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Structural characterization of exopolysaccharides from biofilm of a cariogenic streptococci

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ABSTRACT

Soluble (EPS-SOL), as well as insoluble extracellular polysaccharide (EPS-INSOL), extracted from biofilm of *Streptococcus mutans*, were analyzed by nuclear magnetic resonance spectroscopy, methylation analysis, and a controlled Smith degradation. EPS-SOL was a branched α-glucan containing a (1 → 6)-and (1 → 3)-linkages. EPS-INSOL was a branched α-glucan with similar linkages, but with a (1 → 3)-linked main-chain partially substituted at O-6 with Glc*p*-(1 → 6)-Glc*p*-side chains. Biofilm EPS had a distinct chemical structure compared with those synthesized by plankton cells or by purified enzymes from *S. mutans*, which could indicate different mechanisms for its degradation.

1. Introduction

Streptococcus mutans is the major etiological agent in dental caries, one of the most common, worldwide oral diseases (Bowen, 1999). It synthesizes extracellular polysaccharides (EPS) that are considered to be key contributors to the development of pathogenic biofilms. *S. mutans* produces at least three enzymes for polysaccharide synthesis. These are glucosyltransferase-B, which synthesizes a polymer of mostly insoluble (1 → 3)-linked α-glucan (Aires, Koo, Sassaki, Iacomini, & Cury, 2010), glucosyltransferase-C, which synthesizes a mixture of insoluble (1 → 3)-linked and soluble (1 → 6)-linked α-glucans (Kopec, Vacca-Smith, & Bowen, 1997), and glucosyltransferase-D, which synthesizes a soluble (1 → 6)-linked α-glucan (Aires et al., 2010).

Although dental plaque is the most familiar human biofilm (Marsh, 2003), the chemical structure of EPS has been well established only for those synthesized by plankton cells (Wiater, Choma, & Szczodrak, 1999), or by purified glucosyltransferases from *S. mutans* (Aires et al., 2010; Kopec et al., 1997). However, EPS from biofilm have distinct patterns of formation, when compared to those from plankton. Similarly, purified enzymes do not mimic the different glucosyltransferase interactions present in biofilm, which could modify the final polymer. Thus, characterization of EPSs that are synthesized uniquely in biofilms could be a determinant factor to identify novel therapeutic agents for cariogenic dental plaque.

Considering that a detailed chemical characterization of EPS may have an impact on strategies that effectively disrupt development of pathogenic dental plaque, we now determine the fine chemical structure of these polysaccharides from *S. mutans* biofilm.

2. Material and methods

2.1. Preparation and collection of S. mutans biofilms

Biofilms of *S. mutans* UA159 were formed over 5 days on glass microscope slides (26 mm × 76 mm × 1.2 mm) in cultures at 37 °C under 5% CO_2. They were grown in buffered tryptone yeast-extract broth containing 1% sucrose, the culture medium being replaced daily (Koo et al., 2003). Each resulting biofilm was then gently dip-washed three times in physiological saline to remove loosely adherent material. Each slide was scraped with a sterile spatula to harvest and pass adherent biofilm cells into 25 mL of sterile saline

Abbreviations: EPS, extracellular polysaccharide; EPS-SOL, soluble extracellular polysaccharide; EPS-INSOL, insoluble extracellular polysaccharide.

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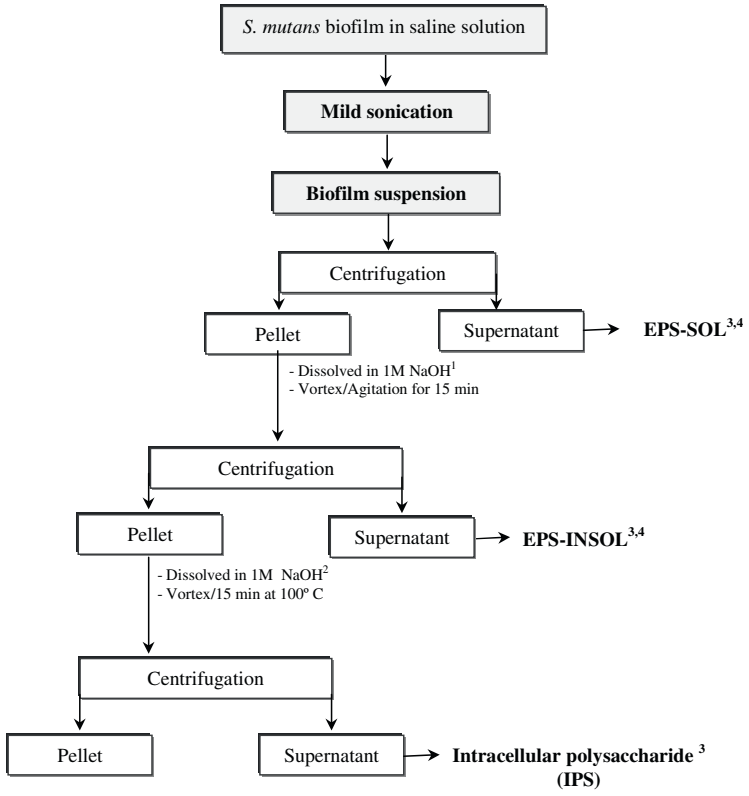

1: Cury *et al.*, 1997

2: Tenuta *et al*., 2006

3: Precipitated with 1:3 v/v EtOH, centrifuged, washed x2 with 70% EtOH, dissolved in 1M aq. NaOH and estimated by phenol-sulphuric acid method (Dubois *et al.*, 1956).

4: Structural characterization

Fig. 1. Isolation of different polysaccharides fractions from *S. mutans* biofilm.

solution. The suspension was centrifuged and the supernatant was discarded in order to eliminate remaining culture medium. The pellet was resuspended in 40 mL of saline solution (biofilm suspension). To estimate biofilm weight, three aliquots from the suspension were collected, centrifuged, and the supernatant was discarded. The pellet was washed with water, centrifuged and the precipitate was dried over P_2O_5 for 24 h and weighed (±0.01 mg). Samples of 1 mL of the suspension were transferred to 1.5 mL sterile microcentrifuge tubes and submitted to polysaccharide and microbiological analysis.

2.2. Polysaccharide and microbiological analysis

Mild sonication can successfully extract soluble EPS that interferes with RNA extraction from *in vitro* biofilms (Cury & Koo, 2007). A Digital Sonifier® Unit, model S-150D (Branson Ultrasonics Corporation, Danbury, CT, USA) was used in all experiments. Different parameters were tested for the sonication of the *S. mutans* UA159 biofilm suspension. These were: (1) power of sonication (4, 7 and 12 W for 10, 30 and 60 s), (2) time of sonication (7 W for 5, 10, 20, 30, 40, and 80 s); (3) number of sonication pulses (1, 2, 4 or 8 pulses), (4) sonication with or without saline replacement after pulses, and (5) volume of biofilm suspension (1, 2, 4, and 8 mL). After sonication, aliquots of 100 and 300 μL from each biofilm suspension were collected for viable bacteria biomass determination and EPS extraction, respectively. A vortexed-only sample was used as a control.

2.3. Polysaccharide extraction

Aliquots of 300 μL (biofilm suspension), of either vortexed or sonicated samples, were centrifuged at 10,000 × *g* for 5 min at 4 °C. The supernatant containing extracted, soluble EPS was collected and transferred to another tube (EPS-SOL, Fig. 1) to which 3 volumes EtOH were added. To the pellet, 400 μL of 1 M NaOH were added for insoluble EPS extraction (Cury, Rebello, & Del Bel Cury, 1997). The tube was vortexed, agitated for 15 min, centrifuged, and the supernatant was transferred to another tube (EPS-INSOL, Fig. 1) to which 3 volumes of EtOH were added. To the microcentrifuge tube containing the residual pellet, 400 μL of 1 M NaOH were added for intracellular polysaccharide (IPS, Fig. 1) extraction (Tenuta, Ricomini Filho, Del Bel Cury, & Cury, 2006). This tube was vortexed, heated for 15 min at 100 °C, centrifuged, and the supernatant was transferred to another tube named IPS to which 3 volumes of EtOH were added.

The tubes containing EtOH plus EPS-SOL, EPS-INSOL, and IPS were maintained for 30 min at −20 °C, centrifuged and the pellets were washed twice with 70% EtOH. The polysaccharides precipitated were resuspended in 250 μL of 1 M NaOH and total carbohydrate was estimated by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), using glucose as standard. The results were normalized by dry weight of biofilm.

2.4. Viable bacteria biomass determination

Aliquots of 100 μL (biofilm suspension) of either vortexed or sonicated samples was diluted in 0.9% NaCl and serial decimal dilu-

Table 1
Viable bacterial biomass, concentration of different polysaccharides after treatment of biofilm with different sonication power (mean ± standard deviation, $n = 3$).

	Conditions	Bacteria (CFU × 106 mg^{-1} dry weight)	EPS-SOL (μg mg^{-1} dry weight)	EPS-INSOL (μg mg^{-1} dry weight)	IPS (μg mg^{-1} dry weight)
	Control vortexedonly	7.9 ± 2.1	2.6 ± 0.1 (1%)[a]	348,1 ± 51.4 (99%)[a]	30.7 ± 3.8
4 W ($n = 3$)	1 pulse: 10 s	34.0 ± 4.1	7,2 ± 0.7 (2%)[a]	314.9 ± 22.3 (98%)[a]	28.0 ± 3.2
	1 pulse: 30 s	52.7 ± 17.9	14.5 ± ±0.9 (4%)[a]	355.5 ± 45.0 (96%)[a]	31.9 ± 2.8
	1 pulse: 60 s	34.1 ± 27.6	18.5 ± 0.7 (6%)	284.3 ± 29.6 (94%)	26.5 ± 3.2
7 W ($n = 3$)	1 pulse: 10 s	51.8 ± 16.8	13.4 ± 1.0 (4%)[a]	338.5 ± 46.4 (96%)[a]	21.3 ± 3.9
	1 pulse: 30 s	75.1 ± 32.3	24.5 ± 5.7 (7%)[a]	348.0 ± 78.6 (93%)[a]	25.8 ± 3.9
	1 pulse: 60 s	96.1 ± 20.9	44.0 ± ±4.6 (11%)[a]	345.4 ± 41.2 (89%)[a]	33.5 ± 12.1
12 W ($n = 2$)	1 pulse: 10 s	77.6 ± 5.2	19.8 ± 3.7(6%)[a]	333.1 ± 17.6 (94%)[a]	34.0 ± 1.0
	1 pulse: 30 s	110.8 ± 26.1	28.0 ± 1.4 (9%)[a]	280.6 ± 12.8 (91%)[a]	31.4 ± 3.5
	1 pulse: 60 s	0.3 ± 0.1	31.1 ± 3.0 (16%)[a]	161.0 ± 45.8 (84%)[a]	18.9 ± 4.2

[a] Relative % of polysaccharide from biofilms, according to treatment.

tions were inoculated in duplicate by the drop-counting technique (Tenuta et al., 2006) in brain heart infusion agar. The plates were incubated under 10% CO_2 at 37 °C for 48 h. The colony-forming units (CFU) were counted and the results expressed in CFU mg^{-1} dental biofilm dry weight. In all experiments, the contamination of extracellular with intracellular components was evaluated by intracellular polysaccharide (IPS) determination and cell lysis was shown by decrease in viable counts.

2.5. Preparation and collection of S. mutans biofilms for chemical characterization

Considering that one pulse of 7 W 60 s^{-1} in 1 mL of saline solution was capable of preserving bacterial viability and recovery in a greater yield of EPS-SOL from biofilm (see results), we chose this sonication parameter to extract polysaccharides for chemical characterization. Fragmentation of EPS after this sonication parameter was checked by high performance steric exclusion chromatography coupled to a multiangle laser light scattering (HPSEC-MALLS). 10 mg of bovine glycogen were resuspended in 1 mL of 0.9% NaCl and submitted to vortex or sonication (7 W 60 s^{-1}). Results revealed that no fragmentation occurred with the sonicated sample (data not shown).

Thus, biofilms of *S. mutans* UA159 were formed for 5 days on glass microscope slides and polysaccharide extraction was performed as described above (Fig. 1). After washed twice with 70% cold ethanol, soluble and insoluble EPS were dried at room temperature and kept in tubes for structural analysis. In order to acquire more sample quantities, the sonication protocol used was 7 W, 8 pulses-60 s in 20 mL of saline solution since that the recovery of soluble EPS is equivalent to parameter 7 W, 1 pulse-60 s in 1 mL of saline solution (data not shown).

2.6. Monosaccharide composition

Monosaccharide composition was performed according to Sassaki et al. (2008). Each polysaccharide (1 mg) was hydrolyzed with 2 M trifluoroacetic acid (0.2 mL) at 100 °C for 8 h, followed by evaporation to dryness. The residue was then dissolved in 0.5 M NH_4OH (100 μL) and held at room temperature for 10 min in reinforced 4 mL Pyrex tubes with Teflon-lined screw cap vessels. $NaBH_4$ (1 mg) was added and the solution was maintained at 100 °C for 10 min, in order to reduce aldoses to alditols. The product was dried and excess $NaBH_4$ was eliminated by addition of HOAc (10 μL), and boric acid, as trimethyl borate, was removed by the addition of methanol (×2) followed by evaporation to remove volatile trimethyl borate. Acetylation was performed in pyridine–Ac_2O (200 μL; 1:1, v/v), heated for 30 min at 100 °C. After addition of ice-water, the resulting alditol acetates were extracted with $CHCl_3$ and analyzed by gas chromatography/mass spectrometry (GC–MS; Varian Saturn-3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225 capillary column (30 m × 0.25 mm i.d) programmed from 50 to 220 °C at 40 °C min^{-1}, with He as carrier gas. Products were identified by their typical retention times and electron ionization spectra.

2.7. Methylation analysis

Methylation analysis was performed according to Aires et al. (2010). Resulting samples containing partially *O*-methylated alditol acetates were then analyzed by GC–MS, using the same conditions described for alditol acetates, except that the final temperature was 215 °C. Components were identified by their typical retention times and electron impact ionization spectra (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.8. Nuclear magnetic resonance analysis

EPS-SOL (55 mg) or EPS-INSOL (69 mg) were dissolved respectively in 600 μL of D_2O and 600 μL of Me_2SO-d_6. Nuclear magnetic resonance (NMR) spectra (^1H, ^{13}C and DEPT-135 experiments) were obtained using a 400 MHz Bruker Avance III spectrometer in 5 mm inverse probe head at 70 °C. Chemical shifts are expressed in δ relative to acetone at δ 32.77 (^{13}C) 2.21 (^1H), based on DSS (sodium 2,2-dimethyl-2-silapentane-3,3,4,4,5,5-d_6-5-sulfonate) at $\delta = 0.00$ for ^{13}C and ^1H in accordance with IUPAC recommendations.

Table 2
Viable bacterial biomass, concentration different polysaccharides after treatment of biofilm with different sonication times (mean ± standard deviation, $n = 3$).

Conditions	Bacteria (CFU × 106 mg^{-1} dry weight)	EPS-SOL (μg mg^{-1} dry weight)	EPS-INSOL (μg mg^{-1} dry weight)	IPS (μ mg^{-1} dry weight)
Control vortexed-only	6.4 ± 1.5	1.4 ± 0.0 (1%)[a]	255.5 ± 63.4 (99%)[a]	23.6 ± 5.8
7 W, 1 pulse 5 s	46.7 ± 12.7	7.1 ± 0.6 (2%)[a]	340.2 ± 20.5 (98%)[a]	32.5 ± 4.3
7 W, 1 pulse 10 s	42.8 ± 7.3	9.1 ± 0.4 (3%)[a]	334.2 ± 60.8 (97%)[a]	31.7 ± 5.6
7 W, 1 pulse 20 s	46.7 ± 7.7	12.4 ± 1.1 (4%)[a]	290.7 ± 22.1 (96%)[a]	28.9 ± 2.2
7 W, 1 pulse 30 s	45.7 ± 13.8	18.7 ± 1.1 (6%)[a]	312.9 ± 38.6 (94%)[a]	33.8 ± 1.8
7 W, 1 pulse 40 s	62.3 ± 16.1	19.2 ± 2.5 (6%)[a]	315.8 ± 22.3 (94%)[a]	31.7 ± 1.8
7 W, 1 pulse 80 s	0.2 ± 0.0	34.6 ± 8.3 (12%)[a]	242.7 ± 37.4 (88%)[a]	28.8 ± 3.9

[a] Relative % of polysaccharide from biofilms according to treatments.

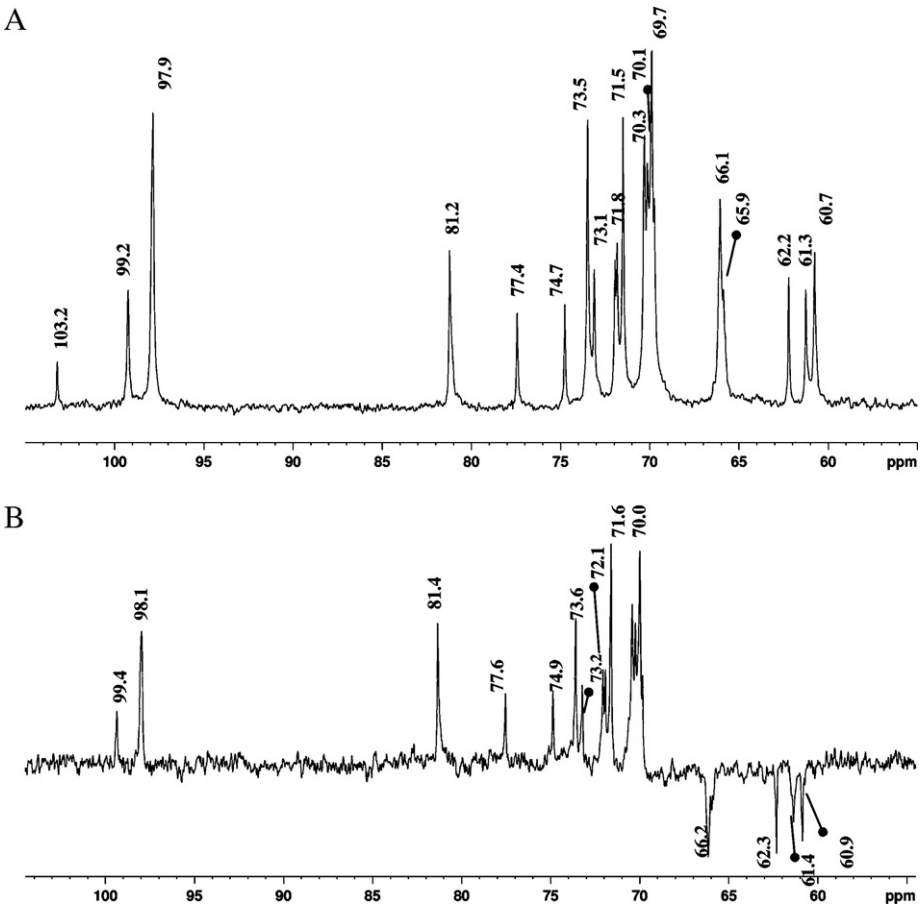

Fig. 2. ^{13}C NMR (A) and DEPT (B) spectra in D_2O of EPS-SOL (chemical shifts are expressed as δ ppm).

2.9. Controlled Smith degradation

EPS-INSOL (70 mg) were submitted to oxidation in 0.1 M $NaIO_4$ (15 mL) for 72 h at 25 °C in the dark. The solution was then dialyzed against tap H_2O for 48 h and treated with $NaBH_4$ for 20 h (Goldstein, Hay, Lewis, & Smith, 1965). The solution was dialyzed again, and freeze-dried. The products were then submitted to partial acid hydrolysis (2 M TFA, pH 2.0, 30 min, 100 °C) (Gorin, Horitsu, & Spencer, 1965), and dialyzed against tap H_2O using membranes with a size exclusion of 2 kDa and retained material was freeze-dried. Preliminary studies showed that EPS-SOL did not provide a residual polymer, indicating that its main chain is not composed of (1 → 3)-linked Glc*p* units (data not shown).

3. Results

3.1. Polysaccharide and microbiological analysis

In relation to sonication power parameters, all the experimental groups had higher viable bacteria counts compared with the control group, except for 12 W 60 s^{-1}, which resulted in loss of bacterial viability. The resulting concentration of EPS-SOL increased with increase of the power and time of sonication. IPS concentration showed similar values for all groups except for the above parameter. The yield of EPS-SOL was greater at 7 W when compared to 4 W and similar to that at 12 W (Table 1).

All experimental groups had a higher viable bacteria biomass compared to a control group, except for the parameter 7 W 80 s^{-1} (Table 2), which resulted in loss of bacterial viability. EPS-SOL concentration increased with increase of sonication time. IPS concentration showed similar values for all groups tested (Table 2).

In relation to number of sonication pulses, all the experimental groups had a greater viable bacterial biomass compared to a control group (data no show). EPS-SOL concentration increased with increase of the number of sonication pulses and IPS concentration was similar for all groups tested (data not shown).

Replacement of saline solution between each sonication pulse did not result in different yield of EPS-SOL. Furthermore, the proportion of EPS-SOL decreased with increase of the volume of biofilm suspension used for sonication.

3.2. Monosaccharide composition

Polysaccharide synthesized by bacteria, and extracted as a biofilm by sonication, was analyzed, the only component of EPS-SOL and EPS-INSOL being glucose units.

3.3. Methylation and nuclear magnetic resonance analysis

Methylation analysis of EPS-SOL provided partially *O*-methylated alditol acetates of 2,3,4,6-Me_4-Glc (21%), 2,4,6-Me_3-Glc (14%), 2,3,4-Me_3-Glc (43%), and 2,4-Me_2-Glc (22%), indicating a branched glucan (Table 3).

The ^{13}C NMR spectrum of EPS-SOL (Fig. 2A) contained C-1 signals at δ 99.2 of non-reducing end and 3-*O*-substituted α-Glc*p* units, as well as these C-1 signal at δ 97.9, corresponding to 6-*O*- and 3,6-di-*O*-substituted α-Glc*p* units.

The glycosidic linkages of this glucan were shown by the presence of 3-*O*-substituted signals at δ 81.2, and *O*-substituted CH_2-6

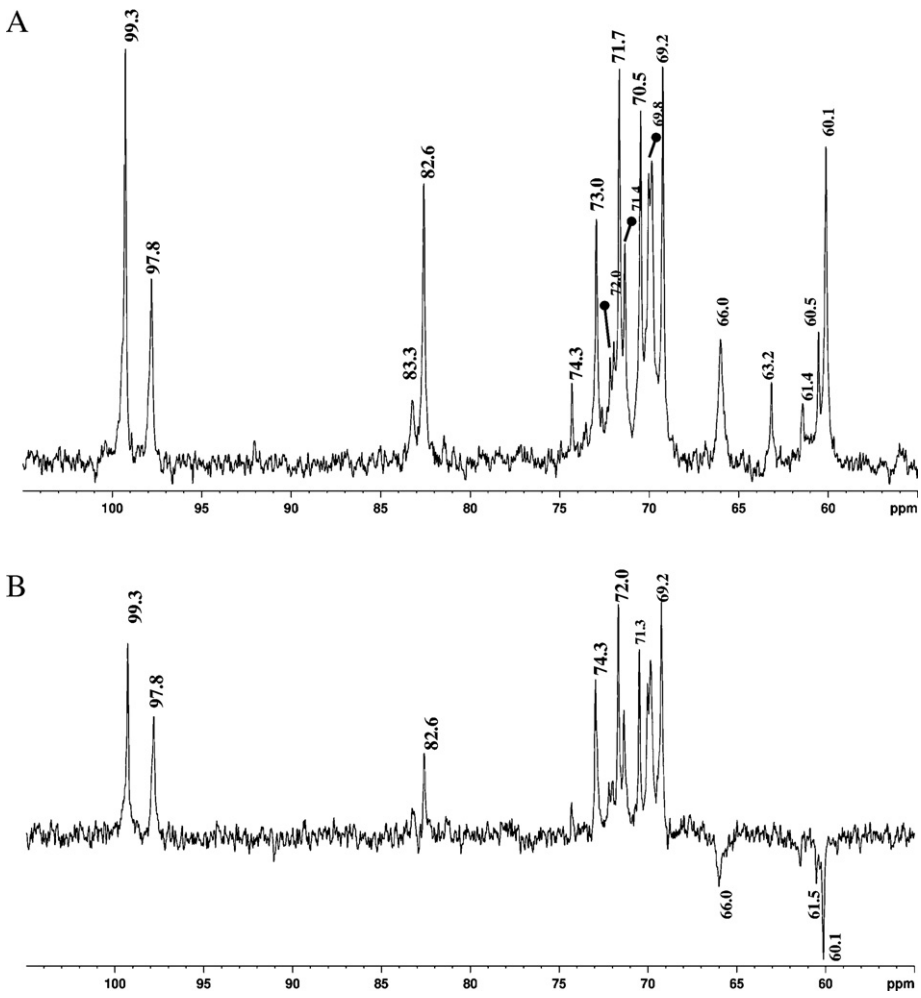

Fig. 3. ^{13}C NMR (A) and DEPT (B) spectra of EPS-INSOL in DMSO-d_6 (chemical shifts are expressed as δ ppm).

signals at δ 66.1 and 65.9 (Fig. 2A), confirmed from inverted DEPT signals (Fig. 2B).

Methylation analysis of EPS-INSOL (Table 3) provided partially *O*-methylalditol acetates of 2,3,4,6-Me$_4$-Glc (19%), 2,4,6-Me$_3$-Glc (31%), 2,3,4-Me$_3$-Glc (34%) and 2,4-Me$_2$-Glc (16%), indicating the presence of a (1 → 3) and (1 → 6) branched glucan. The ^{13}C NMR spectrum of EPS-INSOL (Fig. 3A) contained signals in the anomeric region at δ 99.3 corresponding to non-reducing end units and 3-*O*-substituted, while those at δ 97.8 are from 6-*O*-substituted (Fig. 3B) and 3,6-di-*O*-substituted residues.

3.4. Controlled Smith degradation

On controlled Smith degradation, EPS-INSOL showed only six signals at δ 99.7, 71.0, 83.0, 69.8, 72.1 and 60.7, arising respectively, from C-1 to C-6 in ^{13}C NMR spectrum (Fig. 4) (Gorin, 1981).

Table 3
Partially *O*-methylalditol acetates and linkage types shown by methylation analysis of soluble (EPS-SOL) and insoluble EPS (EPS-INSOL) from biofilm of *S. mutans*.

Partially *O*-methylated alditol acetate[a]	Linkage type[b]	% Peak area	
		EPS-SOL	EPS-INSOL
2,3,4,6-Me$_4$-Glc	Glcp-(1→	21	19
2,4,6-Me$_3$-Glc	→3)-Glc*p*-(1→	14	31
2,3,4-Me$_3$-Glc	→6)-Glcp-(1→	43	34
2,4-Me$_2$-Glc	→3,6)-Glcp-(1→	22	16

[a] Analyzed by GC–MS, after per-*O*-methylation, total acid hydrolysis, reduction, and acetylation.
[b] Based on derived *O*-methylalditol acetates.

4. Discussion

Extracellular polysaccharides play a major role in the pathogenesis of dental caries, by promoting bacterial adherence and accumulation on tooth surfaces, causing biochemical and structural changes in the matrix of the biofilms (Paes Leme, Koo, Bellato, Bedi, & Cury, 2006). Thus, from a global perspective, the structure of polysaccharide may contribute to an explanation of the virulence of cariogenic biofilms, which could help to elaborate strategies for its degradation.

Although a monospecies biofilm model does not mimic the complex microbial community found in dental plaque, we now register for the first time a detailed characterization of EPS from cariogenic biofilm. EPS from biofilm involves a concerted action of all glucosyltransferases, and this could be verified using the present model.

The structure of EPS-SOL from biofilm was composed of (1 → 6) and (1 → 3)-linked α-glucan, containing (1 → 6)-linkages in its main chain. In contrast, EPS-INSOL contained a (1 → 3)-linked main-chain partially substituted at O-6 by Glc*p*-(1 → 6)-Glc*p* side-chains. Wiater et al. (1999) also observed the presence of α-(1 → 6) bound monomers as part of the predominantly α-(1 → 3) linked glucan produced by *S. mutans* and *S. sobrinus* in a plankton cell culture. We can thus suppose that the soluble portion may be trapped in a matrix of insoluble glucans, which was homogenized

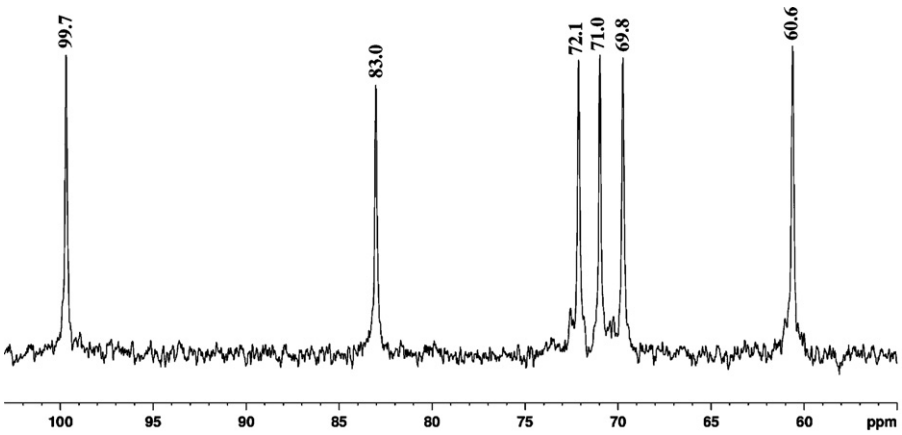

Fig. 4. ^{13}C NMR spectrum of (1 → 3)-linked α-glucan, derived by controlled Smith degradation of EPS-INSOL, in Me_2SO-d_6 at 70 °C (chemical shifts are expressed as δ ppm).

by the mild sonication. Another hypothesis, as the soluble portion might act as acceptor for synthesis of insoluble polysaccharide could explain the presence of α-(1 → 6) portions in insoluble EPS should be investigated in future works.

Present study showed that are differences between EPSs produced by purified enzymes and those synthesized from bacteria in biofilm. According to methylation data, soluble α-glucan from purified enzymes contained 60% of (1 → 6)-linkages while soluble EPS from biofilm presented 43%. For the insoluble one, purified enzymes synthesized an EPS composed of 57% of (1 → 3)-linkages (Aires et al., 2010) while insoluble EPS from biofilm showed 31% of this linkage. These could be due to modification that one glucosyltransferase promote in the product of another (Russel, 2009). In addition, the antagonistic action of both synthesizing enzyme and degrading enzyme could influence the nature of the final EPS formed (Hayacibara et al., 2004). In this view, strategy to degrade EPS from biofilm should be distinct to those applied for EPS from planktonic cells or purified enzymes.

Polysaccharides cannot be characterized in presence of clumps. Thus, a method to disperse the aggregate formed by biofilm formation was performed by mild sonication and feasible effects were observed. This method promotes dispersion of the microorganisms, preserves bacterial viability, extract soluble EPS from biofilm and preserve polysaccharide integrality.

Considering that this polymer is an important virulence factor of microorganisms and that up to 40% of the dry weight of dental biofilm is composed of polysaccharides (Paes Leme et al., 2006), strategies to degrade EPS from dental plaque seems be important for caries prevention. However, considering that EPS from biofilm differ to those synthesized by planktonic cells or purified enzymes from *S. mutans*, distinct strategies for its degradation should be elaborated. This is crucial for development of future therapeutics agents.

Disclosure statement

The authors of the present study declare that there are no conflicts of interest in relation to this work.

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