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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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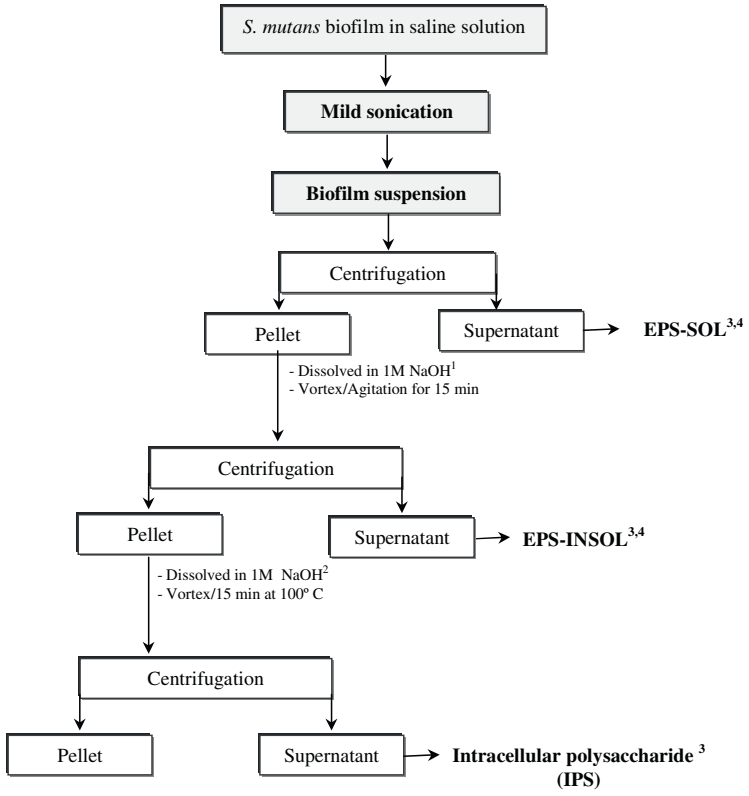


**1: Cury *et al.*, 1997**

**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-