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Studies on Dental Caries Prevention by Traditional Medicines. VIII. Inhibitory Effect of Various Tannins on Glucan Synthesis by Glucosyltransferase from *Streptococcus mutans*[1)]

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Hydrolyzable tannins from crude drugs were examined for inhibitory activity against glucosyltransferase from a cariogenic bacterium, *Streptococcus mutans*. Pentagalloylglucose showed a strong inhibitory effect, whereas ellagitannins stimulated the glucan synthesis in 10% ethanol. All the gallotannins were more potent than chlorhexidine gluconate in an aqueous reaction mixture. Among them, penta- and hexagalloylglucose were the most potent, being inhibitory at a concentration of 10^{-3} M. The inhibitory character of gallotannins depended on the number of galloyl residues.

Keywords——enzyme inhibition; dental caries; tannin; galloylglucose; glucosyltransferase; *Streptococcus mutans*

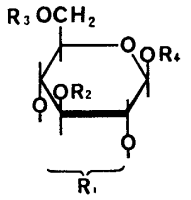
Introduction

In the course of a survey of antiplaque agents for the purpose of dental caries prevention, we have screened various traditional Chinese medicines for antibacterial action against *Streptococcus mutans*, a primary cariogenic bacterium, and for ability to inhibit the adherence of the cells to smooth surfaces.[2-4)] In a previous paper, we have reported that the extracts of Galla Rhois, Paeoniae ruburae Radix and Uvae ursi Folium, which are rich in gallotannins cause relatively strong inhibition of the adherence of *S. mutans* cells to glass surfaces, while those of Chebulae Fructus and Geranii Herba, which are rich in ellagitannins, cause no inhibition at concentrations of 0.1—1.0 mg/ml.[2)] In the present paper, we report the effects of various tannins isolated from these crude drugs on glucan synthesis by glucosyltransferase from *S. mutans*.

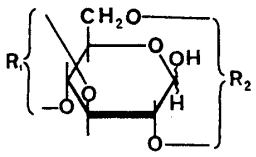
Materials and Methods

Chlorhexidine gluconate was purchased from Sumitomo Chemical Industry Co., Ltd. (Osaka). Uniformly labeled (^{14}C)sucrose was obtained from New England Nuclear. Bovine serum albumin was purchased from Seikagaku Kogyo Co., Ltd. Sucrose was obtained from Wako Pure Chemical Industry. A scintillation counter, ACS-II, was purchased from Amersham. Toyo 51A paper was used for paper chromatography. A crude glucosyltransferase preparation from *S. mutans* strain OMZ 176 (*serotype d*) was prepared by the modified procedure of Mukasa and Slade.[5,6)]

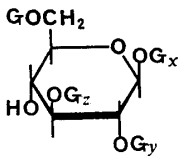
Tannins——Trigalloylglucose and tetragalloylglucose were isolated from the leaves of *Arctostaphylos uvae-ursi*.[7)]



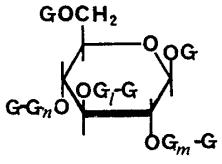
	R_1	R_2	R_3	R_4
chebulinic acid	A	G	G	G
chebulagic acid	A	(*R*) HHDP		G
granatin A	(1′*S*) DHHDP	H	(*S*) HHDP	
granatin B	(1′*S*) DHHDP	(*R*) HHDP		G
geraniin	(1′*R*) DHHDP	(*R*) HHDP		G



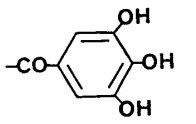
	R_1	R_2
punicalin	H	B
punicalagin	H	B



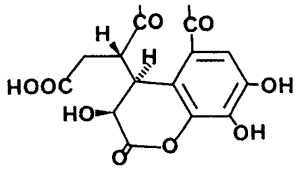
trigalloylglucose	$x+y+z=2$
tetragalloylglucose	$x+y+z=3$



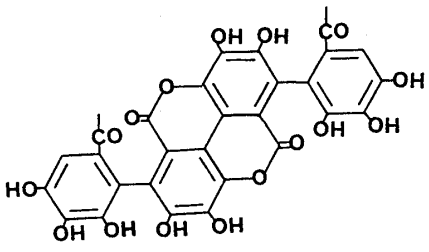
pentagalloylglucose	$l+m+n=0$
hexagalloylglucose	$l+m+n=1$
heptagalloylglucose	$l+m+n=2$
octagalloylglucose	$l+m+n=3$



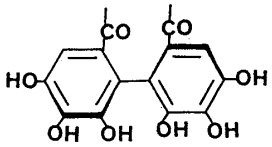
G



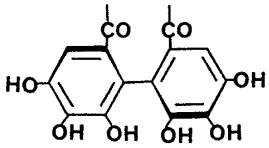
A



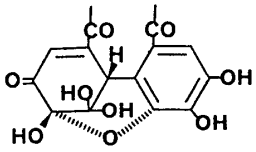
B



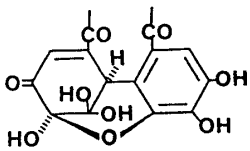
(*S*) HHDP



(*R*) HHDP



(1′ *S*) DHHDP



(1′ *R*) DHHDP

Fig. 1. Structure of Tannins

Pentagalloylglucose, hexagalloylglucose, heptagalloylglucose and octagalloylglucose were isolated from Galla Rhois.[8] Chebulinic acid and chebulagic acid were isolated from the fruit of *Terminalia chebula*.[9] Geraniin was obtained from the leaves of *Geranium thunbergii*.[11,12] Granatin A, granatin B, punicalin and punicalagin were obtained from the fruit peel of *Punica granatum*.[10,13,15] The structures of these compounds are shown in Fig. 1.

Enzyme Assay——Standard enzyme assay was performed in a reaction mixture (10 μl) which contained (^{14}C)sucrose (1 mM, 100 μCi/ml), potassium phosphate buffer (0.1 M, pH 6.8), 5 μl of an aqueous solution of tannins or 1 μl of an ethanol solution of tannin and glucosyl transferase (0.34 mg protein/ml for an aqueous reaction mixture, or 2.2 mg protein/ml for a 10% ethanolic mixture). After a 60-min incubation, the reaction mixture was chilled in ice water, then applied to a filter paper. The tube was rinsed with 10 μl of water. The rinsing was also applied to the filter paper. The paper was developed three times with pyridine–water–butanol (6 : 3 : 4). Radioactive spots were detected by autoradiography, and counted in a liquid scintillation counter. The result was expressed as a percentage of control (^{14}C)sucrose incorporation. The assay was repeated 3 to 6 times, and the results were averaged.

Results

Dilution of tannins was first done with ethanol because the ellagitannins were hardly soluble in water, and the glucosyltransferase-catalyzed reaction was carried out in the presence of 10% ethanol. Ethanol itself, however, has inhibitory activity on GTase reaction. For instance, the extent of the enzyme reaction at the concentration of 2.2 mg/ml in 10% ethanol was one-seventh of that under aqueous conditions. Figure 2 presents the effect of various tannins and reference compounds on glucan synthesis. Among these tannins,

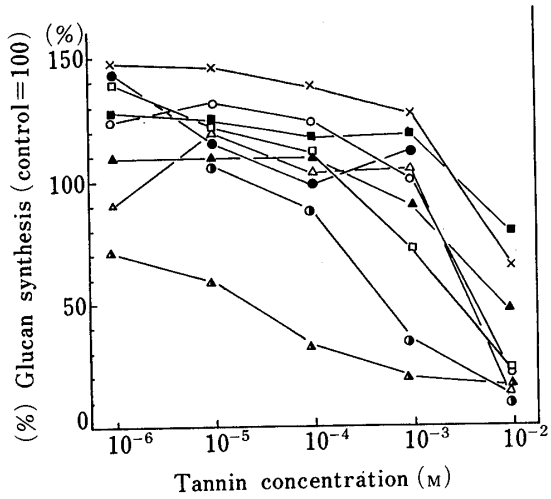


Fig. 2. Effect of Increasing Concentrations of Tannins on Glucosyltransferase Activity in 10% Ethanol

Chebulinic acid (○), chebulagic acid (●), granatin A (△), granatin B (▲), geraniin (□), punicalin (×) punicalagin (■), pentagalloylglucose (◑), chlorhexidine (◭).

Table I. % Inhibition of Glucan Synthesis Catalyzed by Glucosyltransferase in the Presence of 10^{-3} M Tannins in 10% Ethanol

Inhibitors	(%) inhibition
Chebulinic acid	−12.7
Chebulagic acid	−1.6
Granatin A	−4.3
Granatin B	8.8
Geraniin	26.3
Punicalin	−28.9
Punicalagin	−19.1
Pentagalloylglucose	64.8
Chlorhexidine	80.6
Ellagic acid	−63.8

Table II. % Inhibition of Glucan Synthesis Catalyzed by Glucosyltransferase in the Presence of 10^{-3} M Tannins in Aqueous Solution

Inhibitors	(%) inhibition
Trigalloylglucose	69.1
Tetragalloylglucose	81.6
Pentagalloylglucose	94.2
Hexagalloylglucose	94.2
Heptagalloylglucose	90.3
Octagalloylglucose	88.8
Chebulinic acid	2.4
Chlorhexidine	65.6
Gallic acid	−34.4
Glucose	−10.0

pentagalloylglucose showed an evident inhibitory effect at concentrations above 10^{-4} M. On the other hand, none of the ellagitannins inhibited the enzyme reaction at these concentrations. In fact, they, especially punicalin, stimulated the reaction. Chlorhexidine, which was reported to be an efficient inhibitor under aqueous conditions by Takemura,[14] was observed to have an inhibitory effect at concentrations of more than 10^{-6} M, and was more potent than the tannins at concentrations from 10^{-6} to 10^{-3} M.

The inhibition of glucan synthesis in the presence of 10^{-3} M tannins in 10% ethanol is presented in Table I. Pentagalloylglucose was the most potent among the tannins. Most ellagitannins had negative values of inhibition. Chebulinic acid, punicalin and punicalagin were remarkably stimulative. Geraniin and its stereoisomer, granatin B, weakly inhibited the enzyme reaction.

Since pentagalloylglucose was found to be an effective GTase inhibitor, other gallotannins were examined. These experiments were performed in aqueous reaction mixtures to eliminate the effect of ethanol. The concentration of the enzyme was reduced to 0.34 mg/ml in this case, to obtain extents of glucan synthesis similar to those in the control reactions (5000—8000 cpm/assay). The concentration–dependent inhibitory effects of gallotannins and reference compounds under the above conditions are shown in Fig. 3. Gallotannins did not show any inhibitory effect at concentrations below 10^{-5} M. Furthermore, octagalloylglucose and heptagalloylglucose had stimulative activity at concentrations below 10^{-5} M. This was also seen in the case of chlorhexidine at 10^{-5} M. Glucan synthesis started dropping when the concentration of gallotannins was increased above 10^{-5} M, and the IC_{50} values were around 10^{-4} M. Hexagalloylglucose was the most potent inhibitor among them at concentrations from 10^{-5} to 10^{-3} M, and was more effective than chlorhexidine.

Table II summarizes the percentages of inhibition of glucan synthesis in the presence of 10^{-3} M gallotannins in an aqueous reaction mixture. All the gallotannins were more potent than chlorhexidine, whereas chebulinic acid which has three galloyl residues in the molecule showed almost no effect. The inhibitory potency decreased in the following order, pentagalloylglucose = hexagalloylglucose > heptagalloylglucose > octagalloylglucose > tetragalloylglucose > trigalloylglucose. Constituents of gallotannins, *i.e.*, glucose and gallic acid, did not inhibit the glucan synthesis significantly at concentrations from 10^{-5} to 10^{-3} M

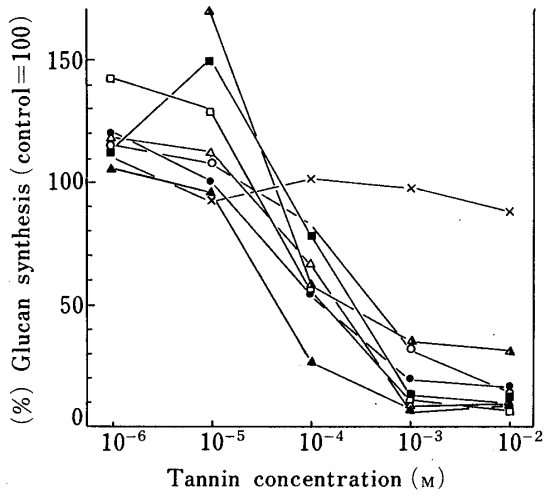


Fig. 3. Effect of Increasing Concentrations of Tannins on Glucosyltransferase Activity in Aqueous Conditions

Trigalloylglucose (○), tetragalloylglucose (●), pentagalloylglucose (△), hexagalloylglucose (▲), heptagalloylglucose (□), octagalloylglucose (■), chebulinic acid (×), chlorhexidine (▲).

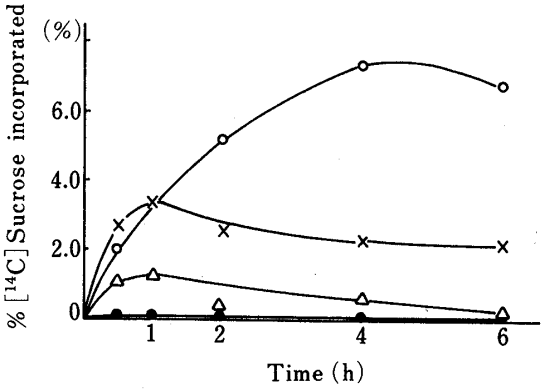


Fig. 4. Time Course of the GTase Reaction in the Presence of Pentagalloylglucose

Control (○), 10^{-5} M pentagalloylglucose (×), 10^{-4} M pentagalloylglucose (△), 10^{-3} M pentagalloylglucose (●).

(data not shown).

Since tannins are defined as polyphenols having special properties, such as precipitating with alkaloids or proteins, it is possible that GTase was inactivated as a result of interaction with gallotannins. However, the glucan synthesis, which was inhibited by 94% by 10^{-3} M pentagalloylglucose, was not restored by the addition of bovine albumin immediately before the enzyme addition at a concentration up to 1.0 mg/ml. This concentration was three times as much as that of the enzyme. When the concentration of sucrose was increased to 20 mM (20 times the standard concentration) the inhibition of glucan synthesis by 10^{-3} M pentagalloylglucose increased from only 5.8% to 13.3% of the control.

Figure 4 shows the time course of glucan synthesis of the control and the presence of pentagalloylglucose at the concentrations of 10^{-3}, 10^{-4} and 10^{-5} M. The control glucan synthesis progressed up to 4 h after the initiation of the reaction, but the reactions with 10^{-4} and 10^{-5} M pentagalloylglucose reached a plateau 1 h after the initiation. The incorporations of (^{14}C)sucrose into glucan after 6 h in the presence of 10^{-5}, 10^{-4}, 10^{-3} M pentagalloylglucose were 36.2%, 3.6% and 0%, respectively, of the control.

Discussion

Although gallotannins have long been known to interact with proteins, detailed examinations of their biological properties have just started because their absolute structures have only been determined in recent years.[8,15,16] The extent of inhibition of the respiration of rat liver mitochondria by gallotannins has been reported to depend on the number of galloyl residues.[17] We found, in this work, that their inhibitory effect on glucosyltransferase also depended on the number of galloyl residues.

We found that penta- and hexagalloylglucose inhibited the glucosyltransferase reaction more potently than chlorhexidine in aqueous solution. As the number of galloyl residues linked to glucose was decreased, the inhibitory potency decreased. A similar result was observed in the study on rat liver mitochondria.[17] Galloylglucoses had a tendency to stimulate this enzyme reaction at lower concentrations, and hepta- and octagalloylglucose were particularly stimulative. The previous findings that gallotannin-containing crude drugs such as Galla Rhois, Paeoniae rubrae Radix and Uvae ursi Folium strongly inhibited cell adherence at higher concentrations but were stimulative at lower concentrations are consistent with the present results.

Ellagitannins were rather stimulative at concentrations below 10^{-3} M in 10% ethanol, though they started precipitating. Again these observations agree with the previous results that the extracts of Chebulae Fructus and Geranii Herba, which contain ellagitannins, were not inhibitory but stimulative. A comparison of activity among tannins with similar structures, (*e.g.*, chebulinic acid with chebulagic acid, granatin A with granatin B, and punicalin with punicalagin) indicated that tannins with hexahydroxydiphenoyl residues were more inhibitory than those without. The inhibition by gallotannins was not affected by the addition of another protein, bovin serum albumin (BSA), which suggested that inhibition was not caused by the non-specific binding of tannins and proteins.

The synthesis of insoluble glucan by cell-bound or extracellular glucosyltransferase is essential for the adherence of *S. mutans* cells to smooth tooth surfaces, during the first stage of dental caries.[5] Therefore glucosyltransferase can be considered as an appropriate target for dental caries prevention. The results obtained in the present examination show that gallotannins are promising as antiplaque agents.

Moreover, Chinese nutgall powder, in combination with a ferrous acetate solution, has been commonly used for tooth staining (called "ohaguro") in Japan for many centuries. "Ohaguro" was believed to prevent tooth decay, and its prevention of acid etching and its

sterilizing effect on teeth have been verified.[18,19] This also indicates that gallotannins may show useful anti-dental caries activity.

References and Notes

1) Part VII: T. Namba, M. Tsunezuka, D. M. R. B. Dissanayake, U. Pilapitiya, K. Saito, N. Kakiuchi, and M. Hattori, *Shoyakugaku Zasshi*, **39**, 146 (1985).
2) T. Namba, M. Tsunezuka, Y. Takehana, S. Nunome, K. Takeda, Y.-Z. Shu, N. Kakiuchi, S. Takagi, and M. Hattori, *Shoyakugaku Zasshi*, **38**, 253 (1984).
3) T. Namba, M. Tsunezuka, K. Bae, and M. Hattori, *Shoyakugaku Zasshi*, **35**, 295 (1981).
4) T. Namba, M. Tsunezuka, and M. Hattori, *Planta Med.*, **44**, 100 (1982).
5) H. Mukasa and H. D. Slade, *Infect. Immun.*, **8**, 555 (1973).
6) H. Mukasa and H. D. Slade, *Infect. Immun.*, **9**, 419 (1974).
7) M. Nishizawa, T. Yamagishi, G. Nonaka, T. Nishizawa, T. Nagasawa, and H. Ohura, Abstracts of the 23rd Symposium of the Chemistry of Natural Products, Nagoya, 1980, p. 514.
8) M. Nishizawa, T. Yamagishi, G. Nonaka, and I. Nishioka, *J. Chem. Soc., Perkin Trans. 1*, **1982**, 2963.
9) T. Yoshida, T. Okuda, T. Koga, and N. Toh, *Chem. Pharm. Bull.*, **30**, 2655 (1982).
10) W. Meyer, A. Gorner, and K. Andra, *Justus Liebigs Ann. Chem.*, **1977**, 1976.
11) T. Okuda, T. Yoshida, and T. Hatano, *Tetrahedron Lett.*, **21**, 2651 (1980).
12) T. Okuda, T. Yoshida, and T. Hatano, *J. Chem. Soc., Perkin Trans. 1*, **1982**, 9.
13) T. Okuda, T. Hatano, H. Nitta, and R. Fujii, *Tetrahedron Lett.*, **21**, 4361 (1980).
14) K. Takemura, *Nippon Shika Hozongaku Zasshi*, **18**, 35 (1975).
15) T. Okuda, T. Yoshida, K. Mori, and T. Hatano, *Heterocycles*, **15**, 1323 (1981).
16) I. Nishioka, *Yakugaku Zasshi*, **103**, 125 (1983).
17) M. Nishizawa, T. Yamagishi, and T. Ohyama, *Chem. Pharm. Bull.*, **31**, 2152 (1983).
18) S. Ai, T. Ishikawa, and A. Seno, *Int. Dent. J.*, **15**, 426 (1965).
19) R. Yamaga and M. Nakayama, *Dent. Survey*, **49**, 70 (1973).