Low-cariogenicity of trehalose as a substrate

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Abstract

Objectives: The effects of trehalose on cariogenesis by mutans streptococci were investigated.

Methods: Inhibited effect of trehalose on water-insoluble glucan (WIG) synthesis from sucrose by glucosyltransferase (GTase) of mutans streptococci was assayed. The acid fermentability of trehalose by mutans streptococci was determined by the measurements of pH, and amounts of lactic acid production. Plaque pH was determined by the measurements of collected plaque from volunteers after sugar mouth-rinse. Rat experimental caries was investigated by feeding a sucrose and/or trehalose diet.

Results: Trehalose was not utilized as a substrate for GTase. In addition, trehalose inhibited synthesis of WIG by GTase in the presence of sucrose. Trehalose showed weaker and slower acid fermentation than sucrose by mutans streptococci. The levels of lactic acid production from trehalose by Streptococcus mutans and Streptococcus sobrinus were 24.2 and 59.8% of those from sucrose, respectively. The minimum plaque pH after sucrose mouth-rinse was lower than those after trehalose mouth-rinse in all subjects. Plaque pH after trehalose mouth-rinse never reached critical pH. The substitution of trehalose for sucrose in the rat diet significantly reduced caries scores. Furthermore, rats fed diets containing sucrose and trehalose had significantly lower caries scores than those fed a sucrose diet.

Conclusions: These results suggested that trehalose might be not only lowly cariogenic but also anti-cariogenic, and is promising as a sugar substitute. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Dental caries; Mutans streptococci; Low-cariogenicity; Trehalose; Glucosyltransferase inhibition

1. Introduction

The consumption of sucrose is considered to be one of the principal dietary factors in promoting dental caries. Mutans streptococci is one of the microorganisms responsible for dental caries [1,2], and its characteristic cariogenicity depends on the availability of sucrose. One of the most effective methods for dental caries prevention, therefore, is to substitute other sweetening substances for sucrose. Several investigators have evaluated the cariogenicity of such sweeteners [3–5].

Trehalose is a disaccharide which consists of two molecules of glucose bound to glucose by an α,α-1,1 linkage. It has recently been developed for mass production with a reasonable price. The resulting trehalose comprises white crystals, is thermostable and more than 98% pure. The sugar is about 45% as sweet as sucrose and is also slightly hygroscopic. It exists in small amounts in mushrooms, bread, beer, soybeans and shrimp [6].

The purpose of the present study was to determine the effects of trehalose as a substrate on the cariogenic activities of mutans streptococci and on the caries-promoting potential of trehalose in specific pathogen-free rats infected with Streptococcus mutans and on the plaque pH response of human dental plaque.

2. Materials and methods

2.1. Carbohydrates

For animal experiments, highly purified trehalose was obtained from Hayashibara (Okayama, Japan). For in vitro experiments, trehalose and other carbohydrates used in this study were obtained as reagent grade products (Wako Pure Chem., Tokyo, Japan).

2.2. Microorganisms

Laboratory stock cultures of streptomycin-resistant (1.0 mg/ml) S. mutans JC-2 and Streptococcus sobrinus OMZ-176 were used in this study.
2.3. Glucosyltransferase preparation and assay

Crude glucosyltransferase (GTase) was prepared according to the previously described methods [7]. In the assay of GTase activity, the reaction mixture in a total volume of 300 μl consisted of 0.3 M acetate buffer (pH 5.5), 0.15 M sucrose and/or 0–0.6 M trehalose and crude GTase preparation. The mixture was incubated at 25°C in microcuvettes, and increases in absorbance at 340 nm as the result of water-insoluble glucan (WIG) production were measured using a Shimadzu UV-160A spectrophotometer (Shimadzu Co., Kyoto, Japan). The activity (ΔA340 per min) was determined from the slope at a linear part of the time course curve. Individual reaction mixtures were heat-inactivated and the turbid materials were precipitated by centrifugation (15,000g x 5 min), and washed three times with distilled water. The total amounts of WIG were measured by the phenol sulphuric acid method and expressed as amounts glucose equivalent (μmol glucose/min).

2.4. Acid fermentability

The rate of fermentation of sucrose or trehalose was examined using *S. mutans* and *S. sobrinus*. The bacteria were cultured at 37°C for 20 h in heart infusion broth (HI, Difco Lab., MI, USA) containing 1% yeast extract (Difco Lab., MI, USA) and 0.25% sucrose (HIYS). The cells were harvested, washed thrice with Stephan’s buffer [8] and used to prepare packed cells (ca. 3.3 x 10^10 colony forming units (CFU/ml)) with the same buffer. The reaction mixture was composed of 0.4 ml of packed cells and 0.2 ml of 0.3 M sucrose or 0.3 M trehalose in Stephan’s buffer, and incubated at 37°C in a water bath with shaking. Aliquots of 20 μl were collected at timed intervals, and pH was measured using a digital pH meter (Shindengen Co., Tokyo, Japan).

2.5. Lactic acid production

Bacteria were cultured at 37°C for 20 h in HIYS. The cells were harvested by centrifugation, washed 5 times and suspended in 40 mM potassium phosphate buffer (pH 6.8). Reaction mixtures of 2 ml were prepared containing the cell suspension (ca. 1.0 x 10^10 CFU), 1% of sucrose or trehalose, 4.5 mM MgCl_2 and 90 mM potassium phosphate buffer (pH 6.8). The reaction mixtures were incubated at 37°C for 30 min and boiled for 5 min. After centrifugation, lactic acid in the supernatant was analyzed by an enzymatic method using the TC ht-Lactic acid kit (Boehringer Mannheim Biochem., Tokyo, Japan).

2.6. Plaque pH measurement

Plaque pH was determined according to the method described previously by Rugg-Gunn et al [9] in accordance with the ethical guidelines for human experiments. Twelve volunteers were chosen from among laboratory staff and dental students with good oral hygiene. Subjects rinsed their mouths for 2 min with 10 ml of 10% sucrose, 10% trehalose or 10% sorbitol at least 2 h after eating or drinking. Experiments with each sugar were performed at one-day intervals. Plaque samples (ca. 1 mg) were collected at 0, 3, 7, 11, 20 and 30 min, dispersed in 20 μl of de-ionized water and pH was measured using a digital pH meter at 90 s after the commencement of collection.

2.7. Experimental rat caries

The experiment was performed according to the procedure described previously [7], in accordance with the ethical guidelines for animal experiments of the Animal Research Center of Nihon University School of Dentistry at Matsudo. Briefly, specific pathogen-free (SPF) Sprague-Dawley rats (17 days old; Japan Clea Laboratory, Tokyo Japan) were isolated individually in a cage. The animals were infected with a streptococmycin-resistant (1 mg/ml) strain of *S. mutans* JC-2 by pipette (50 μl of a suspension of 1 x 10^11 CFU/ml). All of the rats were fed a modified diet containing different sugars in place of sucrose in diet 2000 [10] as follows: 21.3% sucrose plus 34.7% starch for group A (7 rats); 21.3% trehalose plus 34.7% starch for group B (7 rats); 30% sucrose plus 26% trehalose for group C (8 rats). Oral swabs were taken to confirm colonization. The rats were sacrificed with dry ice gas at 77 days old. Mandibular caries were scored using the Keyes procedure [11]. During the experimental period, the rats were given access to diet and water ad libitum.

2.8. Statistical analysis

The caries scores and in vitro data were analyzed statistically by calculating means and standard deviations of the mean. Differences between means of the experimental and control groups were evaluated by Welch’s *t*-test (two group *t*-test: unpaired) using Stat View ver 5.0 (SAS Institute Inc.).
3. Results

### 3.1. Inhibition of GTase activity

Table 1 shows the inhibitor effect of trehalose on GTase activity in the absence and presence of sucrose. Trehalose was not utilized as a substrate for GTase activity. When the ratio of the amount of trehalose to sucrose was equimolar, the enzyme activities both from *S. mutans* and *S. sobrinus* were inhibited by approximately 10%. With a ratio of trehalose to sucrose of 2:1 or 4:1, the GTase activity of *S. mutans* was inhibited by 35.0 or 63.5%, respectively. The inhibitory effect of trehalose on the enzyme activity from *S. sobrinus* was weaker than that on the activity from *S. mutans*. In the presence of a 1:4 ratio of sucrose and trehalose, the activity was inhibited to 37.3%.

### 3.2. Acid fermentability

The rate of fermentation of trehalose was examined using *S. mutans* (mean $1.32 \times 10^{10}$ CFU) and *S. sobrinus* (mean $1.34 \times 10^{10}$ CFU). The time course curves of pH reduction are shown in Fig. 1. When sucrose was used for acid fermentation, the pH of the reaction mixture dropped linearly and reached critical pH (5.5), after incubation for 4.65 min in *S. mutans* and 3.13 min in *S. sobrinus*, respectively. On the other hand, trehalose was very slowly fermented in this system. The time to reach critical pH was 11.0 min for *S. mutans* and 7.25 min for *S. sobrinus*, respectively.

### 3.3. Lactic acid production

Fig. 2 shows the amounts of lactic acid formed by the cell suspensions of *S. mutans* (mean $1.30 \times 10^{10}$ CFU) and *S. sobrinus* (mean $8.5 \times 10^{9}$ CFU). Both mutants streptococci cell suspensions produced large amounts of lactic acid from sucrose. Although *S. mutans* and *S. sobrinus* produced some
lactic acid from trehalose, the amounts were about 24.2 and 58.9% of those obtained with sucrose, respectively.

3.4. Plaque pH measurement

The plaque pH curves for a 2-min 10% sugar challenge for the 12 subjects are shown in Fig. 3. With a least-significant difference of 0.003, the subjects could be divided into two groups according to their pH minima by sucrose-rinse; low-pH subjects (minimum pH 5.28 at 3 min, Fig. 3A) and higher-pH subjects (minimum pH 6.13 at 7 min, Fig. 3B). The mean minimum pH values were significantly lower in sucrose-rinse than in trehalose-rinse groups. The minimum pH following trehalose-rinse was 6.50 at 7 min in the low-pH group and 6.98 at 7 min in the higher-pH group. Minimum pH was reached the fastest in the low-pH group with sucrose-rinse, i.e. after 3 min. Sorbitol mouth-rinse did not affect the plaque pH.

3.5. Rat experiments

To establish the in vivo caries-promoting ability of trehalose, SPF rats were infected with S. mutans JC-2 (Table 2). The mean ± S.D. total caries scores of the sucrose and trehalose groups were 75.7 ± 28.1 and 27.7 ± 4.0, respectively with significant differences (p < 0.01). Group C, fed a diet containing 30% sucrose plus 26% trehalose, showed a total caries score of 59.5 ± 5.5. This score was lower than in group A with 21.3% sucrose only. The buccal caries scores of group C given a diet containing a sucrose plus trehalose were lower than those of group A given sucrose without trehalose-containing diet. The average body weights of the rats and number of S. mutans per mandible were not significantly different between groups.

4. Discussion

Use of alternative sweeteners has been suggested as an effective measure for the prevention of caries [3–5]. Trehalose is widely distributed in nature, especially in mushrooms, shrimp, seaweed and yeast [6]. It is a natural sweetening agent, has a good taste, is thermostable, and the crystals are not hygroscopic. An industrial process for manufacturing this compound has recently been developed.

WIG synthesis by GTase causes accumulation of dental plaque on the tooth surface. Trehalose was not utilized as a substrate for WIG synthesis by GTase obtained from S. mutans and S. sobrinus. However, the addition of trehalose to sucrose inhibited WIG synthesis by GTase of S. mutans and S. sobrinus by 10–64% (Table 1). This phenomenon is similar to those seen with maltose, glucose, fructose [12], low-molecular weight dextran [13] and coupling sugar [14]. Imai et al. [15] reported that maltose and maltosylfructoside inhibit GTase synthesis, and might act as acceptors in the transglycosylation reaction. Although the mechanism of inhibition of trehalose has not been clarified, trehalose is a disaccharide and may act in a similar manner to maltose that utilizes glucosyl acceptors for synthesis of WIG from sucrose.

S. mutans and S. sobrinus were both capable of acid

![Fig. 3. Mean plaque pH following a two-minute mouth-rinse with 10% sugar. A, low-pH group; B, high-pH group; ■, sucrose; □, trehalose; ◦, sorbitol. Bars indicate the standard deviations of the mean.](image_url)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>No of rats</th>
<th>Mean caries score ± SD</th>
<th>No. of S. mutans per mandible (×10^6)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Buccal</td>
<td>Sulcal</td>
</tr>
<tr>
<td>A</td>
<td>21.3% sucrose + 34.7% starch</td>
<td>7</td>
<td>14.9 ± 15.5</td>
<td>56.9 ± 13.3</td>
</tr>
<tr>
<td>B</td>
<td>21.3% trehalose + 34.7% starch</td>
<td>7</td>
<td>2.6 ± 2.9</td>
<td>25.1 ± 5.3</td>
</tr>
<tr>
<td>C</td>
<td>30% sucrose + 26% trehalose</td>
<td>8</td>
<td>6.5 ± 4.8</td>
<td>50.0 ± 9.3</td>
</tr>
</tbody>
</table>

* Mean CFU ± SD.
fermentation from trehalose, although to a much lesser degree than sucrose (Fig. 1). The activity or amount of trehalase that hydrates trehalose to glucose and supplies them to bacteria may be less than those of invertase. Lactic acid production from bacteria in dental plaque is the main cause of enamel demineralization [16,17]. In the present study, *S. mutans* and *S. sobrinus* showed lower levels of production of lactic acid from trehalose compared with those from sucrose (Fig. 2). With respect to the acid fermentation of trehalose and sucrose by these bacteria, their abilities may just reflect the lactic acid production. At the same cell concentrations, acid fermentation and lactic acid production by strain OMZ-176 were faster and greater than those by strain JC-2. These results suggested that cariogenicity by *S. sobrinus* is stronger than that by *S. mutans* [18].

Plaque pH is used as a predictor of the cariogenic potential of sugars. In the plaque pH measurement experiment, the subjects were divided into two groups according to their pH minima following sucrose-rinse. In this regard, mutants streptococci was found to form less than 0.3% of total cultivable bacteria from any subjects (data not shown). Although there are many kinds of microorganisms in the dental plaque, the population of bacteria, which ferment sucrose such as *S. mutans* may have been greater in low-pH dental plaque samples than in those from the higher-pH group. The pH reduction by sucrose-rinse was lower and faster than that by trehalose-rinse. Trehalose produced smaller pH drops to 6.50 and 6.98 compared to sucrose, which produced reductions to 5.28 and 6.13 in low- and in higher-pH groups, respectively. Plaque pH by sucrose-rinse dropped quickly to 5.3 and slowly returned to the baseline value, producing a typical Stephan curve. These results are compatible with those indicating acid fermentation ability and lactic acid production (Figs. 1 and 2). Therefore, trehalose has reduced acidogenic potential.

It is better to confirm anti-cariogenicity of sugars by in vivo experiments. Animal experiments indicated that trehalose promotes significantly less caries formation compared with sucrose (Table 2). Rats fed a diet containing trehalose develop significantly fewer and less severe carious lesions compared with those fed an equivalent diet containing sucrose. Dental caries induced by sucrose was also inhibited by trehalose (group C in Table 2). These results were presumed to be due to GTase inhibition, and weak acid fermentation and production from trehalose by *S. mutans*. Although sugar alcohols cause undesirable phenomena such as diarrhoea [19,20], we observed that rats fed a diet containing trehalose remained in good health and free of diarrhoea.

The results of the present study confirmed that: (1) exposure of intra-oral and tooth surface to trehalose shows obviously slower pH response than exposure to sucrose; (2) trehalose undergoes less acid fermentation and production by mutants streptococci; and (3) rat dental caries was reduced by the presence of trehalose in the diet. These in vitro and rat experiments indicated that trehalose may be of value in controlling dental caries, in addition to being a sweetening agent.

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References

[17] Kleinberg I. Regulation of the acid–base metabolism of the

