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Purification and structural characterisation of (1→3;1→6)-β-D-glucans (botryosphaerans) from *Botryosphaeria rhodina* grown on sucrose and fructose as carbon sources: a comparative study

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Abstract

Two botryosphaerans, exopolysaccharides (EPS) secreted by the ascomyceteous fungus *Botryosphaeria rhodina*, when grown on sucrose and fructose as sole carbon sources, were structurally compared after their isolation from the culture medium. Both EPS were submitted to trypsin digestion, and eluted as a single peak on gel filtration. Total acid hydrolysis yielded only glucose, and data from methylation analysis and Smith degradation indicated that both EPS constituted a main chain of glucopyranosyl β(1→3) linkages substituted at O-6. The products obtained after partial acid hydrolysis demonstrated side chains consisting of glucosyl- and gentiobiosyl- linked β(1→6) residues. ^{13}C-NMR spectroscopy studies showed that all glucosidic linkages were of the β-configuration. The carbon source affected the side chain structures of botryosphaeran but not the main chain makeup. Sucrose produced less branching (21%) than fructose (31%).

Keywords: *Botryosphaeria rhodina*; Exopolysaccharides; β(1→3;1→6)-D-Glucans; Carbon source; Structural variation

1. Introduction

Industrial applications of polysaccharides have relied mainly upon raw materials from plants and marine algae until recently (Tharanathan, 1995). Nowadays, fungal polysaccharides are receiving increased attention due to their peculiar physicochemical and rheological properties, concomitant with novel functionality (Lowman, Ferguson, & Williams, 2003; Ricciardi et al., 2002; Sandula, Kogan, Kacuráková, & Machová, 1999; Selbmann, Onofri, Fenice, Federici, & Petruccioli, 2002). Consequently, microbial polysaccharides as biomaterials have found a wide range of new applications including use in pharmaceutical therapy due to their unique physiological activities; as antitumor, antiviral and anti-inflammatory agents (Ishibashi et al., 2004; Kumar, Joo, Choi, Koo, & Chang, 2004; Li et al., 2004; Tsiapali et al., 2001). Many of the biological activities of exopolysaccharides (EPS) such as the β-glucans are attributable to the (1→3) type of linkage (Alquini, Carbonero, Rosado, Consentino, & Iacomini, 2004; Bao, Zhen, Ruan, & Fang, 2002; Engstad, Engstad, Olsen, & Osterud, 2002; Falch, Espevik, Ryan, & Stokke, 2000; Kulicke, Lettau, & Thielking, 1997; Willment, Gordon, & Brown, 2001). They are usually structurally complex homopolymers comprised of glucose that are generally isolated from yeasts and fungi. Different physicochemical parameters of β-glucans, such as solubility, primary structure, molecular weight, extent of

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branching by side-chain substituents (Bohn & BeMiller, 1995), and the charge on the polymer, all appear to influence their biological activity (Vetvicka & Yvin, 2004). Schizophyllan, one of the β(1→3)-D-glucans known to have immunomodulating potential and antitumor activity, has been used clinically as an immunopotentiator against some types of cancer, chiefly leukocytopenia (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Kidd, 2000; Kubala et al., 2003).

Although many fungal β(1→3)-glucans have been described, only a few have been rigorously characterised. Consequently, their chemical diversity and potentially useful functional properties are still poorly understood (Schmid et al., 2001). Since these EPS occur in Nature as mixtures of heterogeneous cellular components or secretions, it is first necessary to isolate, purify and structurally characterise these polysaccharides, before proceeding with detailed investigations of their properties and applications (Kim et al., 2003; Sutherland, 1998).

The ascomyceteous fungus *Botryosphaeria* sp. (isolate MAMB-05) has been studied as a laccase producer since 1995 (Barbosa, Dekker, & Hardy, 1996; Barbosa, Dekker, Kurtböke, & Hardy, 1995; Dekker, Vasconcelos, Barbosa, Giese, & Paccola-Meirelles, 2001), and was only recently classified to the species level as: *Botryosphaeria rhodina* (Garcia, Vilas-Boas, Dekker, Fungaro, & Barbosa, 2004) using molecular biology techniques. However, when this ligninolytic fungus was grown on glucose as sole carbon source, an EPS of the β-glucan type was secreted in the culture medium (Dekker & Barbosa, 2001). The structure of this glucan was characterised (Barbosa, Steluti, Dekker, Cardoso, & Corradi da Silva, 2003) recently as a (1→3;1→6)-β-D-glucan with approximately 22% side branching at C-6. The branches consisted of single (1→6)-β-linked glucosyl, and (1→6)-β-linked di-glucosyl (gentiobiose) residues. This β-glucan type was subsequently named botryosphaeran in accordance with the fungus producing this EPS type.

A comparison of botryosphaeran production by *B. rhodina* on several carbohydrate carbon sources has recently been described (Steluti et al., 2004). With the exception of mannitol, the fungus produced EPS on all of the carbon sources examined, with highest yields occurring with sucrose followed by glucose and fructose. According to Jin et al. (2003) and Zhang, Yang, Ding, and Chen (1995), different carbon sources generate similar bioactive polymers with different degrees of branching and distinct polymerisation, producing biopolymers that are more or less water-soluble, and as a consequence, may possess higher or lower biological activity.

The work reported herein discusses the purification and structural characterisation of the botryosphaerans produced by *B. rhodina* when the fungus was grown on fructose (EPS_{FRU}) and sucrose (EPS_{SUC}). ^{13}C-NMR spectroscopy studies showed that all glucosidic linkages were of the β-configuration and that the carbon source affected only the side chain structures of the botryosphaerans but not the main chain constitution.

2. Materials and methods

2.1. Microorganism, culture media and growth conditions

Botryosphaeria rhodina was maintained at 4 °C on potato-dextrose-agar (Barbosa et al., 1996). Inoculum was prepared by growing *B. rhodina* on minimum salts medium (Vogel, 1956) containing agar (20 g/L) and glucose (10 g/L). After 5 days growth (28 °C), mycelial fragments were transferred to four 125 mL Erlenmeyer flasks containing 25 mL of minimum salts medium and glucose (0.5 g/L), and incubated at 28 °C for 48 h on a rotary shaker (180 rpm). The mycelia were homogenised for 30 s at maximum speed. Then, the cell homogenate was centrifuged for 10 min at 1250×*g*, the mycelium recovered, and diluted with sterilised physiological saline solution to an absorbance between 0.4 and 0.5 at 400 nm. Four millilitre-aliquots of the suspension were used to inoculate Erlenmeyer flasks (1 L) containing 200 mL of minimum salts medium and sole carbon sources (sucrose and fructose, 5 g/L). Cultures were grown in submerged cultivation (180 rpm) for 72 h at 28 °C.

2.2. Preparation and dissolution of exopolysaccharides

Cell-free extracellular fluid was obtained after removal of the mycelium by centrifugation (5500×*g*/10 min) at 4 °C. The supernatant was treated with 3 volumes of absolute ethanol, the precipitate recovered and dissolved in distilled water, followed by extensive dialysis against frequent changes of distilled water, and then freeze-dried. This preparation was used to determine the carbohydrate and protein content.

2.3. Analytical techniques

Total sugars were determined by the phenol–sulphuric acid method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956) and reducing sugars were quantified by the cupro-arsenate method of Somogyi–Nelson (Nelson, 1944). Glucose was used as the standard in both assay procedures. Protein was measured by the Bradford method (1976) using bovine serum albumin as standard, and spectrophotometrically at 280 nm.

2.4. Purification of botryosphaerans

A sample of each botryosphaeran (100 mg; EPS_{SUC} and EPS_{FRU}) was dissolved in 0.1 M sodium phosphate buffer (150 mL, pH 7.6) followed by the addition of 5 mg of trypsin (Sigma) enzyme (Krcmar, Novotny, Maraias, & Joseleau, 1999) and the solutions incubated at 37 °C for

48 h. After the digestion step, the samples were extensively dialysed against frequent changes of distilled water and freeze-dried. The procedure was repeated twice, and protein, total and reducing sugars were determined on these samples to assess homogeneity. The EPS preparations were then dissolved in water (1 g/L) and purified by gel permeation chromatography on a column of Sepharose CL-4B (43.0 cm × 1.0 cm) and eluted with water (0.3 mL/min). Fractions (2.6 mL) were collected and analysed for carbohydrate and monitored at 280 nm for protein. Fractions corresponding to peaks were pooled and freeze-dried.

2.5. Monosaccharide analysis

Polysaccharide samples (0.050 mg) were hydrolysed in 0.5 mL of 5 M trifluoroacetic acid (TFA) at 100 °C for 16 h (Barbosa et al., 2003). After hydrolysis, the solutions were evaporated under vacuum, and the residue dissolved in 0.5 mL of water and evaporated again. The dissolution–evaporation cycle was repeated twice. Finally, the residue was dissolved in 0.2 mL of water, and 0.025 mL aliquots used for High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection (HPAEC/PAD); Dionex Chromatograph DX 500 (Fan, Namiki, Matsuoka, & Lee, 1994; Weitzhandler et al., 1993). Neutral monosaccharides were separated isocratically (0.014 M NaOH) using a CarboPac PA-10 (Dionex Chromatography) column (4 × 250 mm) equipped with a PA-10 guard column at a flow rate of 1.0 mL/min (Elífio, Corradi da Silva, Iacomini, & Gorin, 2000; Weitzhandler et al., 1993). Elution was performed using water (eluent 1) and 0.2 M NaOH (eluent 2). The column was regenerated after 20 min using 100% of eluent 2 for 15 min, followed by a return to 0.014 M NaOH. Monosaccharide quantification was carried out from peak area measurements using response factors obtained from standard monosaccharides.

2.6. Methylation–acid hydrolysis–acetylation

Fractions of each botryosphaeran (10 mg) were solubilised in dimethyl sulfoxide (1 mL), methylsulfinylcarbanion (0.5 mL) was added, and the mixture sonicated for 50 min at ambient temperature followed by the gradual addition of methyl iodide (0.3 mL). The methylated polysaccharides were extracted with chloroform (4 mL), and the chloroform phase evaporated until completely dry (Barbosa et al., 2003). Second and third methylation runs were performed as above. The permethylated polysaccharides were solubilised in 72% (v/v) H_2SO_4 (0.5 mL) in an ice bath for 2 h, and then water was added (4 mL) to obtain a final concentration of 1.4 M and kept under reflux at 100 °C for 18 h (Bouveng & Lindberg, 1965; Corradi da Silva, Iacomini, Jablonski, & Gorin, 1993). The resulting partially methylated sugars were reduced with $NaBH_4$, acetylated, and analysed by gas liquid chromatography (GLC) on a Varian Model 3300 Gas Chromatograph. A conventional column DB-225 (medium-polar column—silicon polymer containing methyl, phenyl and nitrite groups) was used. The injection temperature was 50 °C with a program to 220 °C (constant temperature) (Woranovicz, Pinto, Gorin, & Iacomini, 1999).

2.7. Smith degradation

Samples (40 mg) of each botryosphaeran were dissolved in 1 M NaOH (7 mL) and 0.5 M HCl added to a final pH of 4.6. Each sample was then oxidised with aqueous 0.05 M $NaIO_4$ (50 mL) for 96 h at 4 °C in the dark (Fabre, Bruneteau, Ricci, & Michel, 1984). The oxidised polysaccharides were next reduced with 1.0 M $NaBH_4$, and a portion subjected to total acid hydrolysis (1.0 M TFA, 100 °C, 6 h). Another portion was subjected to mild acid hydrolysis (1.0 M H_2SO_4, 24 h, 50 °C) to remove the oxidised sugar residues attached to the polysaccharide chain (Smith degradation), followed by neutralisation, dialysis against water and freeze-drying. A sample of each oxidised botryosphaeran product (5 mg) was methylated twice as described above, and another portion (6.0 mg) was subjected to ^{13}C-NMR spectroscopy.

2.8. ^{13}C-NMR spectroscopy

^{13}C-NMR spectra were obtained on a Bruker DRX-400 NMR Spectrometer with each botryosphaeran (10–12 mg) sample in dimethyl sulfoxide at 400 MHz (30 °C). Chemical shifts were referred to tetramethylsilane (Gorin, 1981). The high viscosity of the EPS presented problems affecting the quality of the NMR spectra.

2.9. Partial acid hydrolysis

Partial acid hydrolysis of each botryosphaeran (30 mg) was performed as described by Ukai, Yokoyama, Hara, and Kiho (1982) using 50% (v/v) H_2SO_4 for 16 h at 4 °C, and then stirred for 1 h at 35 °C. The hydrolysed material was neutralised with barium carbonate, de-ionised with Amberlite IR-400 (carbonate) resin, and the products separated on a column of Sephadex G-15 (103 cm × 0.8 cm) calibrated with starch, melibiose and glucose. Water was used as eluent (0.18 mL/min) and fractions collected were analysed for carbohydrate by the phenol–sulphuric acid method. Fractions containing the modified poly-, oligo- and monosaccharides were analysed by HPAEC/PAD (Lee & Rice, 1993; Rice & Corradi da Silva, 1996). Mono- and oligosaccharides were separated on a CarboPac PA-100 column (4 × 250 mm) and guard column (4 × 50 mm) at a flow rate of 1 mL/min. The column was equilibrated in 0.1 M NaOH (97%) and 0.5 M sodium acetate (3%). After 15 min, a 0–0.25 M sodium acetate gradient was applied over a 60 min interval, while the concentration of NaOH remained at 0.1 M. Monosaccharides and oligosaccharides were detected by Pulsed Amperometric Detection (Dionex DX-500)

without the addition of post-column alkali. Monosaccharides and oligosaccharides in the experimental samples were identified by comparison to known retention times (min). The retention times (T_R) of the standard sugars were: glucose (2.86 min), gentiobiose (4.58 min), laminaribiose (7.50 min), gentiotriose (11.55 min) and laminaritriose (15.01 min).

3. Results and discussion

Quantification of total sugars (an indication of EPS content), reducing sugars and protein in the botryosphaeran samples, EPS_{FRU} and EPS_{SUC}, revealed a high content of carbohydrate in relation to protein. The carbohydrate/protein ratios for EPS_{FRU} and EPS_{SUC} were 1:0.035 and 1:0.123, respectively. It is not uncommon for EPS preparations to contain protein, which co-precipitates with polysaccharides during extraction with ethanol, and can be removed by digestion with proteases. This was the case for a crude EPS preparation from *Phlebia radiata* containing 20% protein and 80% carbohydrate, and was purified following trypsin digestion resulting in a protein-free material characterised (Krcmar et al., 1999) as a (1→3;1→6)-β-D-glucan. Both botryosphaerans from *B. rhodina* when subjected to trypsin digestion resulted in highly enriched carbohydrate-containing material following this treatment, and gave a carbohydrate/protein ratio of 1:0.003 and 1:0.001, respectively. Aliquots of the trypsin-digested botryosphaeran samples fractionated by gel permeation chromatography on a Sepharose CL-4B column resulted in each EPS eluting as a single peak (Fig. 1) indicating homogeneity. These preparations were then considered adequately pure for structural characterisation. After total acid hydrolysis of both purified botryosphaeran fractions, only glucose was detected by HPAEC analysis. This finding was similar to the EPS produced when *B. rhodina* was grown on glucose as sole carbon source (Barbosa et al., 2003).

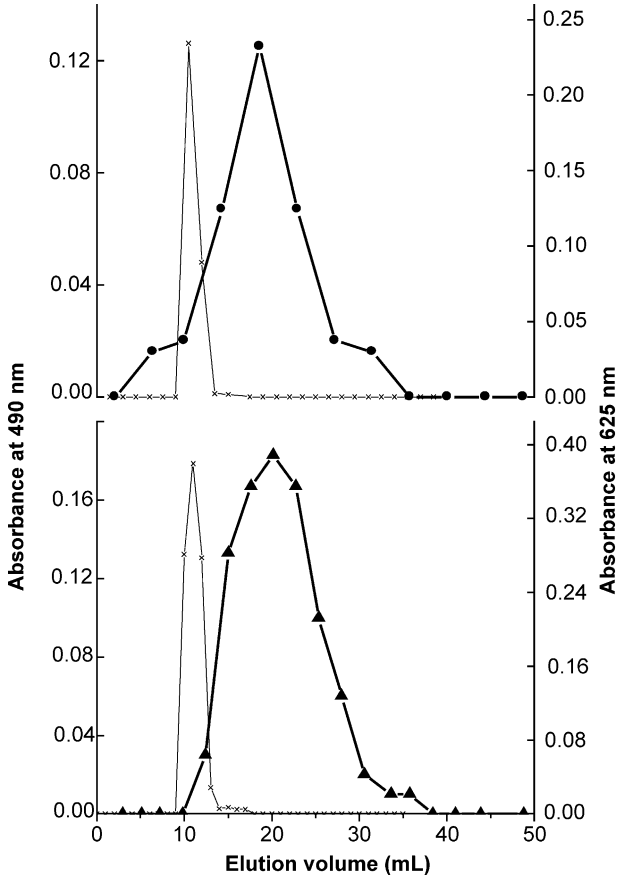

Fig. 1. Gel permeation chromatography profile of botryosphaerans EPS_{FRU} and EPS_{SUC} from *Botryosphaeria rhodina* on a Sepharose CL 4B column after trypsin digestion. The column (43.0 cm × 1.0 cm) was eluted with water at a flow rate of 0.3 mL/min. (•) A_{625}: Blue Dextran; (×) A_{490}: EPS_{FRU}; (▲) A_{490}: EPS_{SUC}.

When submitted to methylation-GLC analysis to determine the nature of the glucosidic linkage, the EPS showed the following methylated sugar derivatives: (I) 2,3,4,6-tetra-*O*-methyl-glucose corresponding to non-reducing terminal units, (II) 2,4,6-tri-*O*-methyl-glucose correlated to 3-O-substituted glucosyl residues, (III) 2,3,4-tri-*O*-methyl-glucose correlated to 6-O-substituted glucosyl residues, and (IV) 2,4-di-*O*-methyl-glucose attributed to 3,6-di-O-substituted units, in different molar proportions for each botryosphaeran sample (Table 1). These results established that both botryosphaerans constituted a (1→3)-linked glucosyl backbone substituted with branch points on C-6. The reduced content of component III suggests that the backbone essentially consisted of consecutive glucose residues (1→3)-linked. The relatively low amount of component II in EPS_{FRU} in comparison to derivatives I and IV, is in agreement with a highly branched structure, while that for EPS_{SUC} was somewhat lower. Structural studies on the extracellular β-D-glucans from *Phytophthora parasitica* (Gandon & Bruneteau, 1998) by the Hakomori method showed similar methylated sugar derivatives in different molar ratios, establishing that these glucans consisted of (1→3)-, (1→6)-, and (1→3;1→6)- linkages, and non-reducing terminal glucopyranoside components having a low proportion of (1→6)-linked residues. The relatively low amount of the 2,4,6-tri-*O*-methyl derivative in comparison to the tetra-*O*-methyl and the di-*O*-methyl derivatives agreed with a highly branched structure, and the low value of

Table 1
Methylation analysis of the botryosphaerans produced by *Botryosphaeria rhodina* cultured on sucrose and fructose as carbon sources

Methylated derivatives	Molar ratios (%)	
	EPS_{FRU}[a]	EPS_{SUC}[a]
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	34	23
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol	20	47
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol	15	9
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl glucitol	31	21

[a] Refers to the exopolysaccharide, Botryosphaeran, arising from the fungus grown on either fructose or sucrose.

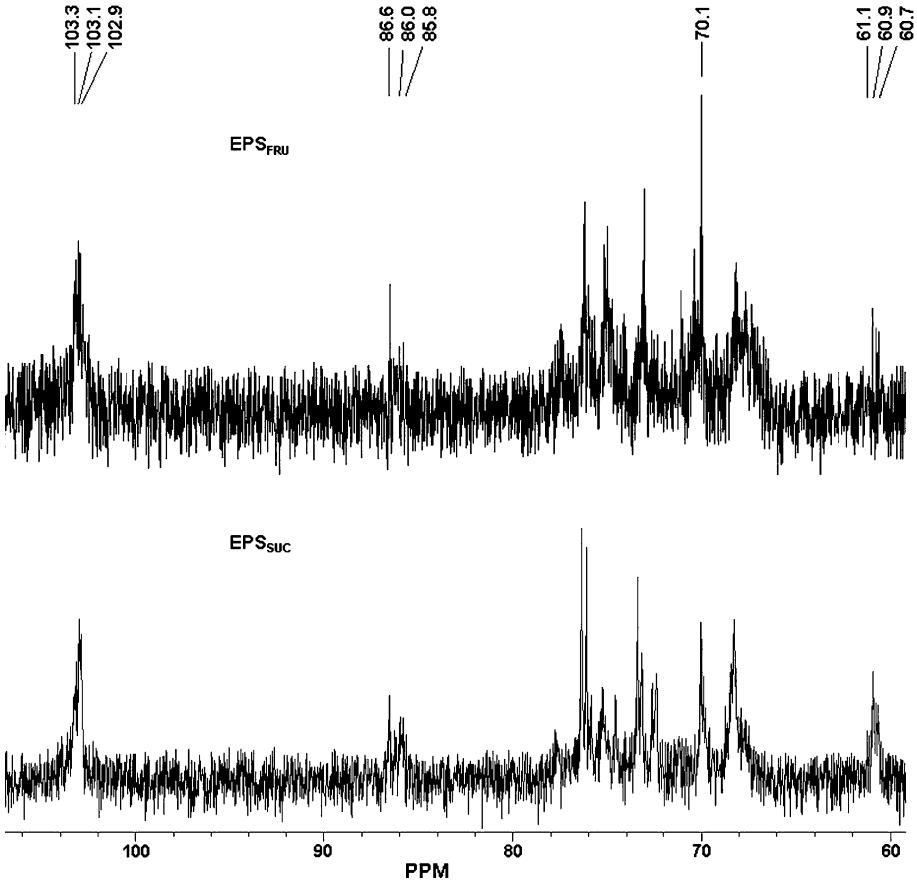

Fig. 2. ^{13}C-NMR spectral assignments of botryosphaerans EPS$_{FRU}$ and EPS$_{SUC}$ produced by *Botryosphaeria rhodina*.

the 2,3,4-tri-*O*-methyl derivative suggested that the backbone chain of this glucan consisted essentially of consecutive (1→3)-linked D-glucose residues. Methylation analysis of the exopolysaccharide from spores of *Ganoderma lucidum* (Bao et al., 2002) yielded three derivatives: 2,3,4-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl glucose (1:4:1), suggesting that this glucan too contained a backbone chain composed essentially of (1→3) linkages with side branching on C-6.

The botryosphaerans were subjected to sequential periodate oxidation and borohydride reduction, followed by total and partial acid hydrolysis. Total hydrolysis of the EPS$_{SUC}$ released glycerol (35%), which corresponded to non-reducing terminal and 6-O-substituted units, and glucose (65%) arising from 3-O- and 3,6-di-O-substituted components that were not oxidised by periodate. The same components were also obtained from EPS$_{FRU}$, but in different amounts, 54 and 46%, respectively. Mild hydrolysis followed by GLC analysis indicated the almost total disappearance of non-reducing terminal units. The evidence of (1→3)-linked glucans was confirmed by the detection of the major component, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol. These structural characteristics have been found mainly in fungal polysaccharides (Krcmar et al., 1999; Woranovicz et al., 1999). The extracellular β-D-glucans derived from *Phytophthora parasitica* (Gandon & Bruneteau, 1998) when subjected to Smith degradation followed by mild acid hydrolysis showed 2,3,4,6-tetra-, 2,4,6-tri, and 2,4-di-*O*-methyl glucose (molar ratios 1:7:1) by GLC analysis of their alditol acetate derivatives, which was in accordance with a (1→3)-linked backbone chain structure.

In accordance with some literature reports, ^{13}C-NMR spectra of the botryosphaerans (Fig. 2, Table 2) showed signals attributable to β(1→3)-glucans O-substituted on C-6 (Gorin, Baron, Corradi da Silva, Teixeira, & Iacomini, 1993), but did not reveal peaks at δ 100.0 ppm that corresponded to the α-configuration of the anomeric carbon (Schmid et al., 2001). Peaks were only visible between δ 103.3 and 102.9 ppm, which strongly indicated that only β-anomeric carbons were present. The signal at δ 103.1 was attributed to the C-1 of 3-O-substituted glucopyranosyl units in comparison to a high rate of 2,4,6 tri-*O*-methylglucitol derivative obtained after Smith degradation. The signal at δ 102.9 was attributable to 3-O-substituted glucose units of the main chain containing branched residues (β-glucopyranosyl and β-di-glucopyranosyl) on C-6, in agreement with the general rule for β-anomeric carbon glycosylation that explained an upfield chemical shift. The signal at δ 103.3 corresponded to the anomeric carbon of non-reducing terminal glucose units.

Table 2
^{13}C NMR spectral assignments of botryosphaerans produced by *Botryosphaeria rhodina* cultured on fructose and sucrose as carbon sources

Linked-glucose residue	Chemical shifts (δ, ppm)											
	EPS_{FRU}						EPS_{SUC}					
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6
Glc*p*-(1→	103.3	73.5	75.5	68.8	76.7	61.1	103.3	73.5	75.5	68.8	76.7	61.1
→6)-Glc*p*-(1→	102.9	73.0	74.8	68.7	76.4	70.1	102.9	73.0	74.8	68.7	76.4	70.1
→3)-Glc*p*-(1→	103.1	73.0	86.6	68.7	76.7	61.1 60.9 60.7	103.1	73.0	86.6	68.7	76.7	61.1 60.9 60.7
→3,6)-Glc*p*-(1→	102.9	73.0	86.0 85.8	68.7	76.4	70.1	102.9	73.0	86.0 85.5	68.7	76.4	70.1
After Smith degradation												
→3)-Glc*p*-(1→	103.1	73.0	86.6	68.7	76.7	61.1	103.1	73.0	86.6	68.7	76.7	61.1

Signals found at δ 70.1 derived from the C-6 carbons, which were O-substituted and disappeared following Smith degradation (Table 2), while unsubstituted C-6 carbons showed signals at δ 61.1–60.7. The signal at δ 61.1 was related to the free C-6 from the principal region of 3-O-substituted glucan. The other signals suggested adjacent glucose residues substituted with β-glucopyranosyl and β-di-glucopyranosyl (gentiobiosyl) branches, respectively.

The 3-O-substituted carbon atoms from the main chain were detected between δ 85.8 and 86.6. The most intensive signal, δ 86.6, was attributed to the main glucan chain C-3 devoid of substitutions. Signals at δ 86.0 and δ 85.8 were attributed to 3,6-di-O-substituted by β-glucopyranosyl and β-di-glucopyranosyl (gentiobiosyl) residues, respectively, and have almost the same intensity, confirming that these ramifications were found in similar ratios in the molecule in accordance with the methylation results. The ^{13}C-NMR spectra of both botryosphaerans were very similar, differing only in the intensity of the signals. In accordance with results from methylation, the most important difference was the higher amount of the 6-O-substituted derivative in EPS_{FRU}. This evidence was confirmed by the intensity of the 70.1 ppm signal that correlated to glucose residues with substitution on C-6 with glucose or gentiobiose.

From the literature there appears to be no evidence that side branched fungal (1→3;1→6)-β-glucans are composed of regular repeating units, and this appears to be the domain of bacterial EPS. Three different strains of *Epicoccum nigrum*, which were cultivated under the same conditions of growth, produced EPS with slight structural variations, mainly in the ramifications on the backbone chain (Schmid et al., 2001). These variations suggested that the frequency and the extent of side branching were determined by the specificities of the side branch-synthesising enzymes responsible for producing these EPS.

The presence of glucopyranosyl and di-glucopyranosyl units branched on C-6 was can be obtained only through original polymer fragmentation, e.g. through partial acid hydrolysis (Bao, Fang, & Li, 2001). Since the β(1→3) glucosidic linkage is more labile to acid hydrolysis than β(1→6) glucosidic linkages, it is possible to obtain gentiobiose and gentiotriose, which corresponded, respectively, to glucose and gentiobiose linked at C-6 to the main chain. The acid hydrolysis products of EPS_{FRU} and EPS_{SUC} were fractionated into four peaks by gel filtration on Sephadex G-15 (Fig. 3). No short chain sugars were detected in Peak I by HPAEC/PAD analysis, probably because it consisted of 'residual polymer' of more than 50 monosaccharide units (Dionex, 1989). Peak II represented a mixture of sugars comprising: laminaribiose (in the proportion of EPS_{FRU} 10.3% and EPS_{SUC} 51.1%);

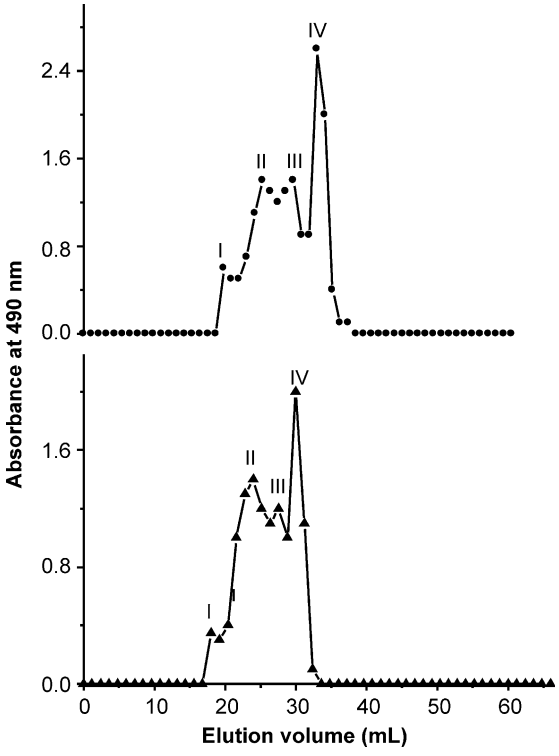

Fig. 3. Gel permeation chromatography profile of botryosphaerans EPS_{FRU} and EPS_{SUC} on a Sephadex G-15 column after partial acid hydrolysis. The column (103.0 cm×0.8 cm) was eluted with water at a flow rate of 0.18 mL/min and fractions of 1.1 mL collected. (•) EPS_{FRU}; (▲) EPS_{SUC}.

gentiotriose (EPS_{FRU} 60.1% and EPS_{SUC} 38.7%); and laminaritriose (EPS_{FRU} 29.5% and EPS_{SUC} 10.1%), respectively, as identified from reference standards (see Section 2). Peak III showed gentiobiose (EPS_{FRU} 60.9% and EPS_{SUC} 45.5%); laminaribiose (EPS_{FRU} 26.4% and EPS_{SUC} 41.5%); gentiotriose (EPS_{FRU} 8.0% and EPS_{SUC} 4.3%); and laminaritriose (EPS_{FRU} 4.6% and EPS_{SUC} 5.7%), while glucose was identified as the main component in Peak IV. The detection of gentiotriose was confirmed by methylation analysis from the derivative 2,3,4 tri-*O*-methylglucitol.

According to the results presented in this work, we concluded that the botryosphaerans produced by *B. rhodina* when grown on fructose and sucrose are β-glucans constituted by a main chain comprising glucopyranosyl units (1→3)-linked and substituted at O-6 by glucosyl and gentiobiosyl units. EPS_{SUC} has 21% side-branching, with a branch point for every five glucosyl residues in the main chain. This value was similar to the botryosphaeran from *Botryosphaeria* sp. (now named *B. rhodina*) when grown on glucose (Barbosa et al., 2003) (22% side branching). The only diversification was the degree of branching with glucosyl and gentiobiosyl units in the main chain. The EPS produced when the fungus was grown on fructose was more branched (31%) than the botryosphaeran produced on sucrose, having approximately a branch point to every three glucose units in the main chain. These results, and those obtained by Barbosa et al. (2003), indicated that it was possible to vary the extent of substitution of repetitive branching residues by modifying the composition of the nutrient medium. However, it was not possible to change the structure or molecular constitution of the botryosphaeran produced. Such an observation has been reported by Margaritis and Pace (1985), who, through manipulating the conditions of microbial culture, demonstrated that this was a possible mechanism to get appropriate polymers for technical applications.

4. Conclusion

It appears that the EPS's produced by *B. rhodina* constitute a family of botryosphaerans that differ only in the degree and frequency of side branching, but no changes appear in the main chain constitution of this exopolysaccharide comprising a backbone of β(1→3)-linked D-glucopyranosyl residues.

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