Calcium binding to *S. mutans* grown in the presence or absence of sucrose

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Abstract: Sucrose is the most cariogenic dietary carbohydrate because it is a substrate for insoluble extracellular polysaccharide (IEPS) production in dental biofilms, which can proportionally decrease bacterial density and, consequently, the number of biofilm calcium (Ca) binding sites. Ca bound to bacterial cell walls can be released into the biofilm fluid during a cariogenic challenge, reducing the driving force for mineral dissolution provoked by the pH drop. Thus, we investigated the effect of an IEPS-rich extracellular matrix on bacterial Ca binding after treatment with Ca solutions. *Streptococcus mutans* Ingbritt 1600 was cultivated in culture broths supplemented with 1.0% sucrose or 0.5% glucose + 0.5% fructose. The IEPS concentration in bacterial pellets was determined after alkaline extraction. Bacterial pellets were treated with 1 mM or 10 mM Ca^{++} solutions at 37°C for 10 to 60 min. Ca binding to bacterial pellets, determined after acid extraction using the Arsenazo III reagent, was fast and concentration dependent. Although the IEPS concentration was approximately ten times higher in bacterial pellets cultivated in sucrose as compared to its monossaccharides, bound Ca concentration after Ca treatment was similar in both conditions. These results suggest that IEPS may not influence the amount of Ca bound to reservoirs of dental biofilms.

Descriptors: Sucrose; *Streptococcus mutans*; Polysaccharides; Calcium; Dental Caries.

Introduction

There is considerable evidence demonstrating that sucrose is the most cariogenic carbohydrate from the human diet,[1] with enhanced cariogenicity as compared to its component monosaccharides, glucose and fructose.[2] The reason for this relies on the fact that sucrose, besides being fermented to acids, is also used by oral bacteria to synthesize extracellular polysaccharides (EPS).[3] These EPS, mainly the insoluble ones (IEPS), play a significant role on the adhesion and accumulation of cariogenic streptococci on the tooth surface, especially *S. mutans*.[4] In addition, they change the biofilm structure, resulting in increased porosity,[5] which allows fermentable substrates to diffuse and be metabolized in the deepest parts of the biofilm.[6]

Furthermore, since IEPS decrease the bacterial density of biofilms,[7,8] they may reduce the amount of biofilm calcium (Ca) binding sites[9], which could also influence biofilm cariogenicity. Ca bound to bacterial cell

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walls might function as a source of mineral ions to the biofilm fluid during a pH drop,[9] acting as a mineral buffer by helping to maintain the saturation of plaque fluid with respect to tooth mineral, a factor that largely governs its dissolution.[10]

Therefore, we hypothesized that the number of binding sites for Ca would be reduced in dental biofilm formed in the presence of sucrose, and we investigated this effect by evaluating the rate of Ca binding to *S. mutans* grown in the presence of sucrose or its component monosaccharides *in vitro*.

Methodology

Experimental design

S. mutans Ingbritt 1600 was cultivated in the presence of sucrose (to allow the production of extracellular polysaccharides) or its component monosaccharides, glucose and fructose (not substrates for EPS synthesis). Bacterial pellets obtained by centrifugation were analyzed for IEPS, bacterial proteins (as an indicator of bacterial density[11]), and baseline Ca. Additional pellets were treated with 1 or 10 mM Ca^{++} ($CaCl_2$), buffered with 0.05 M PIPES (piperazine-N, N'-bis [2-ethanesulphonate]; Sigma Biochemicals), pH 7.0, at 37°C for 10, 30, or 60 min. The Ca concentrations used, 1 and 10 mM, represent the resting Ca biofilm fluid concentration[12] and the high Ca concentration found in biofilm fluid after a pH drop, respectively.[10] After the specified equilibrium time, the bacteria were separated from the test solution by centrifugation, bound Ca was extracted from the bacterial pellet with acid treatment, and its concentration was determined.

Bacterial preparation

S. mutans Ingbritt 1600 was cultivated in Todd-Hewitt broth (THB) (Difco Labs., Detroit, USA) supplemented with 1% sucrose or 0.5% glucose + 0.5% fructose for 18 h at 10% pCO_2 and 37°C. Bacterial pellets were separated by centrifugation. In order to remove remnants of culture broth and unbound Ca, the pellets were sequentially washed using sonication (Vibra Cell sonicator, Sonics and Materials, Danbury, USA) at 7 W for 1 min, first in 0.05 M PIPES buffer, pH 7.0, followed by 0.01 M EDTA solution, and again in PIPES buffer.[9] Between each washing, the pellet was recovered by centrifugation. After this procedure, the pellet was spread on filter paper to remove excess moisture. Aliquots were transferred to microcentrifuge tubes for IEPS and protein determination and baseline Ca and Ca-binding analyses.

IEPS and protein determination in bacterial pellets

To extract EPS, aliquots (n = 3) of bacterial pellets were weighed (± 0.01 mg), suspended in 0.9% NaCl solution (1 mL/mg wet weight), sonicated at 7 W for 60 s, and centrifuged at 10,000 *g* for 5 min at 4°C to remove soluble EPS.[13] IEPS was alkaline extracted from the remaining pellet[14] and precipitated with ethanol. The carbohydrate concentration in the IEPS extract was estimated by the phenol-sulfuric acid method.[15]

To determine bacterial proteins, aliquots of bacterial pellets (n = 3) were first treated with a mild alkaline solution[16] to remove extracellular proteins, and the supernatant obtained by centrifugation was discarded. The precipitate was treated with a hot alkaline solution to extract bacterial proteins,[13] whose concentration was determined by the Lowry method.[17]

Ca-binding assessment

Aliquots of *S. mutans* pellets were exposed to PIPES buffer (1.5 mL/10 mg of bacteria), pH 7.0, containing 1 or 10 mM Ca, for 10, 30, or 60 min (n = 2 for each time point). The high dilution was used to maintain a stable Ca concentration during the experiment. At each time point, the pellets were collected by centrifugation (21,000 *g* for 5 min), and the supernatant was carefully vacuum-aspirated with a micropipette under a microscope to remove any treatment solution residue. The efficacy of residual Ca removal was validated by a preliminary experiment (data not shown) in which the amount of Ca remaining in the bacterial pellet was confirmed to be bound and not simply trapped in the bacterial pellet fluid.

Determination of Ca bound to bacterial cells

Bound Ca was extracted from the bacterial pel-

lets by treatment with 0.5 M HCl (0.1 mL/10 mg bacterial wet weight) for 3 h.[14] The acid extract was collected after centrifugation, and the Ca concentration was measured using the Arsenazo III colorimetric reagent, after neutralization with 2.5 M NaOH.[18] For the analyses, Ca standards contained HCl and NaOH in the same proportion as the samples. The absorbance of the mixtures was read in 96-well microplates, using a Multiskan Spectrum (Thermo Scientific) microplate reader at 650 nm.

Statistical analyses

Data of IEPS, bacterial proteins, and baseline Ca in untreated pellets on biofilms cultivated in the presence of sucrose or glucose + fructose were compared by the *t*-test. Ca binding to the bacterial pellets after treatment with Ca solutions at different times were compared by split plot ANOVA, using cultivation conditions (sucrose or glucose + fructose) as plots and Ca concentration in the treatment solution (1 or 10 mM) and time (10, 30, or 60 min) as subplots. The normality of error distribution and the homogeneity of variance were checked for each response variable using the SAS/LAB package (SAS software, version 8.01, SAS Institute Inc., Cary, USA), and data were transformed as suggested by the software, according to Box *et al.*[19] The SAS system (version 9.2) was used in the analyses, and the significance level was set at 5%.

Results

Comparisons of the composition of bacterial pellets cultivated in the presence of sucrose or glucose + fructose revealed significantly ($p < 0.05$) higher EPS and lower bacterial protein and baseline Ca concentrations for the former condition (Table 1).

Figure 1 shows that the rate of Ca binding to the bacterial pellets cultivated either in the presence of sucrose or glucose + fructose was fast, with no significant difference in bound Ca concentration among the treatment times ($p > 0.05$). Higher Ca concentrations were found in bacteria treated with 10 mM Ca, as compared to those treated with

Table 1 - Insoluble extracellular polysaccharide (IEPS), protein concentration (mg/g wet weight, mean ± SD; n = 3), and baseline Ca (μmol/g wet weight, mean ± SD, n = 4) in bacterial pellets according to the carbohydrate source used for *S. mutans* growth.

S. mutans carbohydrate source	IEPS*	Bacterial protein	Baseline Ca
Sucrose	52.5 ± 2.5	39.3 ± 1.8	0.4 ± 0.1
Glucose + fructose	6.0 ± 0.2	75.1 ± 0.5	1.7 ± 0.3

* An inverse transformation of the IEPS data was performed to fit the assumptions of the *t*-test. Significant differences were observed between the treatments for all variables analyzed ($p < 0.05$).

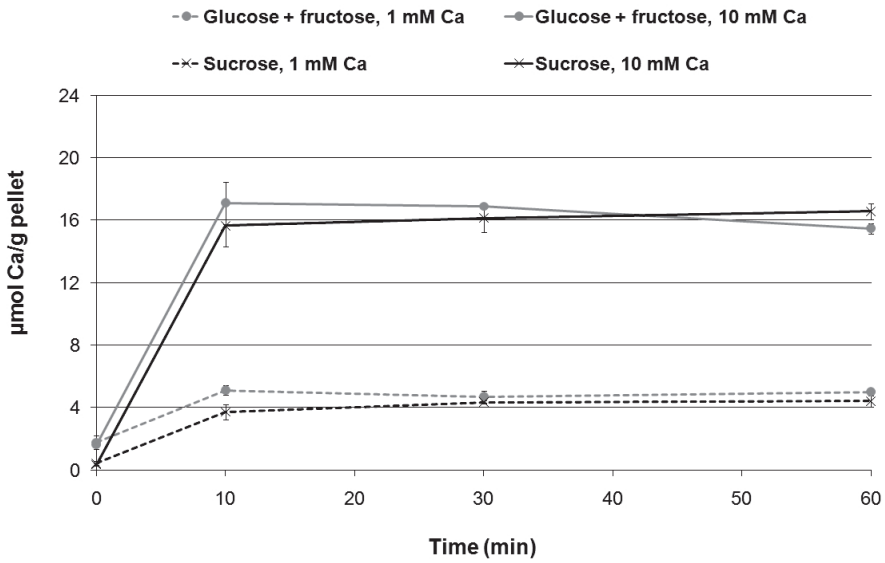

Figure 1 - Bound Ca concentration (μmol/g wet weight, mean ± SD; n = 2 for each time point) in bacterial pellets as a function of time, carbohydrate source, and Ca concentration in the treatment solution. Time 0 represents the baseline Ca amount found in bacterial pellets (n = 4). Data were transformed to $\log_{10}$ units for statistical analysis. Only the effect of Ca treatment solutions was found to be significant (split-plot ANOVA, $p < 0.05$).

1 mM Ca ($p < 0.05$). However, the amount of bound Ca was not significantly different between the two bacterial growth conditions (presence or absence of sucrose) ($p > 0.05$).

Discussion

In the present study, the concentration of IEPS was approximately 10 times higher in the bacterial pellets cultivated in the presence of sucrose when compared to its monosaccharides (Table 1). These results confirm the ability of this *S. mutans* strain to produce EPS in the presence of sucrose,[6,8] as a model that simulates what happens in dental biofilm.[2] Also, the lower protein concentration in bacterial pellets (Table 1) is in agreement with van Houte *et al.*,[8] who reported decreased bacterial cell density when this *S. mutans* strain was grown in THB supplemented with 2% sucrose as compared to the same medium with a ten times lower concentration. Considering that proteins from bacterial cell walls are Ca-binding sites,[20] and EPS does not have this property,[9] it would be expected that bacterial pellets grown in the presence of sucrose would have a lower ability to bind Ca per weight of bacteria. Although this was observed for the baseline Ca concentration in the bacterial pellets (Table 1), the results of Ca binding after treatment with Ca-containing solutions did not support this hypothesis (Figure 1), and the theoretical support to explain these results is presented below.

The concentration of bound Ca found after treatment with Ca solutions is consistent with previous studies of Ca binding to streptococci strains from solutions with varying Ca concentrations,[21-23] in which glucose was used as the carbohydrate source for bacterial growth. However, these authors did not evaluate the effect of different sugars used to cultivate bacteria on Ca binding, especially sucrose, which significantly affects the biofilm extracellular matrix composition.[2,3] This was the aim of the present study.

Moreover, the results agree with those of previous studies[2,12,24] that did not find a significant difference in total Ca concentration in biofilms formed *in situ* in the presence of glucose + fructose when compared to sucrose, suggesting that the IEPS-rich extracellular biofilm matrix is not able to affect whole biofilm Ca binding. In this regard, it is noteworthy that IEPS concentration represents approximately 5% of the total biofilm wet weight, in agreement with previous *in situ* studies.[2,13,14,24-26] This low percentage, supposedly relevant to induce differences between the bacterial pellets on residual Ca-binding capacity, might not be able to significantly affect bound Ca concentration once the bacteria are treated with Ca-containing solutions (i.e., 1 or 10 mM). Furthermore, the results suggest that the EPS concentration is not the reason for the low concentration of inorganic ions (Ca, inorganic phosphorus, and fluoride) found in dental biofilms formed under exposure to sugars,[2,3,12-14,24,26] suggesting that further research should be done to explain this biological phenomenon.

Nevertheless, an alternative hypothesis to explain our findings is based on the effect of sucrose to enhance the number of Ca-binding sites in bacterial plaque formed in its presence. *In vivo* studies have shown that dental plaque formed in the presence of sucrose has a higher amount of lipoteichoic acid,[27] which might enhance Ca-binding capacity because phosphate groups have a higher affinity for Ca ions than carboxyl groups from proteins present in streptococcus cell walls.[20] Therefore, in the presence of sucrose, a higher concentration of bacterial cell wall components with higher Ca-binding capacity could be expressed and compensate for the lower bacterial density.

Our Ca-binding kinetics results are consistent with those by Tatevossian,[28] who studied the kinetics of Ca binding in a pool of bacterial plaque using Ca ion-selective electrodes *in vitro*; this study showed that the binding was rapid and almost reached saturation within 10 min. Although the experimental design used in the present study did not allow for reaction rate determination at times less than 10 min with good precision, the high dilution of the bacterial pellet used ensured that the concentration of the treatment solution did not change as binding occurred.

It should be noted that the importance of bacterial-bound Ca as a source of Ca to the biofilm fluid during a pH drop is subject to further confirmation.

Thus, not only the binding capacity, but also the kinetics of Ca release as a function of pH should be studied in further detail to provide a better understanding of these phenomena in dental plaque *in vivo*.

Conclusion

Our data suggest that Ca binding to bacterial surfaces is Ca concentration-dependent, but it is not affected by the presence of EPS in the biofilm matrix.

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