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Effect of antiplaque compounds and mouthrinses on the activity of glucosyltransferases from *Streptococcus sobrinus* and insoluble glucan production

A. Furiga[1], M. Dols-Lafargue[2], A. Heyraud[3], G. Chambat[3], A. Lonvaud-Funel[2], C. Badet[1,2]

[1]Laboratoire Odontologique de Recherche, UFR d'Odontologie, Université Victor Segalen Bordeaux 2, Bordeaux, France, [2]UMR 1219 Œnologie, Université Victor Segalen Bordeaux 2, Talence, France, [3]Centre de Recherche sur les Macromolécules Végétales, ICMG/CNRS, Grenoble, France

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Introduction: The development of therapeutic agents inhibiting the activity of glucosyltransferases (GTF) and their production of glucans is a potential strategy to reduce dental decay. The aim of this study was first to characterize a GTF preparation from *Streptococcus sobrinus* ATCC 33478 and then to evaluate the effects of select compounds and mouthrinses on insoluble glucan (ISG) formation by combined GTFs.
Methods: The purity of the crude GTF mixture was assessed by electrophoresis. The effects of pH, temperature, sucrose, and dextran T10 concentrations on GTF activity were analyzed and the chemical structure of the products was investigated. Finally, the inhibition of GTF by commercial mouthrinses used in oral hygiene and their active components (chlorhexidine, polyphenolic compounds, fluoride derivatives, polyols, cetylpyridinium chloride, and povidone iodine) was analyzed through the reductions in the overall reaction rate and the quantity of ISG synthesized.
Results: The *S. sobrinus* ATCC 33478 crude GTF preparation obtained contains a mixture of four different GTFs known for this species. For optimal adherent ISG formation, the reaction parameters were 37°C, pH 6.5, sucrose 50 g/l, and dextran T10 2 g/l. Under these conditions, the most effective agents were chlorhexidine, cetylpyridinium chloride, and tannic acid. Eludril®, Elmex®, and Betadine® were the most effective inhibitors of all the mouthrinses tested.
Conclusion: As the formulation of commercial products considerably influences the efficiency of active components, the fast representative ISG inhibition test developed in this study should be of great interest.

Key Words: dental plaque; glucosyltransferases; insoluble glucan; mouthrinses; *Streptococcus sobrinus*

Cecile Badet, Laboratoire Odontologique de Recherche, UFR d'Odontologie, Université Victor Segalen Bordeaux 2, 16 cours de la Marne, 33082 Bordeaux Cedex, France
Tel.: + 33 5 57 57 30 07;
fax: + 33 5 57 57 30 10;
e-mail: cecile.badet@odonto.u-bordeaux2.fr
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Streptococcus mutans and *Streptococcus sobrinus* play major roles in the development of oral diseases like dental caries. One of the important virulence properties of these organisms is their ability to colonize dental surfaces in the presence of dietary sucrose (13, 51). This process is dependent in part on the synthesis of glucans by extracellular glucosyltransferases (GTFs). This contributes significantly to the formation of dental plaque in which the accumulation of metabolic acids produced by bacteria leads to local demineralization of the tooth (28, 31, 37, 56). A primary means of controlling bacterial colonization and the accumulation of plaque would be to inhibit the functions of these enzymes, and in particular the insoluble glucan (ISG) production (12).

While *S. mutans* has been shown to be more prevalent than *S. sobrinus* in dental

plaque samples, several epidemiological studies have shown that the prevalence of *S. sobrinus* is more closely associated with high caries activity (4, 14, 23, 31, 40). This species possesses four *gtf* genes, *gtfI*, *gtfU*, *gtfT*, and *gtfS*, corresponding respectively to the enzymes GTF-I, GTF-S_1, GTF-S_2, and GTF-S_3 (26, 39). These four enzymes present a highly homologous primary structure and all belong to the glucansucrase glycoside-hydrolase family 70 (E.C. 2.4.1.5). The multistep polymerization mechanism catalyzed by these enzymes has been recently elucidated (36). It proceeds in two steps: glucosyl enzyme formation and glucosyl transfer, which represents the limiting step (35). In the absence of primer, two molecules trigger polymerization by glycosyl transfer: sucrose itself and the glucose produced by sucrose hydrolysis. Elongation then occurs by transfer of the glucosyl residues originating from sucrose to the non-reducing end of the products initially formed (36). Depending on the enzyme, (i) the elongation step is more or less activated by added glucan, and (ii) the nature of the glucosidic linkage formed varies, as well as the size, degree of branching, and solubility of the products. GTF-I (also referred to as GTF-P3, 175 kDa) synthesizes significant amounts of α-1,3 water-insoluble glucan only in the presence of added water-soluble glucan. In the absence of added primer, it mainly hydrolyzes sucrose without synthesizing water-insoluble glucan (19, 26). GTF-S_3 (GTF-S or GTF-P2, 155 kDa) hydrolyzes sucrose and synthesizes water-soluble α-1,6 isomalto-oligosaccharides without the need of a primer (57). GTF-S_1 (GTF-P4 or GTF-U) is activated by the addition of water-soluble glucan whereas GTF-S_2 (GTF-P1 or GTF-T) activity is primer-independent. These two enzymes can hydrolyze sucrose and synthesize water-soluble glucan containing both α-1,3 and α-1,6 linkages, with the GTF-P4 glucan being more branched than the GTF-P1 glucan (19).

The glucans synthesized by isolated and purified GTF display a structure and properties different from the products formed by the crude enzyme preparations and from the glucans naturally occurring in dental plaque (25). In the case of *S. sobrinus* AHT, the combined action of the four GTFs described above mainly leads to the formation of water-insoluble, adherent glucan containing about 77% α-1,3 glucosidic bonds (19). Without the cooperative action of a GTF synthesizing ISG and a GTF producing soluble glucan, it would not be possible for this organism to have any degree of adhesion. Indeed, the synthetic product of each purified enzyme alone is unable to adhere to the glass of a test tube (15, 25).

The development of therapeutic agents inhibiting the combined action of *S. sobrinus* GTFs is therefore an important strategy to reduce dental decay. However, enzymatic inhibitors have often been studied on highly purified and poorly representative GTF preparations (53, 55). Several classes of compounds are used as antiplaque agents in dentistry (33). These molecules mainly include bis-biguanides, quaternary ammonium salts, fluoride, and iodine derivatives. Other agents, like polyols and polyphenolic compounds, have more recently been studied and have shown an interesting antiplaque activity (10, 55, 58).

The aim of this study was first to characterize a representative GTF preparation from *S. sobrinus* ATCC 33478 and then to evaluate the effects of some GTF inhibitors and mouthrinses on ISG formation by combined GTFs.

Materials and methods

Reagents

Chlorhexidine digluconate (Sigma, St Louis, MO) was obtained as a 20% [weight/volume (w/v)] aqueous solution. Sorbitol, xylitol, cetylpyridinium chloride, sodium fluoride, and tannic acid were purchased from Sigma and prepared in purified water at a concentration of 1% (w/v).

Mouthrinses chosen for testing were commercially available over-the-counter preparations of the following oral hygiene products: Eludril® mouthwash [Pierre Fabre Medicament, Castres, France; containing chlorhexidine digluconate 0.1% (w/v), chlorobutanol hemihydrate 0.5% (w/v), ethanol 42.8% (v/v), glycerol, docusate sodium, mint essence, levomenthol, cochineal red A, purified water]; Elmex® mouthwash [GABA Laboratories, Bois-Colombes, France; containing amine fluoride (olafluor) 0.13% (w/v), sodium fluoride 0.033% (w/v), water, polyethyleneglycol-40 (PEG-40), hydrogenated castor oil, aroma, polyaminopropyl biguanide, potassium acesulfame, hydrochloric acid]; Meridol® mouthwash [GABA Laboratories; containing amine fluoride (olafluor) 0.1641% (w/v), stannous fluoride 0.0523% (w/v), water, xylitol, polyvinylpyrrolidone, PEG-40, hydrogenated castor oil, aroma, sodium saccharin, CI 42051]; Listerine® Coolmint mouthwash [Pfizer, Paris, France; containing eucalyptol 0.092% (w/v), thymol 0.064% (w/v), menthol 0.042% (w/v), methyl salicylate 0.06% (w/v), ethanol 21.6% (v/v), water, sorbitol, poloxamer 407, benzoic acid, aroma, sodium saccharin, sodium benzoate, CI 42053]; Alodont® mouthwash [Pfizer, Paris, France; containing cetylpyridinium chloride 0.005% (w/v), chlorobutanol hemihydrate 0.05% (w/v), eugenol 0.04% (w/v), ethanol 21% (v/v), polyoxyethylene hydrogenated castor oil, sodium hydroxide, citric acid, mint essence, sodium saccharin, patent blue V (E131), purified water]; and Betadine® mouthwash 10% (Meda Pharma SAS, Cergy, France; containing povidone iodine 10% (w/v), glycerol, sodium saccharin, sodium hydroxide, aroma, purified water].

Bacterial strain and culture conditions

S. sobrinus ATCC 33478 was grown anaerobically for 18 h at 37°C in brain–heart infusion (Difco, Sparks, MD) supplemented with glucose at 10 g/l. A precultured broth (18 h old, 17 ml) of bacteria grown at 37°C in the same medium was used for flask inoculation.

Preparation of crude GTF

After 18 h, the culture broth was centrifuged (13,500 ***g***, 30 min, 4°C) and the proteins were precipitated from the supernatant by addition of ammonium sulfate (50% saturation). After 30 min of agitation at 4°C, the precipitate was collected by centrifugation (13,500 ***g***, 30 min, 4°C), dissolved in 10 mM K_2HPO_4 buffer (pH 6.5) and dialyzed against the same buffer for 18 h at 4°C to give crude cell-free GTF from *S. sobrinus*. Protein concentrations were determined by the method of Lowry et al. (32) with bovine serum albumin as a standard.

Enzyme assay

The standard reaction mixture contained crude GTF solution (0.1 U/ml), 65 mM K_2HPO_4 buffer (pH 6.5), 50 g/l sucrose, 2 g/l dextran T10 and 0.1 g/l NaN_3. Incubation was carried out at 37°C. For investigation purposes, the pH, temperature, and sucrose and dextran T10 concentrations were modified as specified in the text. Just after sampling, the reaction was stopped by heating the samples at 100°C for 10 min.

The initial reaction rate was determined by measuring the reducing sugar concentration increase over the first 3 h of reactions by dinitrosalicylic acid assay, with

fructose as a standard (47). One unit of total GTF activity (U) was defined as the amount of enzyme that catalyzed the release of reducing sugars equivalent to 1 μmol of fructose from sucrose per min under standard conditions. Appropriate substrate and enzyme blanks were included to correct for any free reducing group not emanating from sucrose transformation.

For more precise substrate and product analysis, the heated samples were centrifuged (17,600 ***g***, 4°C, 30 min). The ISG was recovered from the pellet, washed three times with distilled water, dried at 65°C for 24 h, and then weighed. From the supernatant, various compounds were analyzed. To determine the amount of soluble glucan, three volumes of cold ethanol were added to the supernatant. The mixture was incubated at 4°C for 16–24 h and centrifuged at 17,600 ***g*** at 4°C for 5 min. The precipitate was washed twice with 80% ethanol and the last precipitate was redissolved in distilled water. The amount of soluble glucan in the supernatant was determined by the phenol–sulfuric acid method with glucose as a standard (11). The amounts of fructose and glucose contained in the supernatant were determined by means of an enzymatic kit (D-Glucose: D-Fructose; R-biopharm, Darmstadt, Germany). Leucrose and sucrose concentrations were measured by reverse phase chromatography (C18 column, Ultrasep 100; Bischoff, Leonberg, Germany) using a Waters system consisting of a pump (Waters 515; Waters, Milford, MA), a manual injection loop (20 μl) and a refractometer (Waters 2414). For routine analysis, the eluant (ultrapure water) had a constant flow rate of 0.5 ml/min at room temperature as described by Remaud-Simeon et al. (43). This high-performance liquid chromatography (HPLC) method also enabled the visualization of peaks corresponding to oligosaccharides. To analyze larger oligosaccharides (degree of polymerization between 6 and 12), the eluant (H_2O/methanol: 99/1) had a constant flow rate of 0.6 ml/min at 45°C (9).

Glucosyltransferases characterization

Electrophoresis analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the method of Laemmli with 10% (w/v) acrylamide gels (29). The enzyme preparation was deposited on minigels (Miniprotean; BioRad, Marnes la Coquette, France). After migration, proteins were stained with Coomassie brilliant blue R-250.

For *in situ* detection of GTF activity, the gel was first washed three times with 65 mM K_2HPO_4 buffer (pH 6.5) containing 0.1 % (v/v) Triton X-100 at 4°C to eliminate the SDS. It was then incubated in the same buffer supplemented with 50 g/l sucrose and 2 g/l dextran T10 at room temperature for 72 h; active bands were detected by the appearance of dextran polymer as described by Miller and Robyt (34). The gel was then stained with Periodic Acid Schiff reagent; the staining phase was started by oxidation with periodic acid 0.7% (w/v) and acetic acid 5% (v/v) for 1 h. The gel was washed six times for 30 min in sodium metabisulphite 0.2% (w/v) and acetic acid 5%, and placed in Schiff's reagent, protected from light, for 50 min, then bleached with sodium metabisulphite 0.5% and acetic acid 5% and visualized.

Glucan synthesis and structural analysis

Glucans were synthesized by incubating a crude GTF preparation in 65 mM K_2HPO_4 buffer (pH 6.5), 50 g/l sucrose, and 0.1 g/l NaN_3 in the absence of dextran T10. After incubation at 37°C for 48 h, the reaction mixture was centrifuged (17,600 ***g***, 4°C, 20 min). The insoluble material was recovered from the pellet, washed twice with ultrapure water, and lyophilized. Soluble dextran was purified from the supernatant by ethanol precipitation at a 75% (v/v) final concentration, washed twice with ultrapure water, and lyophilized.

The glucans mixture was subjected to structural analysis. Each glucan was methylated twice (to ensure complete methylation) as previously described (17). The sample (4–7 mg) was solubilized in dimethylsulfoxide (1 ml). Methylsulfinyl anion (0.5 ml) was added to the solution and the mixture was stirred under nitrogen for 16 h at 20°C. Methyl iodide (1 ml) was then added over a 2-h period. The excess methyl iodide was evaporated and the mixture was dialyzed and freeze-dried. A second methylation was performed under the same conditions on the entirety of the freeze-dried material. The permethylated polysaccharides were then hydrolyzed with formic acid (0.8 ml, 90% at 100°C by mg of samples) for 1 h. After drying, further hydrolysis with trifluoroacetic acid (1 ml, 2 M, 100°C) was performed for 3 h. Samples were then reduced with a sodium borohydride solution for 16 h at room temperature. Borate compounds were eliminated by methanol/HCl 1% treatment. The reduced samples were acetylated with acetic anhydride/pyridine (v/v) at 100°C for 1 h (44). Alditol acetate derivatives were analyzed by gas chromatography equipped with a flame-ionization detector using a SP2380 macrobore column (0.53 mm × 30 m) in a Hewlett Packard 5890A system using various methylated alditol carbohydrates as controls. The carrier gas was high-purity nitrogen and the injector port and detector were heated to 260°C and 280°C, respectively. For sample separation the following conditions were applied: 3 min at an initial temperature of 165°C followed by an incremental increase (2.5°C/min) to a final temperature of 225°C for 3 min. Complementary identification was obtained by gas chromatograph mass spectrometry (Delsi GC coupled to a Nermag R10-10C mass analyzer) using the chromatographic protocol described above.

The soluble glucan fraction was analyzed using an Autoflex time-of-flight (TOF) mass spectrometer (Bruker, Billeria, MA) equipped with a nitrogen laser (l = 335 nm). Experiments were performed in the reflection mode. Mass spectra were acquired in the positive-ion mode. The sample was prepared in a solution of 2000 p.p.m. in ultrapure water and the dried-droplet deposit method was used with 2,5-dihydroxybenzoic acid as matrix.

The ^{13}C nuclear magnetic resonance spectra of the glucans were recorded with a Bruker AC 300 spectrometer operating at a frequency of 75,468 MHz. Samples were examined as solutions in deuterium oxide (10–15 mg in 0.35 ml of solvent) at 70°C in 5-mm diameter spinning tubes (internal standard: tetramethylsilan). Quantitative ^{13}C spectra were recorded using the INVGATE Bruker sequence, with a 90-pulse length (6.5 ms), a 15,000-Hz spectral width, 8000 data points, a 0.54-s acquisition time, and a relaxation delay of 1.5 s; 100,000 scans were accumulated.

Yield calculations

The percentages of glucosyl residues coming from sucrose incorporated into free glucose ($\%_{\text{sucrose hydrolyzed}}$), leucrose ($\%_{\text{leucrose}}$) and insoluble and soluble glucans ($\%_{\text{glucan}}$) were calculated using the following formulae:

$$\%_{\text{sucrosehydrolyzed}} = ([\text{glucose}_{tf}] - [\text{glucose}_{t0}] \times 342)/([\text{sucrose}_{t0}] - [\text{sucrose}_{tf}] \times 180)$$

$$\%_{\text{leucrose}} = ([\text{leucrose}_{tf}] - [\text{leucrose}_{t0}])/([\text{sucrose}_{t0}] - [\text{sucrose}_{tf}])$$

$$\%_{glucan} = ([glucose_{t0}] - [glucose_{tf}] \times 342)/([sucrose_{t0}] - [sucrose_{tf}] \times 162)$$

where $[glucose_{tf}]$, $[leucrose_{tf}]$, and $[sucrose_{tf}]$ correspond to the final concentrations of glucose, leucrose and sucrose, respectively, at the end of the reaction, and $[glucose_{t0}]$, $[leucrose_{t0}]$, and $[sucrose_{t0}]$ correspond to the initial concentrations.

Inhibition assays

For inhibition assays, the compounds dissolved at 1% (w/v) in ultrapure water were added at the indicated concentrations to the reaction mixture composed of the crude GTF preparation, 65 mM K_2HPO_4 buffer (pH 6.5), 50 g/l sucrose, 0.1 g/l NaN_3, and 2 g/l dextran T10. The mouthrinses were used undiluted, except for Eludril® and Betadine®, which were previously diluted in ultrapure water 2.5-fold and 50-fold, respectively, according to the manufacturer's instructions. Then, 1 ml of the mouthrinses was added to 2 ml of the standard reaction mixture [33% (v/v) final concentration]. The inhibition was analyzed through the reduction of the initial overall reaction rate (determined by measuring the increase in concentration of the reducing sugars over the first 3 h of reaction), and through the reduction of the quantity of ISG synthesized over 24 h. The principal active molecules present in the mouthrinses tested were analyzed in pure form in parallel, except for the povidone iodine present in the Betadine® mouthwash because no excipient could influence its effectiveness.

Statistical analysis

All results are expressed as means of triplicate experiments. Student's *t*-test was used to calculate the significance of the difference between the mean effect of a given compound or mouthrinse compared with no treatment. Statistically significant values were defined as $P < 0.05$.

Results

GTF concentration and purification

After 18 h of growth (optical density at 650 nm = 1.365), the total GTF activity measured in the culture supernatant of *S. sobrinus* ATCC 33478 was 61 mU/ml and its specific activity was 4.5 mU/mg. After ammonium sulfate precipitation (50%) and dialysis, 50 ml of concentrated GTF (652 mU/ml) was obtained, with a specific activity of 434 mU/mg of protein. More than 35% of the enzyme initially present in an active form was collected and its specific activity had increased 96-fold.

The culture supernatant and the crude GTF preparation were analyzed by 10% SDS–PAGE. The GTF concentration in the supernatant was too low and no band could be observed either by Coomassie brilliant blue staining or by *in situ* glucan synthesis (data not shown). The results obtained with crude GTF are presented in Fig. 1. Three bands were obtained after staining with Coomassie brilliant blue (Fig. 1, lane 2); two bands corresponding to proteins with a molecular weight of 174 and 165 kDa produced significant amounts of glucan after incubation in the presence of 50 g/l sucrose for 72 h at room temperature (Fig. 1, lane 3). A third minor band corresponding to a protein with a molecular weight of 160 kDa was detected after Schiff staining (Fig. 1, lane 4). In contrast, the protein with a molecular weight of 64 kDa did not present any glucansucrase activity under the assay conditions. No band appeared after incubation with sucrose, even after revelation by Schiff staining.

Kinetic analysis of sucrose transformation under various reaction conditions

The progress curve of sucrose transformation by the crude GTF preparation in standard conditions is presented in Fig. 2. During the first 4 h of the reaction, all the sucrose consumed was converted into fructose and ISG. Between 4 and 48 h, the enzyme converted 70% of the sucrose consumed into ISG and fructose; 12% of the sucrose was transformed into leucrose (α-D-glucopyranosyl-1,5-D-fructopyranose) (1), and 16% of the sucrose consumed was hydrolyzed, thus releasing glucose and fructose. The soluble glucan concentration slightly decreased, indicating a partial insolubilization of the dextran T10 initially introduced. A similar kinetic was observed at 25°C, except that the enzymatic reaction proceeded less quickly than at 37°C (data not shown).

At 37°C, but in the absence of dextran T10, the reaction also proceeded more slowly than under standard conditions. Moreover, the nature of the products synthesized was slightly modified (Table 1). First, the crude GTF preparation synthesized less ISG. Second, isomalto-oligosaccharides were precipitated from the supernatant, although they were much less abundant than the ISG and hardly detectable either by alcohol precipitation or HPLC. The side reactions, i.e. sucrose hydrolysis and leucrose synthesis, were more intense than in the presence of dextran T10.

The influence of pH, dextran T10, and sucrose initial concentrations were also investigated (Fig. 3). Release of reducing sugars and synthesis of ISG from sucrose were only slightly sensitive to variations in pH between 5 and 7.5. The pH range for maximal activity was 6.5 (Fig. 3A). While dextran T10 was not essential for *S. sobrinus* glucansucrase activity, the latter increased with its addition (Fig. 3B). The reducing sugars releasing activity

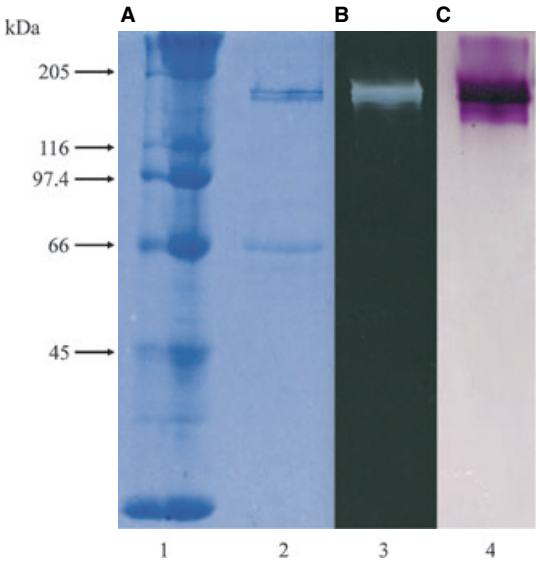

Fig. 1. SDS-PAGE of the crude GTF preparation. (A) PAGE gel stained with Coomassie brilliant blue. (B) PAGE gel incubated for 72 h at room temperature with 50 g/L sucrose and 2 g/L dextran T10. Bands were revealed by *in situ* glucan synthesis. (C) PAGE gel after Periodic Acid Schiff staining. Lane 1, molecular weight marker; lanes 2, 3 and 4, crude GTF preparation.

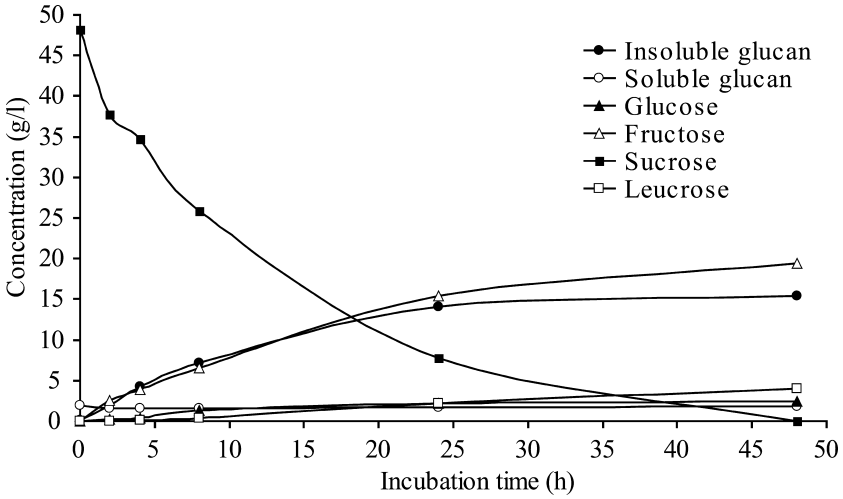

Fig. 2. Progress curve of sucrose transformation by the GTF crude preparation. The reaction mixture contained 0.1 U/mL crude GTF solution, 65 mM K_2HPO_4 buffer pH 6.5, 50 g/L sucrose and 0.1 g/L NaN_3. Reactions were run at 37°C. The insoluble glucan (filled circles) was weighed, soluble glucan (open circles) concentration was determined by the phenol sulphuric method, glucose (filled triangles) and fructose (open triangles) concentrations were determined with an enzymatic kit, and sucrose (filled squares) and leucrose (open squares) concentrations were analyzed by HPLC.

Table 1. Comparison of product yields after 48 h of reaction in the presence and absence of dextran T10

	Dextran T10 (2 g/l)	Without dextran T10
Sucrose conversion after 48 h	100%	89%
Insoluble glucan yield	88%	63%
Soluble glucan yield	−3%	4%
Leucrose yield	8%	12%
Sucrose hydrolyzed	10%	15%

The reaction mixture contained 0.1 U/ml crude glucosyltransferase solution, 65 mM K_2HPO_4 buffer pH 6.5, 50 g/l sucrose, and 0.1 g/l NaN_3 with or without 2 g/l dextran T10. The insoluble glucan was weighed, soluble glucan concentration was determined by the phenol sulphuric method, glucose and fructose concentrations were determined with an enzymatic kit, and sucrose and leucrose concentrations were analyzed by high-performance liquid chromatography. The percentages were calculated from the means of concentrations of triplicate experiments according to formulas described (2.5.3).

reached a maximum at 2 g/l of dextran T10, whereas the formation of ISG increased gradually up to 4 g/l dextran T10. On the other hand, the absence of dextran T10 had a greater effect on the quantity of ISG formed than on the overall initial reaction rate. Between 0 and 50 g/l of sucrose, the overall initial reaction rate and the ISG concentration increased with the initial concentration (Fig. 3C). The apparent Michaelis constant (K_m) for the overall initial reaction rate was 4 g/l and the apparent K_m for ISG synthesis was 20 g/l. Beyond 50 g/l, the rate of reducing sugar release and ISG synthesis were slightly inhibited.

Glucan structure analysis

Glucose was the only monosaccharide observed after hydrolysis of either soluble or insoluble glucan. It accounted for 100% of the insoluble glucan mass and 77.5% of the soluble glucan mass (Table 2). The two glucans were analyzed by nuclear magnetic resonance (data not shown). For soluble glucan, the ^1H spectrum analysis indicated a majority of α-anomers of glucose and the presence of oligosaccharides. The ^{13}C spectrum unambiguously showed that the linkages were mainly α-1,6 but that they were not exclusive. For insoluble glucan, only one ^{13}C spectrum was interpretable in DMSO in the presence of NaOH. Although the resolution was low, the spectrum clearly indicated the presence of α-1,3-glucosidic linkages and a lower but significant rate of α-1,6 linkages. To confirm these observations and to decide whether the structure was alternate or branched, the two samples were methylated (Table 2). This indicated the presence of dimethylated derivatives and branched glucosidic residues. Nevertheless, their low proportion and the ratio of the two types of connections (1,6 : 1,3 ratio 11) indicated a very weakly branched structure. For insoluble glucan, a ratio for 1,3 : 1,6 linkages of 3.8 was obtained, indicating a more branched polymer structure, with very short side chains considering the abundance of the tetra-methylated derivative corresponding to non-reducing ends. Furthermore, methylation analysis suggested that the branches consisted of α-1,3-glucosidic linkages for soluble glucan and of α-1,6-glucosidic linkages for the ISG. Mass spectrometry (by matrix-assisted laser desorption/ionization time-of-flight) performed on soluble glucan showed a distribution of the degree of polymerization between 6 and 15, with an average of 9 (Fig. 4).

Inhibition of GTF activity

The inhibition by various compounds of ISG synthesis by the crude GTF preparation was investigated at pH 6.5 in the presence of 2 g/l dextran T10 and 50 g/l sucrose. These were the most favorable conditions for ISG synthesis. The inhibition was analyzed through the reduction of the initial overall reaction rate (through the reducing sugar concentration increase over the first 3 h of reactions) and through the reduction of the quantity of ISG synthesized over 24 h.

Concerning the reduction in ISG synthesis (which is the first effect required), the most effective inhibitors were cetylpyridinium chloride and tannic acid (Table 3). Low concentrations of these compounds (0.005–0.01%) strongly inhibited crude GTF activity in a dose-dependent manner. On the other hand, 0.01% cetylpyridinium chloride nearly abolished ISG synthesis with the overall reaction rate being reduced more than twofold. Higher concentrations of chlorhexidine digluconate (0.1–0.2%) were necessary to obtain a significant reduction (40%) of ISG synthesis. Sodium fluoride (0.22–0.33%, corresponding to 0.1 and 0.15% fluoride, respectively) had a negligible effect on crude GTF activity. At these concentrations, it inhibited ISG synthesis by less than 15%, and the overall reaction rate was unchanged, indicating that side reactions and soluble glucan formation were slightly stimulated. At the highest concentration tested (20%), ethanol significantly inhibited ISG synthesis (15% reduction). The non-cariogenic sweeteners sorbitol and xylitol had no appreciable effect on overall GTF

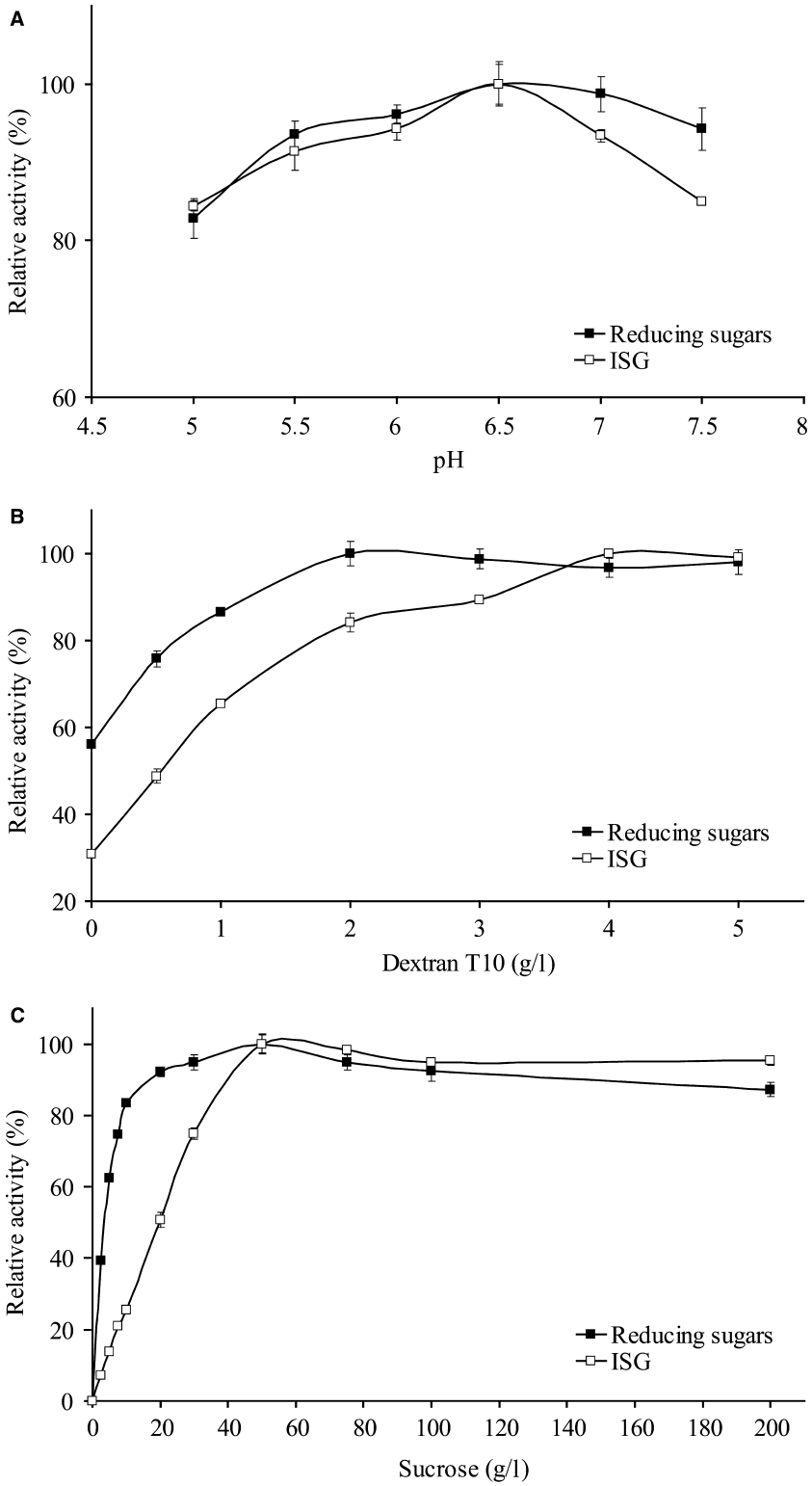

Fig. 3. Influence of pH (A), dextran T10 (B) and sucrose (C) concentrations on GTF activity. The standard reaction mixtures contained 0.1 U/mL crude GTF solution, 65 mM K_2HPO_4 buffer pH 6.5, 50 g/L sucrose, 2 g/L dextran T10 and 0.1 g/L NaN_3. The reactions were run at 37°C. Enzymatic activity (U/mL) was determined by measuring the amount of reducing sugars (filled squares) released after 3 h incubation. The amount of insoluble glucan (ISG, open squares) synthesized was also measured after 24 h incubation. The references used for calculation of the relative activities were the activities measured with the corresponding enzymes mixture at : (A) 50 g/L sucrose (0.7 U/mL, ISG 14.6 g/L); (B) 2 g/L dextran (0.7 U/mL) and 4 g/L dextran (ISG 17.4 g/L); (C) pH 6.5 (0.7 U/mL, ISG 14.6 g/L). Results are expressed as means and standard deviations of triplicate experiments.

activity. However, they inhibited ISG synthesis by 5–10% depending on the concentration used, although the overall reaction rate was not significantly modified.

The effects on crude GTF activity of several mouthrinses that contain potentially active agents were also analyzed (Table 4). Of all the commercial solutions tested, Betadine® was the most effective inhibitor and inhibited more than 90% of the overall reaction, with almost no ISG synthesized over 24 h. Four of the other mouthrinses presented intermediate inhibitory activity, with residual ISG synthesizing activity ranging from 54% with Elmex® to 84% with Alodont®. However, Alodont® inhibited the overall reaction more than ISG synthesis. Of all the mouthrinses tested, Meridol® showed the lowest inhibition. In its presence, the enzyme preparation retained at least 90% of its residual ISG synthesizing activity. Moreover, it inhibited the overall reaction more than the ISG synthesis.

The possible transfer of glucosyl residues onto the inhibitors was then analyzed by HPLC (data not shown). None of the molecules tested displayed any acceptor behavior in the presence of the *S. sobrinus* crude GTF preparation because no additional peak could be detected in conditions that routinely enable the visualization of oligosaccharides.

Discussion

The inhibition of adhesion is an attractive target for the development of new therapies in the prevention of bacterial dental infections. The synthesis of glucans by glucosyltransferases from *Streptococcus* sp. contributes significantly to the formation of dental plaque (31). Various studies have examined the effects of inhibitors on GTF activity (5, 10, 53, 55). However, the results are not easily comparable because the purification methods employed are very different, which leads to very distinct GTF preparations. For example, chlorhexidine digluconate (1.25 mM) had no effect on glucan synthesis by GTF-B purified from *Streptococcus milleri* KSB8 by hydroxyapatite column chromatography (53), but it inhibited (at 2 mM) the glucan synthesis (99.7% of inhibition) by GTFs (unfractionated enzymes) purified from *S. mutans* 6715 by several ultrafiltrations and ethanol precipitation (5). Indeed, schemes for the purification of GTF from *Streptococcus* sp. culture fluids are as varied as the number of laboratories involved in purification. Different and conflicting

Table 2. Linkage analysis of soluble and insoluble glucans

Compounds	Glucose rate (%)	Methylated glucitol acetate (%)			
		2,3,4,6	2,4,6	2,3,4	2,4
Soluble glucan	77.5	20.7	5.8	64.0	9.5
Insoluble glucan	100.0	14.5	54.6	14.2	16.7

Linkage analysis of soluble and insoluble glucans synthesized by *S. sobrinus* ATCC 33478 crude glucosyltransferase (GTF) preparation. Glucans were obtained after 48 h incubation at 37°C of the reaction mixture containing 0.1 U/ml crude GTF solution, 65 mM K_2HPO_4 buffer pH 6.5, 50 g/l sucrose and 0.1 g/l NaN_3. After centrifugation, insoluble glucan was recovered from the pellet and the soluble glucan was purified from the supernatant by ethanol precipitation. Both were washed, lyophilized, methylated twice, and then analyzed by gas chromatography. Glucose rate (%), % of glucose observed after hydrolysis of glucans.

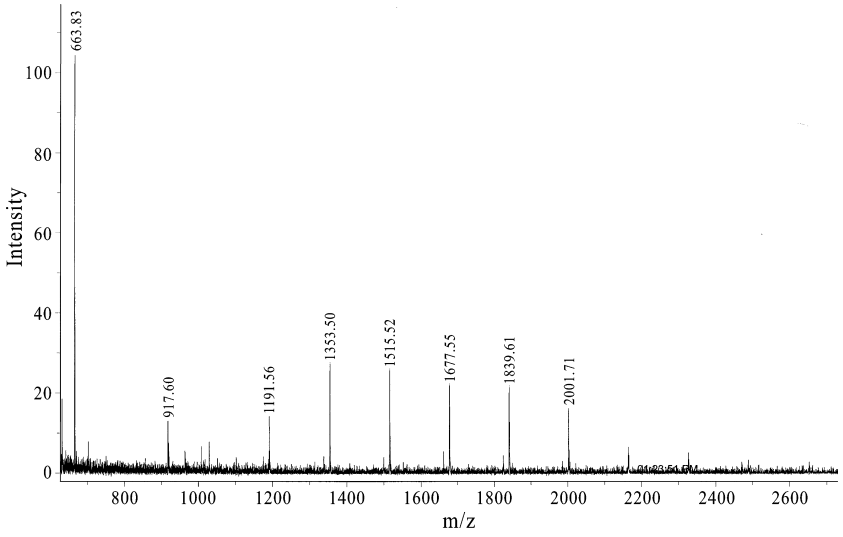

Fig. 4. Mass spectrometry (MALDI Tof) of soluble glucan. Soluble glucan was obtained after 48 h incubation by centrifugation and lyophilization of the reaction mixture containing 0.1 U/mL crude GTF solution, 65 mM K_2HPO_4 buffer pH 6.5, 50 g/L sucrose and 0.1 g/L NaN_3. The mass spectrum showed a distribution of degree of polymerization (DP) between 6 to 15 with an average DP of 9 with a quasi-molecular ion $[M+K]^+$ at m/z 1515.52.

Table 3. Effect of selected compounds on GTF activity

Compounds	Concentration		Residual activity (/control)	
	mM	% (weight/volume)	Reducing sugars	Insoluble glucan
Cetylpyridinium chloride	0.06	0.002	90.7 ± 1.1[1]	82.5 ± 1.3[1]
	0.1	0.005	83.5 ± 2.8[1]	40.5 ± 6.5[1]
	0.3	0.01	39.1 ± 1.0[1]	5.0 ± 3.6[1]
Tannic acid	0.03	0.005	78.5 ± 0.9[1]	48.3 ± 2.3[1]
	0.06	0.01	63.7 ± 3.7[1]	16.1 ± 1.0[1]
Chlorhexidine digluconate	0.1	0.01	99.3 ± 1.2	90.0 ± 0.6[1]
	1.1	0.1	85.2 ± 1.1[1]	63.2 ± 1.3[1]
	2.2	0.2	73.7 ± 0.4[1]	57.9 ± 2.4[1]
Sodium fluoride	2.4	0.01 (0.005)	99.3 ± 1.2	97.3 ± 1.2
(fluoride iron)	52.4	0.22 (0.1)	98.5 ± 1.3	90.2 ± 0.5[1]
	78.6	0.33 (0.15)	97.0 ± 1.3[1]	87.7 ± 1.0[1]
Ethanol	2171	10	87.3 ± 1.1[1]	94.8 ± 0.5[1]
	4341	20	75.2 ± 1.0[1]	83.1 ± 0.2[1]
Xylitol	1.3	0.02	100.7 ± 1.2	94.8 ± 0.5[1]
	6.6	0.1	96.7 ± 1.1	90.9 ± 1.0[1]
Sorbitol	1.1	0.02	98.7 ± 1.2	92.2 ± 0.7[1]
	5.5	0.1	96.0 ± 3.5	93.2 ± 0.6[1]

Enzymatic activity was analyzed, in the presence or in the absence of drug, through the initial overall reaction rate determined by measuring the amount of reducing sugars released over the first 3 h of reaction, and through the quantity of insoluble glucan synthesized over 24 h. Results are presented as percentage of enzymatic activity with respect to control (without any compound). Results are expressed as means and standard deviations of triplicate experiments.

[1]Statistical differences ($P < 0.05$) between glucosyltransferase assay and control ($n = 3$ experiments).

characteristics reported for purified GTF preparations are, in part, the result of the use of different *Streptococcus* sp. strains, different growth conditions, different purification methods, different GTF assays with or without primer. This is why in this work, we analyzed and compared the effects of several compounds and mouthrinses used in dentistry on the same GTF preparation after its characterization and under the most favorable conditions for ISG synthesis.

In the growth conditions used, *S. sobrinus* ATCC 33478 produced at least three glucansucrases that (i) were co-purified by ammonium sulfate precipitation and (ii) had sufficient primer-independent activity to produce a detectable glucan on SDS–PAGE (dextran T10 did not diffuse in the gel). The recovery level obtained was very high compared to those described by others (19, 49, 54). The molecular weights of the GTFs were in the range of 160–175 kDa generally described for this family of enzymes (8). *S. sobrinus* AHT and BN13 were shown to produce four distinct glucansucrases (57). The obtaining of water-insoluble glucan suggests that strain ATCC 33478 also produced a distinct primer-independent high-molecular-weight glucan synthase, and so a total of four different glucansucrases. The two major bands should correspond to GTF-S_1 and GTF-S_2. In fact, two bands were also obtained by Nanbu et al. (39) with *S. sobrinus* recombinant GTF-S. The minor band could correspond to the isomalto-oligosaccharide synthesizing enzyme GTF-S_3: it is smaller than the others and isomalto-oligosaccharides are small products weakly retained in the gel (21). In contrast, GTF-I, which is strictly primer-dependent, could not be detected. Finally, the high recovery level and the presence of several bands corresponding to glucansucrases suggest that the crude GTF preparation obtained is representative of the four GTFs excreted by *S. sobrinus* ATCC 33478 that are generally described and, because our study design is fast and easy, little denaturation can occur in the obtained preparation. The *S. sobrinus* crude GTF preparation contains a mixture of at least four different glucansucrases. Therefore, its kinetic behavior is complex. Konishi et al. and Takehara et al. (26, 49) showed that the hydrolysis of low concentration sucrose and in the absence of dextran T10 was characteristic of *S. sobrinus* GTF, and especially GTF-I. Sucrose hydrolysis was promoted at low sucrose concentrations. Other authors have established that in the absence of primer,

Table 4. Effect of mouthrinses on glucosyltransferase activity

Mouthrinses	Composition principal compounds	Final concentration (% weight/volume)	Residual activity (/control)	
			Reducing sugars	Insoluble glucan
Betadine®	Povidone iodine	0.07	8.7 ± 4.3[1]	0.7 ± 0.6[1]
Elmex®	Olafluor	0.04 (fluoride 0.003)	75.8 ± 5.4[1]	54.0 ± 2.5[1]
	Sodium fluoride	0.01 (fluoride 0.005)		
Eludril®	Chlorhexidine digluconate	0.01	71.1 ± 1.5[1]	68.1 ± 1.3[1]
	Chlorobutanol hemihydrate	0.07		
	Ethanol	5.7		
Listerine®	Eucalyptol, thymol, menthol	0.03, 0.02, 0.01	96.0 ± 2.0[1]	74.6 ± 2.5[1]
Coolmint	Methyl salicylate	0.02		
	Ethanol	7.2		
Alodont®	Cetylpyridinium chloride	0.002	78.5 ± 0.9[1]	84.0 ± 1.2[1]
	Chlorobutanol hemihydrate	0.02		
	Eugenol	0.01		
	Ethanol	7		
Meridol®	Olafluor	0.05 (fluoride 0.004)	97.3 ± 2.3	100.0 ± 0.3
	Stannous fluoride	0.02 (fluoride 0.004)		

Enzymatic activity was analyzed, in the absence or in the presence of 33% volume/volume (final concentration) of the mouth rinses, through the initial overall reaction rate determined by measuring the amount of reducing sugars released over the first 3 h of reaction, and through the quantity of insoluble glucan synthesized over 24 h. Results are presented as percentage of enzymatic activity with respect to control (without any compound). Results are expressed as means and standard deviations of triplicate experiments.
[1]Statistical differences ($P < 0.05$) between treated samples and water controls.

the various GTF-S have to synthesize their product to enable the action of GTF-I. In the absence of primer, the activity of GTF-S_1 is lower (35). Our results generally confirm these findings. Indeed, influence of sucrose concentration analysis suggests that the low initial sucrose concentration promoted the side reactions (sucrose hydrolysis and leucrose synthesis) at the expense of ISG synthesis. Furthermore, in the absence of dextran T10, the overall reaction rate was lower and the side reactions (particularly sucrose hydrolysis) were greater. Nevertheless, once the soluble glucan had been synthesized, the glucosyl transfer from sucrose to these primers by GTF-I was very efficient because no high-molecular-weight soluble glucan could be detected in the reaction medium, whatever the reaction conditions.

The enzymatic reaction of the crude GTF preparation leads to two glucans that differ greatly in their solubility, perhaps as a result of their very different structures. The low concentration of soluble glucan made its recovery difficult, leading to a lower level of purity than for the ISG. This might explain the presence of constituents other than glucose in the polymer preparation. The water-insoluble glucan obtained mainly contains α-1,3 linkages (about 70%), whereas the water-soluble glucan comprises low-molecular-weight products mainly containing α-1,6 linkages. The structure of the adhesive insoluble polymer described here contains the same level of α-1,6 linkages as that described for *S. sobrinus* AHT crude GTF (19). However, it is twice as branched. This could be because of different relative abundances of the various GTFs excreted by the different strains and concentrated in the crude enzyme preparation. In fact, the *S. sobrinus* crude GTF ISG has been described as a tree (20), or a comb-like structure (6, 7, 46), depending on the abundance and the length of the side chains. The high proportion of α-1,3 ISG indicated the abundance of GTF-I in the enzyme preparation. The presence of soluble oligosaccharides confirmed the presence of GTF-S_3 in the crude preparation. These products could be less efficient primers for GTF-I than the high-molecular-weight soluble glucans produced by the other GTF-S, but the latter were not detected in the crude GTF reaction medium. The ISG obtained in this study strongly adhered to glass surfaces, regardless of the presence or not of dextran T10. This is in contradiction with the results of Inoue et al. (24) who found that insoluble glucan synthesized by GTF-I and GTF-S was adherent to glass in the absence of dextran T10 but not in its presence.

The effectiveness of the compounds tested on GTF activity is very variable from one component to another. Moreover, many antiplaque compounds may be ineffective when incorporated into mouthrinse formulations, because their activity is inhibited by other ingredients (22). We therefore tested the effects on crude GTF activity of several mouthrinses that contain potentially active agents. The bad inhibition concerning polyols matched the data of Wunder et al. (55). This is not an unexpected finding because neither sugar appears to act as a substrate for the enzyme (3). Inhibition by ethanol was evaluated because some of the mouthrinses contain it in their formulation. Indeed, a high concentration of ethanol significantly inhibited ISG synthesis, but this was not sufficient to explain all the inhibition observed when introduced into the reaction medium with 33% (v/v) mouthrinses.

Sodium fluoride also had a negligible effect on crude GTF activity. It might be active only on GTF-I, and not on the other GTFs present in the crude preparation. In fact, low fluoride concentrations (0.001–0.01%) were shown to display a stimulatory effect on the synthesis of soluble and insoluble polysaccharides by *S. mutans* (52). In the Meridol® and Elmex® mouthrinses, the active molecules are fluoride derivatives. The inefficiency of Meridol® compared to the effective Elmex® could be the result of the following. It could be a difference in the associated molecules: they both contain olafluor at similar concentrations but Elmex® contains sodium fluoride whereas Meridol® contains stannous fluoride. Nevertheless, the results with sodium fluoride when employed pure indicate that fluoride derivatives are poor GTF inhibitors. Alternatively, there could be a difference resulting from an alteration in the fluoride once it is in the mouthrinse formulation.

High concentrations of chlorhexidine digluconate (0.1–0.2%) were necessary to obtain a significant reduction of ISG synthesis by the crude GTF preparation. In contrast, *in vitro* studies with bacteria have shown that chlorhexidine, in the same concentration range, has the greatest inhibitory effect on the development of biofilm

(16, 45). This suggests that it exerts its outstanding antiplaque effect by others mechanisms. The effectiveness of Eludril® (chlorhexidine 0.01% final concentration) compared to chlorhexidine alone (0.01%) indicates that the mouthrinse formulation keeps the molecule in its active form. Moreover, part of the inhibition could be attributed to ethanol or chlorobutanol hemihydrate.

With regard to the high activity of cetylpyridinium chloride, our results are in agreement with the data of Ciardi et al. (5), but contradict those of Pinheiro et al., who showed that this compound (1.47 mM) had a moderate effect on ISG synthesis by a mixture of GTFs isolated from dental plaque (42). This could be explained by the fact that GTFs directly extracted from dental plaque were more resistant to agents than were GTFs synthesized by bacteria from the American Type Culture Collection. Alodont® (cetylpyridinium chloride 0.002%) and cetylpyridinium chloride used pure (0.002%) had the same effect on ISG synthesis (84% and 82.5% of inhibition, respectively). However, Alodont® inhibited the overall reaction more than cetylpyridinium chloride. This could be attributed to ethanol or chlorbutanol hemihydrate.

Both the hydrophobic and electrostatic properties of the surface-active nitrogen compounds contribute to the inhibition of glucan synthesis (5). The significant inhibition of the crude GTF preparation by basic nitrogen compounds with the hydrophobic groups such as chlorhexidine (bisbiguanides) and cetylpyridinium chloride (quaternary ammonium salts), confirm this assumption. The lack of inhibition by sodium fluoride supports the hypothesis that hydrophobic groups of the surface-active compounds are necessary to inhibit combined GTF activity.

Very low concentrations of tannic acid (0.005–0.01%) strongly inhibited crude GTF activity. In many studies, polyphenolic compounds were found to inhibit *S. mutans* GTF activity (38, 48). This work also shows that tannic acid is a good inhibitor of the combined action of *S. sobrinus* GTFs and especially of GTF-I. The Listerine® Coolmint mouthwash contains what is essentially a phenolic flavour mixture consisting of eucalyptol, thymol, and menthol. These polyphenolic compounds may be responsible for the intermediate inhibition observed in ISG synthesis. The nature and level of purity of the polyphenolic compounds is very likely responsible for the low level of inhibition observed (74.6% residual activity) compared to that obtained with tannic acid (16.1–48.3% residual activity).

Of all the commercial solutions tested, Betadine® was the most effective inhibitor. The only active molecule in the Betadine® mouthwash is povidone iodine prepared in water (10% w/v). The high inhibition capacity of iodine derivatives was previously underlined by Tam et al. (50). Unlike many antibacterial agents, iodine does not possess a positive charge so may bind differently to GTFs. In the commercial preparation, no additional compound interferes with it.

In conclusion, a crude GTF preparation highly representative of the *S. sobrinus* ATCC 33478 GTF complex was studied and the effect of various agents on its activity was determined. The reaction parameters of optimal adherent ISG formation were identified and used for inhibition assays. In the study conditions, the most effective inhibitors were cetylpyridinium chloride (0.01%), tannic acid, and povidone iodine, which significantly reduced both insoluble and soluble glucan synthesis by the crude GTF preparation. Inhibition by polyphenols like tannic acid was effective at very low concentrations (0.005%) and was observed with GTFs of various origins and purity (30, 41), in contrast to cetylpyridinium chloride, whose activity was debated (42). Moreover, the principal advantage of the phenolic compounds is the complete absence of side effects, in contrast for example to the povidone iodine (brown-yellow tooth coloration, allergy). The inhibition assays performed in the presence of mouthrinses indicate that the commercial product formulation has a great impact on the efficiency of the active molecules. However, even if a large proportion of other works have focused mainly on GTF activity in solution (18, 30, 38), other data have shown that pellicle-absorbed enzymes have more resistance to agents than those in solution (53, 55). Various phenomena (saliva, clearance of the active molecule, food, posology, etc.) could also interfere *in vivo* with the molecules and influence their effectiveness (2, 27). This must be taken into account for the formulation of new mouthrinses containing active natural polyphenols. For this purpose, the fast and representative ISG inhibition test developed in this study should be helpful.

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