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Effect of carbohydrate fatty acid esters on *Streptococcus sobrinus* and glucosyltransferase activity

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Abstract—Mutans streptococci are oral bacteria with a key role in the initiation of dental caries, because their glucosyltransferases synthesize polysaccharides from sucrose that allow them to colonize the tooth surface. Among the strategies to prevent dental caries that are being investigated are (1) the inhibition of bacterial growth of mutans streptococci or (2) the inhibition of glucosyltransferases involved in polysaccharide formation. Pure fatty acid esters of sucrose, maltose and maltotriose were synthesized by an enzyme-catalyzed process and tested as inhibitors of two glucosyltransferases of great homology, those from *Streptococcus sobrinus* and *Leuconostoc mesenteroides* NRRL B-512F. In spite of having their nonreducing end glucose blocked at 6-OH, they did not inhibit dextran synthesis. However, their effect on the growth of *S. sobrinus* in the solid and liquid phase was notable. 6-*O*-Lauroylsucrose, 6′-*O*-lauroylmaltose and 6″-*O*-lauroylmaltotriose at 100 μg/mL showed complete inhibition of *S. sobrinus* in agar plates. Consequently, these nontoxic derivatives are very promising for inclusion in oral-hygiene products aimed at disrupting plaque formation and preventing caries.

Keywords: Mutans streptococci; Glycosyltransferases; Sugar esters; Antimicrobials; Dental caries; Dextransucrase

1. Introduction

Oral bacteria referred to as mutans streptococci are remarkable for their pivotal role in the initiation of dental caries. Two α-haemolytic 'mutans group' streptococci, *Streptococcus sobrinus* and *Streptococcus mutans*, are directly involved in this process.[1]

Glucosyltransferases (GTFs, EC 2.4.1.5) from mutans streptococci are able to synthesize extracellular polysaccharides (glucans) from dietary sucrose in situ, which serve as adherence sites for streptococci.[2] In the protected environment conferred by the glucans, the mutans streptococci and other microorganisms form a stable and protected community (dental plaque) and may release sufficient quantities of metabolic acids to demineralize tooth enamel and initiate dental caries. Mutans streptococci secrete at least three glucosyltransferases: two synthesize α-(1→6)-linked water-soluble glucans (dextrans), which differ in glucan affinity and degree of α-(1→3)-branching, and a third synthesizes an α-(1→3)-linked water-insoluble glucan (mutan).[3]

GTFs belong to the family of glycansucrases (glucansucrases and fructansucrases), bacterial enzymes acting on sucrose and utilizing it as the sole energy source for sugar synthesis.[4] All glycansucrases share the same substrate specificity, but catalyze the synthesis of a variety of oligo- and polysaccharides. Dextransucrase (sucrose: α-(1→6)-D-glucan 6-α-D-glucosyltransferase, EC 2.4.1.5) is a GTF closely related to GTFs from mutans streptococci. Dextransucrase polymerizes the

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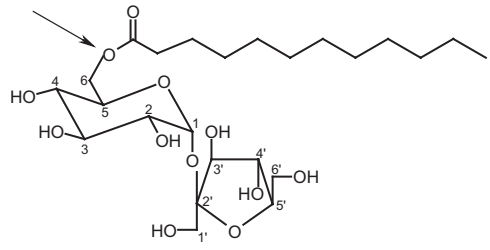
glucosyl moiety of sucrose to form a dextran with additional α-(1→2), α-(1→3), or α-(1→4) branch linkages, depending on the bacterial strain.[5]

To prevent the dental caries due to cariogenic bacteria, different alternatives such as inhibition of GTF activity by specific enzyme inhibitors,[6] inhibition of cell adhesion by mono- and polyclonal antibodies,[7] inhibition of cell growth of mutans streptococci by antibacterial agents,[8,9] or hydrolysis of the glucan by commercial enzymes[10] are being investigated.

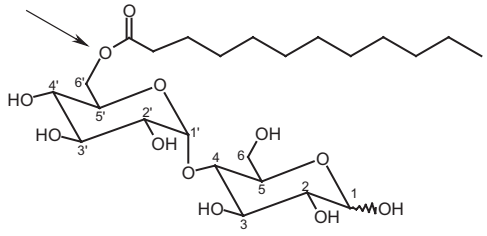
Sugar esters are biodegradable, nontoxic and nonionic surfactants whose properties may be modulated by controlling the degree of esterification and the nature of fatty acid and sugar. They are currently being employed in the food, cosmetics and detergent industries.[11] Sucrose esters, by far the most developed derivatives of this group, are being produced at about 4000 ton/year.[12] Current chemical production of sucrose esters has a low selectivity and yields coloured derivatives as side-products. Regioselective acylation of carbohydrates is not straightforward due to their intrinsic multifunctionality.[13] We recently developed an enzyme-catalyzed process for the synthesis of sugar esters, which provides regioselective products.[14]

The antimicrobial features of carbohydrate esters have been previously reported.[15] It has been demonstrated that commercial sucrose fatty acid esters can decrease acid production from sugar by oral bacteria[16] and also reduce the development of dental caries in rats when added to sucrose-rich diets.[17] Use of fatty acids and their sugar esters potentially represent a virtually nontoxic and nonallergenic means of controlling the acidogenic organisms associated with dental caries.[16]

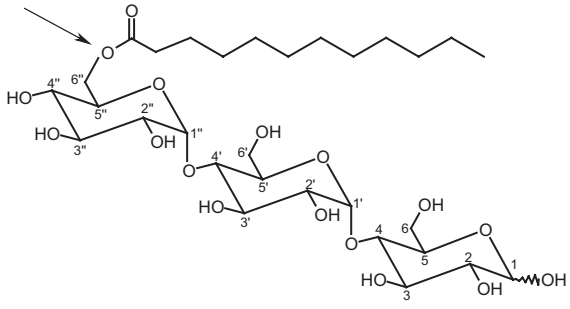
In the present study, we have investigated the effect of pure, enzymatically obtained, carbohydrate fatty acid monoesters (blocked at the 6-OH of glucose at the nonreducing end) on (1) glucansucrase activity (dextran synthesis) and (2) bacterial growth of *S. sobrinus*.



6-*O*-lauroylsucrose



6'-*O*-lauroylmaltose



6''-*O*-lauroylmaltotriose

2. Results

2.1. Inhibition of glucosyltransferases by carbohydrate esters

We synthesized a series of carbohydrate esters using an enzymatic method developed previously in our laboratory.[14] In the case of sucrose, NMR analysis showed that the 6-OH group on the glucose ring had been selectively acylated. When considering maltose and maltotriose, the 6-OH position of glucose at the nonreducing end was acylated with high regioselectivity. The structure of the three compounds assayed in this study are given below.

In a first attempt, we studied the effect of pure sugar esters on the enzymes responsible for glucan synthesis, namely glucosyltransferases from *S. sobrinus*. Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F was also assayed, since it belongs to the same group of glycansucrases and shares the same EC number (EC 2.4.1.5). Based on the amino acid sequence similarities within the catalytic domains, both enzymes have been assigned to the family 70 of glycoside hydrolases and transglycosidases.[18] In fact, dextransucrases show a sequence homology close to 50% with *Streptococcal* glucansucrases, and therefore great similarities in the main catalytic features of both enzymes are expected.

The active site of *L. mesenteroides* B-512F dextransucrase is described as having two sucrose binding sites and one acceptor binding site. For the transfer of glucose units to yield dextrans (with the concomitant release of fructose), the formation of two covalent glucosyl–enzyme complexes constitutes the first step.[5] A nucleophilic attack of the hydroxyl group located on C-6 of the nonreducing end to C-1 at the reducing end of the second glucosyl–enzyme complex is responsible for the dextran chain elongation. In this context, we

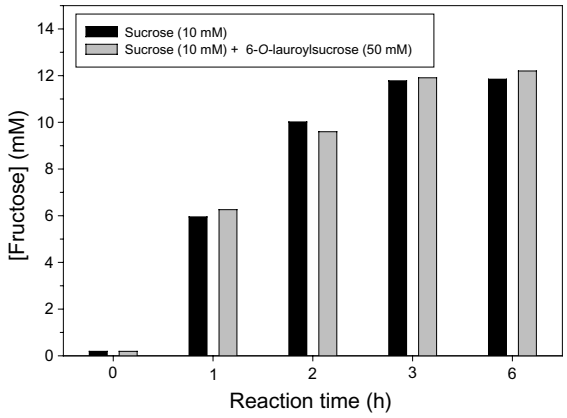


Figure 1. Effect of 6-*O*-lauroylsucrose on fructose formation using dextransucrase from *L. mesenteroides* NRRL B-512F. Conditions described in the Experimental Section.

hypothesize that a sucrose derivative in which the OH at the C-6 of glucose is blocked will inhibit dextran formation. 6-*O*-Lauroylsucrose was evaluated for its inhibitory activity on dextransucrase using sucrose as substrate. The time course of fructose appearance, in the presence and absence of 6-*O*-lauroylsucrose (molar ratio sucrose:ester 1:5), was analyzed by HPLC to assess GTF activity. As shown in Figure 1, no significant differences were observed, suggesting that the affinity of 6-*O*-lauroylsucrose for the sucrose binding sites of the enzyme was negligible. The ability of 6-*O*-lauroylsucrose to act as a dextransucrase substrate in the absence of sucrose was also evaluated. Interestingly, 6-*O*-lauroylsucrose was consumed by dextransucrase very slowly and never reached total depletion. This implies that acylation of sucrose at the 6-OH position nearly eliminates it as a dextransucrase substrate, although the derivative that is obtained is not able to inhibit dextran synthesis.

A similar noninhibitory phenomenon was found while studying the glucosyltransferase from *S. sobrinus* by means of a ^{14}C-isotope–transfer assay that followed ^{14}C flow from sucrose to dextran. None of the three sugar esters tested (6-*O*-lauroylsucrose, 6′-*O*-lauroylmaltose and 6″-*O*-lauroylmaltotriose) was able to inhibit glucosyltransferase activity when present in the range of 5–500 mM. Table 1 summarizes the activity using the highest concentrations assayed (100–500 mM). As shown, the activities are even higher than those in the absence of sugar ester. This indicates that *Streptococcal* GTFs are able to utilize sugar esters as substrates more efficiently than dextransucrase.

2.2. Antimicrobial activity of carbohydrate esters against *S. sobrinus*

We tested the effect of the pure sugar esters on the growth of *S. sobrinus* on agar plates. *S. sobrinus* bacterial cultures were grown to a stationary phase (14 h) in brain–heart infusion broth (BHI) and used for these experiments. Interestingly, no bacterial growth was observed on BHI plates supplemented with the three inhibitors at the assayed concentrations (0.1–1 mg/mL). Figure 2 shows the Petri dishes using 100, 200 and 400 μg/mL of 6″-*O*-lauroylmaltotriose and a control in the absence of inhibitor. In the control plate, bacterial colonies were too crowded to count the number. No bacterial colonies were observed in the plates containing 6″-*O*-lauroylmaltotriose.

Experiments were also carried out in liquid BHI broth cultures supplemented with inhibitors. No bacterial growth was detected in the liquid culture medium supplemented with 6″-*O*-lauroylmaltotriose (0.5 mg/mL) and with 6′-*O*-lauroylmaltose (1 mg/mL). In the case of

Table 1. Effect of 6-*O*-lauroylsucrose, 6′-*O*-lauroylmaltose and 6″-*O*-lauroylmaltotriose on glucosyltransferase activity

Concentration (mM)	Glucosyltransferase activity (μmol/min mg)[a,b]
Enzyme control	2.75
6-O-lauroylsucrose	
100 mM	4.56
300 mM	4.62
500 mM	4.65
6′-O-lauroylmaltose	
100 mM	5.49
300 mM	5.77
500 mM	7.65
6″-O-lauroylmaltotriose	
100 mM	5.16
300 mM	5.48
500 mM	5.48

[a] Represents average of duplicates.
[b] Amount of labelled glucan was measured.

Figure 2. Cell growth of *S. sobrinus* in the presence of 100, 200 and 400 μg/mL of 6″-*O*-lauroylmaltotriose in agar plate. The values given in the figure correspond to 25 mL of medium.

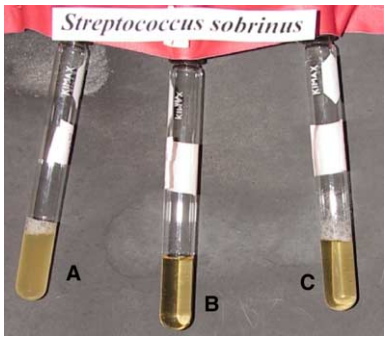


Figure 3. Cell growth of *S. sobrinus* in liquid medium in the presence of 0.5 mg/mL of 6″-*O*-lauroylmaltotriose (**B**) and 1 mg/mL of 6′-*O*-lauroylmaltose (**C**). The control without inhibitor is also shown (**A**).

6-*O*-lauroylsucrose, no bacterial growth was observed in the medium supplemented with 2 mg/mL (Fig. 3).

3. Discussion

Surface-active compounds synthesized from renewable resources, such as fatty acids and polyols, continue to receive widespread attention owing to their superior performance and compatibility in the health and environmental arenas compared to the petroleum-derived products. Esterification of fatty acids with polyhydroxylic alcohols, such as glycerol or sucrose, enhances their antimicrobial effect.[19] In general, Gram-positive bacteria are more susceptible to the effects of these compounds than Gram-negative bacteria, and fungal growth is more susceptible to carbohydrate esters than yeasts.[15]

Most of the previous studies on antimicrobial properties of sugar esters have been performed using commercial derivatives.[15,20,21] These are complex mixtures of monoesters, diesters, triesters, etc. containing different regioisomers.[22] To our knowledge, the present work is one of the first to examine antimicrobial properties with pure carbohydrate fatty acid esters. Watanabe et al. studied the growth-inhibitory effect of a series of monosaccharide esters synthesized by lipases and proteases on *Streptococcus mutans*.[23] Only galactose and fructose laurates suppressed the growth of *S. mutans* to a significant extent.

In the present study, we evaluated the effect of sugar head groups (sucrose, maltose, maltotriose) on inhibition of the microorganism and its related enzymatic activities. In contrast, with numerous studies on the properties of sucrose-based esters, there is not much knowledge concerning other di- and trisaccharide esters, probably due to the lack of appropriate synthetic methods. According to the glucansucrase mechanism,[5] we proposed that a di- or trisaccharide esterified at the 6-OH in the nonreducing end glucose could inhibit dextran formation. In fact, 6-deoxy-6-fluorosucrose, 6-deoxysucrose and 6-thiosucrose are the most efficient competitive inhibitors described.[24–26] The inhibition data indicate that the replacement of the hydroxyl group at C-6 by another group may give rise to a strong competitive inhibitor. However, these compounds are difficult to use as anticaries agents due to their toxicity and complex synthesis. Our experiments using dextransucrase and glucosyltransferase from *S. sobrinus* indicate that the affinity of 6-*O*-lauroylsucrose, 6′-*O*-lauroylmaltose and 6″-*O*-lauroylmaltotriose for the sucrose binding sites is very low.

We also evaluated the antimicrobial activity of our derivatives against *S. sobrinus*. These experiments were successful, with MIC values lower than 100 μg/mL in the solid phase and in the range of 0.5–2.0 mg/mL in liquid medium. Watanabe et al. reported MIC values in the range of 50–200 μg/mL for several monosaccharide esters.[23] However, from the practical point of view, monosaccharide derivatives are notably less soluble in water than the corresponding di- and trisaccharide monoesters, as a consequence of their higher hydrophobicity.

Although the concentrations of sugar esters that inhibit *S. sobrinus* in liquid phase may be considered high, it is important to take into account that fatty acid esters of sucrose are commercially used in foods at concentrations as high as 10 mg/mL (Ryoto Sugar Ester Technical Information, Mitsubishi-Kagaku Foods Co., 1998). Sucrose esters are tasteless and odourless.[27,28] Sucrose esters are digested by pancreatic lipase to their components (sugar and fatty acids), which are metabolized in the normal way.[29] However, they are resistant to degradation in the saliva due to the absence of lipases. Sucrose esters are stable at pH values between 4 and 8, and up to 180 °C. In fact, sucrose esters are being used as emulsifiers in the manufacture of oral-care products such as toothpaste, due to the following features: (a) low irritation to the oral membrane, (b) appropriate foamability, (c) little effect on the sense of taste after washing.[27]

The mechanism of antimicrobial action of these carbohydrate esters has not been elucidated. Tsuchido and co-workers observed that sucrose esters cause changes in cellular morphology of growing-cells of *Bacillus subtilis*, which induce autolytic processes.[30,31] It is feasible that sugar esters disrupt the cell membrane, thereby altering its permeability and causing a selective leakage of glycolytic intermediates.[16]

Enzymatically synthesized disaccharide and trisaccharide esters in the present study showed interesting properties. In particular, our results indicate that 6″-*O*-lauroylmaltotriose has a notable antimicrobial activity. This could be of great value in identifying novel inhibitors for inclusion in oral-hygiene products aimed at disrupting plaque formation and preventing disease.

4. Experimental

4.1. Materials

S. sobrinus 6715-7 (serotype g) stock cultures were obtained from the American Type Culture Collection (Rockville, MD, USA). Difco Brain Heart Infusion (BHI) dehydrated medium was obtained from Becton Dickinson Microbiology Systems (Sparks, MD). Bacto-agar was obtained from Fischer Scientific Co. (Fair Lawn, NJ, USA). UNIFLO, 3-mm, 0.2-μm sterile nonpyrogenic disposable syringe filters were obtained from Schleicher & Schuell (Keene, NH, USA). Dextransucrase from *L. mesenteroides* NRRL B-512F was supplied by Prof. P. Monsan (INSA, Toulouse) and was obtained as described.[32] Glucosyltransferase from *S. sobrinus* was purified as previously described.[33] Glucose, maltose and maltotriose were from Sigma Chemical Co. Sucrose and Celite® were from E. Merck. Vinyl laurate was from Fluka Chemical Co. Lipase from *Thermomyces lanuginosus* (Lipolase 100L) was kindly donated by Novozymes A/S (Denmark). All other reagents and solvents were of the highest available purity and used in accordance with the recommendations of the supplier.

4.2. Enzymatic synthesis of sugar esters

Synthesis of 6-*O*-lauroylsucrose, 6′-*O*-lauroylmaltose and 6″-*O*-lauroylmaltotriose was carried out according to an enzymatic method previously developed in our laboratory.[14] The synthesis was performed by transesterification of the sugar with vinyl laurate in a medium of 2-methyl-2-butanol (*tert*-amyl alcohol) containing 20% of dimethyl sulfoxide. The lipase from *Thermomyces lanuginosus* was immobilized on diatomaceous earth (Celite®), and used as a biocatalyst. Reactions were performed at 40 °C with magnetic stirring in the presence of 3 Å molecular sieves. The products were isolated by column chromatography and/or solvent precipitation and fully characterized by chromatography and spectroscopic techniques (HPLC, NMR, IR, HRMS) as described.[14,34]

4.3. Dextransucrase inhibition assay

Dextransucrase (0.5 enzyme units measured in the dinitrosalicylic acid assay)[35] was incubated at 30 °C in 20 mM sodium acetate buffer, pH 5.4, containing 0.05 g/L $CaCl_2$, 10 mM sucrose and 50 mM 6-*O*-lauroylsucrose. At different times (0–6 h), 290 μL aliquots were extracted from the reaction mixture. The reaction was stopped by incubation at 90 °C for 5 min and centrifuged for 7 min at 5000 × *g* using an Eppendorf tube that contained a 0.45-μm nylon filter. The samples were analyzed by HPLC using a system equipped with a SP 8810 pump (Spectra-Physics, Inc., San Jose, CA), a Nucleosil 100-C18 column (4.6 × 250 mm) and a refractive index detector (Waters, model 2410). Ultrapure water was used as the mobile phase (flow rate 0.5 mL/min).

4.4. Glucosyltransferase inhibition assay

Glucosyltransferase (from *S. sobrinus*) activity was measured by means of a dextran assay based on ^{14}C–isotope transfer from uniformly labelled sucrose to glucan.[36] Reaction mixtures (50 mL) were buffered in 0.1 M sodium phosphate, pH 6.5. Different concentrations of carbohydrate fatty acid esters (5, 20, 50, 100, 170, 250, 300, 365 and 500 mM) were tested to see if they inhibited dextran synthesis. Four 10-μL aliquots were removed at one-min intervals, transferred to a 0.5 × 1 cm filter paper strip (Whatman No. 1 filter paper), and immersed in gently stirred cold MeOH. The filter paper strips were washed in cold MeOH for 30 min at 10-min intervals with fresh MeOH. Radioactivity on dried paper strips was measured with a Beckman model LS 8000 liquid scintillation counter. Glucosyltransferase activity was calculated based on the amount of labelled glucan. All reactions were carried out at 27 °C in duplicate.

4.5. Bacterial growth on agar plates

S. sobrinus growth was assayed on BHI agar medium with and without the inhibitors. BHI agar medium supplemented with inhibitors (25 mL) was added to each Petri dish. The final inhibitor concentrations tested were 0.1, 0.2, 0.3, 0.4 and 1 mg/mL. Each inhibitor was dissolved in Omnisolv water (suitable for HPLC, Spectrophotometry, Gas Chromatography) and was filter sterilized with UNIFLO 0.2-μm disposable syringe filters. A required amount of the filter sterilized inhibitor solution was added to each 25-mL aliquot of medium so as to get the required final concentrations. For each inhibitor, controls were maintained with water. A loopful of bacterial suspension from a stationary phase culture was streaked on each Petri dish. The plates were incubated in the dark at 37 °C for bacterial growth for one week. Each experiment was carried out in duplicate.

4.6. Bacterial growth in liquid media

Bacterial growth was also assayed in liquid BHI broth supplemented with inhibitors (2.5, 5 and 10 mg). BHI broth cultures (5 mL) supplemented with inhibitors (final concentrations 0.5–2 mg/mL) were inoculated with 0.05 mL of bacterial suspension from a stationary phase culture of *S. sobrinus*. Cultures were further incubated in the dark at 37 °C for bacterial growth for one week.

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