its decomposed derivatives are widely used in foods, cosmetics and pharmaceutical industries (Ci et al, 1999; Johnson et al, 1997). Alginate hydrolysates exhibit many bioactivities, such as stimulating human keratinocytes, accelerating plant root growth and enhancing penicillin production from cultures of *Penicillium chrysogenum* (Ariyo et al, 1998; Kawada et al, 1997; Natsume et al, 1994). We have clarified that alginic acid with lowered molecule (mean molecular weight about 55,000) has suppressive effects on the elevation of blood glucose and insulin secretion (Oku et al, 1997) and improves defecation ant the fecal conditions (Oku et al, 1998).

Morus alba has traditionally been cultivated in China, Korea and Japan to use its leaves to feed silkworms. Recently, health benefits of *Morus alba* have been clarified and naturally occurring 1-deoxynojirimycin (DNJ) was isolated from its roots (Yagi et al, 1976). DNJ is glucose analogue with a secondary amine group instead of an oxygen atom in the pyranose

ring of glucose. Then, DNJ has also been found in the leaves and fruits of *Morus alba* (Asano et al, 1994, 2001). Ever since, preventive effect of *Morus alba* on diabetes by α-glucosidase inhibitor has been extensively studied. Furthermore, *Morus alba* has been clarified multiple biological and physiological effects, as well as hypoglycemic, anti-oxidant and decrease in serum triacylglycerol (TG) level (Kojima et al, 2010). In a long term treatment study, intake of *Morus alba* does not cause harmful effects (Kimura et al, 2007).

The roots and stems of *Salacia* species plants have been used in the Ayurvedic system of Indian medicine to treat diabetes mellitus (DM) (Li et al, 2008). *Salacia* is a woody climbing plant belonging to the Celastrceae family that is found in limited regions of India and Sri Lanka. Currently, extracts of *Salacia* are consumed in commercial foods and food supplements in Japan for the treatment of diabetes and obesity. The water soluble portion of the methanolic extract inhibits α-glucosidase. Moreover, Beppu et al have reported that *Salacia reticulata* has improvement effect of fasting blood glucose and HbA1c levels in human including mild type 2 diabetics and has no toxicity (Beppu et al, 2006). The potential genotoxicity of *Salacia oblonga* extract was evaluated and it was determined not to be genotoxic (Flammang et al, 2006).

These phytochemicals, Alg53, ELM and ES competitively inhibit sucrase, maltase and trehalase of vesicles of the brush border membrane of rat intestine. The activity of GTase is inhibited by some polyphenols. We hypothesized that phytochemicals that inhibit α-glucosidases such as sucrase may also inhibit the synthesis of glucan from sucrose by GTase, because the latter is also a type of enzyme related to carbohydrate metabolism. Conversely, acarbose and DNJ are known to competitively inhibit sucrase and GTase, and suppress the postprandial elevation of blood levels of glucose and insulin (Newbrun et al, 1983). These chemicals are used as medicine for the treatment of DM.

We have investigated the anticariogenic effect of some phytochemicals using simple *in vitro* methods. The inhibitory effect of phytochemicals on the production of glucan from sucrose by GTase can be used for screening of the anticariogenic effects of natural materials. Surveys of natural materials with anticariogenic effects are important for reduction of the development of dental caries. Discovering new materials to prevent dental caries could expand the repertoire of the development of functional foods for oral health. These functional foods for oral health should be used in combination with different types of materials because the development of dental caries is related to multiple factors.

In this chapter, we introduce the procedures employed to evaluate the anticariogenic effects of phytochemicals. We also discuss the properties of three phytochemicals, Alg53, ELM and ES. Although many natural materials have been studied for anti-cariogenic effect, in our knowledge, this is the first and unique report that α-glucosidase inhibitor also inhibit GTase activity in natural materials. That is to say, Alg53, ELM and ES are expected as multiple functional food materials which have the effects of prevention to dental caries, diabetes and obesity. Moreover, if we search for natural materials that inhibit GTase, it might be a key point that certain materials have inhibition of α-glucosidase activity.

2. Evaluation of the anticariogenic effects of phytochemicals

Evaluation of the anticariogenic effects of phytochemicals is often tested on animals and eventually in humans. The *in vitro* experiments should be first done using a simple method.

Because the inducing factor of dental caries is complicate, some types of *in vitro* experiments have to be carried out and judged carefully to recognize characteristics of phytochemicals. Several typical methods are described below.

2.1 *In-vitro* experiments to evaluate anticariogenic effects

In vitro experiments to evaluate the anticariogenic effects of phytochemicals include observations of pH decline by acid production, inhibitory effects on glucan production by GTase from mutans streptococci and sucrose-dependent cell adhesion on smooth surfaces by mutans streptococci. In addition, antibacterial effect and the evaluation of plaque accumulation or enamel demineralization by using artificial mouth also have been investigated (Hinoide et al, 1984; Pigman et al, 1952). We have evaluated the anticariogenic effects of phytochemicals based on pH decline, glucan production by GTase and sucrose-dependent cell adhesion.

2.1.1 Evaluation of inhibitory effects on pH decline by acid production

Oral bacteria produce organic acids from sugars. If oral pH declines to approximately 5.5 due to organic acids produced, enamel demineralization of teeth begins. If organic acids produced from sugars by mutans streptococci are omitted or decreased and oral pH does not decline to 5.5, enamel demineralization is prevented by buffering action of saliva. Therefore, the degree of pH decline of the culture medium is measured during the incubation with oral bacteria (especially mutans streptococci) in the presence and absence of test substance with anticariogenic effects. Our procedure to measure pH decline is rapid and reliable. Cell suspension and phytochemical solution are added to 20 mM glucose solution in Stephan's buffer (pH 7.0) in a test tube (total volume, 1.0–2.0 mL), and incubated for 10–60 min at 37°C under anaerobic conditions after mixing. During incubation, the pH of the reaction medium is periodically measured with a portable pH meter (Hashiguchi-Ishiguro et al, 2009). Another method uses an incubation period of 24-48 h. But oral pH declines to about 4 immediately after the ingestion of sugars as glucose and sucrose (Lingström et al, 2000). Actually, the pH of reaction medium resulted in an immediate decline within 30 min in our study. In practical eating, if we intake sugars from meals or snacks, the food is masticated and swallowed within a few minutes and the pH in oral cavity declines. Therefore, the rapid and reliable method is suitable for *in vitro* assay.

2.1.2 Evaluation of inhibitory effects on glucan production by GTase

Water-insoluble and water-soluble α-linked glucans produced from sucrose due to the action of GTases adhere to the surfaces of teeth and promote the development of dental caries. GTase inhibitors disturb the production of these glucans and prevent the development of dental caries. The inhibitory effect of test substances on GTase has been evaluated by partially purified GTase from mutans streptococci, particularly *Streptococcus mutans* and *Streptococcus sobrinus,* which are considered to be the primary causative agents of dental caries in humans. Partially purified GTase can be conveniently used to evaluate the inhibitory effects of test substances on glucan production because it is stable and readily administered after preparation. If *S. mutans* and *S. sobrinus* are directly used to evaluate the inhibitory effects of test substances on glucan production, the assay is complicated and additional effort is required

for the storage and administration of mutans streptococci. Hence, we are using a partially purified GTase from *S. mutans* and *S. sobrinus* to evaluate the inhibitory effects on glucan production. *S. sobrinus* GTase which synthesizes the water-insoluble glucan is released into the reaction medium during culture and that of *S. mutans* is localized mainly on the cell surface (Furuta et al, 1985). The properties and preparation method of GTase have been described (Baba et al, 1986; Furuta et al, 1985; Hamada et al, 1989).

An outline of our procedure to evaluate the inhibitory effect of phytochemicals on glucan production by GTase is given here. To measure the inhibition by phytochemicals for the synthesis of glucan from sucrose, 1 mL of 3% sucrose solution, 0.3 mL of GTase solution, 0.3 mL of test solution, and 1.4 mL of 0.1 M phosphate buffer are mixed and incubated at an angle of 20° for 24 h at 37°C. After the reaction is stopped in boiling water, the reaction mixture is centrifuged to separate water-insoluble and water-soluble glucans. The amount of total carbohydrate is measured using the phenol-sulfuric acid method (Dubois et al, 1956).

The phenol-sulfuric acid method is a popular and simple method for the determination of glucan produced by GTase (Koo et al, 2002). However, this method randomly determines the amount of whole carbohydrate in the sample without a clear difference between glucan production and the structure of phytochemicals in the reaction medium. Therefore, if the structure of test phytochemicals is similar to glucose polymer, alternative method is recommended to evaluate the inhibitory effect on glucan production. One method is the determination of radioactive carbon (^{14}C) transferred to glucan from ^{14}C-sucrose by GTase. Briefly, ^{14}C-sucrose is added to the reaction mixture mentioned above and incubated under identical conditions. After incubation, the amount of ^{14}C incorporated into glucan is measured by a liquid scintillation counter. This method requires specific facilities, but can specifically determine glucose incorporated to glucan fraction by GTase.

The preparation of GTase to evaluate inhibitory effects upon glucan production was carried out in our experiments. Briefly, to prepare partially purified GTase, after *S. sobrinus* 6715 was grown for 24 h at 37°C in 2 L of Brain Heart Infusion (Difco, Franklin Lakes, NJ, USA), the supernatant containing GTase was precipitated with 60% saturated $(NH_4)_2SO_4$ for 24 h at 4°C. Low-molecular weight (<30,000) proteins contained in the precipitate were removed using an ultrafiltration system (Millipore, Billerica, MA, USA). The crude GTase obtained was further purified by chromatography using a Bio-gel hydroxyapatite (BioRad, Hercules, CA, USA) column. GTase fractions eluted to 0.5 M with a linear gradient from 0.1 M to 0.6 M phosphate buffer (pH 6.8) were used for the assay of inhibitory effects of phytochemicals (Venkitaraman et al, 1995; Yanagida et al, 2000).

After *S. mutans* MT8148 was grown for 24 h at 37°C in 2 L of Brain Heart Infusion (Difco), the collected cells were suspended with 8 M urea to obtain the cell-extract solution. Low-molecular weight (<30,000) proteins contained in the solution were removed using an ultrafiltration system (Millipore). The resulting solution was used to evaluate the inhibitory effects on glucan production (Yanagida et al, 2000).

2.1.3 Evaluation of inhibitory effects on sucrose-dependent cell adhesion on smooth surfaces by mutans streptococci

When mutans streptococci are cultured in a medium containing sucrose, they strongly adhere on smooth surfaces. Dental biofilms are formed on teeth surface by interaction

between glucan and oral bacteria. Therefore, the measurement of sucrose-dependent cell adhesion is used to evaluate the formation of biofilms on teeth surface. If sucrose-dependent cell adhesion on the smooth surface is inhibited by phytochemicals, the test substance indicates the possibility to reveal anticariogenic effects.

This procedure has been described by Hamada and Torii (Hamada and Torii, 1978). After mutans streptococci (*S. sobrinus* or *S. mutans*) are grown in the medium, the collected cells are resuspended in the medium. The cell suspension (0.5 mL), sucrose solution (final concentration, 1%) and 0.3 mL of phytochemical solution are mixed in a new glass test tube, and incubated at angle 20° for 24 h at 37°C. After incubation, the reaction mixture containing nonadherent cell is gently removed by a Pasteur pipette. The glass test tube upon which mutans streptococci adhere to the surface is gently washed with distilled water at angle 20°. In the control, cell and glucan are not peeled by washing because of those are tightly adhere on test tube. Then, the cell and glucan are suspended in 1 N NaOH to measure absorbance at 550 nm.

We have tried to evaluate the inhibitory effect of phytochemicals using this method, described below (Hashiguchi-Ishiguro et al, 2011). The degree of inhibitory effects on cell adhesion correlated roughly with inhibition of glucan production by phytochemicals. Anticariogenic effects of extractives from red wine, apple polyphenols and propolis are evaluated using this method (Furiga et al, 2008; Hayacibara et al, 2005; Yanagida et al, 2000). In this method, the surface of glass test tube is used for smooth surface model of teeth. The glass test tube used in this method must be new one that has very smooth surface with no flaw. Human saliva-coated hydroxyapatite beads are used as teeth surface model in other method (Venkitaraman et al, 1995), because teeth consists mainly of hydroxyapatite. The model of latter method reflects the situation of oral environment. Koo et al have reported the details of the procedure (Koo et al, 2002, 2010).

2.2 *In-vivo* experiments to evaluate anticariogenic effects

If anticariogenic effects of phytochemicals are demonstrated by some *in vitro* experiments as described above, an *in vivo* experiment using experimental animals (e.g., rats) is carried out to confirm that the test material has anticariogenic effects in the systemic body. The animal experiment to evaluate anticariogenic effects consumes much expense and time. In other method, human plaque is also used the *in vivo* experiment. "Touch electrode method" and "plaque sampling method" are used for the purpose of measuring human plaque pH (Frostell, 1970; Stephan, 1940). Mühlemann et al develop " indwelling plaque pH telemetry method" (Graf H, et al 1966). In this section, we show an outline on animal experiment.

2.2.1 Animals and diets to evaluate anticariogenic effects

Fifteen-day-old specific pathogen-free Sprague–Dawley (SD) rats are suitable for caries studies. The first and second molars are coming through at this age. Mutans streptococci are inoculated to animals during this period. If inoculation lags behind, the prevalence of dental caries is reduced (Ooshima et al, 1994). The number of mutans streptococci that must be inoculated to definitely cause dental caries is very important. The breeding period after inoculation with mutans streptococci is about 55 days. Diet #2000 is a popular diet in animal experiments on caries (Keyes and Jordan, 1964) and contains 56% sucrose. If the percentage

of sucrose is reduced, the prevalence of dental caries is also reduced. Phytochemicals are commonly added to the diet to evaluate anticariogenic effects. After breeding, the molar is removed and the degree of dental caries is scored. The details of the experimental protocol have been described (Ooshima et al, 1981; Tsunehiro et al, 1997). The typical procedure of caries scoring is the Keyes Caries Score (Keyes, 1958).

According to the established method, we have carried out animal experiments using phytochemicals that revealed anticariogenic effects *in vitro*. Young rats were fed with diet #2000 containing phytochemicals with anticariogenic effects for 60 days. However, our results were inconclusive. Therefore, we would like to describe some key points for the planning of animal experiments based on our experience.

2.2.2 Amount of mutans streptococci

If oral infection by mutans streptococci is not sufficient, dental caries is not induced in the experimental animal despite feeding with a caries-inducing diet. The amount of mutans streptococci adhered on teeth also influences the development of caries. Accordingly, the amount of mutans streptococci in the oral cavity should be measured periodically until the end of the experimental schedule. According to several studies, dental caries is definitely induced if the amount of mutans streptococci is $>10^5$ colony-forming units (CFU)/mL (Ooshima et al, 1993; Tsunehiro et al, 1997).

2.2.3 Amount of sucrose intake and texture of diets

Dental caries is positively correlated with the amount of sucrose intake (Sreebny, 1982). Therefore, the amount of diet that the animals ingest needs to be equal among feeding groups. Furthermore, the Vipeholm Dental Caries Study clarified that the texture of food containing sucrose influences the occurrence of dental caries (Gustafsson et al, 1954). In that study, subjects ate several foods (e.g., bread, chocolate, caramel) containing sucrose. The incidence of caries was higher in the group consuming “gooey" foods between meals than in the control group. Namely, the ingestion of sucrose that causes the adhesion to the teeth surface becomes a high risk of dental caries induction. Therefore, the texture and configuration of test materials containing phytochemicals added to the animal experimental diet are important to get significance. If the texture and taste of test substances are unique and likely to influence intake and adhesion, the method to reduce these factors should be implemented.

2.2.4 The indirect effect of anticariogenic substances on body except for tooth

The test substance might have multiple functions apart from anticariogenic effects. Test substances such as ELM, ES and Alg53, which have been used in our experiments, have inhibitory effect on α-glucosidase. Therefore, if experimental animals are given an α-glucosidase inhibitor and sucrose, the latter is not digested by intestinal disaccharidases and reaches the large intestine, where it is fermented by microbiota. These intestinal microbiota produce short-chain fatty acids, CO_2, NH_4 and H_2 (Oku, 2005). This action is similar to that of prebiotics such as non-digestible oligosaccharides and sugar alcohols ingested orally. These short-chain fatty acids are energy sources for the host and improve intestinal microflora. In this way, sucrose (the digestion of which in the small intestine is inhibited by

α-glucosidase) provides many beneficial effects. However, if a large amount of sucrose and α-glucosidase are ingested simultaneously, transient diarrhea is caused because of an increase in osmotic pressure in the large intestine. This mechanism is thought to identical to that of lactose intolerance.

When a diet containing ELM or ES is added to Diet #2000 and given to rats, most of the rats suffer osmotic diarrhea during the experimental period and growth is slightly suppressed. ELM and ES strongly inhibit sucrase activity. Hence, a lot of sucrose of Diet #2000 is transferred to the lower intestine and may cause osmotic diarrhea. Osmotic diarrhea may reduce the immune response, and disturb anticariogenic effect of phytochemicals in experimental animals. If experimental animals catch illness except for dental caries during the experiment, the risk of dental caries infection may increase. Therefore, the properties and functional effects of test substance apart from anticariogenic effects need to be examined, and the concentration and form of test substance added to diets should be investigated carefully before carrying out animal experiments.

3. Preparation and property of phytochemicals with anticariogenic effect

We have investigated the anticariogenic effects of phytochemicals such as ELM, ES and Alg53. Each of phytochemicals has unique properties and structure.

3.1 Extractive from the leaves of *Morus alba* (ELM)

Morus alba has been used for centuries in Japan as a tea infusion. *Morus alba* contains DNJ and some of its derivatives, which are well known as α-glucosidase inhibitors, as shown in Fig. 1 (Asano et al, 1994). D-glucose analogs such as voglibose, miglitol and acarbose, with nitrogen-in-rings, have been used for the treatment of DM (Drent et al, 2002; Raimbaud et al, 1992; Yasuda et al, 2003).

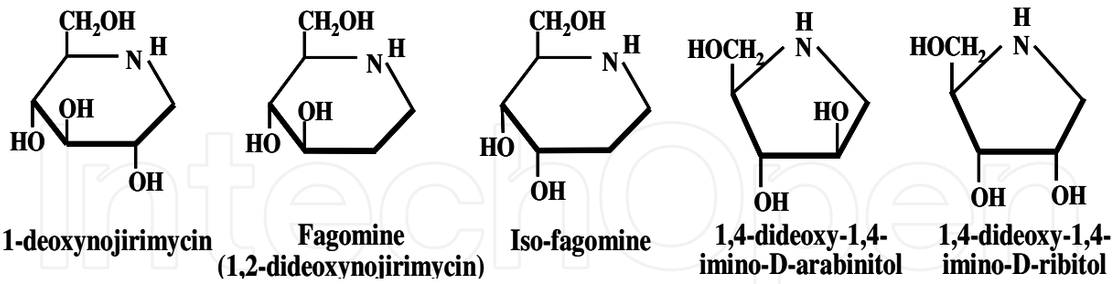


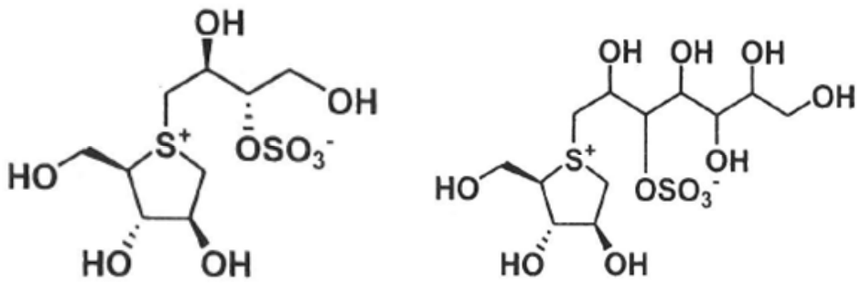
Fig. 1. Chemical structures of components of the extractive from the leaves of *Morus alba*, 1-deoxynojirimycin and its derivatives

We have clarified that ELM competitively inhibits the activity of sucrase, maltase, and isomaltase using human and rat intestinal homogenates, and significantly suppresses the increment in blood glucose levels, when ELM is administered with sucrose to rats (Oku et al, 2006). In addition, we found that confections with ELM effectively suppress the post-prandial blood levels of glucose and insulin in healthy humans (Nakamura M et al, 2009). We suppose that confections with ELM can contribute to the prevention and the quality-of-life for pre-diabetic and diabetic patients.

To prepare the ELM solution, the leaves are extracted with 50% ethanol, and ethanol is removed with a rotary evaporator. ELM used in this study is kindly provided by Toyotama Healthy Food Co., Ltd. (Tokyo, Japan). The original extract solution contains 0.24% DNJ. A small amount of several types of DNJ derivative is measured using liquid chromatography-mass spectrometry (LC-MS). It has been clarified that this extraction is not associated with toxicity or hematologic, blood biochemical, or pathologic abnormalities in rats (Miyazawa et al, 2003).

3.2 Extractive from *Salacia chinensis* (ES)

The stems of *Salacia* species plants are pulverized and extracted with methanol for 3 h at 80°C. After filtration, the extract is evaporated to obtain a powder. The powder of the methanol extract is dissolved and purified using Sephadex LH-20 column chromatography. ES used in this study is kindly provided by Kobayashi Pharmaceutical Co., Ltd. (Osaka, Japan). Two compounds isolated from *Salacia* extracts, salacinol and kotalanol, strongly inhibit sucrase. Their structures are quite unique, bearing thiosugar sulfonium sulfate inner salt comprising a 1-deoxy-4-thio-D-arabinofranosyl cation and 1-deoxyaldosyl-3-sulfate anion (Fig. 2) (Shimoda et al, 1998; Yosikawa et al, 2001). It has been clarified that this extraction is not associated with toxicity or with blood biochemical or pathologic abnormalities in rats and other animals.



Salacinol **Kotalanol**

Fig. 2. Chemical structure of components of the extractive from *Salacia chinencis* (ES)

3.3 Partially decomposed alginate by *Vibrio alginolyticus* SUN53 (Alg53)

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 brown seaweeds (Fig. 3) (Wong et al, 2000). We demonstrated that partially decomposed alginate by *Vibrio alginolyticus* SUN53 (Alg53) had a competitive inhibitory effect for sucrase of the vesicles of the intestinal brush border membrane of rats. The procedure for the preparation of Alg53 has been described (Nakamura S et al, 2008).

Alginate (0.5%) (mean M.W., 55,000) partially hydrolyzed by HCl is incubated with *Vibrio alginolyticus* SUN53 (10^6 CFU/mL) in culture medium (pH 7.0) containing 0.025% yeast extract, 0.05% peptone, 1% NaCl and 0.01% $FePO_4$ for 5 days at 25°C. After incubation, the supernatant is treated with 3 times-volume (75%) of ethanol to obtain low-M.W. hydrolyzed alginate. It is dried by freezing after ethanol is evaporated with a rotary evaporator. The

mean M.W. of partially decomposed alginate is approximately 1,000 by column chromatography using a Sephadex G-15 column. Tseng et al reported that alginate lyase isolated from *Vibrio alginolyticus* (ATCC17749) has specificity for polymannuronic blocks, and Haug et al reported that depolymerizing alginate by lyase yields a product containing deoxyuronic acid (Tseng et al, 1992; Haug et al, 1967). Therefore, it is considered that Alg53 also comprises penta- or hexa-mannuronic acid with deoxymannuronic acid as the non-reducing terminal moiety. The conversion ratio of Alg53 by *Vibrio alginolyticus* SUN53 is very low, so we could not obtain a sufficient amount of Alg53 for *in vivo* experiments using animals. We have to develop a culture condition in which Alg53 is produced effectively.

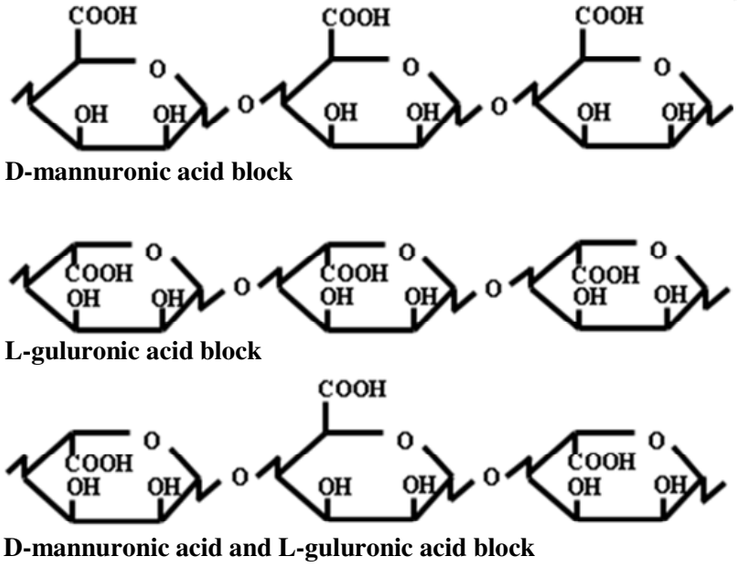


Fig. 3. Chemical structure of components of alginate

4. *In vitro* evaluation of the anticariogenic effects of phytochemicals

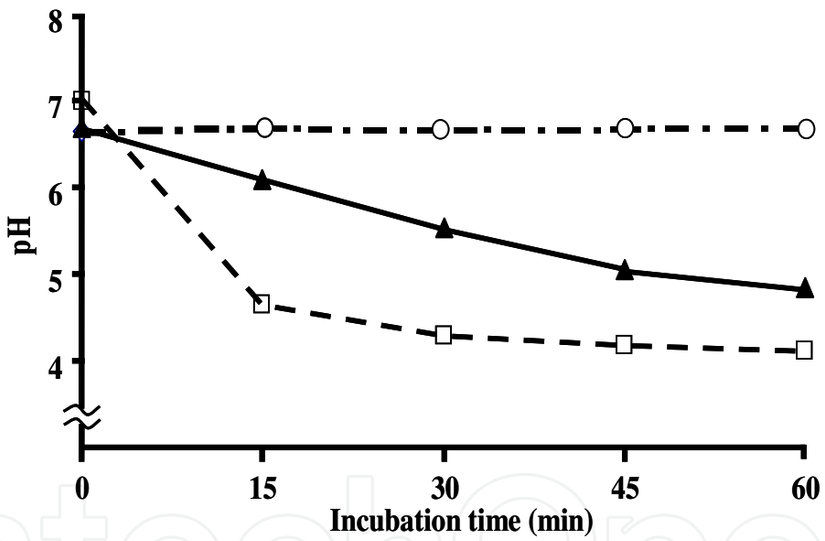
In this section, we introduce the anticariogenic effects evaluated using the three *in vitro* methods described above. The phytochemicals used in our experiments were Alg53, ELM, ES, and oolong as the positive control.

4.1 Effects of Alg53 on acid production by *S. sobrinus* 6715

The production of organic acids by *S. sobrinus* 6715 is illustrated in Fig. 4. The positive control maintained the initial pH. The results indicated that Alg53 disturbed the conversion of the substrate to organic acids. 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 acids was dependent upon the concentrations of Alg53 in the reaction mixture (Fig. 5). The inhibitory effect on pH decline was also investigated using ELM, ES and oolong, but these phytochemicals did not inhibit the pH reduction by *S. sobrinus* 6715.

4.2 Effects of ELM, ES, Alg53 and oolong on glucan production by GTase from *S. sobrinus* 6715 and *S. mutans* MT8148

Oolong has been used as a functional food to prevent dental caries. Oolong was therefore used to compare the inhibitory effects of other phytochemicals on glucan production by GTase. The inhibitory effect of phytochemicals on water-insoluble glucan synthesis by GTase from *S.sobrinus* 6715 is illustrated in Fig. 6A. The original ELM solution reduced the production of water-insoluble glucan to 66% of that of the control (ELM-free). ES also significantly reduced the synthesis of water-insoluble glucan. The inhibitory effect of ES was remarkable compared with that of ELM. The inhibitory effect of oolong on the production of water-insoluble glucan by GTase was stronger than that of ELM and of a similar level to that of ES. Fig. 6B shows water-insoluble glucan synthesis by GTase from *S. mutans* MT8148. ELM significantly inhibited the glucan production by GTase from *S. mutans* MT8148, and the ratio of inhibition of production of water-insoluble glucan was 64% that of the control (ELM-free). The inhibitory effect of ES and oolong on glucan production by GTase from *S. sobrinus* 6715 was stronger than that by GTase from *S. mutans* MT8148. The inhibitory effect of ELM was of a similar level on glucan production by GTase from *S. sobrinus* 6715 and *S. mutans* MT8148.

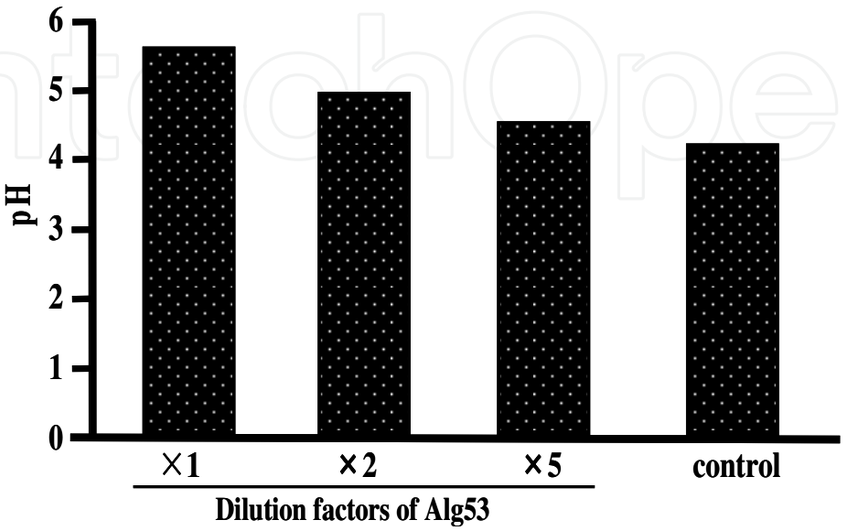


Open circle, positive control (no production of acid); open square, negative control (no inhibition); closed triangle, with Alg53
In the positive control (no production of acid), Stephan's buffer (pH 7.0) was added instead of glucose. In the negative control (no inhibition), distilled water was added instead of Alg53. Data are mean values of duplicate assays (Hashiguchi-Ishiguro et al, 2009).

Fig. 4. Time-course of pH decrease with acid production by *S. sobrinus* 6715 from glucose with and without partially decomposed alginate by *V. alginolyticus* SUN53

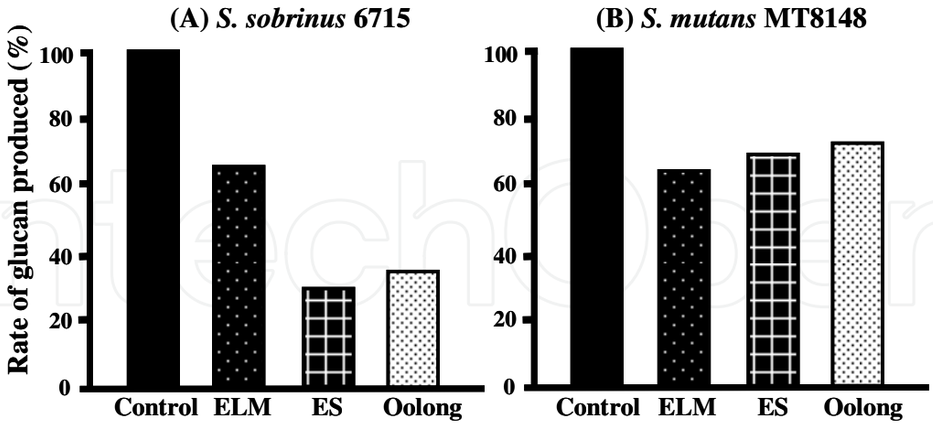
The inhibitory effect of Alg53 on water-insoluble and water-soluble glucan synthesis by GTase from *S.sobrinus* 6715 is illustrated in Fig. 7. The original Alg53 solution and a ten-fold dilution of Alg53 solution reduced the production of water-insoluble glucan to 21% and 23%, respectively. However, Alg53 barely affected the production of water-soluble glucan by GTase. These results demonstrated that Alg53 clearly inhibits the synthesis of water-

insoluble (but not water-soluble) glucan by GTase from *S. sobrinus* 6715. Water-insoluble glucan is closely associated with the formation of biofilms on teeth surface. In addition, Alg53 has inhibitory effects on acid production and synthesis of glucan by mutans streptococci. That is, Alg53 has two types of anticariogenic effects. Accordingly, Alg53 may demonstrate considerable anticariogenic effects.



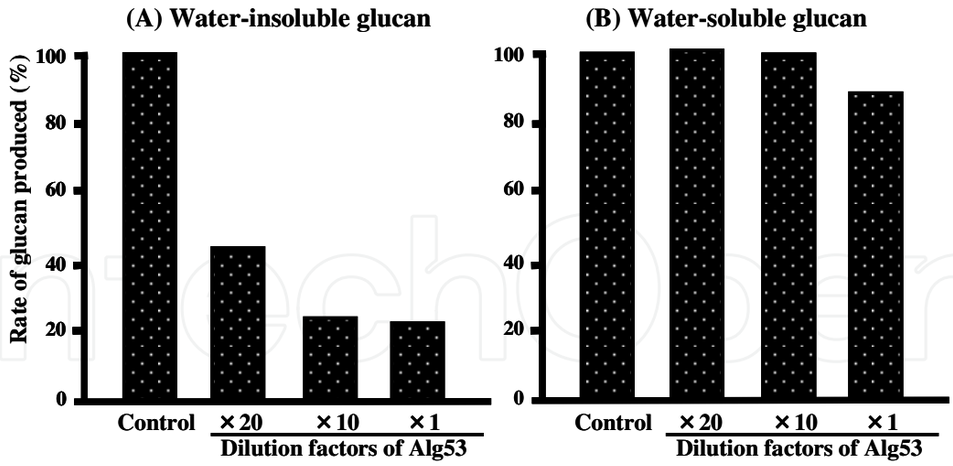
pH was measured after incubation for 1 h.
Control: water was added instead of Alg53
(Hashiguchi-Ishiguro et al, 2009)

Fig. 5. Inhibitory effect by different concentrations of partially decomposed alginate by *V. alginolyticus* SUN53 on acid production from glucose by *S. sobrinus* 6715



ELM, extractive from the leaves of *Morus alba*; ES, extractive from *Salacia chinensis*
Reaction mixture [GTase, 0.2 mL; sucrose solution, 0.31 mL (includes ^{14}C-sucrose, 20 μCi); test substance, 0.1 mL] incubated at 20° for 24 h at 37°C. The final concentration of sucrose in the reaction mixture was 1%. Glucan was expressed as the relative amount (%) of glucan produced as compared with the amount produced with the negative control (distilled water) (Hashiguchi et al, 2011).

Fig. 6. Inhibitory effects of the extractive from the leaves of *Morus alba*, *Salacia chinensis* and oolong on insoluble glucan produced by GTase



Reaction mixture [3% sucrose (final concentration, 1%) in 0.1 M phosphate buffer (pH 6.8), 1 mL; GTase from *S. sobrinus*, 0.3 mL; 0.1 M phosphate buffer (pH 6.8), 1.4 mL; Alg53, 0.3 mL] incubated at 20° for 24 h at 37°C. Glucan was expressed as the relative amount (%) of glucan produced as compared with the amount produced in the absence of Alg53. The amount of total carbohydrate was measured at 490 nm by the phenol-sulfuric acid method. Dates are expressed as mean values of duplicate assays (Hashiguchi-Ishiguro et al, 2009).

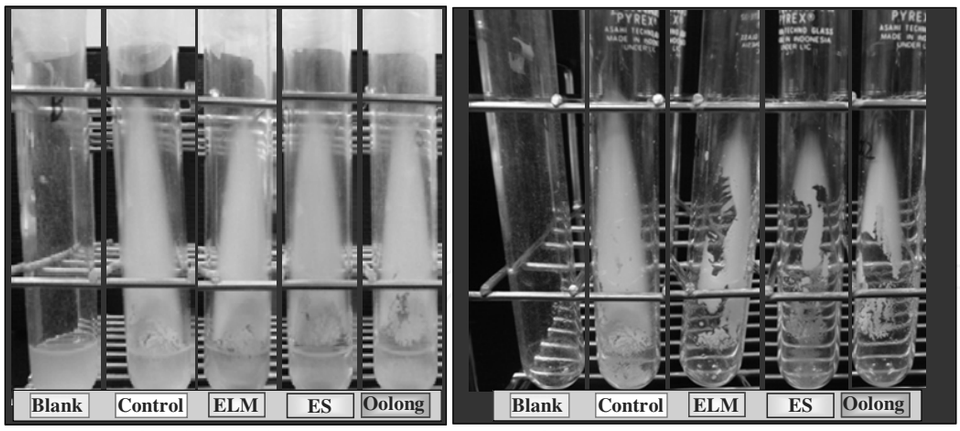
Fig. 7. Inhibitory effect of partially decomposed alginate by *V. alginolyticus* SUN53 on water-insoluble and water-soluble glucan produced by GTase from *S. sobrinus*

4.3 Effects of ELM and ES on sucrose-dependent cell adhesion on smooth surfaces

The inhibitory effect of ELM on sucrose-dependent adherence of cells onto the surface of glass test tubes was examined using growing cells of *S. sobrinus* 6715. Fig. 8A shows that cells adhered to the surface of glass test tubes after incubation. The cells grew well and adhered to the glass surface of the control (no phytochemical), ELM, ES and oolong. However, cells and glucan did not adhere to the glass surface of blank test tubes (sucrose-free). Fig. 8B shows the conditions of test tubes in which the reaction mixture was removed by pipetting, and then washed gently with distilled water. As shown clearly in Fig. 8B, cell adhesion was very strong in control test tubes, but was feeble in ELM, ES and oolong tubes; cells were removed by washing. The results demonstrate that ELM and ES inhibit the adhesion of cells to the glass surface. Adhered cells that remained on the surface of glass test tubes after washing were suspended with 1 N NaOH and absorbance measured at 550 nm (Fig. 9). The cell number was 60% for ELM and 21% for ES compared with that of the control.

5. Potential of phytochemicals as anticariogenic materials

The main finding of this study is that three phytochemicals, partially decomposed alginate by SUN53 (Alg53), the extractive from the leaves of *Morus alba (*ELM) and the extractive from *Salacia chinencis* (ES), have inhibitory effects on glucan synthesis by GTase. It may become a key point that certain phytochemicals have inhibitory effects on α-glucosidase when we screen natural materials which inhibit Gtase activity. However, the degree of inhibitory effect is not always similar for sucrase and GTase. The inhibitory effect of ELM and ES on sucrase was very strong. The inhibitory constant (*K*i) of ELM and ES for sucrase was 2.1×10^{-4} mM and 6.7×10^{-4} mM, respectively (Oku et al, 2006).



A: Right after incubation **B: After washing**

ELM, extractive from the leaves of *Morus alba*; ES, extractive from *Salacia chinensis*
Reaction mixture [cell solution, 0.5 mL; 2% sucrose (final concentration, 1%) in BHI, 0.8 mL; test substance 0.3 mL] incubated at 20° for 24 h at 37°C. In the blank, distilled water was added instead of sucrose. In the negative control, distilled water was added instead of test substance (Hashiguchi et al, 2011).

Fig. 8. Adhesion of *S. sobrinus* 6715 and glucan on smooth surfaces of glass after incubation with sucrose

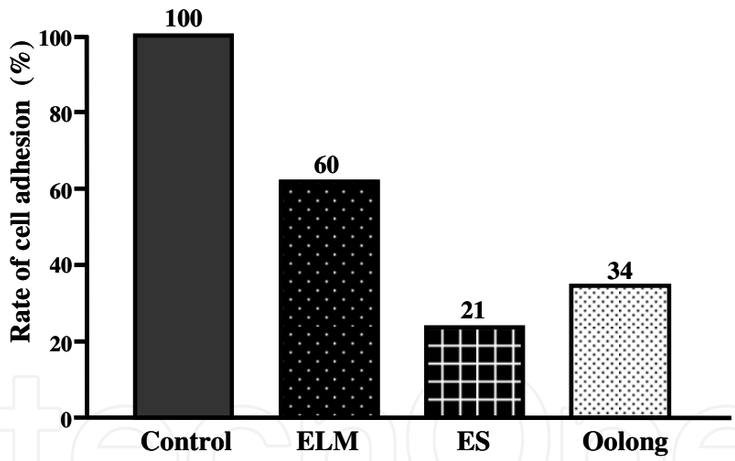


Fig. 9. Inhibitory effects of the extractive from the leaves of *Morus alba* and *Salacia chinensis* on the adhesion of *S. sobrinus* 6715 and glucan (Hashiguchi et al, 2011)

In addition, Alg53 suppressed pH decline by the production of organic acids from glucose, whereas ELM and ES could not suppress pH decline as well as oolong. For the prevention of dental caries, Alg53 may be useful as a functional food that has two types of inhibitory effects on the synthesis of glucan by GTase and acid production. Alternative sweeteners for sucrose, such as sugar alcohols and oligosaccharides, are not used as substrates for acid production by mutans streptococci, so pH decline does not occur. However, alternative sweeteners cannot inhibit the production of organic acids from sugars. Therefore, we recommend that ELM and ES are used in a combination of sugar alcohols or oligosaccharides to prevent dental caries.

6. Future prospects of functional foods for the prevention of dental caries

In Japan, various types of functional foods have been developed and widely consumed for health promortion. Some of them have been advanced for prevention of dental caries. GTase inhibitors and sugar substitutes that are not the direct cause of dental caries are actively developed and utilized as preventive foods for dental caries. Recently, functional materials which enhance the defence system of host on dental caries are further added to those foods. For example, there are functional materials that promote re-mineralization of tooth and stimulate saliva excretion to block oral pH decline. The probiotics which improves oral bacterial flora may also be expected to be one of these functions. If functional materials that have different types of preventive effect for dental caries are combined in a functional food, the potential of functional foods may expand the food market for health promotion.

It is important that intraoral pH does not decline for prevention of dental caries among children, aduts and elderlies. So, sweeten confections between meals must have a resistance for utilization by mutans streptococci. However, dental caries cannot be completely prevented by the utilization of functional foods, although the risk of dental caries is decreased. Therefore, the combination of teeth brushing after meal and functional foods is very important for prevention of dental caries among all people. Furthermore, consumers need to pay attention, when they utilize some functional foods containing nondigestible oligosaccharide which improves the intestinal microbiota, because it produces acids to decrease oral pH.

7. Conclusion

We found that three phytochemicals that had inhibitory effects upon α-glucosidase also had inhibitory effects on glucan synthesis by GTase. This time we introduced partially decomposed alginate by SUN53, the extractive from the leaves of *Morus alba* and the extractive from *Salacia chinensis*. And Alg53 suppressed pH decline by the production of organic acids from glucose. Therefore, these phytochemicals are expected to be used as multiple functional food materials that can prevent the development of dental caries. Furthermore, these results may suggest that we propose the screening steps, another phytochemicals that have anticariogenic effects but haven't clarified yet.

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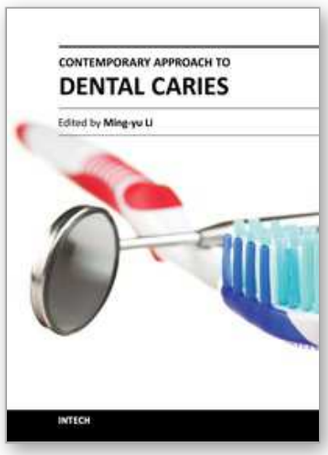

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