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Original Article

Potent Inhibitory Effects of D-tagatose on the Acid Production and Water-insoluble Glucan Synthesis of *Streptococcus mutans* GS5 in the Presence of Sucrose

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We examined and compared the inhibitory effects of D-tagatose on the growth, acid production, and water-insoluble glucan synthesis of GS5, a bacterial strain of *Streptococcus mutans*, with those of xylitol, D-psicose, L-psicose and L-tagatose. GS5 was cultured for 12h in a medium containing 10% (w/v) of xylitol, D-psicose, L-psicose, D-tagatose or L-tagatose, and the inhibitory effect of GS5 growth was assessed. Each sugar showed different inhibitory effects on GS5. Both D-tagatose and xylitol significantly inhibited the acid production and water-insoluble glucan synthesis of GS5 in the presence of 1% (w/v) sucrose. However, the inhibitory effect of acid production by D-tagatose was significantly stronger than that of xylitol in presence of sucrose.

Key words: *Streptococcus mutans*, D-tagatose, xylitol, acid production, water-insoluble glucan

The definition of 'rare sugars' is monosaccharaides and their derivatives that are not commonly found in nature. The biological effects of rare sugars have not been investigated in detail, partly because they are difficult to obtain and partly because their synthesis is laborious, time-consuming and inefficient [1, 2]. Kagawa University Rare Sugar Research Center established a simple method to produce rare sugars in large amounts from inexpensive D-glucose and D-fructose [3]. Most types of rare hexoses are now available for experiments to determine their functional characteristics. For example, various favorable effects on human health have been reported for D-psicose, a non-calorie sweetener and the most extensively examined rare sugar, including anti-diabetes and anti-obesity effects [4–7].

The Gram-positive bacterium *Streptococcus mutans* plays one of the most important roles in the development of dental caries, which is the most common oral infectious disease in the world [8]. As part of the mechanism of dental caries, *S. mutans* adheres to an acquired pellicle formed on the tooth surface, synthesizes glucan by the action of glucosyltransferases (gtf), and promotes the formation of dental plaque [9]. In the dental plaque, *S. mutans* produces organic acids as a result of carbohydrate metabolism. These organic acids induce demineralization of the tooth surface, resulting in dental caries [10].

Xylitol is a well-known sugar that prevents dental

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caries [11]. However, the preventive effect of xylitol has been shown to be remarkably inhibited by the existence of another monosaccharide that can be metabolized by *S. mutans* [12]. In the present study, we examined the effects of 4 rare ketohexoses (D-psicose, L-psicose, D-tagatose, and L-tagatose) on the growth, acid production and water-insoluble glucan synthesis of *S. mutans*. We used xylitol, a 5-carbon polyalcohol, as a control monosaccharide since xylitol is known to have inhibitory effects on the growth, acid production and water-insoluble glucan synthesis of *S. mutans*. The applications of xylitol includes hygiene, nutraceutical formulations and products such as anti-cariogenic gums. It is roughly as sweet as sucrose with 33% lower calories.

Fig. 1 shows the structural differences of four rare sugars including D-fructose and xylitol. D-psicose and D-tagatose are C-3 and C-4 epimers of D-fructose, respectively (Fig. 1). L-psicose and L-tagatose are the optical isomers of D-psicose and D-tagatose, respectively. All of these rare monosaccharides can be synthesized according to the production strategy of Izumoring, and D-psicose can be produced from D-fructose by the enzymatic reaction of D-tagatose 3 epimerase [3]. D-tagatose is very similar in texture to sucrose and is 92% as sweet, but with only 38% of the calories and with a very low glycemic index. The properties and functions of L-psicose and L-tagatose have hardly been researched because of their limited availability. The present study is the first to examine the effects of these rare monosaccharides on a typical bacterial strain of *S. mutans*, GS5.

Materials and Methods

Reagents. Rare sugars were supplied by the Rare Sugar Research Center of Kagawa University (Kagawa, Japan). The TY medium was prepared from 1.4% tryptone and 0.8% yeast extract. Tryptone, yeast extract and other chemicals were purchased from Wako Pure Chemical Industries (Tokyo, Japan). We found that it was difficult to reveal the different effects of each rare sugar with a rich-sugar medium, and thus we use a low-sugar TY medium.

Bacterial strains and culture conditions. *S. mutans* GS5, a clinically isolated strain commonly used in dental research [13], was kept at −80℃ until use. GS5 was grown in TY medium at 37℃ in static culture in air-sealed tubes for 24 h, and it was diluted with TY medium at an optical density of $OD_{600} = 0.3$. Based on the results of previous studies, we selected the static culture because there was no difference between the static culture and the anaerobic culture and because a caries induction test was possible with the static culture [14]. Two mL of TY medium containing 200 mg (10% w/v) xylitol, D-psicose, L-psicose, D-tagatose or L-tagatose was prepared and divided into two 1-mL solutions in the tubes.

Some of the rare sugars show insolubility when the concentration is greater than 15%. In addition, most of the soft drinks available to the public contain less than 10% (10 g/100 mL solution) sugar [15]. We therefore decided to use various sugars at 10% concentration in the present experiment. Ten mg of sucrose (1% w/v) was added to one 1-mL solution, and no sucrose was added to the other 1-mL solution.

To start the growth of GS5, 50 μL of the bacterial solution was inoculated into the TY medium, and the tubes were sealed by Parafilm (Pechiney Plastic Packaging Co., Batavia, IL, USA) for culture at 37℃. The optical density at 600 nm was measured at 4, 8, 12, and 20 h after inoculation. The pH and the

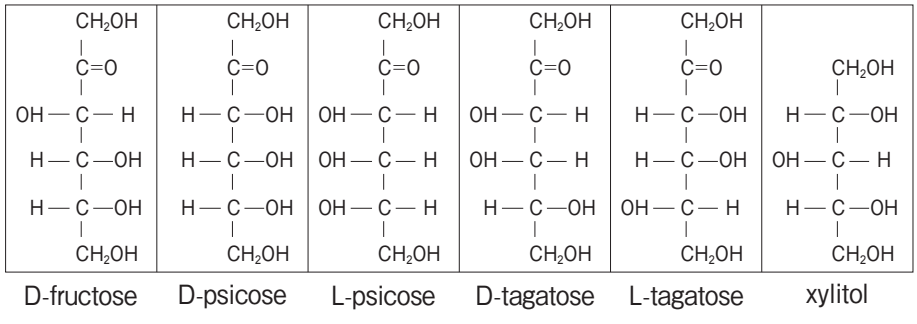

Fig. 1 Chemical structures of the monosaccharides used in this study.

water-insoluble glucan production were measured at 12 h.

Bacterial growth assay. Bacterial growth was assessed by measuring the turbidity of the solution. In the presence of sucrose, GS5 formed many bacterial aggregates containing insoluble glucan that were difficult to homogenously dissolve. Thus, turbidity measurement was performed only for the groups without sucrose supplementation at 4, 8, 12 and 20 h after inoculation. To determine the solution turbidity, optical density was measured using spectrophotometry at 600 nm (Gene Quant Pro, Amersham, Tokyo, Japan).

Measurement of pH. The ability of GS5 to produce acid at 12 h after inoculation was evaluated by measuring the pH of the solution using the CHECKER pH tester (Hanna Instruments, Tokyo, Japan). The initial pH of the TY medium in the presence of various sugars was 7.4.

Measurement of water-insoluble glucan. The production of water-insoluble glucan was measured at 12 h after inoculation. Water-insoluble glucan and bacteria lumps were collected by centrifugation at 3,500 × g for 10 min. The pellets were then washed with 0.5 mL of phosphate-buffered saline (pH 6.8), then subsequently re-centrifuged at 3,500 × g for 10 min. The precipitate was further washed twice with distilled water, and the insoluble glucan in the precipitate was dissolved in an aqueous solution of 1 N NaOH. Separation and removal of bacteria required additional centrifugation at 3,500 × g for 10 min. The amount of total water-insoluble glucan in the supernatant was measured by the phenol sulfuric acid method [16].

Statistical analysis. All experiments were performed in triplicate. Each value represents the mean ± SD of triplicate results from a representative experiment. Statistical significance was determined by Student's *t*-test. *P*-values < 0.05 were accepted as significant.

Results

Bacterial growth assay. Fig. 2A shows the time-dependent increase of the turbidity of the culture medium caused by the increase in the amount of *S. mutans* in the first 8–12 h of incubation; after that, the turbidity was observed to decrease progressively until the 20th hour. The turbidity in D-tagatose was the least among the sugars and also lower than xylitol throughout the experiment, to the 20th hour.

GS5 grew rapidly in the control TY medium, which contained no monosaccharide. The inhibition of GS5 growth by xylitol, D-psicose, L-psicose, D-tagatose and L-tagatose groups was significant ($p < 0.05$) compared to the control group. Only D-tagatose had a significantly stronger inhibition of bacterial growth than xylitol ($p < 0.05$).

Decrement of pH. The decrease in pH along with bacterial growth in the control medium and in the xylitol-, D-psicose-, L-psicose-, D-tagatose- and L-tagatose-containing media was measured in the absence and presence of 1% sucrose (Fig. 3). In the absence of sucrose, only xylitol significantly inhibited the pH decrement. It the others showed only a tendency to inhibit the pH decrement.

In the presence of sucrose, all of the sugars significantly inhibited the pH decrement compared to the change in the control group pH ($p < 0.05$). Interestingly, both D-tagatose and D-psicose showed significantly stronger inhibition of pH reduction compared to xylitol ($p < 0.05$). L-psicose and L-tagatose did not show stronger inhibition of pH reduction than xylitol.

Measurement of water-insoluble glucan. Fig. 4 shows the amount of water-insoluble glucan produced in the absence or presence of sucrose after 12 h of incubation. There were no significant differences between the control group and any of the other sugar groups. However, when sucrose was added to the media, the amount of water-insoluble glucan was markedly increased. Both the xylitol and D-tagatose groups showed the strongest inhibition of water-insoluble glucan synthesis induced by the addition of sucrose to the medium ($p < 0.05$). The other three sugar groups showed a non-significant trend of moderate inhibition on bacterial glucan synthesis.

Discussion

Table 1 summarizes the inhibitory effects of the monosaccharides on bacterial growth, acid production and water-insoluble glucan synthesis of *S. mutans* GS5. Although for many years xylitol has been believed to be useful as a tooth-friendly sweetener with an anti-caries effect, 2 recent systematic reviews of clinical trials could not find conclusive evidence that xylitol

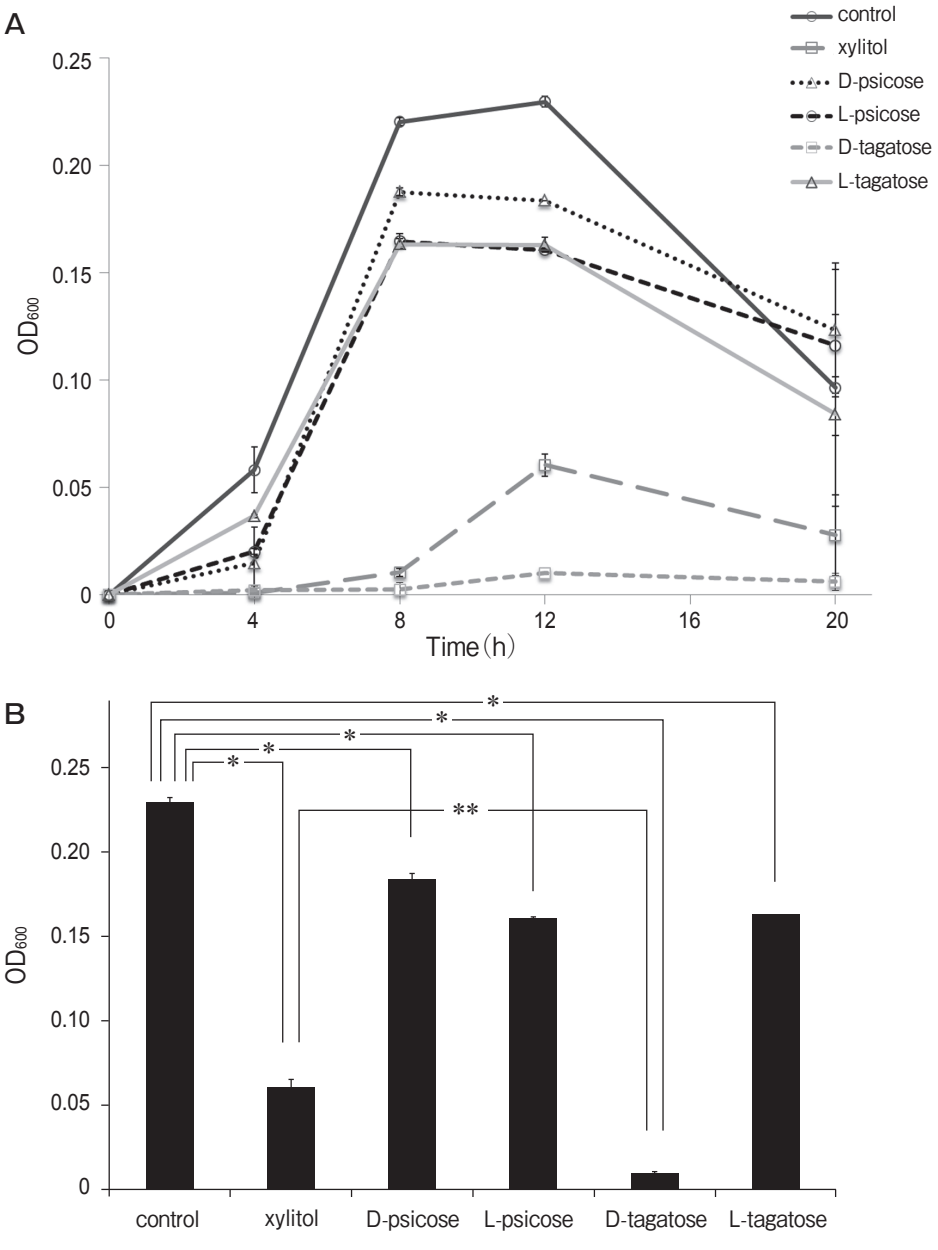

Fig. 2 Inhibition of *S. mutans* GS5 growth by various monosaccharides. **A**, Time course of turbidity of 10% xylitol, 10% D-psicose, 10% L-psicose, 10% D-tagatose and 10% L-tagatose solution containing GS5 was measured in the absence of 1% sucrose as described in the Materials and Methods section; **B**, The ability of 10% xylitol, 10% D-psicose, 10% L-psicose, 10% D-tagatose and 10% L-tagatose to inhibit GS5 growth at 12 h after inoculation. $^{*}p < 0.05$ for the mean values vs. the control group. $^{**}p < 0.05$ for the mean values vs. the xylitol group.

was indeed superior to other polyols such as sorbitol [17] or equal to topical fluoride in its anti-caries effect [18]. In addition, a 33-month xylitol caries trial for adults at an elevated risk of developing caries, in which participants were given either 5 g of xylitol or a placebo, found no statistically or clinically significant reduction in 33-month caries increment [19]. In the present study, it is also interesting to note that unlike D-tagatose, the superior suppression of acid production by xylitol in the absence of sucrose

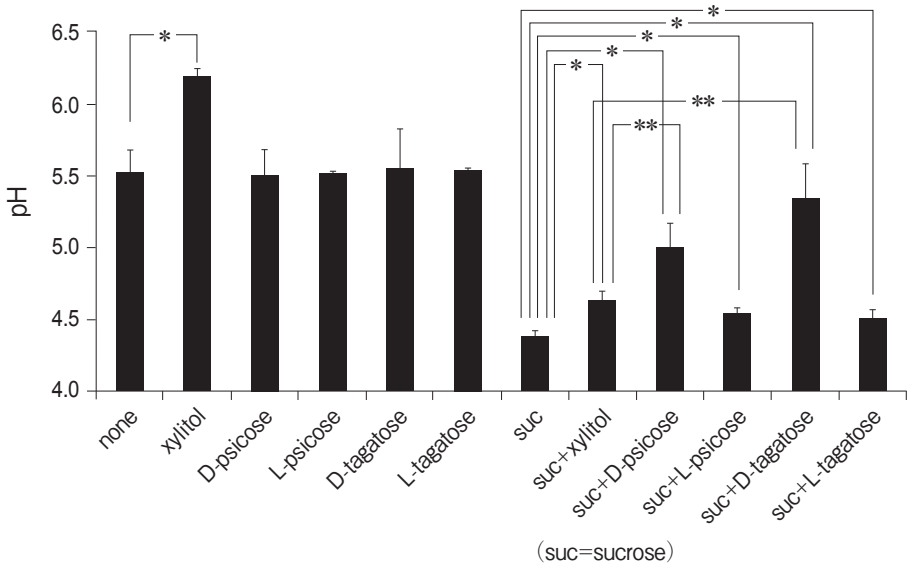

Fig. 3 Inhibition of acid production by various monosaccharides. The decrease in pH along with bacterial growth in the control medium and in xylitol-, D-psicose-, L-psicose-, D-tagatose- and L-tagatose-containing media in the absence and presence of 1% sucrose was measured. $^{*}p < 0.05$ for the mean values vs. the respective control group. $^{**}p < 0.05$ for the mean values vs. the xylitol group.

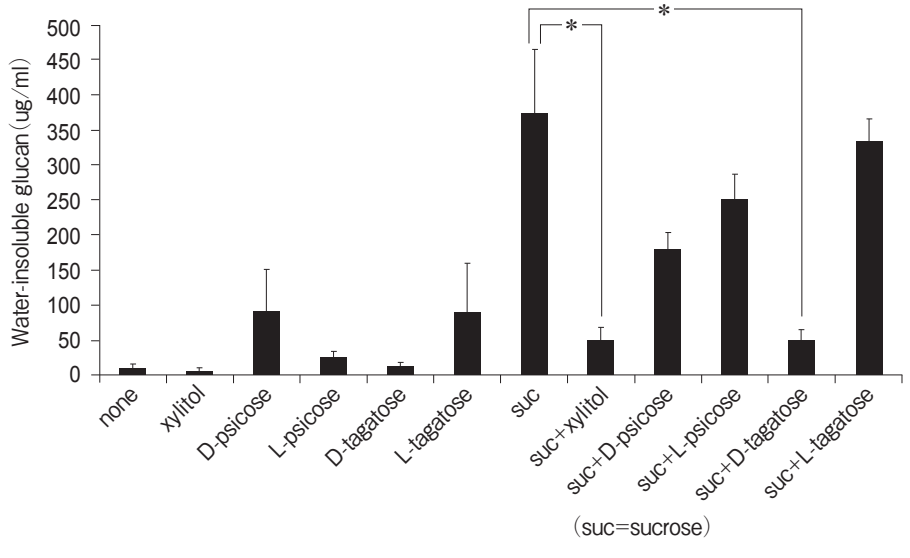

Fig. 4 Inhibition of water-insoluble glucan by various monosaccharides. The amount of water insoluble glucan of control, 10% xylitol, 10% D-psicose, 10% L-psicose, 10% D-tagatose and 10% L-tagatose in the absence and presence of 1% sucrose 12h after incubation. $^{*}p < 0.05$ for the mean values vs. the sucrose groups.

disappeared when sucrose was added (Fig. 3). The U.S. Food and Drug Administration (FDA) announced that D-tagatose did not reduce plaque pH below 5.7 and therefore did not promote demineralization of dental enamel.

Our present research is the first to demonstrate the suppression of water-insoluble glucan by D-tagatose. As shown in Fig. 2A, D-tagatose and xylitol clearly suppressed the growth of *S. mutans* more than other sugar groups. The inhibition of acid production and

Table 1 Summary of the inhibitory effects of various sugars

	Bacterial growth	Acid production (+sucrose)	Water insoluble glucan synthesis (+sucrose)
Xylitol	++	++	+
D-psicose	+	+++	−
L-psicose	+	+	−
D-tagatose	+++	+++	+
L-tagatose	+	+	−

The inhibitory effects of the five sugars as determined by statistical analysis are shown as −, no inhibition; +, slight inhibition; ++, moderate inhibition; +++, strong inhibition.

water-insoluble glucan synthesis may be due to the reduction of bacterial growth. Unlike xylitol, D-tagatose can be incorporated into the human body, and it then inhibits the activity of sucrase [20]. It was also reported that D-tagatose can be catabolized by a variety of lactic bacteria [21]. Although sucrase in *S. mutans* may not be the same as that in the human body [22], it is likely that incorporated D-tagatose into *S. mutans* can inhibit the activity of this enzyme.

We found that the inhibitory effect of D-psicose on *S. mutans* growth was weaker than that of xylitol (Fig. 2A), and that its inhibitory effect on acid production in the presence of sucrose was stronger than that of xylitol (Fig. 3). Our preliminary study showed that D-psicose, like D-tagatose, inhibited intestinal sucrase (unpubl. data), and D-psicose may work similarly to *S. mutans*. Further studies are necessary to elucidate the inhibitory mechanisms of D-psicose as well as D-tagatose.

S. mutans adheres to an acquired pellicle formed on the tooth surface, and it synthesizes glucan by the action of gtf [9]. In a study using reverse transcription-polymerase chain reaction (RT-PCR), we investigated whether these sugars could alter the expression of gtf genes. We found that D-tagatose had a tendency to suppress the expression of the *gtfB* gene in GS5 (data not shown). We will continue to do further research to elucidate the effect of D-tagatose on gtf expression.

However, the present study is the first to obtain evidence that D-tagatose inhibits *S. mutans* growth, acid production and water-insoluble glucan synthesis, especially in the presence of sucrose. D-tagatose is a monosaccharide classified as a ketohexose, and it has a structure similar to that D-fructose except for the epimeric structure of the hydroxyl group on carbon 4. D-tagatose has already attained Generally Recognized As Safe (GRAS) status under FDA regulations, thereby permitting its use as a sweetener in food and beverages [23]. D-tagatose is 92% as sweet as sucrose but has fewer calories at 1.5 kcal/g compared to xylitol (3.0 kcal/g) and sucrose (4.0 kcal/g), and it can thus be used as a low-calorie sweetener which may have a stronger anti-cariotic activity than xylitol. Although further studies to clarify the precise mechanisms of D-tagatose are necessary, D-tagatose seems to be beneficial for oral health control.

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