Similarly, minimum pH with a cutoff value at 5.0 showed a high sensitivity (0.950) and screened 95% of subjects with caries activity, although specificity was low (0.391 in Fig 4b) and 60.9% of subjects without caries risk may be identified as false positives. Because of the multifactorial etiology of dental caries, accurate prediction of dental caries using a single measure seems unreasonable.[25] Both lactic acid production and minimum pH can be used as primary screening tests for caries risk of preschool children, but may need to be combined with an additional test.

Conversely, the correlation of ΔDFT with minimum pH and lactic acid production was unclear (see Table 2), and predictive power for caries increment was not high (see Figs 4d to 4f). A correlation was identified between ΔDFT and dft at the first oral examination (see Table 2), supporting the notion that dft is a caries predictor for permanent teeth, as reported previously.[26]

Recent studies using schoolchildren as subjects[27,28] have reported that plaque acidogenicity after sugar exposure is unrelated to caries incidence. This may be due to differences in subject age: The acidogenicity of dental plaque may vary among individuals during preschool age and correlate with caries incidence more closely, but during and after school age, the acidogenicity of dental plaque may become similar among individuals. Our observation that minimum pH values obtained at the second oral examination showed a narrower range and smaller standard deviation than those at the first oral examination (see Table 1) may support this speculation.

CONCLUSIONS

It is important for patients and their parents to understand the involvement of dental plaque and its acidogenicity in dental caries etiology to improve lifestyles for dental caries prevention. The method for evaluating dental plaque acidogenicity developed in this study can offer a practical and useful method for this purpose, since the technique is simple to perform chairside in dental clinics and requires only 15 to 20 minutes to show results to patients or their parents, and both minimum pH and lactic acid production show significant correlations with caries experience. In addition, both minimum pH and lactic acid production can be applicable as primary screening tests for preschool children with primary caries risk, although these tests need to be combined with an additional test for the improvement of accuracy.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Science Research (B) no. 14370687 and no. 16390601 from the Ministry of Education, Science, Sports, and Culture, Japan.

REFERENCES

1. Socransky SS. Caries susceptibility tests. Ann New York Acad Sci 1968;153:137–146.
2. Margolis HC, Duckworth JH, Moreno EC. Composition of pooled resting plaque fluid from caries-free and caries-susceptible individuals. J Dent Res 1988;67:1468–1475.
3. Margolis HC, Moreno EC. Composition of pooled plaque fluid from caries-free and caries-positive individuals following sucrose exposure. J Dent Res 1992;71:1776–1784.
4. Lingström P, van Ruyven FOJ, van Houte J, Kent R. The pH of plaque in its relation to early enamel caries and dental plaque flora in humans. J Dent Res 2000;79:770–777.
5. Geddes DAM. Acids produced by human dental plaque metabolism in situ. Caries Res 1975;9:98–109.
6. Carter WM J, Dunn JK, Fosdick LS, Moore BW. The formation of lactic acid in dental plaque. I. Caries-active individuals. J Dent Res 1956;35:778–785.
7. Margolis HC, Moreno EC. Composition and cariogenic potential of dental plaque fluid. Crit Rev Oral Biol Med 1994;5:1–25.
8. Margolis HC, Zhang YP, van Houte J, Moreno EC. Effect of sucrose concentration on the cariogenic potential of pooled plaque fluid from caries-free and caries-positive individuals. Caries Res 1993;27:467–473.
9. Gao XJ, Fan Y, Kent RL Jr, van Houte J, Margolis HC. Association of caries activity with the composition of dental plaque fluid. J Dent Res 2001;80:1834–1839.

10. Greene JC, Vermillion JR. The Simplified Oral Hygiene Index. J Am Dent Assoc 1964;68:7–13.
11. Federation Dentaire Internationale Technical Report No. 31. Review of methods of identification of high caries risk groups and individuals. Int Dent J 1988;38:177–189.
12. Ansai T, Tahara A, Ikeda M, Katoh Y, Miyazaki H, Takehara T. Influence of colonization with mutans streptococci on caries risk in Japanese preschool children: 24-month survival analysis. Pediatr Dent 2000;22:377–380.
13. Okazaki Y, Higashi T, Murakami T, et al. A study of the newly developed salivary buffer capacity test. Part 2—Effectiveness on caries screening with combination of the CAT21 test and the CAT21 Buf test. Jpn J Pediatr Dent 2002;40:140–147.
14. Nomura Y, Tsuge S, Hayashi M, et al. A survey on the risk factors for the prevalence of dental caries among preschool children in Japan. Pediatric Dent J 2004;14:79–85.
15. Takahashi M, Ozaki M, Imamura M, Kuboyama H, Kyougoku E, Motokawa W. Study of the assessment of caries risk for the primary dentition period. Comparison of caries activity tests and consideration for clinical application. Jpn J Pediatr Dent 2001;39:13–19.
16. Tsubouchi J, Higashi T, Shimono T, Domoto PK, Weinstein P. A study of baby bottle tooth decay and risk factors for 18-month-old infants in rural Japan. ASDC J Dent Child 1994;61:293–298.
17. Tsubouchi J, Yamamoto S, Shimono T, Domoto PK. A longitudinal assessment of predictive value of a caries activity test in young children. ASDC J Dent Child 1995;62:34–37.
18. Clarke P, Fraser-Lee NJ, Shimono T. Identifying risk factors for predicting caries in school-aged children using dental health information collected at preschool age. ASDC J Dent Child 2001;68:373–378.
19. Nishimura M, Bhuiyan MM, Matsumura S, Shimono T. Assessment of the caries activity test (Cariostat) based on the infection levels of mutans streptococci and lactobacilli in 2- to 13-year-old children's dental plaque. ASDC J Dent Child 1998;65:248–251.
20. Jensen ME, Polansky PJ, Schachtele CF. Plaque sampling and telemetry for monitoring acid production on human buccal tooth surfaces. Arch Oral Biol 1982;27:21–31.
21. Chida R. Measurement of acid production using an in-dwelling electrode in the plaque of children and adults. II. Evaluation of the acidogenicity of lactose and the microbial analysis of the plaque on a pH-electrode. Jpn J Pediatr Dent 1984;22:811–827.
22. Chida R, Igarashi K, Kamiyama K, Hoshino E, Esashi M. Characterization of human dental plaque formed on hydrogen-ion-sensitive field-effect transistor electrodes. J Dent Res 1986;65:448–451.
23. Distler W, Kröncke A. The acid pattern in human dental plaque. J Dent Res 1983;62:87–91.
24. Higham SM, Edgar WM. Human dental plaque pH, and the organic acid and free amino acid profiles in plaque fluid, after sucrose rinsing. Arch Oral Biol 1989;34:329–334.
25. Hausen H. Caries prediction—state of the art. Community Dent Oral Epidemiol 1997;25:87–96.
26. Vanobbergen J, Martens L, Lesaffre E, Bogaerts K, Declerck D. The value of a baseline caries risk assessment model in the primary dentition for the prediction of caries incidence in the permanent dentition. Caries Res 2001;35:442–450.
27. Fejerskov O, Scheie AA, Manji F. The effect of sucrose on plaque pH in the primary and permanent dentition of caries-inactive and -active Kenyan children. J Dent Res 1992;71:25–31.
28. Dong YM, Pearce EI, Yue L, Larsen MJ, Gao XJ, Wang JD. Plaque pH and associated parameters in relation to caries. Caries Res 1999;33:428–436.

DENTAL MATERIALS XXX (2009) XXX–XXX

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Fluoride released from glass-ionomer cement is responsible to inhibit the acid production of caries-related oral streptococci

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ARTICLE INFO

Article history:
Received 6 March 2008
Received in revised form
12 September 2008
Accepted 30 October 2008

Keywords:
Glass-ionomer cement (GIC)
Eluate
Fluoride
Acid production
Streptococcus

ABSTRACT

Objectives. Glass-ionomer cements (GICs) are known to have inhibitory effects on bacterial growth, but the biochemical mechanism of this property has not been fully understood. This study aimed to evaluate inhibitory effects of GIC on the acid production of caries-related oral streptococci, and to identify the components responsible for the inhibition.

Methods. An eluate was prepared by immersing set GIC in phosphate-buffered saline at 37 °C for 24 h. Fluoride and other elements in the eluate were quantified by fluoride ion electrode and atomic absorption photometry, respectively. *Streptococcus mutans* NCTC 10449 and *Streptococcus sanguinis* NCTC 10556 were used to evaluate the pH fall and the rate of acid production after the addition of glucose in the presence or absence of the eluate. Acidic end products from glucose were also assayed by carboxylic acid analyzer.

Results. The eluate contained silicon (1.24 ± 0.26 mM), fluoride (0.49 ± 0.02 mM) and aluminum (0.06 ± 0.00 mM), and inhibited the pH fall and the acid production rate of both streptococci at acidic pH, with a concomitant decrease in lactic acid production. These effects were comparable to those of a potassium fluoride solution containing the same concentration of fluoride as the eluate.

Significance. These results indicate that the GIC eluate inhibits the acid production of caries-related oral streptococci at acidic pH and that the effect is due to fluoride derived from the GIC. Thus, adjacent to GIC fillings, bacterial acid production and the subsequent bacterial growth may decrease, establishing a cariostatic environment.

1. Introduction

Fluoride-releasing restorative materials, including conventional glass-ionomer cements (GICs), are frequently used for restorations of permanent or deciduous teeth for the patients with high caries-risk because of their biocompatibility and cariostatic properties.

The eluate of GICs has been found to have no effect on the viability of mouse odontoblast-like cells [1], a weak inhibitory effect on the protein synthesis of human gingival fibroblasts

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doi:10.1016/j.dental.2008.10.014

Please cite this article in press as: Nakajo K, et al. Fluoride released from glass-ionomer cement is responsible to inhibit the acid production of caries-related oral streptococci. Dent Mater (2009), doi:10.1016/j.dental.2008.10.014

[2] and a weak damaging effect on the morphology of human gingival fibroblasts [3]. In addition, GICs are free of cytotoxic components such as 2-hydroxyethyl methacrylate (HEMA), usually contained in resin-modified glass-ionomer [4]. Thus, the available evidence indicates that GICs are less toxic to the cells compared with resin-based materials.

Furthermore, enamel and dentin surfaces adjacent to GIC fillings are protected from acid attack [5,6], indicating that GICs can inhibit acid-induced demineralization of teeth. Furthermore, the demineralized dentin layer adjacent to GIC fillings turns acid resistant with an increase in mineral content such as calcium, and becomes harder [7], suggesting that GICs can promote tooth remineralization [8]. It is considered that these cariostatic properties are due to the fluoride released from GICs; the fluoride can be incorporated in a hydroxyapatite crystal lattice and form fluoroapatite, which has a low acid-solubility [9].

GICs have also been found to have antibacterial effects. It was reported that the population of *Streptococcus mutans* on the surface of GIC fillings was lower than on composite fillings [10,11]. In addition, the pH fall after sucrose fermentation for 4 or 20 h by *S. mutans* cells layered on the surface of a GIC disc was smaller than that on the composite disc [12,13]. These results suggest that GICs inhibit the acid production of *S. mutans* and subsequently decrease the bacterial population. However, the identity and mechanism of this component of GICs is unclear. The observation that the pH of dental plaque decreases within a few minutes after sugar intake [14,15] suggests that the short-term measurement of pH is needed to evaluate the inhibitory effect on microbial acid production.

Therefore, the hypothesis tested was that GIC eluate has a short-term inhibitory effect on the pH fall and the acid production rate of caries-related oral streptococci, *S. mutans* and *Streptococcus sanguinis*, under anaerobic conditions mimicking those in a deep layer of dental plaque, where tooth demineralization occurs. An attempt was also made to identify the components of the GIC eluate that were responsible for these effects.

2. Materials and methods

2.1. *Preparation of GIC eluate and determination of composition*

The powder and liquid of GIC (Fuji IX, GC, Tokyo, Japan) were mixed at the ratio of 1:3.6 for 30 s, and placed into a cylindrical mold (height, 2 mm; diameter, 10 mm). The specimen was allowed to set for 30 min at 25 °C, and immersed in 5 mL of Dulbecco's phosphate-buffered saline containing 154 mM sodium ion, 4 mM potassium ion, 139 mM chloride ion, 10.75 mM hydrogen ion and 9.6 mM phosphate ion (D-PBS; Invitrogen Corp., Rockville, MD, USA) using multi-plates (6 wells). After storage at 37 °C for 24 h, the eluate was collected and the concentration of fluoride was measured using a fluoride ion electrode (F-53, Horiba, Ltd., Kyoto, Japan) after buffering the solution with total ionic strength adjustment buffer (TISAB; Orion Research Inc., Cambridge, MS, USA) at pH 5.3. The concentrations of silicon, aluminum, strontium, magnesium, and calcium in the eluate were determined using atomic absorption spectrometry (AA-6800F, Shimadzu Corp., Kyoto, Japan).

2.2. *Bacterial strains and growth conditions*

S. mutans NCTC 10449 and *S. sanguinis* ATCC 10556 were used. Basal culture medium contained 1.7% tryptone (Difco Laboratories, Detroit, MI, USA), 0.3% yeast extract (Difco Laboratories), and 0.5% NaCl. 0.5% glucose and 50 mM potassium phosphate buffer were added through a sterile membrane filter (pore size 0.22 μm, Millipore Corp., Bedford, Mass, NY, USA) to the autoclaved basal culture medium. This culture medium was kept in an anaerobic chamber (ANB-180L, Hirasawa Works, Tokyo, Japan: 80% N_2, 10% H_2 and 10% CO_2) for at least 3 days before use to remove oxygen. Each strain was pre-cultured in the medium at 37 °C overnight, transferred (5% inoculum size) to medium at pH 7.0 and incubated at 37 °C until the late-log growth phase (O.D. = 0.9–1.0 at 660 nm) in the anaerobic chamber. Bacterial cells were harvested and washed 3 times by centrifuging (21,000 × *g* for 7 min at 4 °C) in D-PBS. Double-sealed centrifuging tubes (Kubota Corp., Tokyo, Japan) were used to protect bacterial cells from air exposure. The cells were suspended in D-PBS in another anaerobic chamber (ANB-180L, Hirasawa Works: 90% N_2 and 10% H_2). The following experiments were performed in this chamber.

2.3. *pH fall by glucose fermentation in the presence of the GIC eluate*

The reaction mixture contained the cell suspension (0.3 mL, O.D. = 10 at 660 nm), D-PBS and the GIC eluate or potassium fluoride (KF) solution. The final concentrations of fluoride in the reaction mixture ranged from 0 to 0.43 mM, which were equivalent to fluoride concentrations in the reaction mixtures for the GIC eluate. The maximum fluoride concentration in the reaction mixture for the GIC eluate containing 0.49 mM fluoride turned to 0.43 mM after mixing with bacterial cell suspension and glucose solution. The reaction mixture was adjusted to pH 7.0, and preincubated at 35 °C for 4 min with agitation by a magnetic stirrer. Then, 10 mM glucose was added to the mixture, and the pH fall was monitored for at least 30 min, using a pH electrode (GS-5015C, Toa Electronics, Tokyo, Japan).

2.4. *Acid production rate from glucose fermentation in the presence of the GIC eluate*

The reaction mixture contained the cell suspension (0.3 mL, O.D. = 3.5 at 660 nm), D-PBS and the GIC eluate or KF solution. The reaction mixture was maintained at pH 5.5 or 7.0 by titration with 60 mM KOH using pH stat (AUT-211S, Toa Electronics) with agitation by a magnetic stirrer, and preincubated at 35 °C for 4 min. The reaction was started by the addition of 10 mM glucose and the acid production was monitored for 10 min.

2.5. *Analysis of acidic end products*

Procedures for analysis of lactic, formic and acetic acids have been detailed previously [16]. At 10 min after the addition of glucose, the reaction mixture was mixed with 0.6N perchloric

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Table 1 – Concentrations of elements in the GIC eluate.

Element	Mean concentration (S.D.)
Fluoride	0.49 (0.02) mM
Silicon	0.82 (0.26) mM
Aluminum	0.01 (0.02) mM
Strontium	<0.001 mM
Magnesium	<0.001 mM
Calcium	<0.001 mM

Data are given in mean (standard deviation) of 3 replicates.

acid. The resultant mixtures were brought out from the anaerobic chamber, filtered through a membrane (pore size 0.20 μm; polypropylene; Toyo Roshi Ltd., Tokyo, Japan) to remove cell debris, diluted with 0.2N HCl and assayed using a carboxylic acid analyzer (Eyela S-3000, Tokyo Rika Co. Ltd., Tokyo, Japan).

2.6. *Statistical analysis*

Statistical significance was assessed by the Dunnett test. A probability of $P < 0.05$ was considered to indicate significance.

3. Results

3.1. *Determination of composition in the eluate from GIC*

The eluate contained silicon, fluoride and aluminum with trace amounts of strontium, magnesium and calcium (Table 1).

3.2. *pH fall by glucose fermentation in the presence of the GIC eluate*

Serial pH falls by glucose fermentation of *S. mutans* are shown in Fig. 1. In all experiments using *S. mutans*, pH started to fall immediately after the addition of glucose. However, 10 min after the addition of glucose, the pH of reaction mixtures differed between the various concentrations of fluoride. At 30 min, in reaction mixtures containing eluate with a fluoride concentration of 0.43 mM or KF solution with a fluoride concentration of 0.43 mM, the pH stabilized at 4.93–5.00, which was significantly higher than the pH values of the control reaction mixture, the reaction mixture containing eluate with a fluoride concentration of 0.043 mM, and the reaction mixture containing KF solution with a fluoride concentration of 0.043 mM (Table 2). After 30 min, in the reaction mixture containing eluate with fluoride concentration of 0.043 mM or KF solution with a fluoride concentration of 0.043 mM, the pH continued to fall and approached the control gradually (date not shown). Similar results were obtained for *S. sanguinis* (Table 2).

3.3. *Acid production rate from glucose fermentation in the presence of the GIC eluate*

The rate of acid production of *S. mutans* at pH 5.5 was inhibited significantly by the GIC eluate in a dose-dependent manner, and decreased to about 10% of the control value at pH 5.5 in the presence of the eluate containing 0.43 mM fluoride (Table 3).

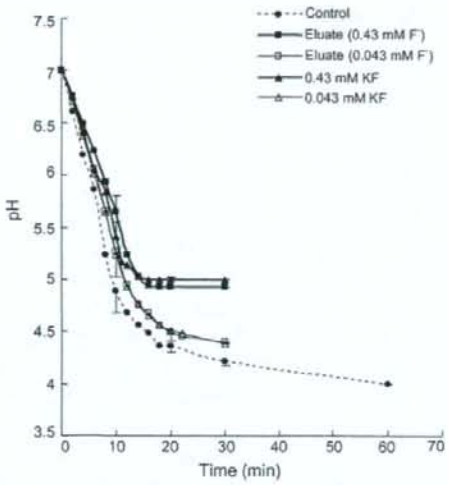

Fig. 1 – Effect of the GIC eluate or KF solution on the pH fall after addition of 10 mM glucose to the cell suspension of *S. mutans* NCTC 10449. The data are the means of 3 independent experiments. Vertical bars indicate standard deviations.

Only a weak inhibition was observed at pH 7.0. All KF solutions inhibited acid production to a degree similar to that of the eluate containing the same concentration of fluoride. There was no significant difference between the Eluate and KF solution containing the same concentration of fluoride. Similar results were obtained for *S. sanguinis* (Table 3).

3.4. *Analysis of acidic end products*

The profiles of acidic end products at pH 5.5 are shown in Fig. 2. As the concentration of fluoride in the eluate or KF solution increased, the proportion of lactic acid decreased, whereas the proportion of formic and acetic acids increased.

Table 2 – pH fall by *S. mutans* and *S. sanguinis* at 30 min after the addition of glucose.

In the presence of:	Mean pH (SD)	
	S. mutans	*S. sanguinis*
None (control)	4.21 (0.04)	4.34 (0.1)
Eluate (0.043 mM F^-)	4.35 (0.06)	4.44 (0.12)
Eluate (0.43 mM F^-)	4.93 (0.10)$^{**,\#\#}$	4.79 (0.09)*
0.043 mM KF	4.38 (0.02)	4.46 (0.08)
0.43 mM KF	5.00 (0.03)$^{**,\#}$	4.98 (0.00)$^{**,\#}$

Data are given in mean (standard deviation) of 3 replicates. Significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$) from the controls. Significant difference ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$) from the eluate (0.043 mM F^-) and KF solution (0.043 mM F^-).

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Table 3 – Acid production rate by *S. mutans* and *S. sanguinis* for 10 min at pH 7.0 and 5.5.

pH	In the presence of	Percentage of acid production rate (S.D.)	
		S. mutans	*S. sanguinis*
7.0	None (control)	100	100
	Eluate		
	0.43 mM F^-	93.4 (4.6)	93.8 (3.9)
	KF		
	0.43 mM	95.4 (4.6)	94.2 (6.2)
5.5	None (control)	61.3 (11.1)*	35.6 (5.4)*
	Eluate		
	0.043 mM F^-	32.5 (5.2)*,**	32.5 (6.1)*
	0.086 mM F^-	28.8 (1.5)*,**	33.4 (1.7)*
	0.215 mM F^-	20.7 (2.6)*,**	15.6 (4.1)*,**
	0.43 mM F^-	6.2 (1.3)*,**	5.4 (3.5)*,**
	KF		
	0.043 mM	31.6 (5.4)*,**	29.0 (8.2)*
	0.086 mM	28.4 (5.5)*,**	28.7 (4.5)*
	0.215 mM	14.4 (2.1)*,**	16.5 (2.2)*,**
	0.43 mM	6.5 (0.6)*,**	4.5(2.0)*,**

Data are given in mean (standard deviation) of 3 replicates. There was no significant difference between the Eluate and KF solution containing same concentration of fluoride.

* Significant difference ($P < 0.01$) from the control at pH 7.0.

** Significant difference ($P < 0.01$) from the controls at pH 5.5.

4. Discussion

In the present study, in addition to fluoride, silicon, aluminum, strontium, magnesium, and calcium were detected in the GIC eluate (Table 1). These elements have previously been reported to leach from GICs [17–19]. The concentrations of these elements are known to differ among GICs [18], probably due to differences in composition. In addition, their concentrations in eluates appear to vary according to eluting conditions. With Fuji IX (a filling cement used in the present study) and Fuji I (a luting cement similar in composition to Fuji IX), the proportion of silicon in the eluate is known to decrease under acidic conditions and increase under neutral pH conditions [17–19]. Consequently, in the present study, D-PBS at pH 7.3 was used for elution, and the proportion of Si in the eluate was relatively high.

The GIC eluate stopped the pH fall completely around pH 4.8–5.0 (Table 2 and Fig. 1) and markedly decreased the rate of acid production at pH 5.5 (Table 3). These findings suggest that streptococcal acid production in areas adjacent to GIC fillings may be inhibited at an acidic pH around the critical pH of tooth demineralization, resulting in a decrease in streptococcal cariogenicity. Furthermore, the present finding that the GIC eluate inhibited acid production to a degree similar to that of the KF solution with the same concentration of fluoride

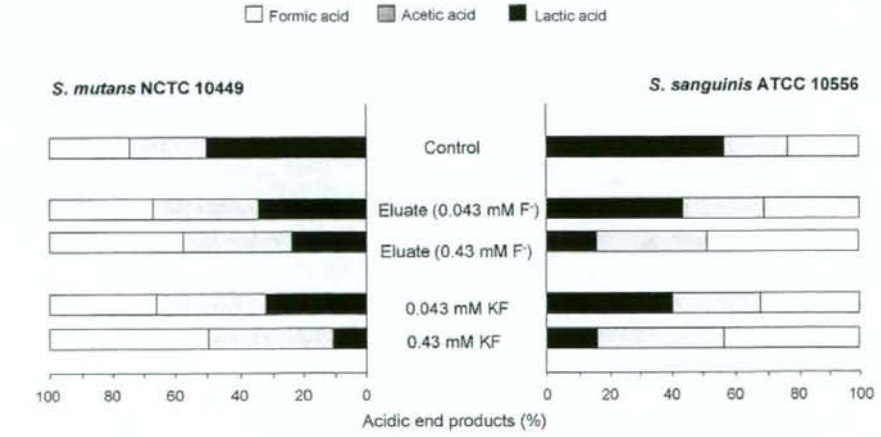

Fig. 2 – Effect of the GIC eluate or KF solution on the profiles of acidic end products for 10 min after addition of 10 mM glucose to the cells of *S. mutans* NCTC 10449 and *S. sanguinis* ATCC 10556 at pH 5.5. The data are the means of 3 independent experiments.

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(Tables 2 and 3, Fig. 1) indicates that this effect of the eluate was due to the fluoride derived from the GIC.

The efficient inhibition of fluoride at acidic pH is due to the fact that hydrogen fluoride (HF) behaves according to the equilibrated reaction, $H^+ + F^- \leftrightarrows HF$ with a pKa value of 3.15. It is possible that fluoride is released from GIC mainly in the form of sodium fluoride (NaF) because the main cation released from Fuji IX is reportedly Na^+ [19], but NaF dissociates to sodium ion (Na^+) and fluoride ion (F^-) immediately at neutral pH. At acidic pH, F^- accepts a proton (H^+) and forms the undissociated form (HF) more easily, according to the equilibrated reaction where the reaction shifts toward the right as the environmental pH is lowered; the concentration of HF is calculated to be about 30 times higher at pH 5.5 than at pH 7.0. Because bacterial cell membranes are more permeable to HF than F^- [20], HF can enter the cells and dissociate into F^- and H^+ again in the relatively alkaline cytoplasm. This intracellular F^- inhibits enolase, a glycolytic enzyme, resulting in a decrease in acid production from glycolysis [21–23]. Thus, an acidic environment can promote entry of HF into bacterial cells and the subsequent inhibition of bacterial acid production via intracellular F^-. Furthermore, H^+ released from HF in the cell can acidify the intracellular pH, leading to a decrease in the entire glycolytic activity. F^- can also inhibit cell membrane-associated H^+-ATPase, a proton pump, resulting in decreased excretion of H^+ from the cells. This can also enhance the acidification of intracellular pH [24–26].

In addition to the inhibitory effect of fluoride, it has been reported that GICs such as Fuji IX are able to neutralize acidic conditions [27], suggesting that there is a buffering potential of released elements from GICs to neutralize acids produced by bacteria.

The present finding, that the proportion of lactic acid was decreased by both the eluate and KF solution (Fig. 2) supports that the eluate and KF solution exert this effect via the same mechanism. Previous researches indicate that this decrease in the proportion of lactic acid coincides with a slowdown in glycolysis through the enolase inhibition by F^- and the subsequent decrease in levels of intracellular glycolytic intermediates [28,29]. A slowdown in glycolysis leads to a decrease in the level of fructose 1,6-bisphosphate (a glycolytic intermediate that activates lactate dehydrogenase), resulting in a decrease in lactic acid production, while the concomitant decrease in levels of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (glycolytic intermediates that inhibit pyruvate formate-lyase), keeps formic and acetic acids production [30].

From the atomic absorption spectrometry, it is not possible to determine the form in which the elements were present in the eluate, i.e. compounds, complexes or ions. Therefore, it is difficult to evaluate the inhibitory effects of elements other than fluoride precisely. However, the most possible ions of the elements in the eluate, SiO_3^- and Al^{3+}, have no inhibitory effects on oral bacterial acid production at the concentrations determined in the present study [31,32]. Although Al^{3+} is known to enhance the inhibitory effects of fluoride on bacterial acid production [33] and H^+-ATPase [25], the concentration of Al^{3+} seemed to be too low to express the synergistic effect. It appears that the concentrations of the other elements were too low to demonstrate the influences on bacterial acid production.

The previous study indicated a possible retention within dental plaque. In the case of resin-modified GIC [8], the plaque fluoride levels increased from the saliva/plaque interface (0.1 mM) towards the plaque/GIC filling interface (1.6 mM). Although fluoride contained in cured GICs is expected to decrease gradually over time [12,34], GIC fillings can undergo a fluoride-recharge by absorbing fluoride from fluoride products such as dentifrices and mouth washes [35,36]. GICs may function as a biomaterial to reserve and release fluoride continuously in the oral cavity.

It is reported that fluoride is bacteriostatic and bactericidal to oral streptococci at concentrations of >15.8 and >160 mM, respectively [37,38]. However, in the current study as well as previous studies [29,39], bacterial acid production from sugar fermentation was inhibited by a lower fluoride concentration, especially at an acidic pH around the critical pH of tooth demineralization. Hallgren et al. [40] observed that dental plaque on the orthodontic brackets retained with GIC had a lower activity of lactic acid production by glucose fermentation as compared to composites. This result supports the antibacterial effect at a low concentration of fluoride as shown in the present study. In addition, they demonstrated that those plaque samples had a lower proportion of mutans streptococci in total viable count [41] and a higher content of fluoride [42].

The inhibition of bacterial acid production not only directly protect against dental caries, but also suppresses the growth of caries-related oral bacteria, which obtain most of the energy for their growth from sugar fermentation. Thus, adjacent to GIC fillings, the acid production and the subsequent growth are expected to decrease. It appears that the inhibition of acid production observed in the present study, together with the inhibition of demineralization and the promotion of remineralization at the tooth surface, would establish a cariostatic environment around GIC fillings *in vivo*. Further study is needed *in vivo* to confirm the expectation.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (Nos. 16390601, 17659659, 17791350, 18659562, and 19209060) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

REFERENCES

[1] Costa CAS, Hebling J, Garcia-Godoy F, Hanks CT. In vitro cytotoxicity of five glass-ionomer cements. Biomaterials 2003;24:3853–8.
[2] Consiglio R, Rengo S, Liguoro D, Riccitiello F, Formisano S, Palumbo G, et al. Inhibition by glass-ionomer cements of protein synthesis by human gingival fibroblasts in continuous culture. Arch Oral Biol 1998;43:65–71.
[3] Caughman WF, Caughman GB, Dominy WT, Schuster GS. Glass ionomer and composite resin cements: effects on oral cells. J Prosthet Dent 1990;63:513–21.
[4] Oliva A, Della Ragione F, Salerno A, Riccio V, Tartaro G, Cozzolino A, et al. Biocompatibility studies on glass ionomer

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cements by primary cultures of human osteoblasts. Biomaterials 1996;17:1351–6.
[5] Francci C, Deaton TG, Arnold RR, Swift Jr EJ, Perdigao J, Bawden JW. Fluoride release from restorative materials and its effects on dentin demineralization. J Dent Res 1991;78:1647–54.
[6] Glasspoole EA, Erickson RL, Davidson CL. Demineralization of enamel in relation to the fluoride release of materials. Am J Dent 2001;14:8–12.
[7] Pereira PN, Inokoshi S, Yamada T, Tagami J. Microhardness of in vitro caries inhibition zone adjacent to conventional and resin-modified glass ionomer cements. Dent Mater 1998;14:179–85.
[8] Yamamoto K, Arai K, Fukazawa K, Fukui K, Nagamatsu K, Kato K, et al. Effect of plaque fluoride released from a glass-ionomer cement on enamel remineralization in situ. Caries Res 2005;39:157–60.
[9] Chow LC, Brown WE. Reaction of dicalcium phosphate dihydrate with fluoride. J Dent Res 1973;52:1220–7.
[10] Forss H, Jokinen J, Spets-Happonen S, Seppä L, Luoma H. Fluoride and mutans streptococci in plaque grown on glass ionomer and composite. Caries Res 1991;25:454–8.
[11] Benelli EM, Serra MC, Rodrigues Jr AL, Cury JA. In situ anticariogenic potential of glass ionomer cement. Caries Res 1993;27:280–4.
[12] Seppä L, Korhonen A, Nuutinen A. Inhibitory effect on S. *mutans* by fluoride-treated conventional and resin-reinforced glass ionomer cements. Eur J Oral Sci 1995;103:182–5.
[13] Seppä L, Torppa-Saarinen E, Luoma H. Effect of different glass ionomers on the acid production and electrolyte metabolism of *Streptococcus mutans* Ingbritt. Caries Res 1992;26:434–8.
[14] Igarashi K, Kamiyama K, Yamada T. Measurement of pH in human dental plaque in vivo with an ion-sensitive transistor electrode. Arch Oral Biol 1981;26:203–7.
[15] Takahashi-Abbe S, Abbe K, Takahashi N, Tamazawa Y, Yamada T. Inhibitory effect of sorbitol on sugar metabolism of *Streptococcus mutans* in vitro and on acid production in dental plaque in vivo. Oral Microbiol Immunol 2001;16:94–9.
[16] Takahashi N, Abbe K, Takahashi-Abbe S, Yamada T. Oxygen sensitivity of sugar metabolism and interconversion of pyruvate formate-lyase in intact cells of *Streptococcus mutans* and *Streptococcus sanguis*. Infect Immun 1987;55:652–6.
[17] Fukazawa M, Matsuya S, Yamane M. Mechanism for erosion of glass-ionomer cements in an acidic buffer solution. J Dent Res 1987;66:1770–4.
[18] Bapna MS, Mueller HJ. Leaching from glass ionomer cements. J Oral Rehabil 1994;21:577–83.
[19] Czarnecka B, Nicholson JW. A preliminary study of the interaction of glass-ionomer dental cements with amino acids. Dent Mater 2006;22:133–7.
[20] Gutknecht J, Walter A. Hydrofluoric and nitric acid transport through lipid bilayer membranes. Biochim Biophys Acta 1981;644:153–6.
[21] Hüther FJ, Psarros N, Duschner H. Isolation, characterization, and inhibition kinetics of enolase from *Streptococcus rattus* FA-1. Infect Immun 1990;58:1043–7.
[22] Kaufmann M, Bartholmes P. Purification, characterization and inhibition by fluoride of enolase from Streptococcus mutans DSM 320523. Caries Res 1992;26:110–6.
[23] Curran TM, Buckley DH, Marquis RE. Quasi-irreversible inhibition of enolase of *Streptococcus mutans* by fluoride. FEMS Microbiol Lett 1994;119:283–8.
[24] Bender GR, Thibodeau EA, Marquis RE. Reduction of acidurance of streptococcal growth and glycolysis by fluoride and gramicidin. J Dent Res 1985;64:90–5.
[25] Sturr MG, Marquis RE. Inhibition of proton-translocating ATPases of *Streptococcus mutans* and *Lactobacillus casei* by fluoride and aluminum. Arch Microbiol 1990;155: 22–7.
[26] Iwami Y, Hata S, Schachtele CF, Yamada T. Simultaneous monitoring of intracellular pH and proton excretion during glycolysis by *Streptococcus mutans* and *Streptococcus sanguis*: effect of low pH and fluoride. Oral Microbiol Immunol 1995;10:355–9.
[27] Nicholson JW, Aggarwal A, Czarnecka B, Limanowska-Shaw H. The rate of change of pH of lactic acid exposed to glass-ionomer dental cements. Biomaterials 2000;21:1989–93.
[28] Hata S, Iwami Y, Kamiyama K, Yamada T. Biochemical mechanisms of enhanced inhibition of fluoride on the anaerobic sugar metabolism by *Streptococcus sanguis*. J Dent Res 1990;69:1244–7.
[29] Maehara H, Iwami Y, Mayanagi H, Takahashi N. Synergistic inhibition by combination of fluoride and xylitol on glycolysis by mutans streptococci and its biochemical mechanism. Caries Res 2005;39:521–8.
[30] Yamada T, Carlsson J. Regulation of lactate dehydrogenase and change of fermentation products in streptococci. J Bacteriol 1975;124:55–61.
[31] Forbes JC, Smith JD. Studies on the effect of metallic salts on acid production in saliva. I. J Dent Res 1951;31:129–31.
[32] Gallagher IH, Cutress TW. The effect of trace elements on the growth and fermantation by oral streptococci and actinomyces. Arch Oral Biol 1977;22:555–62.
[33] Hayacibara MF, Rosa OP, Koo H, Torres SA, Costa B, Cury JA. Effects of fluoride and aluminum from ionomeric materials on *S. mutans* biofilm. J Dent Res 2003;82:267–71.
[34] Gandolfi MG, Chersoni S, Acquaviva GL, Piana G, Prati C, Mongiorgi R. Fluoride release and absorption at different pH from glass-ionomer cements. Dent Mater 2006;22: 441–9.
[35] Strother JM, Kohn DH, Dennison JB, Clarkson BH. Fluoride release and re-uptake in direct tooth colored restorative materials. Dent Mater 1998;14:129–36.
[36] Preston AJ, Agalamanyi EA, Higham SM, Mair LH. The recharge of esthetic dental restorative materials with fluoride in vitro-two years' results. Dent Mater 2003;19:32–7.
[37] Mayhew RR, Brown LR. Comparative effect of SnF_2, NaF, and $SnCl_2$ on the growth of *Streptococcus mutans*. J Dent Res 1981;60:1809–14.
[38] Maltz M, Emilson CG. Susceptibility of oral bacteria to various fluoride salts. J Dent Res 1982;61:786–90.
[39] Harper DS, Loesche WJ. Inhibition of acid production from oral bacteria by fluorapatite-derived fluoride. J Dent Res 1986;65:30–3.
[40] Hallgren A, Oliveby A, Twetman S. L(+)-Lactic acid production in plaque from orthodontic appliances retained with glass ionomer cement. Br J Orthod 1994;21:23–6.
[41] Hallgren A, Oliveby A, Twetman S. Caries associated microflora in plaque from orthodontic appliances retained with glass ionomer cement. Scand J Dent Res 1992;100:140–3.
[42] Hallgren A, Oliveby A, Twetman S. Fluoride concentration in plaque adjacent to orthodontic appliances retained with glass ionomer cement. Caries Res 1993;27:51–4.

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Original Paper

Caries Research

Caries Res 2009;43:17–24
DOI: 10.1159/000189703

Received: May 21, 2008
Accepted after revision: September 29, 2008
Published online: January 9, 2009

Effects of α-Amylase and Its Inhibitors on Acid Production from Cooked Starch by Oral Streptococci

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Key Words
α-Amylase · Acarbose · Acid production · Maltotriitol · Oral streptococci · Starch · Xylitol

Abstract
This study evaluated acid production from cooked starch by *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus sanguinis* and *Streptococcus mitis*, and the effects of α-amylase inhibitors (maltotriitol and acarbose) and xylitol on acid production. Streptococcal cell suspensions were anaerobically incubated with various carbohydrates that included cooked potato starch in the presence or absence of α-amylase. Subsequently, the fall in pH and the acid production rate at pH 7.0 were measured. In addition, the effects of adding α-amylase inhibitors and xylitol to the reaction mixture were evaluated. In the absence of α-amylase, both the fall in pH and the acid production rate from cooked starch were small. On the other hand, in the presence of α-amylase, the pH fell to 3.9–4.4 and the acid production rate was 0.61–0.92 μmol per optical density unit per min. These values were comparable to those for maltose. When using cooked starch, the fall in pH by *S. sanguinis* and *S. mitis* was similar to that by *S. mutans* and *S. sobrinus*. For all streptococci, α-amylase inhibitors caused a decrease in acid production from cooked starch, although xylitol only decreased acid production by *S. mutans* and *S. sobrinus*. These results suggest that cooked starch is potentially acidogenic in the presence of α-amylase, which occurs in the oral cavity. In terms of the acidogenic potential of cooked starch, *S. sanguinis* and *S. mitis* were comparable to *S. mutans* and *S. sobrinus*. α-Amylase inhibitors and xylitol might moderate this activity.

Once the cariogenicity of monosaccharides and disaccharides were recognized [Scheinin et al., 1976], strategies to prevent caries have included controlling the intake of these sugars and using nonfermentable sugar substitutes. However, the cariogenicity of starch contained in rice, wheat and potatoes, which are eaten frequently not only as staple foods but also as snacks, has yet to be determined [Glor et al., 1988; Lingström et al., 2000].

Raw starch has been used in most animal experiments, and results have demonstrated that raw starch is not cariogenic, as it cannot be easily degraded by salivary amylase [Havenaar et al., 1984; Grenby, 1990]. It has also been shown that oral bacteria such as *Streptococcus mutans*, *Streptococcus mitis* and *Actinomyces viscosus* do not cause a fall in the pH due to raw starch [Ellen and Onose, 1978]. Furthermore, it has been reported that acid production

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from raw starch in the oral cavity is too small to be able to demineralize the surfaces of the teeth [Brudevold et al., 1985]. However, it is not realistic to consider evaluating raw starch cariogenicity in modern society since cooked starch rather than raw starch is used today, and cooked starch is easily degraded by amylase.

In the animal experiments, cooked starch has been shown to be noncariogenic in monkeys [Beighton and Hayday, 1984], although it is cariogenic in rats [Firestone et al., 1982, 1984]. The discrepancy for cooked starch cariogenicity may be due not only to the type of animal species used, which involves saliva properties and bacterial flora, but also the experimental conditions, such as the quantity and frequency of intake and the physical properties of the foods, such as the food shape [Lingström et al., 2000]. In humans, it has been reported that upon intake of cooked starch, dental plaque is able to lower the pH to levels that allow enamel demineralization [Lingström et al., 1994; Pollard, 1995; Lingström et al., 2000]. Thus, since starchy foods such as potato chips, popcorn and rice crackers are now being consumed as snacks between meals around the world, it is necessary to evaluate the acidogenic potential of cooked starch in human oral bacteria, including mutans streptococci. Previous studies [Clarkson et al., 1987; Duarte et al., 2008] suggested that *Streptococcus mutans* produces acid from starch in the presence of α-amylase.

Therefore, the present study attempted to evaluate the acid production by representative oral streptococci when exposed to cooked starch in the presence of salivary α-amylase. In addition, α-amylase inhibitors, such as acarbose, maltotriitol and xylitol, were examined in order to determine whether they can disturb the starch metabolism of oral streptococci. Acarbose is an inhibitor of α-glucosidases including α-amylase [Truscheit et al., 1981], and is used as a medicine for diabetes by retarding the digestion of carbohydrates and absorption of glucose [Mörmann et al., 1983; Raimband et al., 1992], while maltotriitol is an indigestible sugar alcohol known to inhibit the maltose metabolism of mutans streptococci [Wursch et al., 1982].

Materials and Methods

Bacterial Strains

Streptococcus mutans NCTC10449, *Streptococcus sobrinus* ATCC6715 were used as representatives of caries-related bacteria. *Streptococcus sanguinis* ATCC10556 and *Streptococcus mitis* NCTC3165, which represented non-mutans streptococci that are predominant in dental plaque, were also used in this study.

Culture Media

Basal culture medium (1 liter) contained 17 g of tryptone (Difco Laboratories, Detroit, Mich., USA), 3 g of yeast extract (Difco) and 5 g of NaCl. After autoclaving, 0.3% glucose and 14 mM K_2HPO_4 were added separately through a sterile membrane filter (pore size 0.22 μm; Pall Gelman Laboratory, Ann Arbor, Mich., USA). This medium was designated as a TYE culture medium.

Anaerobic Procedure

All the experiments for bacterial cultivation and metabolism were conducted under strictly anaerobic conditions. The storage and cultivation of bacteria were performed in an anaerobic chamber (type NHC, gas phase: N_2, 80%; H_2, 10%; CO_2, 10%; Hirasawa Works, Tokyo, Japan). Preparation of bacterial cell suspensions and bacterial metabolism experiments were carried out in another anaerobic chamber (type NH, gas phase: N_2, 90%; H_2, 10%; Hirasawa Works). During centrifugation outside of the anaerobic chamber for harvesting and washing, the bacterial cells were protected from exposure to air by double-sealed centrifuge tubes (Kubota Commercial Affairs, Tokyo, Japan) [Miyasawa et al., 2003]. To remove oxygen, culture media and solutions were kept in the anaerobic chambers for at least 3 days prior to use, while powdered reagents and experimental instruments were placed in the anaerobic chamber for at least 1 day prior to use.

Bacterial Growth Conditions

The bacterial strains, which were cultured on blood agar plates and stored at 4°C in the anaerobic chamber, were inoculated into 8 ml of TYE culture media, and then incubated anaerobically at 37°C overnight. The bacterial suspension was subcultured in another 100 ml of TYE culture medium overnight, and then further cultured in 800 ml of TYE culture medium. Bacterial growth was monitored by measuring the optical density at 660 nm with a spectrophotometer (model UV-160, Shimadzu, Kyoto, Japan). The cells were harvested at the early exponential phase of growth by centrifugation (6,500 *g* for 15 min at 4°C). The cells were washed twice with 2 mM potassium phosphate buffer solution (pH 7.0) containing 150 mM KCl and 5 mM $MgCl_2$ (4°C). Washed cells were finally resuspended in the same solution at a concentration of 1.9 mg dry weight of cells per ml. The cell suspensions were stored at 4°C under anaerobic conditions until the following experiments were performed.

Preparation of Starch Solution and Salivary α-Amylase

Starch powder prepared from cooked potatoes (Wako Pure Chemical Industries, Osaka, Japan) was suspended in deionized water at a concentration of 5%, and heated at 95°C for 15 min. To remove small oligosaccharides that coexisted in the starch solution, the solution was place in a cellulose tube (Viskase Sales, Willowbrook, Ill., USA) and dialyzed against deionized water.

Human stimulated saliva was collected from 2 people. The samples were centrifuged and then filtered through a membrane filter, pore size 0.20 μm (Advantec, polypropylene; Toyo Roshi, Tokyo, Japan). The saliva samples were stored at 4°C and used within 1 h. The α-amylase activity in the saliva was measured by using an assay kit (amylase test, Wako). Commercially available human salivary α-amylase (type VIII-A, Sigma, Tokyo, Japan) was suspended in deionized water at the same enzymic activity. In the following experiments, the salivary α-amylase solution was used.

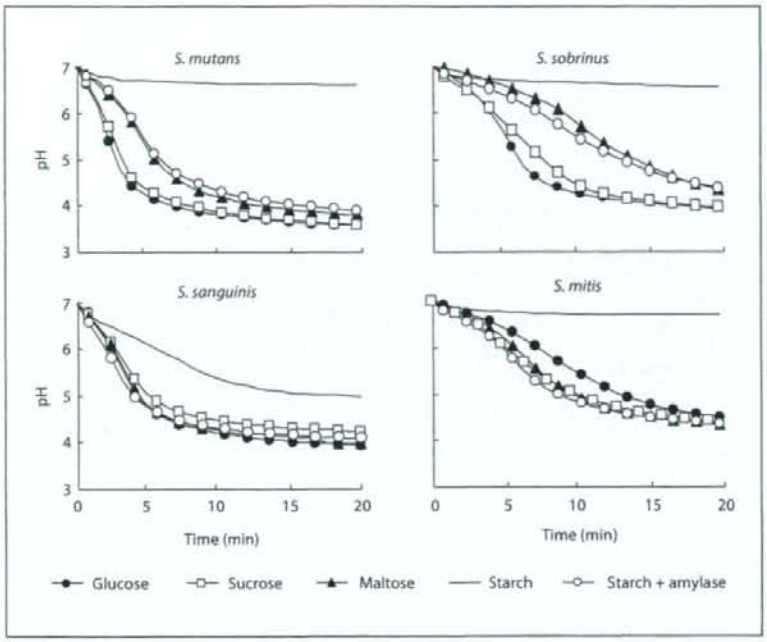

Fig. 1. The pH fall by the acid production from various sugars and cooked starch for 20 min. The results were similar among 3 independent experiments.

Measurement of pH Fall and Acid Production

Cells suspended in 2 mM potassium phosphate buffer solution (pH 7.0) containing 150 mM KCl and 5 mM $MgCl_2$ as described above (2.70 ml) were preincubated at pH 7.0 and 35°C for 4 min by titration with 60 mM KOH using a pH stat (Auto pH Stat; model AUT-211S, Toa Electronics, Tokyo, Japan) with a magnetic stirrer. The reaction was started in the cell suspensions by addition of a mixture (0.30 ml) containing 0.5% glucose, maltose, sucrose or cooked starch in the presence of 0.10 ml of α-amylase solution or deionized water. The pH fall from the initial pH 7.0 was monitored for 20 min by using a pH electrode without pH titration. The acid production rate was monitored at pH 7.0 for 10 min by using an automatic pH titration. The experiments were carried out 3 times independently.

Analysis of Acidic End Products from Various Sugar Fermentations

At 10 min after the addition of various sugars in the pH titration experiment, the cell suspensions (1.00 ml) were sampled and mixed immediately with 0.10 ml 6 N perchloric acid. The resultant mixtures were brought out from the anaerobic chamber and filtered (pore size 0.20 μm; Advantec, polypropylene) to remove cell debris. The cell-free filtrates were diluted with 0.2 N HCl and stored at 4°C for the assay of acidic end products. Acidic end products, which included lactic, acetic, formic and pyruvic acids, were quantified with a carboxylic acid analyzer (model Eyela S-3000X, Tokyo Rikakikai, Tokyo, Japan), as described previously [Takahashi et al., 1987].

Effects of Acarbose, Maltotriitol and Xylitol

As α-amylase inhibitors, acarbose (LKT Laboratories, St. Paul, Minn., USA) and maltotriitol (a gift from Mitsubishi Foodtech, Tokyo, Japan) were examined. Furthermore, we also used xylitol (Wako), which is known to be non-fermentable by oral bacteria [Ghring et al., 1974; Trahan, 1995] and to disturb the sugar metabolism of the mutans streptococci [Kakuta et al., 2003; Miyasawa et al., 2003]. In addition, we used an assay kit (amylase test, Wako) to determine whether acarbose, maltotriitol and xylitol could inhibit α-amylase activity.

Statistical Analysis

All numerical data are given as means ± standard deviations. Comparison between substrates was made by the one-way repeated-measures ANOVA, and significance was examined by the Scheffé post hoc test. Statistical analysis was performed using StatFlex software version 5.0 (Artech, Osaka, Japan). Differences were considered significant at the level $p < 0.05$.

Results

pH Fall in Cell Suspensions

After addition of glucose and sucrose to *S. mutans* suspensions there was a rapid fall in pH, which reached 3.58 at 20 min (fig. 1). While the pH fall from cooked starch in the absence of α-amylase was negligible, in the pres-

Table 1. Acid production rate from various sugars and cooked starch by streptococcal cells

Strains	Substrate							
	glucose	sucrose	maltose	starch	starch + amylase	starch + amylase + acarbose	starch + amylase + maltotriitol	starch + amylase + xylitol
S. mutans								
Acid production rate, μmol/min/mg	0.87 ± 0.43	0.78 ± 0.35	0.62 ± 0.26	0.09 ± 0.02	0.69 ± 0.28	0.19 ± 0.03	0.20 ± 0.06	0.22 ± 0.03
Glucose rate, %	100[a]	90 ± 2[a]	73 ± 5[b]	11 ± 2[c]	81 ± 6[b]			
Starch + amylase rate, %					100[a]	38 ± 1[b]	43 ± 17[b]	52 ± 12[b]
S. sobrinus								
Acid production rate, μmol/min/mg	1.17 ± 0.12	1.01 ± 0.06	0.58 ± 0.08	0.05 ± 0.01	0.61 ± 0.08	0.18 ± 0.03	0.39 ± 0.10	0.42 ± 0.07
Glucose rate, %	100[a]	87 ± 4[b]	49 ± 4[c]	4 ± 0[d]	52 ± 4[c]			
Starch + amylase rate, %					100[a]	30 ± 6[b]	65 ± 12[b]	70 ± 5[b]
S. sanguinis								
Acid production rate, μmol/min/mg	0.89 ± 0.25	0.76 ± 0.27	0.61 ± 0.22	0.30 ± 0.21	0.92 ± 0.31	0.27 ± 0.09	0.64 ± 0.19	0.96 ± 0.32
Glucose rate, %	100[a]	85 ± 6[a]	67 ± 16[a]	32 ± 17[b]	103 ± 5[a]			
Starch + amylase rate, %					100[a]	30 ± 4[b]	70 ± 9[a]	83 ± 7[a]
S. mitis								
Acid production rate, μmol/min/mg	0.47 ± 0.03	0.51 ± 0.03	0.52 ± 0.03	0.09 ± 0.02	0.61 ± 0.05	0.17 ± 0.02	0.27 ± 0.17	0.56 ± 0.06
Glucose rate, %	100[a]	108 ± 12[a]	111 ± 12[a]	20 ± 6[b]	131 ± 1[a]			
Starch + amylase rate, %					100[a]	27 ± 3[b]	42 ± 26[a]	91 ± 3[a]

Data presented are means ± SD for 3 experiments. Acid production rates from all the substrates with and without inhibitors were statistically analyzed in the same group.

A set of experiments was performed with the different substrates at the same time using the same culture of bacteria.

Data with different superscript letters in the same row are statistically different ($p < 0.05$).

ence of α-amylase the pH fall was almost equal to that from maltose, which reached 3.80 at 20 min. The pH value at 20 min observed for both glucose and sucrose was significantly larger ($p < 0.01$) than that for maltose and cooked starch in the presence of α-amylase. Similar results were obtained for *S. sobrinus*, with the exception that the pH fall noted with cooked starch in the presence of α-amylase was a little larger than that for maltose.

On the other hand, the pH values at 20 min after the addition of glucose, sucrose and maltose to *S. sanguinis* suspensions were 3.95, 4.25 and 4.00, respectively, with none of the differences exhibiting significance. In the absence of α-amylase, an obvious pH fall was observed for the cooked starch, though it was smaller than that observed for glucose, sucrose and maltose. In the presence of amylase, the pH fall was closer to that observed for glucose, sucrose and maltose. Similar results were obtained in *S. mitis* with the exception that the pH fall noted for cooked starch in the absence of α-amylase was negligible, while the pH fall for cooked starch in the presence of α-amylase was comparable to the other carbohydrates.

The Rate of Acid Production by Cell Suspensions at pH 7.0

With the rate of acid production from glucose regarded as being 100%, the rates for sucrose, maltose and cooked starch in the absence of α-amylase by *S. mutans* were 90 ± 2, 73 ± 5 and 11 ± 2%, respectively (table 1). In the presence of α-amylase, the acid production from cooked starch was 81 ± 6%, which was almost the same as seen for maltose. Similar results were obtained for *S. sobrinus*.

Similarly, the acid production rates for cooked starch observed in *S. sanguinis* and *S. mitis* were increased by the presence of α-amylase and exceeded those seen for glucose, sucrose and maltose.

Acidic End Products

For *S. mutans*, approximately one half of the total amount of the acidic end products from glucose and sucrose was lactic acid. In contrast, there was a low proportion of lactic acid in the acidic end products from cooked starch, which was the same for maltose (fig. 2). Similar

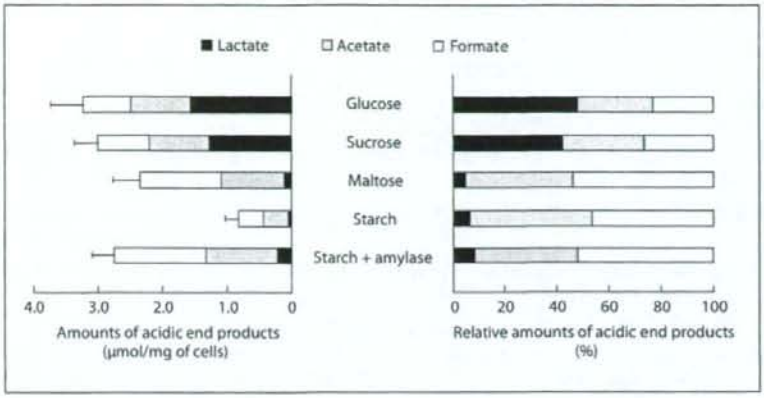

Fig. 2. Amounts of acidic end products and relative amounts of acidic end products for 10 min by the cells of *S. mutans* NCTC 10449 at pH 7.0. The data are the means of 3 independent experiments. Error bars indicate standard deviations.

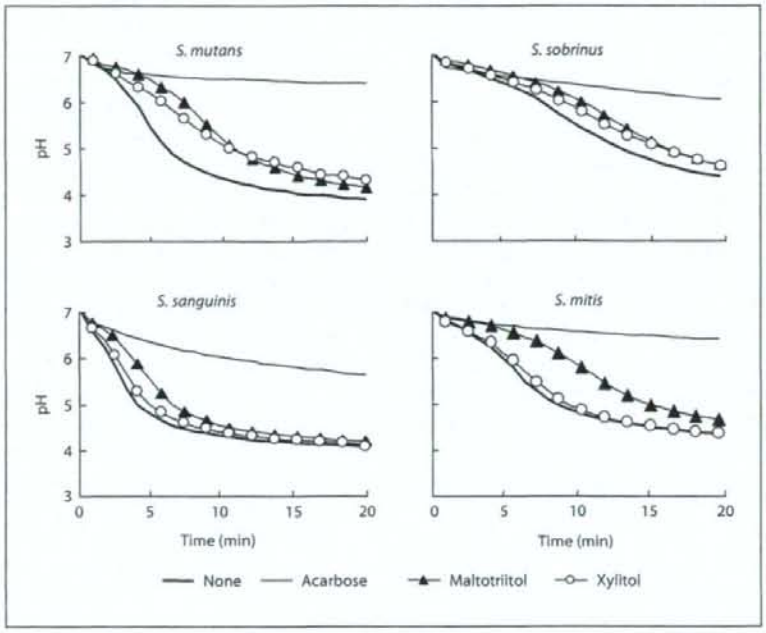

Fig. 3. Effects of acarbose, maltotriitol and xylitol on the pH fall by the acid production from cooked starch in the presence of amylase for 20 min. The results were similar among 3 independent experiments.

results were obtained in *S. sobrinus*, *S. sanguinis* and *S. mitis* (data not shown).

Effects of α-Amylase Inhibitors and Xylitol on pH Fall and Acid Production Rate from Cooked Starch

The addition of 1 mM acarbose inhibited the pH fall from cooked starch in the presence of α-amylase (fig. 3). While 60 mM maltotriitol also inhibited the pH fall, the inhibitory effect was lower than that seen for 1 mM acarbose. In *S. mutans* and *S. sobrinus*, 60 mM xylitol moderately inhibited the pH fall, while no effect was observed with *S. sanguinis* and *S. mitis*.

Acarbose, maltotriitol and xylitol inhibited the acid production rate of *S. mutans* from cooked starch in the presence of α-amylase (table 1). Similar results were obtained in *S. sobrinus*. While acarbose and maltotriitol inhibited the acid production by *S. sanguinis* and *S. mitis*, xylitol only had a small effect.

While acarbose and maltotriitol only had small inhibitory effects on the acid production rates by *S. mutans*

from glucose (5 ± 6%, 4 ± 4%, respectively), sucrose (16 ± 13%, 4 ± 4%, respectively) and maltose (8 ± 2%, 10 ± 1%, respectively), xylitol efficiently inhibited the acid production from glucose (21 ± 10%), sucrose (31 ± 9%) and maltose (47 ± 5%). *S. sobrinus* exhibited similar results. In addition, *S. sanguinis* and *S. mitis* showed similar results except that xylitol had no inhibitory effect (data not shown).

Effects of Acarbose, Maltotriitol and Xylitol on α-Amylase Activity

There were 87 and 32% inhibitions of α-amylase activity (619 Caraway unit) by 1 mM acarbose and 60 mM maltotriitol, respectively. Xylitol, however, exhibited no inhibitory effect on α-amylase activity.

Discussion

It is known that α-amylase hydrolyzes cooked starch mainly to maltose and maltotriose in addition to various other low-molecular-weight dextrins [Mörmann and Mühlemann, 1981; Kashket et al., 1996]. Oral streptococci are known to possess a multiple sugar transport system that transports low-molecular-weight dextrins into cells [Russell et al., 1992; Tao et al., 1993]. This allows for the cells to grow by utilizing maltotriose, maltotetraose, maltopentaose and maltohexaose [Glor et al., 1988; Russell et al., 1992; Tao et al., 1993]. In the present study, the acid production from cooked starch in the presence of α-amylase tended to be higher than that observed for maltose (table 1, fig. 1), suggesting that the oral streptococci incorporate low-molecular-weight dextrins derived from cooked starch more efficiently than maltose, and are thus able to convert them into acids.

S. sanguinis was the only strain that showed both a pH fall and acid production from cooked starch in the absence of α-amylase, suggesting that this strain possesses an extracellular α-glucosidase activity, but this activity was much smaller than that observed for the salivary α-amylase. This result supports a previous study that some oral streptococcal strains have α-amylase activity [Glor et al., 1988].

Both the pH fall and the acid production from glucose and sucrose by *S. mutans* and *S. sobrinus* exceeded those by *S. sanguinis* and *S. mitis* (table 1, fig. 1). This clearly indicates that mutans streptococci are more acidogenic than the non-mutans streptococci. However, with cooked starch in the presence of α-amylase, *S. sanguinis* and *S. mitis* were acidogenic as compared to *S. mutans* and *S. sobrinus*. These results suggest that there is involvement of non-mutans streptococci in the cariogenic potential of cooked starch.

The main end products from maltose and cooked starch are formate and acetate, while lactate is the main end product from sucrose and glucose (fig. 2), suggesting that there is a difference in metabolic regulation between maltose/cooked starch and sucrose/glucose. Oral streptococci metabolize carbohydrates to pyruvate through the Embden-Meyerhof-Parnas pathway, and convert pyruvate into lactate via lactate dehydrogenase. This enzyme is activated by fructose 1.6-bisphosphate (FBP), one of the glycolytic intermediates [Brown and Wittenberger, 1972; Yamada and Carlsson, 1975]. Intracellular accumulation of FBP has been observed during streptococcal glucose and sucrose metabolism [Yamada and Carlsson, 1975], which explains the lactic acid production observed during glucose and sucrose metabolism. The profile of intracellular glycolytic intermediates during maltose and cooked starch metabolism might be different, i.e. there might be low levels of intracellular FBP present, and, if so, pyruvate would be converted into formate and acetate by a series of reactions initiated by pyruvate formate lyase instead of by lactate dehydrogenase [Abbe et al., 1982]. This speculation may explain the observation that acetate and formate accumulated with lactate in the retained particles of starchy food (potato chips) in the dentition [Kashket et al., 1996], while only lactate increased after sucrose intake [Gao et al., 2001]. It is suggested that high pK_a acids, such as acetic acid, diffuse efficiently in the unionized form into the tooth surface layer [Featherstone and Rodgers, 1981; Geddes et al., 1984]. If there was then dissociation of the unionized acetic acid, this could result in the release of protons inside the tooth, thereby promoting the formation of subsurface demineralization [Featherstone and Rodgers, 1981].

In the current study, 1 mM acarbose effectively inhibited the metabolism of cooked starch by oral streptococci, particularly by the mutans streptococci, while 60 mM maltotriitol only moderately inhibited the metabolism (table 1, fig. 3). Since acarbose and maltotoriitol did not inhibit streptococcal sugar fermentation by themselves, but only exhibited inhibition when α-amylase was present, the inhibitory effects can be considered to be indirect and occur mainly through α-amylase inhibition. Acarbose has been reported to inhibit the incidence of caries in rats fed a processed starch diet [Mörmann et al., 1983], while maltotriitol has been reported to inhibit acid production in human dental plaque in the presence of saliva [Wursch and Koellreutter, 1982]. Thus, it is possible that

α-amylase inhibitors such as acarbose and maltotriitol are capable of moderating starch cariogenicity in humans.

The acarbose inhibition in *S. sanguinis* was weaker than for the other streptococci. This could possibly be attributed to the starch-degrading activity of *S. sanguinis* (fig. 1), which might tolerate acarbose. Meanwhile, xylitol inhibited the starch metabolism by the mutans streptococci through a direct inhibition of bacterial glycolysis. It has been reported that xylitol is incorporated into mutans streptococci as xylitol 5-phosphate and inhibits several intracellular glycolytic enzymes including glucose phosphate isomerase and phosphofructokinase, which leads to inhibition of glycolysis [Trahan, 1995; Miyasawa, 2003]. Xylitol did not inhibit starch metabolism of the non-mutans streptococci, as xylitol has no effect on either the glycolysis of the non-mutans streptococci [Vadeboncoeur et al., 1983] or the activity of α-amylase as shown in the present study.

In conclusion, the present study clearly showed that cooked starch in the presence of salivary α-amylase was a potential source of acids that could be produced by oral streptococci. In addition, non-mutans streptococci as well as mutans streptococci can be significant acid producers from cooked starch since they have similar acidogenicity. The acidogenicity might be controlled by α-amylase inhibitors and xylitol.

Because of the adhesiveness of starchy foods, they are easily retained in the dentition [Lingström and Birkhed, 1993], and thus a continuous supply of carbohydrate derived from salivary starch digestion could help to maintain a low plaque pH for a long time [Lingström and Birkhed, 1993; Kashket et al., 1994]. To address the adhesiveness of starchy foods as well as the diffusion of starch and acidic end products within dental plaque, research using biofilm models could be valuable [Kashket et al., 1994; Duarte et al., 2008].

In modern society, there are many opportunities to consume starchy foods not only as staple foods, but also as snacks. Therefore, people with root surfaces exposed to the oral cavity, those with hyposalivation and those who frequently consume starchy foods such as snacks need to pay particular attention to the acidogenic potential of these cooked starch products.

Acknowledgment

This study was supported in part by Grants-in-Aid for Scientific Research B (No. 16390601 and 19390539 to N.T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Abbe K, Takahashi S, Yamada T: Involvement of oxygen-sensitive pyruvate formate-lyase in mixed-acid fermentation by *Streptococcus mutans* under strictly anaerobic conditions. J Bacteriol 1982;152:175–182.

Beighton D, Hayday H: The establishment of the bacterium *Streptococcus mutans* in dental plaque and the induction of caries in macaque monkeys (*Macaca fascicularis*) fed a diet containing cooked-wheat flour. Arch Oral Biol 1984;29:369–372.

Brown AT, Wittenberger CL: Fructose-1,6-diphosphate-dependent lactate dehydrogenase from a cariogenic streptococcus: purification and regulatory properties. J Bacteriol 1972;110:604–615.

Brudevold F, Goulet D, Tehrani A, Attarzadeh F, van Houte J: Intraoral demineralization and maltose clearance from wheat starch. Caries Res 1985;19:136–144.

Clarkson BH, Krell D, Wefel JS, Crall J, Feagin FF: In vitro caries-like lesion production by *Streptococcus mutans* and *Actinomyces viscosus* using sucrose and starch. J Dent Res 1987;66:795–798.

Duarte S, Klein MI, Aires CP, Cury JA, Bowen WH, Koo H: Influences of starch and sucrose on *Streptococcus mutans* biofilms. Oral Microbiol Immunol 2008;23:206–212.

Ellen RP, Onose H: pH measurements of *Actinomyces viscosus* colonies grown on media containing dietary carbohydrates. Arch Oral Biol 1978;23:105–109.

Featherstone JDB, Rodgers BE: Effects of acetic, lactic and other organic acids on the formation of artificial caries lesions. Caries Res 1981;15:377–385.

Firestone AR, Schmid R, Mühlemann HR: Cariogenic effects of cooked wheat starch alone or with sucrose and frequency-controlled feedings in rats. Arch Oral Biol 1982;27:759–763.

Firestone AR, Schmid R, Mühlemann HR: Effect of the length and number of intervals between meals on caries in rats. Caries Res 1984;18:128–133.

Gao XJ, Fan Y, Kent RL Jr, Van Houte J, Margolis HC: Association of caries activity with the composition of dental plaque fluid. J Dent Res 2001;80:1834–1839.

Geddes DA, Weetman DA, Featherstone JDB: Preferential loss of acetic acid from plaque fermentation and its hydrolytic products by oral bacteria. J Dent Res 1988;67:75–81.

Ghring F, Makinen KK, Larmas M, Scheinin A: Turku sugar studies. IV. An intermediate report on the differentiation of polysaccharide-forming streptococci (*S. mutans*). Acta Odontol Scand 1974;32:435–444.

Glor EB, Miller CH, Spandau DF: Degradation of starch and its hydrolytic products by oral bacteria. J Dent Res 1988;67:75–81.

Grenby TH: Snack foods and dental caries: investigations using laboratory animals. Br Dent J 1990;168:353–361.

Havenaar R, Drost JS, de Stoppelaar JD, Huis in't Veld JH, Dirks OB: Potential cariogenicity of Lycasin 80/55 in comparison to starch, sucrose, xylitol, sorbitol and L-sorbose in rats. Caries Res 1984;18:375–384.

Kakuta H, Iwami Y, Mayanagi H, Takahashi N: Xylitol inhibition of acid production and growth of mutans streptococci in the presence of various dietary sugars under strictly anaerobic conditions. Caries Res 2003;37:404–409.

Kashket S, Yaskell T, Murphy JE: Delayed effect of wheat starch in foods on the intraoral demineralization of enamel. Caries Res 1994;28:291–296.

Kashket S, Zhang J, Van Houte J: Accumulation of fermentable sugars and metabolic acids in food particles that become entrapped on the dentition. J Dent Res 1996;75:1885–1891.

Lingström P, Birkhed D: Plaque pH and oral retention after consumption of starchy snack products at normal and low salivary secretion rate. Acta Odontol Scand 1993;51:379–388.

Lingström P, Birkhed D, Ruben J, Arends J: Effect of frequent consumption of starchy food items on enamel and dentin demineralization and on plaque pH in situ. J Dent Res 1994;73:652–660.

Lingström P, van Houte J, Kashket S: Food starches and dental caries. Crit Rev Oral Biol Med 2000;11:366–380.

Miyasawa H, Iwami Y, Mayanagi H, Takahashi N: Xylitol inhibition of anaerobic acid production by *Streptococcus mutans* at various pH levels. Oral Microbiol Immunol 2003;18:215–219.

Mörmann JE, Mühlemann HR: Oral starch degradation and its influence on acid production in human dental plaque. Caries Res 1981;15:166–175.

Mörmann JE, Schmid R, Mühlemann HR: Effect of alpha-amylase and alpha-glucosidase inhibitors on caries incidence and plaque accumulation in rats. Caries Res 1983;17:353–356.

Pollard MA: Potential cariogenicity of starches and fruits as assessed by the plaque-sampling method and an intraoral cariogenicity test. Caries Res 1995;29:68–74.

Raimbaud E, Buleon A, Perez S: Molecular modelling of acarviosine, the pseudo-disaccharide moiety of acarbose, and other inhibitors of alpha-amylases. Carbohydr Res 1992;227:351–363.

Russell RR, Aduse-Opoku J, Sutcliffe IC, Tao L, Ferretti JJ: A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. J Biol Chem 1992;267:4631–4637.

Scheinin A, Mäkinen KK, Ylitalo K: Turku sugar studies. V. Final report on the effect of sucrose, fructose and xylitol diets on the caries incidence in man. Acta Odontol Scand 1976;34:179–216.

Takahashi N, Abbe K, Takahashi-Abbe S, Yamada T: Oxygen sensitivity of sugar metabolism and interconversion of pyruvate formatelyase in intact cells of *Streptococcus mutans* and *Streptococcus sanguis*. Infect Immun 1987;55:652–656.

Tao L, Sutcliffe IC, Russell RR, Ferretti JJ: Cloning and expression of the multiple sugar metabolism (*msm*) operon of *Streptococcus mutans* in heterologous streptococcal hosts. Infect Immun 1993;61:1121–1125.

Trahan L: Xylitol: a review of its action on mutans streptococci and dental plaque – its clinical significance. Int Dent J 1995;45:77–92.

Truscheit E, Frommer W, Junge B, Müller L, Schmidt DD, Wingender W: Chemistry and biochemistry of microbial α-glucosidase inhibitors. Angew Chem 1981;20:744–761.

Vadeboncoeur C, Trahan L, Mouton C, Mayrand D: Effect of xylitol on the growth and glycolysis of acidogenic oral bacteria. J Dent Res 1983;62:882–884.

Wursch P, Koellreutter B: Maltitol and maltotriitol as inhibitors of acid production in human dental plaque. Caries Res 1982;16:90–95.

Yamada T, Carlsson J: Regulation of lactate dehydrogenase and change of fermentation products in streptococci. J Bacteriol 1975;124:55–61.

厚生労働科学研究費補助金
（循環器等生活習慣病対策総合研究事業）

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平成 20 年度
総括研究報告書

平成 21 年4月

発行責任者：厚生労働科学研究
「フッ化物応用の総合的研究」班
主任研究者　眞木吉信

印刷：千葉孔版(株)