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Structural investigation of an extracellular polysaccharide produced by the cariogenic bacterium *Streptococcus mutans* strain UA159

Bo Li[a,*], Justyna M. Dobruchowska[b], Michel A. Hoogenkamp[c], Gerrit J. Gerwig[b]

[a] *School of Food Science, Henan Institute of Science and Technology, Hualan Road, 453003 Xinxiang, China*
[b] *Department of Microbiology, University of Groningen, Nijenborgh 7, 8747 AG Groningen, The Netherlands*
[c] *Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, Gustav Mahlerlaan 3004, 1081 LA Amsterdam, The Netherlands*

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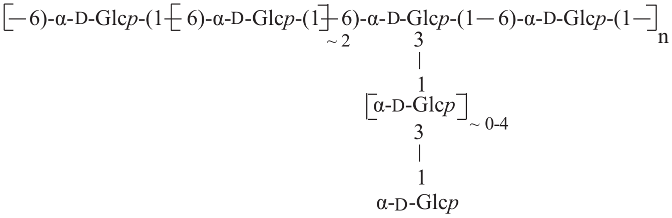
ABSTRACT

The structure of an extracellular polysaccharide EPS159 produced from sucrose by *Streptococcus mutans* UA159 was investigated through the main oligosaccharides obtained from partial acid hydrolysis, monosaccharide/methylation analysis, and 1D/2D ^{1}H NMR spectroscopy. The results showed that EPS159 contained terminal, 3-substituted, 6-substituted, and 3,6-disubstituted α-D-glucopyranose residues in a molar percentage of 14, 18, 54, and 14%. The backbone of EPS159 was composed of →6)Glc*p*(1→ residues, and about 20% of the →6)Glc*p*(1→ residues was substituted at 3-OH by →3)Glc*p*(1→ and/or Glc*p*(1→ residues to form side chains. A composite model of EPS159, that includes all identified structural features, was formulated:



$$\left[-6)\text{-}\alpha\text{-}\mathrm{D}\text{-Glc}p\text{-}(1-\left[6)\text{-}\alpha\text{-}\mathrm{D}\text{-Glc}p\text{-}(1\right]_{\sim 2}-6)\text{-}\alpha\text{-}\mathrm{D}\text{-}\underset{\substack{3\\|\\1\\[\alpha\text{-}\mathrm{D}\text{-Glc}p]_{\sim 0\text{-}4}\\3\\|\\1\\\alpha\text{-}\mathrm{D}\text{-Glc}p}}{\text{Glc}p}\text{-}(1-6)\text{-}\alpha\text{-}\mathrm{D}\text{-Glc}p\text{-}(1-\right]_n$$

1. Introduction

Oral diseases related to dental biofilms afflict the majority of the world's population, and dental caries is still the single most prevalent and costly oral infectious disease, next to chronic inflammatory periodontal disease (Beikler & Flemmig, 2011; Jeon, Rosalen, Falsetta, & Koo, 2011). *Streptococcus mutans* is a key contributor to the formation of the extracellular polysaccharide (EPS) matrix in dental biofilms (Koo, Xiao, Klein, & Jeon, 2010; Loesche, 1986; Nicolas & Lavoie, 2011). Glucans, synthesized from dietary sucrose by the glucosyltransferases (GTFs) of *S. mutans*, have been recognized as virulence factors in the etiology and pathogenesis of dental caries (Koo et al., 2010). Glucans promote the adherence and accumulation of cariogenic streptococci on the tooth surface and play an essential role in the development of pathogenic dental plaque related to caries-forming activity (Bowen & Koo, 2011). *S. mutans* produces at least three GTFs, acting together: GTF B, which synthesizes a polymer of mostly insoluble (α1→3)-linked glucan; GTF C, which synthesizes a mixture of insoluble (α1→3)-linked glucan and soluble (α1→6)-linked glucan; and GTF D, which synthesizes soluble (α1→6)-linked glucan (Hanada & Kuramitsu, 1989). Structural analyses of EPS, formed by incubating streptococci culture filtrates with sucrose, have typically shown that they contain glucose residues with (α1→6) and (α1→3) linkages and have a comb-like, highly branched form. In general, the insoluble glucans are predominantly (α1→3) linked, whereas the soluble glucans are comprised basically of (α1→6)-linked glucose residues. Although there have been many studies on the linkage analyses of both soluble and insoluble glucans produced by different *S. mutans* strains, their structures remain unclear (Aires, Koo, Sassaki, Iacomini, & Cury, 2010; Colson, Jarrell, Lamberts, & Smith, 1979; Davis, Boyko, & Edwards, 1986; Davis, Hines, & Edwards, 1986; Higton & Weigel 1980; Kopec, Vacca-Smith, & Bowen, 1997; Shibata, Goldstein, & Kirkland, 1983; Trautner, Birkhed, & Svensson, 1982;

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* Corresponding author at: School of Food Science, Henan Institute of Science and Technology, Hualan Road, 453003 Xinxiang City, Henan Province, China.
Tel.: +86 373 3040777; fax: +86 373 3040777.
E-mail address: libowuxi@yahoo.com.cn (B. Li).

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Wiater, Choma, & Szczodrak, 1999). The genome of *S. mutans* UA159, a serotype c strain, has been completely sequenced and is composed of 2,030,936 bp (Ajdić et al., 2002). This strain has been used to study the influences of starch and sucrose on *S. mutans* biofilms (Duarte et al., 2008). The purpose of this study was to investigate the structure of an exoglucan produced by *S. mutans* UA159 incubated with sucrose.

2. Materials and methods

2.1. Preparation of exopolysaccharide

EPS159, an exopolysaccharide produced by *S. mutans* UA159, was prepared as followed: *S. mutans* UA159 (ATCC 700610) was incubated overnight on BHI broth, containing 20% sucrose, under anaerobic conditions (10% H_2, 10% CO_2 in N_2) at 37 °C. Bacterial cells were removed by centrifugation for 10 min at 11,000 × *g*. The supernatant was collected and proteins and other media components were removed by TCA precipitation (4% TCA, 2–3 h, at 4 °C), followed by a centrifugation step (2500 × *g*, 20 min at 4 °C). The EPS was precipitated overnight at 4 °C by adding 2 volumes of ice-cold ethanol (96%) to 1 volume of supernatant. After centrifugation (2500 × *g*, 20 min at 4 °C) and dissolving the pellet in distilled water (25 mL) by extensive sonication (3 h), the solution was dialyzed (12–14 kDa cutoff) against distilled water for 48 h. Subsequently, EPS was precipitated again overnight at 4 °C by adding 2 volumes of ethanol (96%) to 1 volume of supernatant. After centrifugation (2500 × *g*, 20 min at 4 °C) and washing the pellet twice with distilled water, dry material of EPS159 was obtained by lyophilization. The total amount of carbohydrate was determined by a phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.2. Monosaccharide composition

EPS159 was subjected to methanolysis (1 M methanolic HCl, 24 h, 85 °C), including Internal Standard (mannitol). After neutralization and evaporation, the sample was trimethylsilylated (hexamethyldisilazane–trimethylchlorosilane–pyridine 1:1:5, 30 min, room temperature), and qualitatively/quantitatively analyzed by GLC on an EC-1 column (30 m × 0.32 mm, Alltech), using a Chrompack CP9002 gas chromatograph with flame-ionization detector (temperature program 140–240 °C, 4 °C/min) (Kamerling & Vliegenthart, 1989). The absolute configuration of the monosaccharides was determined by the method of Gerwig, Kamerling, and Vliegenthart (1978).

2.3. Partial acid hydrolysis and fractionation

EPS159 (119 mg) was hydrolyzed in 3 mL of 0.5 M TFA for 3 h at 90 °C. The hydrolysate was centrifugated (1500 × *g*, 5 min), and the supernatant was taken out. The precipitation was cleaned twice with 2 mL of water with intermediate centrifugation. The combined supernatant was evaporated to dryness using a Rotavap. After dissolving in water, the product was fractionated on a Bio-Gel P-2 column (48 cm × 1.1 cm), eluted with 10 mM ammonium bicarbonate at a flow rate of 10 mL/h. Fractions of 10 min were collected and checked by MALDI-TOF-MS for oligosaccharide mass distribution.

2.4. High-pH anion-exchange chromatographic fractionation

High-pH anion-exchange chromatography (HPAEC) was performed on a Dionex DX500 workstation, equipped with an ED40 PAD system. Pooled Bio-Gel P-2 fractions of partial hydrolyzed EPS159 were fractionated on a CarboPac PA-1 column (250 mm × 9 mm, Dionex), using different linear gradients of 0–300 mM NaOAc in 100 mM NaOH (3 mL/min), depending on the sample (see Section 3). Collected fractions were immediately neutralized with 4 M NaOAc, desalted on CarboGraph SPE columns (150 mg graphitized carbon, Alltech) eluted with 1:3 acetonitrile:H_2O, and lyophilized.

2.5. Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry

Molecular masses of products in the Bio-Gel P-2 fractions from EPS159 partial hydrolysate were determined by MALDI-TOF-MS, using an Axima™ mass spectrometer (Shimadzu Kratos Inc., Manchester, UK) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Samples were prepared by mixing on the target 0.5 μL of sample solution with 0.5 μL of 2,5-dihydroxybenzoic acid (DHB) (10 mg/mL) as matrix solution.

2.6. Linkage analysis

EPS159 samples were permethylated using CH_3I and solid NaOH in DMSO (Ciucanu & Kerek, 1984; Needs & Selvendran, 1993). After hydrolysis with 2 M TFA (2 h, 120 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ (2 h, room temperature). Then, after neutralization with HOAc and removal of boric acid by co-evaporation with methanol, followed by acetylation with 1:1 acetic anhydride:pyridine (30 min, 120 °C), the mixture of partially methylated alditol acetates was analyzed by GLC–EI-MS on an EC-1 column (30 m × 0.25 mm) using a GCMS-QP2010Plus instrument (Shimadzu, Japan) and a temperature program of 140–250 °C at 8 °C/min (Kamerling & Vliegenthart, 1989).

2.7. Nuclear magnetic resonance spectroscopic analysis

Resolution-enhanced 1D/2D 500-MHz ^1H NMR spectra (including TOCSY, ROESY, HSQC) of intact EPS159 and its oligosaccharides obtained by partial acid hydrolysis were recorded in D_2O on a Varian Inova Spectrometer at a probe temperature of 300 K. Before analysis, samples were exchanged twice in D_2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D_2O. Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225 for ^1H and δ 31.08 for ^{13}C).

3. Results

3.1. General

EPS159, an exopolysaccharide produced from sucrose by *S. mutans* UA159, was obtained by ethanol precipitation. After centrifugation, the pellet was redissolved in distilled water by extensive sonication (3 h) and non-dissolved material was removed by centrifugation. The solution was dialyzed (12–14 kDa cut-off) against distilled water. Subsequently, EPS was precipitated again with ethanol. After centrifugation, the pellet was intensively washed with distilled water to remove all water-soluble material. Then, dry EPS was obtained by lyophilization. Thereafter, the obtained EPS159 (MW > 14 kDa) was only soluble, in low concentration, in water after extensive ultrasonication.

Monosaccharide (including D/L) analysis of EPS159 revealed only the presence of D-glucose as carbohydrate constituent (96%, w/w), indicating a glucan polymer. Methylation analysis of EPS159 (Table 1) showed the presence of terminal, 3-substituted, 6-substituted, and 3,6-disubstituted glucopyranose in a molar percentage of 14, 18, 54, and 14%, respectively, indicating a branched glucan.

1D/2D ^1H NMR spectroscopy of EPS159 (Fig. 1, included HSQC spectrum) demonstrated α-anomeric configuration for the glucose

Table 1
Methylation analysis results of EPS159 and some fractions isolated after partial hydrolysis of EPS159.

PMAA	Rt	Linkage type	EPS159	PII$_9$	PII$_{10}$a	PII$_{10}$b	PII$_{11}$a	PII$_{12}$a	PII$_{13}$a	PII$_{14}$b	PIV$_{22}$
2,3,4,6-Me$_4$-Glc-ol	1.00	Glc*p*(1→	14	22	69	18	53	34	35	23	12
2,4,6-Me$_3$-Glc-ol	1.16	→3)Glc*p*(1→	18	–	–	–	–	–	34	77	–
2,3,4-Me$_3$-Glc-ol	1.21	→6)Glc*p*(1→	54	78	–	82	25	51	31	–	88
2,4-Me$_2$-Glc-ol	1.37	→3,6)Glc*p*(1→	14	–	31	–	22	15	–	–	–

Rt, GLC relative retention time to 2,3,4,6-Me$_4$-1,5-Ac$_2$-Glc-ol on EC-1 column with temperature program of 140–250 °C at 8 °C/min.

residues (coupling constants $^3J_{1,2} < 3.0$ Hz). Of these residues, 79% is involved in (α1→6) linkages ($\delta_{\text{H-1}} \sim 4.98$) and 21% in (α1→3) linkages ($\delta_{\text{H-1}} \sim 5.32$), which is in agreement with the linkage distribution found by methylation analysis. TOCSY experiments (20–200 ms, spectra not shown) indicated overlap of two signals at $\delta_{\text{H-1}} \sim 4.98$, stemming from two Glc residues, denoted **B** [→3,6)Glc*p*(1→6] and **C** [→6)Glc*p*(1→6]. The signal at $\delta_{\text{H-1}} \sim 5.32$ also comprises two anomeric signals stemming from a terminal

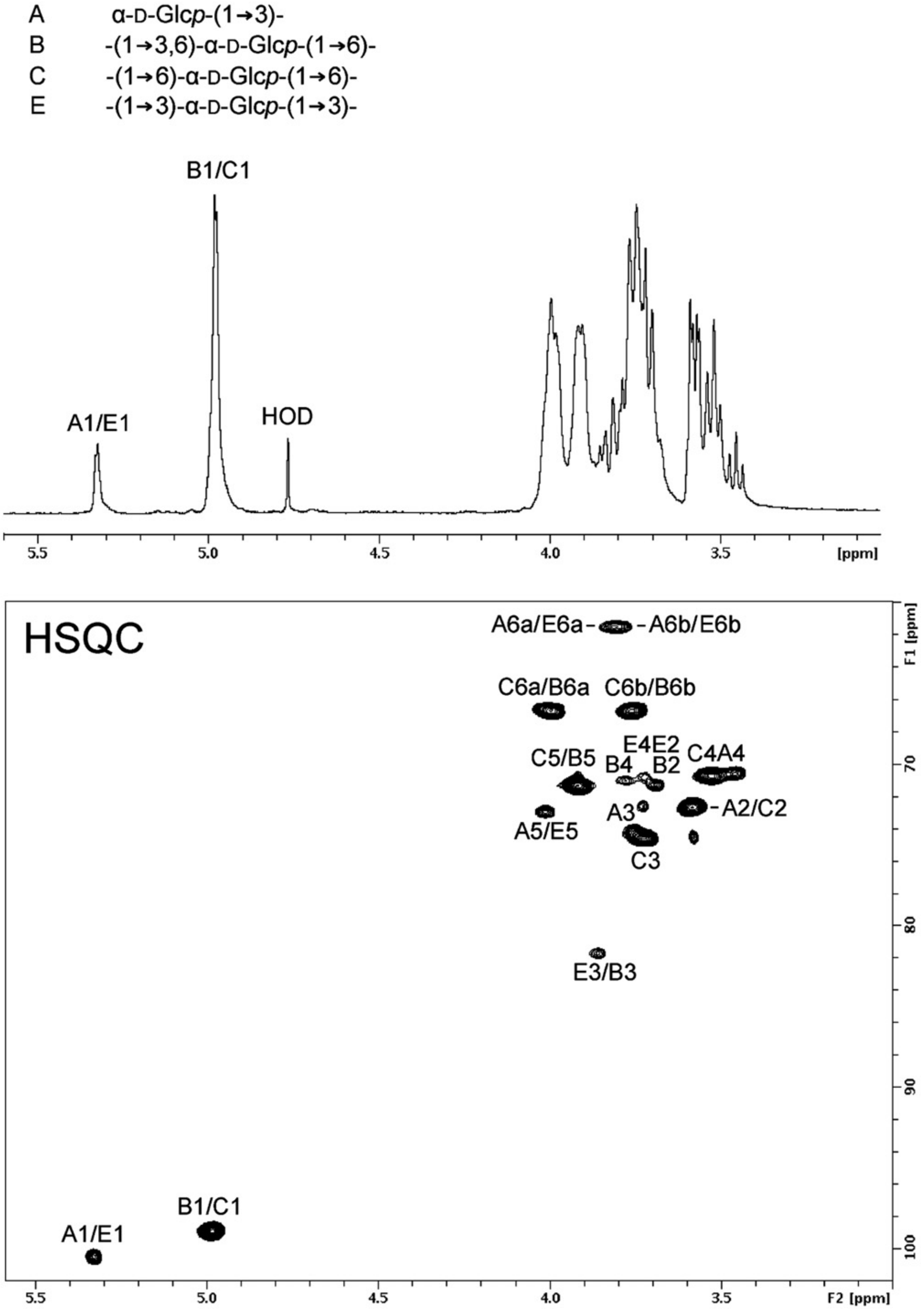


Fig. 1. 1D ^{1}H NMR and HSQC spectrum of EPS159, recorded in D$_2$O at 300 K.

Table 2
The ^{1}H and ^{13}C NMR chemical shifts[a] of glucose residues in EPS159 and derived oligosaccharides, compared to free Glc*p* α/β, recorded in D_2O at 300 K.

Residue	H-1C-1	H-2C-2	H-3C-3	H-4C-4	H-5C-5	H-6aC-6	H-6b
Free Glc*p* α	5.22	3.54	3.72	3.43	3.84	3.85	3.76
	93.1	72.5	73.8	70.7	72.4	61.8	
Free Glc*p* β	4.63	3.23	3.50	3.43	3.46	3.91	3.73
	96.8	75.2	76.8	70.7	76.8	61.8	
A α-D-Glc*p*-(1–3)-	5.33	3.59	3.75	3.46	3.98	3.86	3.79
	100.0	72.7	73.9	70.6	72.8	61.5	
B -(1–3,6)-α-D-Glc*p*-(1–6)-	4.98	3.68	3.87	3.78	3.91	3.98	3.75
	99.0	71.0	81.7	70.8	71.2	66.7	
C -(1–6)-α-D-Glc*p*-(1–6)-	4.98	3.58	3.73	3.53	3.92	3.99	3.76
	98.9	72.6	74.5	70.7	71.4	66.8	
D α-D-Glc*p*-(1–6)-	4.96	3.57	3.73	3.43	3.74	3.85	3.76
	98.8	72.4	74.1	70.6	72.5	61.6	
E -(1–3)-α-D-Glc*p*-(1–3)-	5.33	3.69	3.91	3.69	4.00	3.86	3.78
	100.1	71.3	81.3	70.7	72.6	61.5	
F -(1–3)-α-D-Glc*p*-(1–6)-	4.97	3.67	3.88	3.69	3.75	3.84	3.77
	98.9	71.0	81.5	70.8	72.5	61.5	
Rα -(1–6)-Glc*p* α	5.24	3.55	3.71	3.52	4.01	3.97	3.75
	93.1	72.4	74.0	70.7	70.9	67.1	
Rβ -(1–6)-Glc*p* β	4.68	3.25	3.50	3.51	3.65	4.00	3.85
	96.9	75.2	76.7	70.6	75.8	69.6	

[a] In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).

Glc*p*(1→3 residue **A** and a →3)Glc*p*(1→3 residue **E**. All ^{1}H and ^{13}C NMR chemical shifts of the glucose residues (Table 2) were determined by TOCSY and HSQC (Fig. 1) experiments and use was made of an earlier developed structural-reporter-group concept for the analysis of α-D-glucans (van Leeuwen, Leeflang, Gerwig, & Kamerling, 2008).

3.2. Partial acid hydrolysis and fractionation

For homopolysaccharides, products of partial acid hydrolysis can provide relevant structural information. Thus, EPS159 was subjected to a partial acid hydrolysis using 0.5 M TFA (3 h, 90 °C). The hydrolysate was fractionated on a Bio-Gel P-2 column, and the molecular masses of the products in the fractions were determined by MALDI-TOF-MS. The Bio-Gel P-2 fractions were divided into four pools based on their fragment sizes. Pool I contained Glc_{2-3} ($[M+Na]^+$ pseudomolecular ions at *m*/*z* 365 and 527), Pool II contained Glc_{4-6} ($[M+Na]^+$ pseudomolecular ions at *m*/*z* 689, 851, and 1013), Pool III contained Glc_{7-10} ($[M+Na]^+$ pseudomolecular ions at *m*/*z* 1175, 1337, 1499, and 1661), and Pool IV contained $Glc_{>10}$.

Pool I was subfractionated by HPAEC on CarboPac PA-1, using a linear gradient of 10–100 mM NaOAc in 100 mM NaOH, yielding free glucose and four extra peaks (profile not shown). ^{1}H NMR spectroscopy (spectra not shown) of two isolated compounds (both $[M+Na]^+$ *m*/*z* 365 by MALDI-TOF-MS) showed them to be Glc(α1→6)Glc (isomaltose) and Glc(α1→3)Glc (nigerose), in accordance to literature spectra (van Leeuwen, Leeflang, et al., 2008). The third peak (PI_4) contained Glc_3 ($[M+Na]^+$ *m*/*z* 527 by MALDI-TOF-MS). The ^{1}H NMR spectrum (Fig. 2A) demonstrated the structure as: Glc(α1→6)Glc(α1→6)Glc (isomaltotriose) (Dobruchowska et al., 2011; van Leeuwen, Kralj, et al., 2008). The fourth peak (PI_5) contained also a Glc_3 ($[M+Na]^+$ *m*/*z* 527 by MALDI-TOF-MS) but the ^{1}H NMR spectrum revealed the structure as Glc(α1→3)Glc(α1→3)Glc (Fig. 2B).

Pools II, III, and IV were also subfractionated by HPAEC on CarboPac PA-1, but using a linear gradient of 0–250 mM NaOAc in 100 mM NaOH (Fig. 3A, C, and D). Nine fractions from Pool II, denoted PII_6–PII_{14}, five fractions from Pool III, denoted $PIII_{15}$–$PIII_{19}$, and five fractions from Pool IV, denoted PIV_{20}–PIV_{24} were isolated for structural characterization.

Some of the fractions from Pool II turned out to be mixtures and were further separated by HPAEC on CarboPac PA-1, using adequate linear solvent gradients; 50–110 mM NaOAc in 100 mM NaOH for PII_{10}, yielding two fractions denoted PII_{10}a and PII_{10}b; 50–150 mM for PII_{11}, yielding PII_{11}a and PII_{11}b; 60–180 mM for PII_{12}, yielding PII_{12}a and PII_{12}b; 50–250 mM for PII_{13}, yielding PII_{13}a and PII_{13}b; and 100–300 mM for PII_{14}, yielding PII_{14}a and PII_{14}b (Fig. 3B).

3.3. Structural analysis of fractions

All obtained fractions were analyzed by MALDI-TOF-MS and NMR spectroscopy. Some fractions were also subjected to methylation analysis (MA) when the 1D ^{1}H NMR spectrum was not straightforward. The structures of the oligosaccharides which could be elucidated are depicted in Table 3.

As a typical example of the structural analysis of an oligosaccharide, the analysis of a hexasaccharide in fraction PII_{12}a is described in detail. MALDI-TOF-MS analysis of PII_{12}a showed an $[M+Na]^+$ pseudomolecular ion at *m*/*z* 1013, corresponding with Hex_6.

The ^{1}H NMR spectrum of PII_{12}a (Fig. 4) revealed the anomeric signals of five distinctive glucose residues, respectively at δ 5.34 ($\mathbf{A}_\alpha$ H-1), 5.32 ($\mathbf{A}_\beta$ H-1), 5.24 ($\mathbf{R}_\alpha$ H-1), 4.98 (**B** H-1, **C** H-1, and **D** H-1), and 4.68 ($\mathbf{R}_\beta$ H-1). All proton and carbon signals were assigned by TOCSY, HSQC, and ROESY experiments (Dobruchowska et al., 2011; van Leeuwen, Kralj, et al., 2008). Residue **A** is a terminal α-D-Glc*p*-(1–3)- unit, according to its set of chemical shifts (van Leeuwen, Leeflang, et al., 2008). The splitting of the **A** H-1 ($\mathbf{A}_\alpha$ 5.34, and $\mathbf{A}_\beta$ 5.32) was due to the influence of the α/β configuration of the reducing Glc*p* residue **R**. Although there was strong overlap at δ 4.98, build-up TOCSY experiments (20, 50, 100, 200 ms) revealed the differences in chemical shift of the ring protons. The set of chemical shifts of **D** H-2, H-3, H-4, H-5, and H-6a/b at δ 3.57, 3.73, 3.43, 3.73, and 3.86/3.77, respectively, corresponds with that of a terminal α-D-Glc*p*-(1→6)- unit. The set of chemical shifts of **C** H-2, H-3, H-4, and H-5 at δ 3.59, 3.73, 3.54, and 3.90, respectively, corresponds with that of an internal -(1→6)-α-D-Glc*p*-(1→6)- unit, whereas the set of chemical shifts of **B** H-2, H-3, H-4, and H-5 at δ 3.69, 3.88, 3.75, and 3.92, respectively, corresponds with that of an internal →3,6)-disubstituted α-D-Glc*p* unit.

The substitutions at positions 3 and 6 of residue **B** were supported by the downfield ^{13}C chemical shifts (deduced from HSQC): **B** C-3 at δ 81.7 and **B** C-6 at δ 66.8. The substitution of residue **C** at position 6 was also supported by a downfield chemical shift of **C** C-6 at δ 66.9 (Dobruchowska et al., 2011). In the ROESY spectrum, inter-residual cross peaks were observed indicating the linkages **B**(1–6)**R**, **C**(1–6)**B**, **D**(1–6)**C**, and **A**(1–3)**B**. Considering

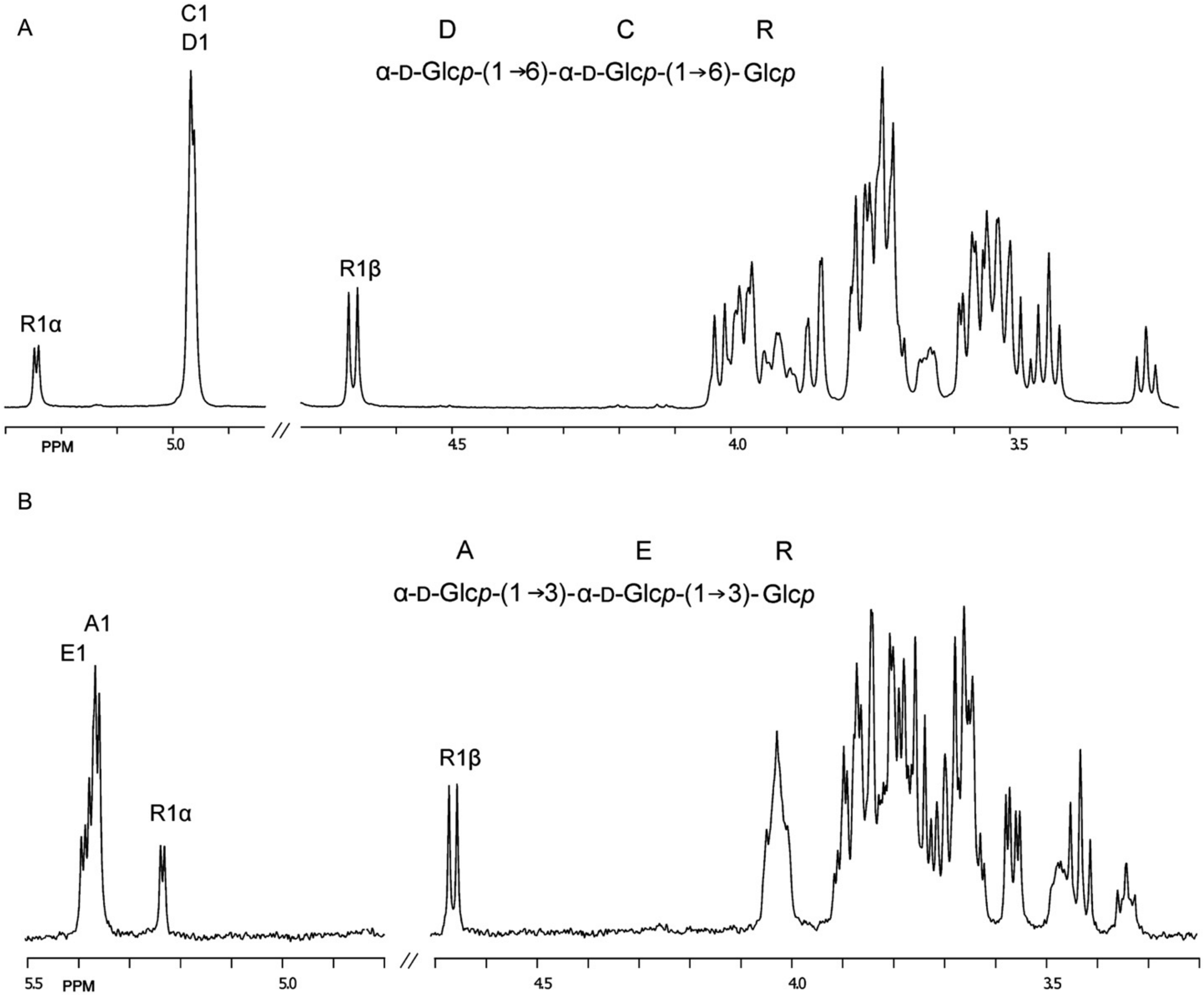
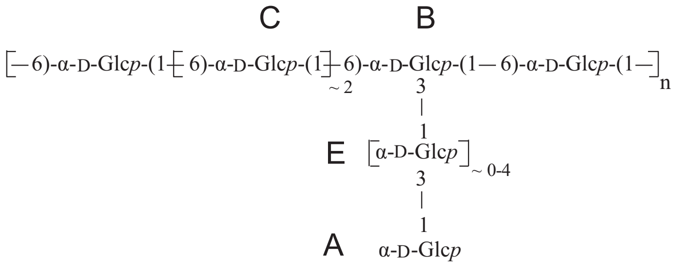


Fig. 2. (A) 1D ^{1}H NMR spectrum of HPAEC fraction PI$_4$ (isomaltotriose), recorded in D$_2$O at 300 K. (B) 1D ^{1}H NMR spectrum of HPAEC fraction PI$_5$ (nigerotriose), recorded in D$_2$O at 300 K.

all analytical data, fraction PII$_{12}$a was identified as **D**1→6**C**1→6**C**1→6[**A**1→3]**B**1→6**R**, *i.e.*, α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→3)]-α-D-Glc*p*-(1→6)-D-Glc*p* (Fig. 4).

3.4. Structure of EPS159

The analysis of oligosaccharide fragments produced by partial acid hydrolysis of EPS159 revealed eleven fractions with only (α1→6) linkages, three fractions with only (α1→3) linkages and four fractions with (α1→6) and (α1→3) linkages. ^{1}H NMR spectroscopic analysis revealed no indications for linear [→3)Glc(α1→6] sequences (van Leeuwen, Leeflang, et al., 2008). Combining the elucidated oligosaccharides with the linkage analysis data and the NMR data of intact EPS159, a composite model of EPS159, that includes all identified structural features can be formulated, however, not showing the definite structure. It indicates that EPS159 contains a long backbone of →6)Glc*p*(1→ residues and about 1/5 of →6)Glc*p*(1→ residues are substituted at 3-OH. The side chains are composed of Glc*p*(1→ or →3)Glc*p*(1→, whose length may vary from 1 to 5 Glc*p* residues. Because (α1→3) linkages are more susceptible to acid hydrolysis with TFA than (α1→6) linkages, the polymer degree of the (α1→3) linked glucose fragments could be higher than five glucose residues.



$$\left[-6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\left[-6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\right]_{\sim 2}6)\text{-}\underset{\substack{3\\|\\1\\ \left[\alpha\text{-D-Glc}p\right]_{\sim 0\text{-}4}\\3\\|\\1\\ \alpha\text{-D-Glc}p}}{\alpha\text{-D-Glc}p}\text{-}(1-6)\text{-}\alpha\text{-D-Glc}p\text{-}(1-\right]_n$$

4. Discussion

Most published data on the structure of glucans produced by *S. mutans* showed that the water-insoluble glucans contain predominantly (α1→3) linkages, whereas the soluble glucans are comprised basically of (α1→6) linkages (Davis, Boyko, et al., 1986; Duarte et al., 2008; Higton & Weigel, 1980; Shibata et al., 1983;

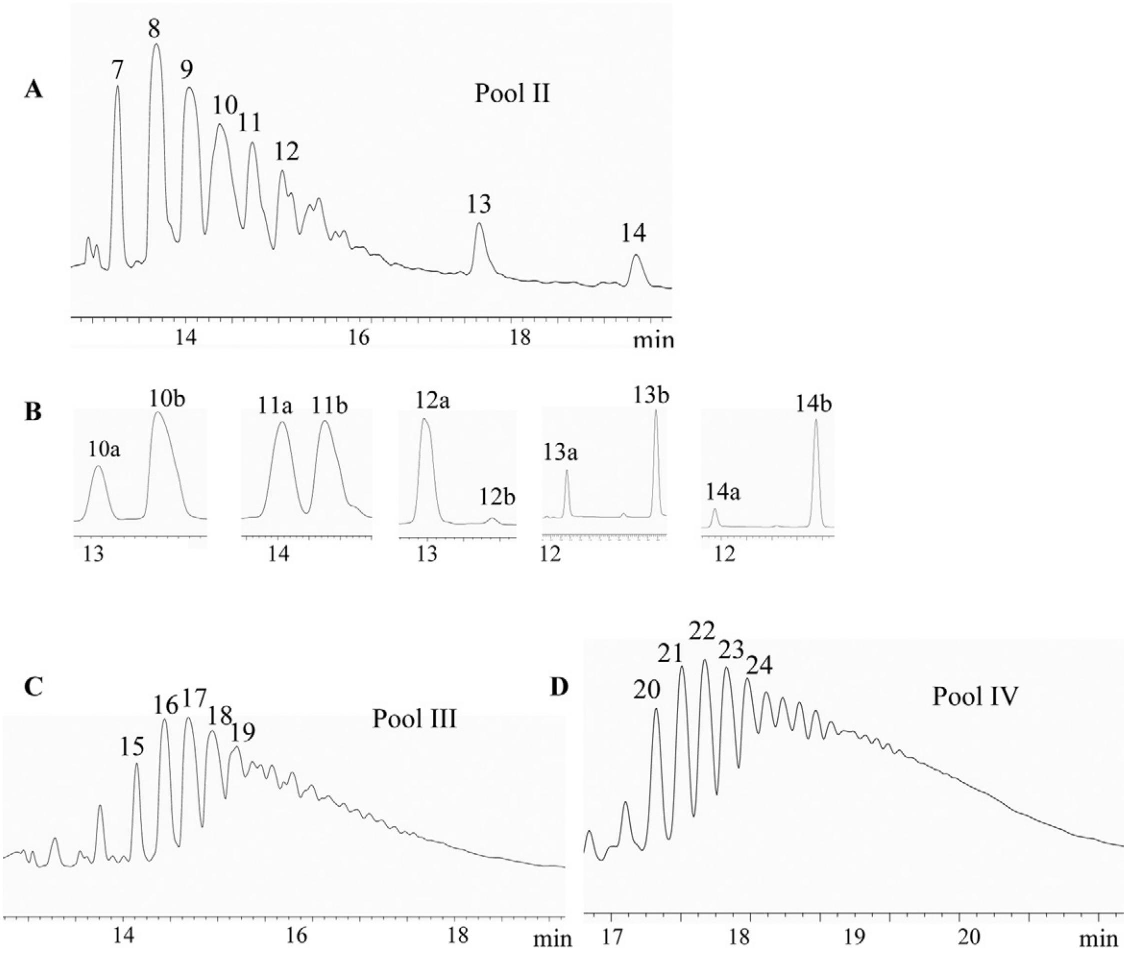


Fig. 3. HPAEC profiles of EPS159 Bio-Gel P-2 Pool II (A), III (C), and IV (D). (B) Subfractionation by HPAEC of fractions PII$_{10}$, PII$_{11}$, PII$_{12}$, PII$_{13}$, and PII$_{14}$.

Table 3
Derived oligosaccharides from partially hydrolyzed EPS159.

Fraction	Sub-fraction	MALDI-TOF-MS [M+Na]$^+$ m/z	MA	NMR	Oligosaccharide structure
PI$_4$		527 (Hex$_3$)		+	G(α1→6)G(α1→6)G
PI$_5$		527 (Hex$_3$)		+	G(α1→3)G(α1→3)G
PII$_6$		n.d.			n.d.
PII$_7$		527 (Hex$_3$)		+	G(α1→6)G(α1→6)G
PII$_8$		689 (Hex$_4$)		+	G(α1→6)[G(α1→6)]$_2$G
PII$_9$		851 (Hex$_5$)	+	+	G(α1→6)[G(α1→6)]$_3$G
PII$_{10}$	PII$_{10}$a	689 (Hex$_4$)	+	+	G(α1→6)G(α1→6)G G(α1→3)$^{/}$
	PII$_{10}$b	1013 (Hex$_6$)	+	+	G(α1→6)[G(α1→6)]$_4$G
PII$_{11}$	PII$_{11}$a	851 (Hex$_5$)	+	+	G(α1→6)[G(α1→6)]$_2$G G(α1→3)$^{/}$
	PII$_{11}$b	1175 (Hex$_7$)		+	G(α1→6)[G(α1→6)]$_5$G
PII$_{12}$	PII$_{12}$a	1013 (Hex$_6$)	+	+	G(α1→6)[G(α1→6)]$_2$G(α1→6)G G(α1→3)$^{/}$
	PII$_{12}$b	n.d.			n.d.
PII$_{13}$	PII$_{13}$a	527 (Hex$_3$)	+	+	G(α1→3)G(α1→6)G
	PII$_{13}$b	689 (Hex$_4$)		+	G(α1→3)[G(α1→3)]$_2$G
PII$_{14}$	PII$_{14}$a	689 (Hex$_4$)			n.d.
	PII$_{14}$b	851 (Hex$_5$)	+	+	G(α1→3)[G(α1→3)]$_3$G
PIII$_{15}$		851 (Hex$_5$)		+	G(α1→6)[G(α1→6)]$_3$G
PIII$_{16}$		1013 (Hex$_6$)	+	+	n.d.
PIII$_{17}$		1175 (Hex$_7$)		+	G(α1→6)[G(α1→6)]$_5$G
PIII$_{18}$		n.d.			n.d.
PIII$_{19}$		n.d.			n.d.
PIV$_{20}$		1499 (Hex$_9$)		+	G(α1→6)[G(α1→6)]$_7$G
PIV$_{21}$		1661 (Hex$_{10}$)		+	G(α1→6)[G(α1→6)]$_8$G
PIV$_{22}$		1823 (Hex$_{11}$)	+	+	G(α1→6)[G(α1→6)]$_9$G
PIV$_{23}$		n.d.			n.d.
PIV$_{24}$		n.d.			n.d.

n.d., not determined, due to too less material or too complex mixture.

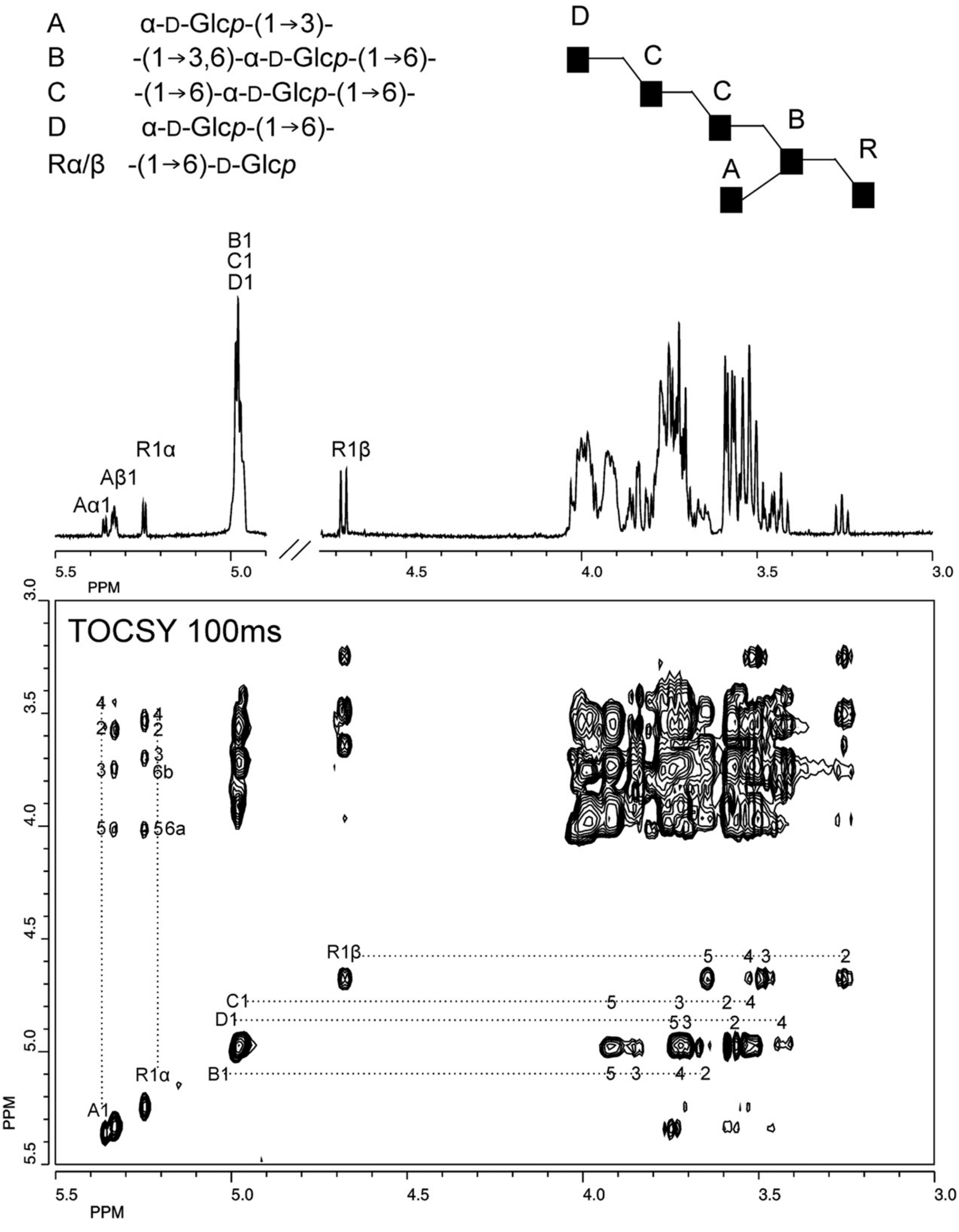


Fig. 4. 1D ^{1}H NMR and 2D ^{1}H–^{1}H TOCSY (100 ms) spectrum of fraction PII$_{12}$a, recorded in D$_2$O at 300 K.

Wiater et al., 1999). In our case, EPS159 is not easily soluble in water and DMSO, only after extensive sonication. However, EPS159 contains 54% of (α1→6) linkages and 18% of (α1→3) linkages. Therefore, the solubility of glucans not only has some connection with the proportion of linkage types but also has to do with the array of these linkages, degree of branching, molecular size, and the overall quaternary structure of the polymer.

Methylation analysis is a primary method to investigate linkage types of polysaccharides. Because EPS159 is difficult to dissolve in DMSO, it increases the complexity of methylation procedure. In our methylation procedure, EPS159 was methylated by adding twice CH_3I (30 min for each time) after extensively sonicating the sample in DMSO for 1 h. Complete methylation was achieved by this treatment based on the equimolar ratio of terminal units to those of branch points. 1D ^{1}H NMR spectroscopy of EPS159 confirmed the methylation result.

Interestingly, the EPS159 composition is similar to that of a water-insoluble glucan produced by *S. mutans* 6715 (Davis, Boyko, et al., 1986; Davis, Hines, et al., 1986). However, we conclude a different structure, containing a (1–6) instead of a (1–3) backbone, similar to EPS from *S. mutans* GS-5 (Aires et al., 2010, 2011; Higton & Weigel, 1980). The chemical compositions and detailed structures of EPS can provide more insight into their *in situ* physical properties and acidogenicity of *S. mutans* as part of the biofilms known as dental plaque and its cariogenicity (Flemming & Wingender, 2010). The degree of branching could contribute to specific physical characteristics of the biofilms.

It has to be noted that dietary combinations of starch and sucrose may be associated with biochemical and structural changes, as well as the total biomass in the biofilms (Duarte et al., 2008). Furthermore, many biological enzymatic interactions occurring within dental plaque including dextranase and mutanase produced by oral microorganisms, and the simultaneous involvement of several proteins play a role in the formation of *S. mutans* biofilm (Hayacibara et al., 2004; Khan, Islam, Khan, & Akram, 2011; Kuramitsu & Wang, 2011). However, knowledge of the detailed structure of exopolysaccharides can contribute to the elucidation of the exact mechanism and interactions that may occur during the formation and maturation of dental plaque, possibly leading to the development of specific inhibitors of glucan formation.

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