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Nigerooligosaccharide acceptor reaction of *Streptococcus sobrinus* glucosyltransferase GTF-I

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Abstract

Nigerose and nigerooligosaccharides served as acceptors for a glucosyltransferase GTF-I from cariogenic *Streptococcus sobrinus* to give a series of homologous acceptor products. The soluble oligosaccharides (dp 5–9) strongly activated the acceptor reaction, resulting in the accumulation of water-insoluble (1→3)-α-D-glucan. The enzyme transferred the labeled glucosyl residue from D-[U-^{13}C]sucrose to the 3-hydroxyl group at the non-reducing end of the (1→3)-α-D-oligosaccharides, as unequivocally shown by NMR ^{13}C–^{13}C coupling patterns. The values of the ^{13}C–^{13}C one-bond coupling constant (^{1}J) are also presented for the C-1–C-6 of the ^{13}C-labeled α-(1→3)-linked glucosyl residue and of the non-reducing-end residue. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Nigerooligosaccharide; Mutan; Mutansucrase; Glucosyltransferase; *Streptococcus sobrinus*; D-[U-^{13}C]sucrose; ^{13}C–^{13}C coupling

1. Introduction

Mutans streptococci, including *Streptococcus sobrinus*, are the major causative organisms of dental caries [1]. *S. sobrinus* secretes three dextransucrases (GTF-S) and one (1→3)-α-D-glucan-synthesizing enzyme [2,3], which cooperatively synthesize an adherent, water-insoluble glucan from sucrose, mediating the accumulation of the organism on smooth tooth surfaces and inducing the development of dental caries. The (1→3)-α-D-glucan is referred to as a mutan [4] and the 'mutansucrase' is usually referred to as a water-insoluble glucan-synthesizing glucosyltransferase GTF-I. Although the mutan-synthesizing activity of purified GTF-I is extremely low, it is highly activated in the presence of dextran by the formation of (1→3) branch-linkages on the dextran, followed by the elongation of (1→3)-α-D-glucan chains [5,6]. However, the water-insoluble mutan itself synthesized by GTF-I did not activate the reaction [7,8]. In this report we therefore prepared nigerooligosaccharides [α-(1→3)-linked glucose oligomers] and studied their possible effects on GTF-I as acceptor and activator, and then determined the direction of the chain elongation by specifically using D-[U-^{13}C]sucrose.

2. Experimental

Carbohydrates and reagents.—Nigerooligosaccharides of dp 2–9 were prepared by partial hydrolysis of mutan (45 g), followed by fractionation on 500 mL of wet activated carbon, as previously reported [9] with some modifications. The 30% EtOH elu-

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ate (1 L), 60% eluate (600 mL) and 90% eluate (500 mL) were concentrated by evaporation under low pressure to 20, 17, and 15 mL, correspondingly. Each concentrate was applied to a column (3.6 × 77 cm) of Sephadex G-25 (ultra fine) and eluted with distilled water. Two microlitres of each fraction (3 mL) was applied and chromatographed on a TLC plate, and sugars were detected, as described below. The fractions of dp 2–9 were each pooled, concentrated to ~15 mL, and further purified by filtration on the same column of G-50 in 20% EtOH in order to prevent co-elution of the next-higher oligomer. The purified nigerooligosaccharides of dp 2–9 obtained were 340, 370, 130, 200, 150, 113, 51, and 7.5 mg. [U-^{13}C]Sucrose (min 99%) was purchased from ISOTEC Inc. (Miamisburg, OH). Leucrose was a gift from Hokuren Federation of Agricultural Cooperatives (Japan). All other reagents were commercially available.

Enzyme.—The starter culture of *S. sobrinus* strain 6715 in 5 mL of Todd–Hewitt broth (Difco) was grown aerobically without stirring at 37 °C in 100 mL of the complete synthetic medium of Terleckyj et al. [10], and then in 5 L of the same medium containing 0.02% PMSF, a serine protease inhibitor, 0.1% Tween 80, and 1.5% fructose instead of glucose. During purification steps and storage, specific care was taken to add 0.01% each of PMSF, merthiolate, and EDTA to all of the buffers used in order to prevent proteolytic degradation of the enzyme [11], since the C-terminal domain of GTF-I may be required for its acceptor and activator reactions [12,13]. The culture supernatant fluid (5 L) was diluted threefold with distilled water and was added to 17 mL of wet Bio-Gel HP (Bio-Rad Laboratories). The hydroxylapatite adsorbing the enzyme was overlaid on top of fresh hydroxylapatite previously packed into a column (1.8 × 14 cm), and the enzyme was eluted with 10–400 mM sodium phosphate buffer (pH 6.5). The fractions of insoluble-glucan-synthesizing activity, as detected by the method described below, were collected and chromatographed on a DEAE-Toyopearl (Tosoh Corporation, Japan) column (1.5 × 4 cm) with 200 mL of a linear gradient of 0–200 mM NaCl in 10 mM sodium phosphate buffer (pH 6.5). The enzyme-containing fractions (total 14 mL) were collected and purified further by preparative isoelectric focusing using a LKB8100 Ampholine electrofocusing column (column volume 110 mL, Pharmacia) containing 0.5% Triton X-100 according to the instruction manual I-8100-E04, as previously reported [14]. Ampholine pH 3.5–5.0 (0.95%) and ampholine pH 3.5–10.0 (0.05%) were used, and the enzyme was focused at a constant power of 8 W for 18 h at near 0 °C. Fractions (each 1 mL) were withdrawn. The purified GTF-I was the intact form of GTF-I with 16.5 kDa and pI 4.9, and was free of GTF-S species (Fig. 1(A,B)) by SDS-PAGE and analytical IEF compared with Bio-Rad high-molecular-mass marker protein standards and the Low pI Calibration Kit (Pharmacia), as described [15].

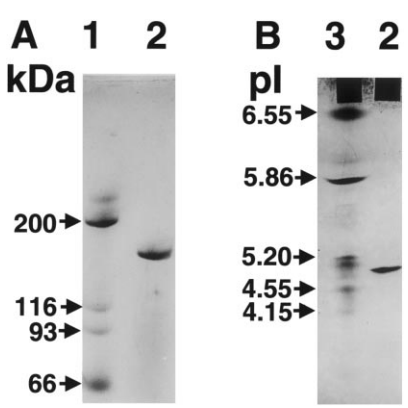

Fig. 1. SDS-PAGE (A) and analytical IEF (B) patterns of the purified *S. sobrinus* GTF-I. Protein was stained with Coomassie Brilliant Blue R-250. Lanes: (1) M_r marker proteins; (2) purified GTF-I (1.6 μg for SDS-PAGE, 1.1 μg for IEF); (3) pI marker proteins.

Reaction conditions and analyses.—All reactions were done at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 0.01% of sodium azide and 0.05% of Triton X-100.

Activation of GTF-I activities with nigerooligosaccharides was studied in 0.2 mL of the buffer added with 10 nM GTF-I, 100 mM sucrose and 1–18 mM glucose equivalent amounts of nigerose to nigerononaose (N2–N9) or dextran T10 (T10). After 15 h, the insoluble glucan and reducing sugars formed were measured by the phenol–H_2SO_4 [16] and Somogyi's methods [17]. One unit (IU) of insoluble-glucan-synthesizing activity or reducing-sugar-release activity was the amount

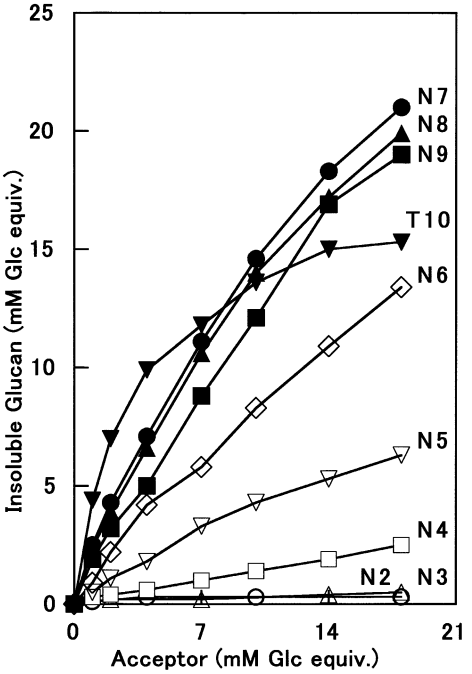

Fig. 2. Stimulation of GTF-I activity by nigerooligosaccharides and dextran T10. Reaction mixtures containing 10 nM GTF-I, 100 mM sucrose, and increasing amounts of nigerose to nigerononaose (N2–N9) or dextran T10 (T10) were incubated for 15 h and the insoluble glucan formed was measured by the phenol–H_2SO_4 method [16].

of enzyme catalyzing the incorporation of 1 μmol of glucose into insoluble glucan or the release of reducing sugar from sucrose per minute. Protein was determined by the Lowry method. The specific activity (IU/mg protein) of reducing-sugar release for GTF-I was 10.5 in the absence of activator, and the increment of the specific activity of reducing-sugar release in the presence of activator was estimated by subtracting 10.5 from each specific activity.

Acceptor products were formed for 1 h in 20 μL of the buffer added with 1 μM GTF-I, 100 mM sucrose and 100 mM acceptor and were separated by thin-layer chromatography (TLC) with three ascents on plates of Silica Gel 70 (Wako Pure Chem., Japan) in 15:3:4 (v/v/v) 1-butanol–pyridine–water. The sugars were visualized by spraying the plates with 80% (v/v) H_2SO_4, followed by heating for 5 min at 120 °C.

The acceptor product for ^{13}C NMR analysis was specifically formed for 1 h in 1 mL of the buffer added with 1 μM GTF-I, 2 mM [U-^{13}C]sucrose (0.67 mg, min 99%), and 40 mM N5 (33.1 mg). The product and the unreacted N5 were adsorbed on 5 mL (wet vol.) of activated carbon powder (Wako), washed with 10% (v/v) EtOH and then eluted with 20 mL of 60% (v/v) EtOH. The total yield after lyophilization was 72%. The sample (24 mg) was dissolved in 0.4 mL of water containing 10% (v/v) D_2O and 1% sodium 4,4-dimethyl-4-silapentane-1-sulfonate (E. Merck). The ^{13}C NMR spectrum was recorded with 163,349 scans at 67.9 MHz on a JNM-GX270 spectrometer (Jeol, Japan), and reported in ppm downfield from the methyl carbon of the internal sulfonate, as previously described [18]. The spectrum of N5 (20 mg) was similarly recorded with 165,468 scans. The assignment for the ^{13}C NMR signals of N5 was deduced from the assignments for reduced N5 [9] and for N2 [19].

The water-insoluble product from the nigeroheptaose acceptor for ^{13}C NMR analysis was also specifically formed for 1 h in 120 μL of the buffer added with 1 μM GTF-I, 60 mM [U-^{13}C]sucrose (2.4 mg), and 8.6 mM N7 (1.2 mg). The insoluble product was collected by centrifugation, washed twice with 360 μL of distilled water, and then reduced with 2 mg of $NaBH_4$ in 1.2 mL of 0.5 M NaOH. After neutralization with 0.5 M HCl and washing with distilled water, the reduced sample (0.81 mg) was dissolved in 0.4 mL of 0.5 M NaOH containing 10% (v/v) D_2O and 1% sodium 4,4-dimethyl-4-silapentane-1-sulfonate. The ^{13}C NMR spectrum was recorded with 29,579 scans, as described above.

3. Results

Activation of S. sobrinus glucosyltransferase GTF-I activities by nigerooligosaccharides.— The GTF-I, purified as shown in Fig. 1, formed a negligible amount of insoluble glucan in the presence of N2 or N3, as was the case in the absence of activator, while the activity was stimulated remarkably by nigerooligosacharides higher than dp 5 (Fig. 2). At a concentration of 18 mM Glc-equivalent of activator, the specific activities (IU/mg protein) of insoluble-glucan synthesis in the presence of N5–N9 were 4.2, 8.9, 14.0, 13.3,

and 12.7, and thus roughly comparable to the increments of the specific activity of reducing-sugar-release of 6.1, 8.6, 16.1, 14.8, and 13.2, correspondingly. Those in the presence of T10 were 10.2 and 9.7, respectively, and the K_m value for T10 was 2.4 mM.

Products of acceptor reaction with nigerooligosaccharides, as revealed by TLC.—GTF-I in the absence of added acceptor transferred the Glc residue from sucrose to water to give glucose and fructose, and also to the 5-hydroxyl group of the resultant fructose to give leucrose plus a little amount of insoluble glucan (Fig. 3, lane 1). When N2 was added at the same molarity as that (100 mM) of sucrose, the products detected were N3, N4, N6, N8, N9, and a small amount of leucrose, approximately one half of N2 remaining unreacted (lane 2). Similarly, when N3 or N4 was added instead of N2, a series of higher nigerooligosaccharides were produced (lanes 3 and 4), indicating that all the nigerooligosaccharides (N2–N4) were good acceptors for the Glc residue from sucrose.

Transfer of [U-^{13}C]Glc residue by GTF-I to N5, as analyzed by NMR.—In this experiment, [U-^{13}C]sucrose was used to distinguish the transferred Glc residue from the Glc residues of the acceptor, and the labeled sucrose was limited to 1/20 of the concentration (100 mM) of N5 in order to obtain only the first acceptor product, since the addition of the equimolar amount of sucrose resulted in the production of a series of homologous oligomers, as shown in Fig. 3. The ^{13}C NMR pattern (Fig. 4(B)) of oligosaccharides in the mixture after reaction revealed that 3.6% of N5 accepted the [U-^{13}C]Glc residue, while the rest (96.4%) remained unreacted. The signals (C), obtained by subtracting (A) from (B), are for the [U-^{13}C]Glc residue transferred to N5, and those for the residues of the N5 portion are not detectable here because of the low content (1.1%) of ^{13}C at natural abundance. As expected, the C-1 and C-6 signals appeared as doublets through one-bond coupling ($^1J_{1,2}$ 44.0, $^1J_{5,6}$ 42.5 Hz), and each signal for C-2–C-5 appeared as a triplet with overlapped signals of C-2 and C-5 (C-2,^1J 42.8; C-3, ^1J 37.6; C-4, ^1J 44.0; C-5, ^1J 42.8 Hz) (Fig. 4(C)). The ppm values at the center of each multiplet for C-1–C-6 were 101.84, 74.50, 75.73, 72.16, 74.50, and 63.23, which corresponded well with the values 101.85, 74.48, 75.66, 72.25, 74.48, and 63.26 of those for C-1–C-6 of the non-reducing-end Glc residue of N5 (Fig. 4(A)). These data unequivocally indicated that GTF-I transferred the Glc residue from sucrose at the 3-hydroxyl group of the non-reducing-end Glc residue of the acceptor N5, resulting in the formation of N6.

Structure of the insoluble glucan from [U-^{13}C]sucrose and nigeroheptaose acceptor.—[U-^{13}C]sucrose was again used to distinguish the newly formed linkage structure of the insoluble glucan resulting from the nigeroheptaose acceptor. The labeled sucrose was increased to 60 mM, which was seven times that of N7 (8.6 mM). In the ^{13}C NMR pattern (Fig. 5) of the insoluble glucan, the C-1 and C-6 signals appeared as doublets ($^1J_{1,2}$ 44.9, $^1J_{5,6}$ 41.8 Hz), and each signal for C-2–C-5 appeared as a triplet with overlapped signals of C-2 and C-4 (C-2, ^1J 39.9; C-3, ^1J 37.7; C-4, ^1J 38.1; C-5, ^1J 40.8 Hz). The ppm values at the center of each multiplet for C-1–C-6 were 102.99, 73.80, 84.99, 73.23, 75.43, and 63.80, which also corresponded well to the values 102.88, 73.76,

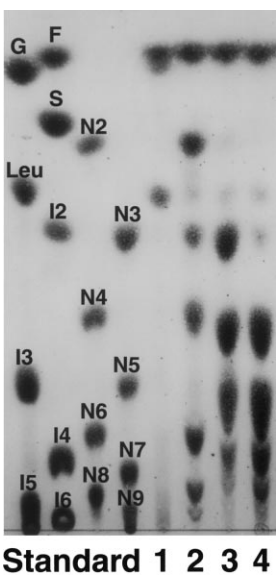

Fig. 3. TLC of acceptor reaction products of GTF-I on nigerooligosaccharides. Standard sugars: F, fructose; G, glucose; S, sucrose; Leu, leucrose; I2–I6, isomaltooligosaccharides of dp 2–6; N2–N9, nigerooligosaccharides of dp 2–9. Reaction mixtures containing 1 μM GTF-I, 100 mM sucrose, and 100 mM acceptor were incubated for 1 h and 2 μL of each mixture was chromatographed. Acceptors: lane 1, none; lane 2–lane 4, N2–N4.

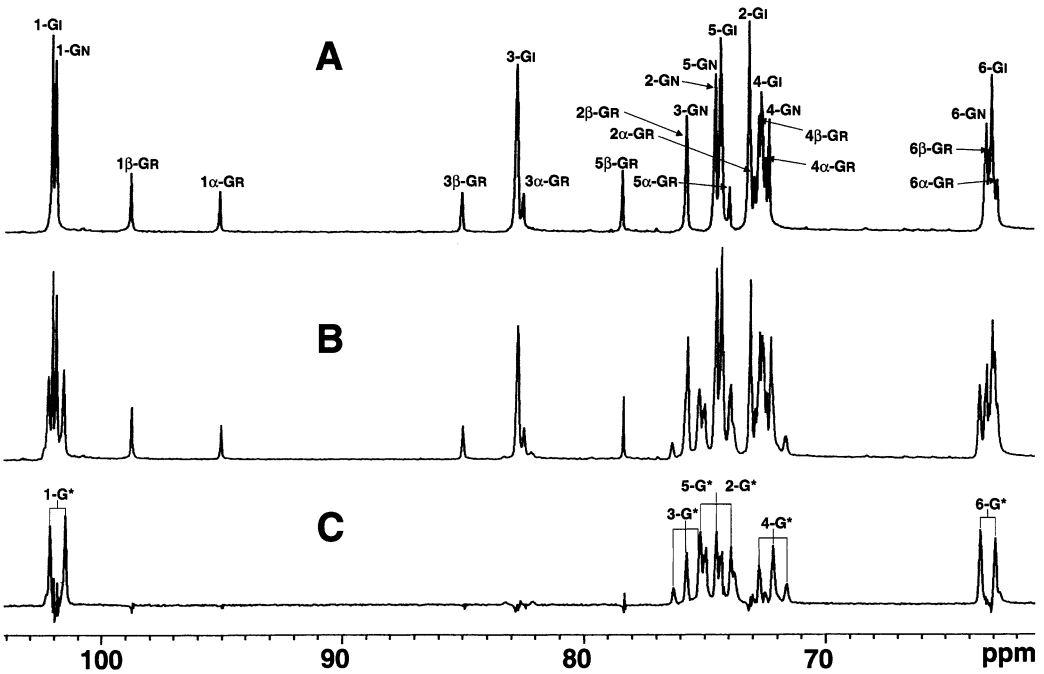

Fig. 4. ^{13}C NMR spectra of nigeropentaose (N5) and the acceptor product of GTF-I on N5. (A) N5: 1α-GR–6α-GR and 1β-GR–6β-GR represent the C-1–C-6 of α and β forms of the reducing terminal Glc residue; 1-GN–6-GN, the C-1–C-6 of the non-reducing terminal Glc residue; 1-GI–6-GI, the C-1–C-6 of the internal Glc residues. (B) Acceptor product and unreacted N5. (C) Acceptor product. This is the difference spectrum of (A) subtracted from (B) at 1.02-fold intensity. 1-G*–6-G*, the C-1–C-6 of the [U-^{13}C] Glc residue transferred from [U-^{13}C]sucrose to the non-reducing end of N5.

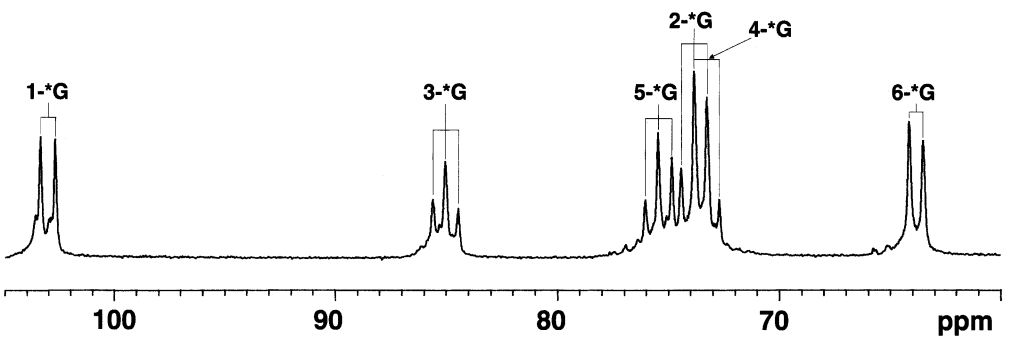

Fig. 5. ^{13}C NMR spectrum of the resulting insoluble glucan from [U-^{13}C]sucrose and N7 acceptor. 1-G*–6-G*, the C-1–C-6 of the [U-^{13}C] Glc residue transferred from [U-^{13}C]sucrose to the N7 acceptor, indicating that the insoluble material is α-(1→3)-linked glucan.

84.89, 73.23, 75.33, and 63.79 of those for C-1–C-6 of the α-(1→3)-linked Glc residues of mutan [20]. This result also clearly indicated that the resulting insoluble material was a α-(1→3)-linked glucan.

4. Discussion

Fu and Robyt [21] reported that *S. sobrinus* GTF-I transferred the Glc residue from sucrose to the non-reducing residue of maltose to give panose (6^2-α-glucosylmaltose). When maltotriose was an acceptor, four tetrasaccharides resulted, with glucose being transferred to the 6-hydroxyl and the 3-hydroxyl groups of both the non-reducing-end and the reducing-end Glc residues. In contrast, in the present study, GTF-I transferred the Glc residues from sucrose to the 3-hydroxyl groups of the non-reducing-end residues of nigerooligosaccharides (dp 2–9), giving the corresponding homologous series. Free glucose and leucrose were scarcely produced (Fig.

3), indicating that all of these nigerooligomers were efficient acceptors for GTF-I. Moreover, the oligomers higher than dp 5 notably activated both the sucrase and insoluble mutan-synthesizing activities, as added dextran T10 did. In this respect, Walker and Schuerch [22] previously suggested that efficient dextrans would contain some α-(1→3)-linked sequences in addition to their α-(1→6)-linked sequences to stimulate GTF-I activities, and that the reaction would be slow until the newly formed side chains possibly consisted of more than three α-(1→3)-linked residues. The activation of GTF-I by higher nigerooligomers may suggest the presence of a binding site best-fitted for about seven α-(1→3)-linked Glc residues, although the K_m values for nigerooligomers were too high to be estimated, as compared with the K_m value (2.4 mM) for dextran T10 (Fig. 2). It is of interest to know if the site for α-(1→3)-Glc residues would be on the catalytic domain, different from the C-terminal domain for the dextran-binding site [13].

The use of [U-^{13}C]sucrose (min. 99% ^{13}C) for the analysis of the first acceptor product was successful (Fig. 4). All the carbons of the [U-^{13}C]Glc residue transferred to the acceptor N5 appeared with the expected multiplicity, so that the transferred residue was not only readily distinguished from the residues of the acceptor, but also its bonding site was rigorously determined. The linkage structure of the resulting insoluble glucan from a nigerooligosaccharide acceptor was also clearly determined as being a typical mutan (Fig. 5). The value of $^1J_{1,2}$ for [1-^{13}C]glucose was reported to be 46.0 Hz [23], which is close to the value of 44.0 Hz of $^1J_{1,2}$ for the [U-^{13}C]Glc residue. As far as we know, this is the first report that uses [U-^{13}C]sucrose in order to elucidate catalytic actions of glycosyltransferases. [U-^{13}C]glucose was also successfully used for studies on the biosynthesis of gallic acid [24] and on the metabolic pathways involved in succinate biosynthesis in vivo [25].

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