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Note

Structural characterization of Botryosphaeran: a (1→3;1→6)-β-D-glucan produced by the ascomyceteous fungus, *Botryosphaeria* sp.

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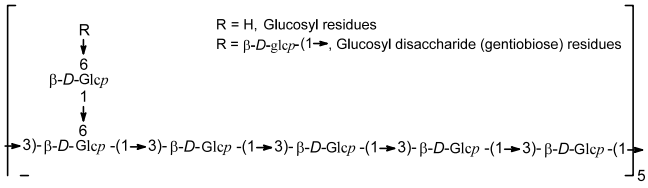
Abstract

The exopolysaccharide, Botryosphaeran, produced by the ligninolytic, ascomyceteous fungus *Botryosphaeria* sp., was isolated from the extracellular fluid by precipitation with ethanol, and purified by gel permeation chromatography to yield a carbohydrate-rich fraction (96%) composed mainly of glucose (98%). Infra-red and ^{13}C NMR spectroscopy showed that all the glucosidic linkages were in the β-configuration. Data from methylation analysis and Smith degradation indicated that Botryosphaeran was a (1→3)-β-D-glucan with approx 22% side branching at C-6. The products obtained from partial acid hydrolysis demonstrated that the side branches consisted of single (1→6)-β-linked glucosyl, and (1→6)-β-linked gentiobiosyl residues.



Keywords: Exopolysaccharide; (1→3;1→6)-β-glucan; Botryosphaeran; *Botryosphaeria* sp.; Ascomyceteous fungus

1. Introduction

Exopolysaccharides (EPS) are widely produced by microorganisms including fungi. Many bacteria have been exploited for the commercial production of polysaccharides which find applications in foods, cosmetic and pharmaceutical products, mainly as viscosity enhancers, and include xanthan, dextran, curdlan, gellan and pullulan..

There is now increasing interest in EPS derived from fungi,[1,2] especially the basidiomycetes, as these fungi were used therapeutically in traditional Chinese and Japanese medicines to treat certain human diseases including cancers.[2] EPS from several fungi have been demonstrated to possess interesting biological activities such as anti-tumor, anti-inflammatory, and immunomodulation (cytokine stimulation).[2,3] One group of carcinostatic substances has been identified in several mushrooms, and were linked to polysaccharides of the β-glucan type, viz., the β-(1→3;1→6)-glucans. These complex polysaccharides have been considered useful in immunotherapy.[2,4] The chemistry and biology of the (1→3)-β-glucans has been discussed by Stone and

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Clarke,[5] and their structure-function activity reviewed.[2,6] Fungal (1→3)-β-glucans are also known to act as phytotoxic agents causing plant diseases,[7] but can also prevent diseases in plants as viral inhibitors.[8]

Fungi belonging to the genus *Botryosphaeria* are well recognised as plant pathogens causing various diseases in plants of commercial importance, including storage rots in preharvested fruits.[9,10] *Botryosphaeria* has been demonstrated as ligninolytic,[11] producing enzymes degrading the lignified plant cell wall.[12] The fungus was reported to produce an EPS tentatively identified as a β-glucan.[13] EPS may be linked directly to the pathogenicity of the fungus, and in woody plants could cause plant vessel blockage through 'gumming' resulting in limb dieback diseases. An EPS from *Botryosphaeria rhodina* was recently reported.[14,15] In the present study, we report on the structural characterization of Botryosphaeran, the exopolysaccharide secreted by the ascomycete, *Botryosphaeria* sp. isolate MAMB-5.

2. Results and discussion

2.1. Analysis and polysaccharide fractionation

The amount of the exopolysaccharide, Botryosphaeran, produced under the culture conditions was 1.7 g/L. The crude EPS freeze-dried preparation contained 86% carbohydrate and 14% protein. Acid hydrolysis showed glucose as the main monosaccharide (98%) with traces of mannose and glucosamine also being present; both probably associated with a glycoprotein. Gel permeation chromatography of the EPS showed two fractions, GP-F_1 and GP-F_2, both eluting after the column void volume (Fig. 1), with GP-F_1 containing the higher proportion of carbohydrate (96%; 4% protein). When this fraction was subjected to DEAE-Sepharose chromatography, the resulting principal fraction (IE-F_2) showed only a minor difference in carbohydrate content (97%; 3% protein) compared to GP-F_1. Its high viscosity prevented obtaining large amounts of fraction IE-F_2, consequently GP-F_1 was chosen as suitable for structural characterization.

Methylation analysis of GP-F_1 (Fig. 2A) showed 2,3,4,6-tetra-*O*-methyl-glucose, 2,4,6-tri-*O*-methyl-glucose, 2,3,4-tri-*O*-methyl-glucose, and 2,4-di-*O*-methyl glucose as the main methylated sugar derivatives in the relative molar proportion of 26:36:16:22, respectively. This demonstrated that the exopolysaccharide was a glucan consisting of a (1→3)-linked glucosyl backbone substituted with approx 22% branch points of C-6. These results were in agreement with other similar β-glucans produced by fungi[16–19] and lichens.[20]

^{13}C NMR studies showed that all carbon lines were resolved and the chemical shifts (Table 1) were in accordance with values reported in the literature.[19,20] The ^{13}C NMR data did not reveal peaks corresponding to the α-configuration of the anomeric carbon; α-glucose anomeric carbons resonate at approx δ 100.0 ppm, and anomeric β-glucose carbons[21] slightly downfield at δ

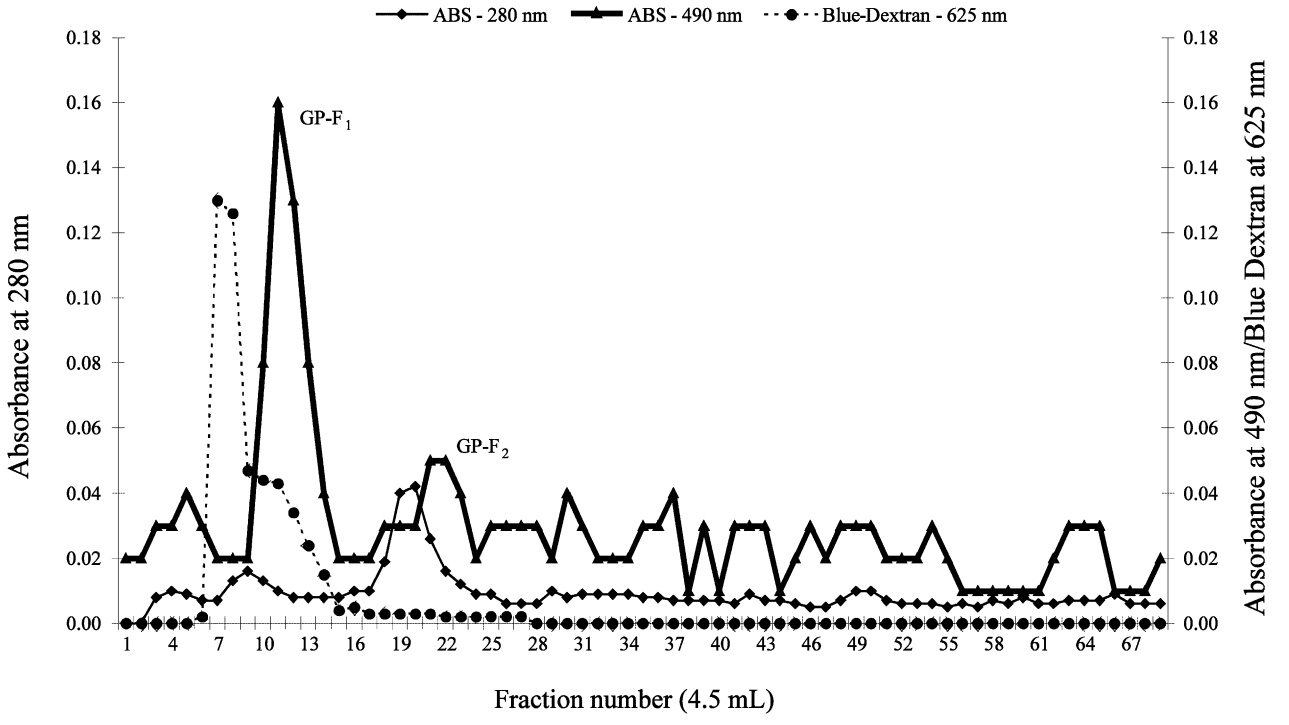


Fig. 1. Gel permeation chromatography profile of EPS from *Botryosphaeria* sp. on a column of Sepharose CL 4B. The column (2.0 × 30.0 cm) was eluted with water at a flow rate of 0.8 mL/min.

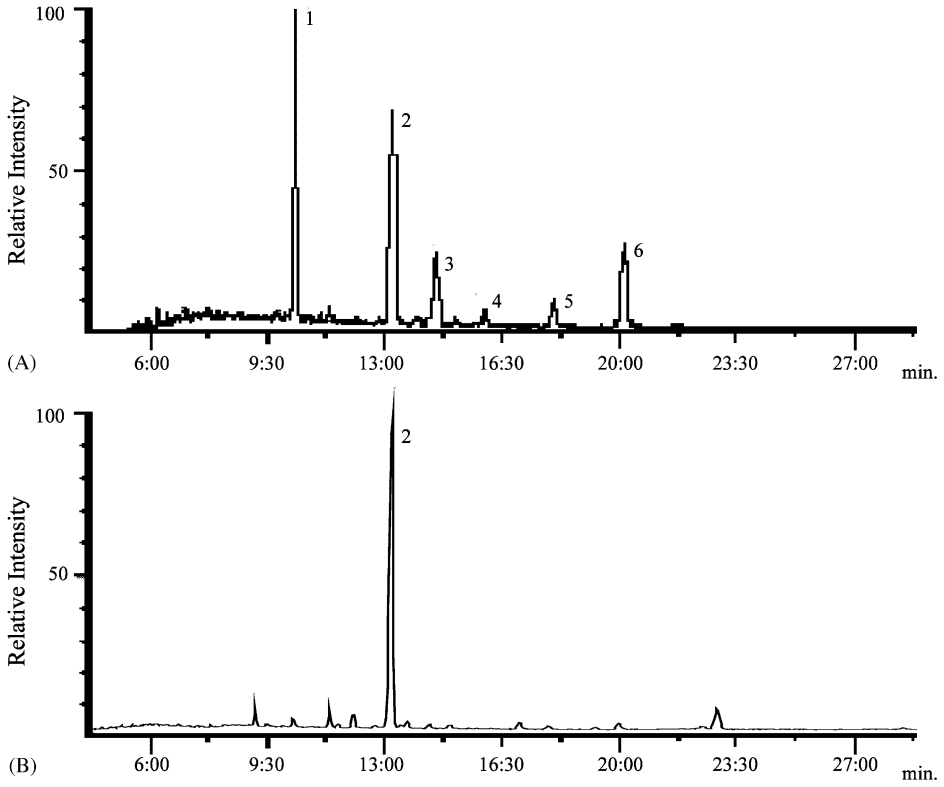


Fig. 2. Methylation analysis (GC–MS) of fraction GP-F_1 (A), and periodate-oxidised GP-F_1 (B) obtained from *Botryosphaeria* sp. Peaks: 1: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol; 2: 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol; 3: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol; 4: not identified; 5: not identified; and 6: 1,3,4,5-tetra-*O*-acetyl-2,4-di-*O*-methylgucitol.

104.0 ppm. Peaks were only visible between δ 103.3 and 102.9 ppm, strongly indicating that only β-anomeric carbons were present. Evidence supporting β-anomeric carbons in the EPS was confirmed by FTIR spectroscopy (data not presented), with bands at 890/cm being typical of (1→3)-β-glucans,[22] and that at 1370/cm being characteristic of β-glucans.[19]

The signal at δ 103.1 was attributed to 3-O substituted glucose anomeric carbon and compared with the signal at C-1 of the residual linear polysaccharide obtained after Smith degradation (Table 1). A general rule for β-anomeric carbon glycosylation explains that there is an upfield chemical shift; in the case of Botryosphaeran, this is shown by a signal at δ 102.9, which is

Table 1
^{13}C NMR spectral assignments of Botryosphaeran produced by *Botryosphaeria* sp. isolate MAMB-5 measured at 30 °C.

	Chemical shifts (δ, ppm)					
Linked-glucose residue	C-1	C-2	C-3	C-4	C-5	C-6
GP-F_1 [a]						
Glc*p*-(1→	103.3	73.8	75.5	68.8	76.7	61.1
→6)-Glc*p*-(1→	102.9	73.0	74.8	68.7	76.3	70.1
→3)-Glc*p*-(1→	103.1	73.0	86.3	68.7	76.7	61.1
						60.9
						60.7
→3,6)-Glc*p*-(1→	102.9	73.0	86.0	68.7	76.3	70.1
			85.5			
After Smith degradation [b]						
→3)-Glc*p*-(1→	103.1	73.0	86.3	68.7	76.7	61.1

[a] EPS fraction obtained by gel permeation chromatography.
[b] Periodate-oxidised GP-F_1.

attributable to 3-O-substituted glucose units of the main chain containing branched residues (β-glucopyranosyl and β-diglucopyranosyl; gentiobiosyl) at C-6.

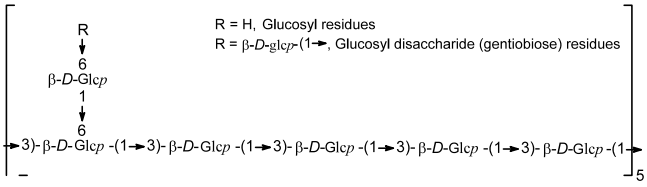
Signals found at δ 70.1 were from the C-6 carbons, which were O-substituted and disappeared following Smith degradation, while unsubstituted C-6 carbons showed signals at δ 61.1–60.7. The signal at δ 61.1 was compared to that obtained after Smith degradation and was attributed to the free C-6 from the principal region of the 3-O-substituted glucan. The others signals at δ 60.9 and 60.7 suggested adjacent glucose residues substituted with β-glucopyranosyl and β-diglucopyranosyl (gentiobiosyl) branches, respectively. The 3-O-substituted carbon atoms from the main chain resonated between δ 85.5 and 86.3. In the ^{13}C NMR spectrum, the signal at δ 86.3 resonated more intensely suggesting that large proportions of regions along the main glucan chain did not carry substitutions. Signals at δ 86.0 and 85.5 were attributed to →3,6)-Glc*p*-(1→ residues, and were substituted with β-glucopyranosyl and β-diglucopyranosyl (gentiobiosyl) residues, respectively. The signal intensities found in C-3 substituted and free C-6 regions suggest a possible random disposition of the glucosyl substituents along the chain, and will be the subject of further investigation

Methylation of the periodate-oxidised polysaccharide showed almost total disappearance of terminal units (Fig. 2B). Dimethylated units practically disappeared following Smith degradation, which removed the oxidised residues attached to the branching points. The linear (1→3)-β-glucan obtained after one-cycle periodate oxidation and Smith degradation suggested that the side chains was constituted of a single glucose residue, and also of a short-chain (1→6)-β-linked glucosyl oligosaccharide. The latter constituent was confirmed by the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol.[17] Furthermore, ^{13}C NMR spectra of the periodate-oxidised polysaccharide showed the disappearance of the signal at δ 70.1, corresponding to a substituted C-6, and a comparison of the intensities from the signals: C-1, C-3 and C-6 (Table 1) showed a linear D-glucan exclusively carrying β-(1→3) linkages. A similar β-glucan produced by *Pestalotia* sp. 815 was described as having a (1→3)-β-linked backbone with three out of every five glucosyl residues substituted at O-6 with either single D-glucosyl groups, or occasional short (1→6)-β-linked oligosaccharides.[23] Total hydrolysis of the oxidised Botryosphaeran released glycerol (45%), which corresponded to mono- (glucose) and oligo-saccharides (glucose-β-(1→6)-glucose) branch residues, and glucose (55%) arising from (1→3)-β-linked glucose and (1→3;1→6)-β-linked glucose, which were not oxidised by periodate.

The partial acid-hydrolysate of Botryosphaeran (fraction GP-F_1) was fractionated by gel filtration on Sephadex G-15 (Fig. 3) into three fractions comprising modified polysaccharide (Fraction A), mixed oligosaccharides (Fraction B) and mainly glucose (Fraction C) with some shorter oligosaccharides. Fractions B and C were analysed by HPAEC/PAD, and the former (Fig. 4) yielded a homologous series of β-(1→3)- and β-(1→6)-linked glucosyl-oligosaccharides; two of which were identified as gentiobiose and gentiotriose, respectively, from reference standards. Considering that Botryosphaeran (GP-F_1) was purified, and gentiotriose was detected as one of the products obtained through partial acid hydrolysis, the above evidence strongly suggests the presence of a (1→6)-β-D-glucopyranosyl disaccharide (gentiobiose) side branch.

Besides methylation evidence, side-chain/branched structure of β-glucans has also been demonstrated by specific enzymatic hydrolysis of the EPS using a fungal (1→3)-β-exoglucanase to isolate appendage mono- and disaccharides,[21] and a β-1,3-glucosidase to distinguish side-chain structure.[15] Additionally, such studies have revealed there was no evidence that fungal (1→3;1→6)-β-glucans (e.g., epiglucan from *Epicoccum nigrum*[21]) were composed of regular repeating structures as found with bacterial EPS. They do, however, show repeated structural features. Probably the structural variations between (1→3;1→6)-β-glucans are determined during fermentation by specific side-branch synthesising enzymes or through modification by hydrolytic enzymes.

From the foregoing observations, it was concluded that Botryosphaeran produced by *Botryosphaeria* sp. isolate MAMB-5 was a (1→3)-β-D-glucan with both single (1→6)-β-D-glucopyranosyl, and β-(1→6)-β-D-diglucopyranosyl (gentiobiose), side-branches attached along the (1→3)-β-D-glucosyl backbone chain at a frequency (approx 22%) of 1 branch point to every 5 glucose residues (Scheme 1).



Scheme 1.

3. Experimental

3.1. Botryosphaeria, culture media and growth conditions

Botryosphaeria sp. isolate MAMB-5 was maintained at 4 °C on potato-dextrose-agar.[11] The inoculum was prepared by growing *Botryosphaeria* sp. on agar plates [Vogel minimum salts medium,[24] agar (20 g/L) and glucose (10 g/L)]. After 5 days growth (28 °C), mycelial fragments were transferred to four 125 mL flasks containing 25 mL of Vogel minimum salts medium

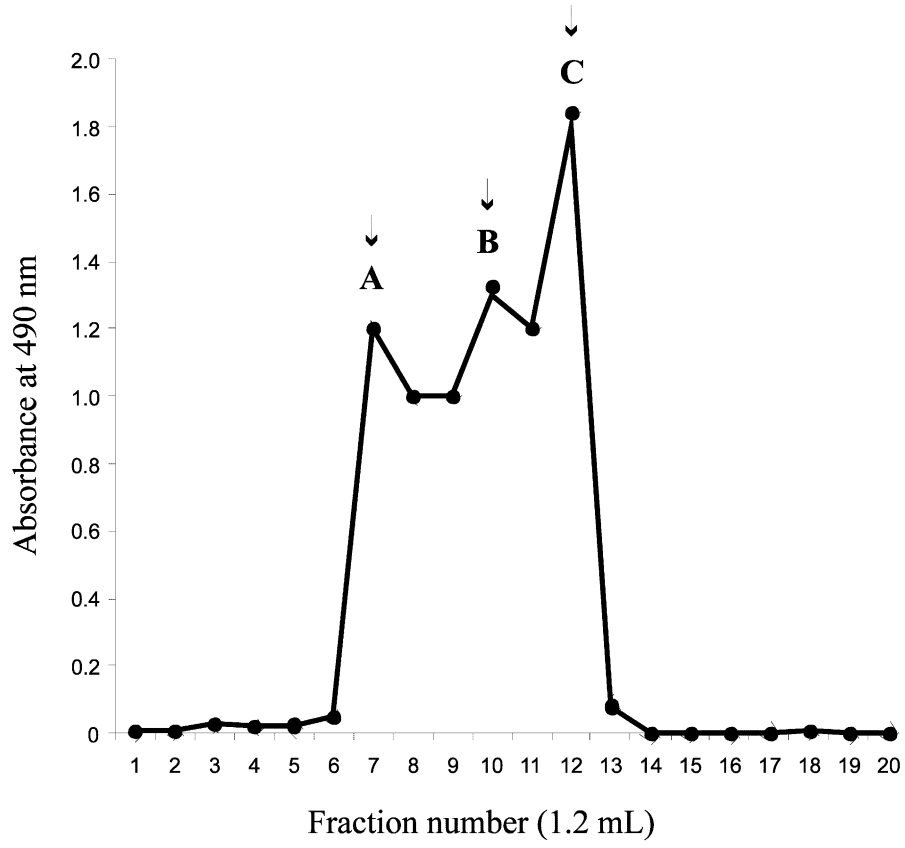


Fig. 3. Gel permeation chromatography profile on Sephadex G-15 after partial hydrolysis of EPS fraction GP-F_1 obtained from *Botryosphaeria* sp. A: modified polysaccharide; B: mixed oligosaccharides; and C: shorter oligosaccharides and glucose (the arrows, from left to right correspond to the standards: starch, melibiose/sucrose/lactose and, glucose). The column (0.8 × 50.0 cm) was eluted with water at a flow rate of 0.13 mL/min.

and glucose (0.5 g/L), and incubated at 28 °C for 48 h on a rotary shaker (180 rpm). The pre-cultures were then homogenised (sterilised chilled Blender) for 0.5 min at maximum speed. The cell homogenate was then centrifuged (1250*g*/10 min), the mycelium recovered, diluted with sterilised physiological saline solution to an absorbance of 0.4–0.5 at 400 nm, and 4 mL aliquots of the suspension used to inoculate Erlenmeyer flasks (1 L) containing 200 mL of Vogel minimum salts medium and glucose (5 g/L). Cultures were grown in submerged cultivation (180 rpm) for 72 h at 28 °C.

3.2. Preparation and dissolution of the exopolysaccharide

Cell-free extracellular fluid was obtained after removal of the mycelium by centrifugation (3000*g*/10 min) at 4 °C. The supernatant was treated with 3 vol of absolute ethanol, and the precipitate recovered, dissolved in deionised water and extensively dialysed against frequent changes of deionised water for 48 h, then frozen and freeze-dried to obtain the crude exopolysaccharide preparation. As the freeze-dried exopolysaccharide preparation showed low solubility in water, complete dissolution was achieved by heating for 2 h at 50 °C (pH 7.0).

3.3. Analytical techniques

Carbohydrate was determined by the phenol–sulfuric acid method of Dubois co-workers[25] with glucose as standard. Protein was measured by the Bradford method[26] using bovine serum albumin as standard.

3.4. Purification of the exopolysaccharide

The exopolysaccharide preparation was dissolved in water (1 g/L) and fractionated by gel permeation chromatography on a column of Sepharose CL-4B (30 × 2.5 cm) eluted with water (0.8 mL/min). Fractions (3.5 mL) were collected and analysed for carbohydrate and monitored at 280 nm for protein. The void volume was determined using blue dextran. Fractions corresponding to peaks (GP-F_1 and GP-F_2) were pooled and freeze-dried.

Fraction GP-F_1 was further fractionated by ion-exchange chromatography. A sample of 1 g/L dissolved in 0.2 M sodium phosphate buffer (pH 6.8) was applied

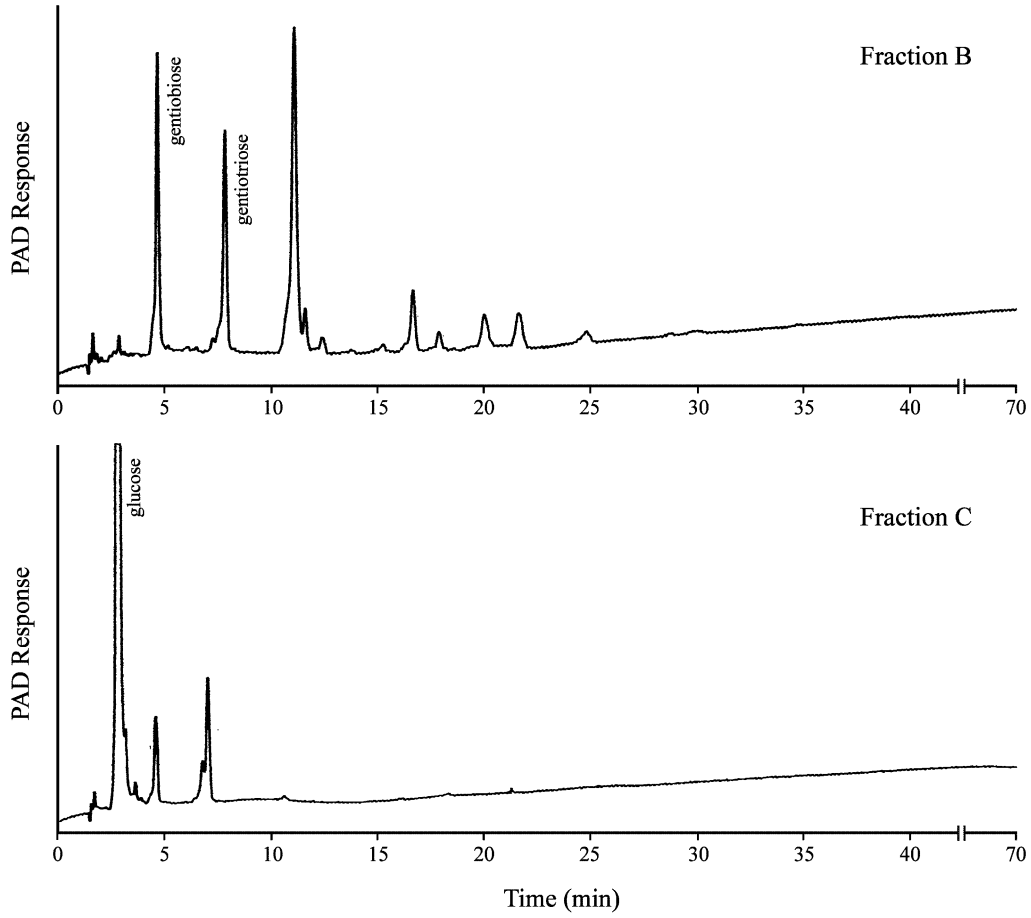


Fig. 4. Analysis of fractions B and C arising from chromatography on Sephadex G-15 (Fig. 3) by HPAEC using a CarboPac PA-100 column.

to a column of DEAE-Sepharose (FF) (12 × 1.8 cm), and developed with 0.2 M sodium phosphate buffer (pH 6.8) as eluent (0.4 mL/min). Fractions (1.0 mL) were collected and analysed for carbohydrate, and read at 280 nm. Fractions corresponding to peak IE-F_2 were pooled, dialysed against deionised water and freeze-dried.

3.5. Monosaccharide analysis

Polysaccharide samples (0.050 mg) were hydrolysed in 5 M trifluoroacetic acid (0.5 mL) for 16 h/100 °C. After hydrolysis, the solution was evaporated under diminished presure and the residue dissolved in 0.5 mL of water and evaporated again. The dissolution and evaporation cycle was repeated twice. Finally, the residue was dissolved in 0.2 mL of water, and 0.025 mL aliquots used for HPAEC/PAD[27,28] (High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection from Dionex DX 500). 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 as previously described.[28,29] Elution conditions were produced 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.

3.6. Methylation–acid hydrolysis–acetylation

GP-F_1 Fraction (5 mg) was dissolved in Me_2SO (1 mL), methylsulfinyl carbanion (0.4 mL) was added and the mixture sonicated for 20 min at ambient temperature. Methyl iodide (0.3 mL) was added gradually. The methylated polysaccharide was extracted with $CHCl_3$ (4 mL), and the chloroform phase evaporated until completely dry.[30] A second and third methylation run was performed as above. The permethylated polysaccharide was hydrolysed with 72% H_2SO_4 at room temperature for 2 h, and then water added (4 mL) and kept under reflux at 100 °C for 18 h.[31,32] The resulting partially methylated sugars were reduced with $NaBH_4$,

acetylated, and analysed by gas liquid chromatography–mass spectrometry (GC–MS) on a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, using He as carrier gas. A capillary column (DB-225; 15 m × 0.25 mm i.d.) was used. The injection temperature was 50 °C with a program to 220 °C (constant temperature).[33]

3.7. Smith degradation

GP-F_1 (50 mg) was oxidised with aqueous 0.05 M $NaIO_4$ (50 mL) for 120 h at 4 °C in the dark.[34] The oxidised polysaccharide was reduced with 1.0 M $NaBH_4$, and a portion subjected to total acid hydrolysis (0.5 M H_2SO_4, 100 °C, 5 h). Another portion was submitted to mild acid hydrolysis (1.0 M H_2SO_4, 24 h, 50 °C) to remove the residues of oxidised sugars attached to the polysaccharide chain (Smith degradation), and then neutralised, dialysed against water, freeze-dried and methylated twice as described above.

3.8. Partial acid hydrolysis

Partial acid hydrolysis of GP-F_1 (30 mg) was performed as described by Ukai co-workers[35] using 50% (v/v) H_2SO_4 for 16 h at 4 °C, and then stirred for 1 h at 35 °C. The hydrolysate was neutralised with Amberlite IR-400 (carbonate) resin, and the products separated on a Sephadex G-15 column (1.0 × 37 cm) calibrated with starch, melibiose and glucose. Water was used as eluent (0.13 mL/min) and fractions collected were analysed for carbohydrate by the phenol–H_2SO_4 method. Fractions containing the modified poly-, oligo- and mono-saccharides were analysed by HPAEC/PAD.[36,37] Mono- and oligo-saccharides 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 AcONa (3%). After 15 min, an acetate gradient was developed over a 60 min period to a limit of 0.25 M AcONa, while the concentration of NaOH remained at 0.1 M. Mono- and oligo-saccharides were detected by Pulsed Amperometric Detection (Dionex DX-500) without the addition of post-column alkali. Mono- and oligosaccharides in the experimental samples were identified by comparing their retention times (min).

3.9. FTIR

FTIR spectroscopy was performed using a Bruker Vector 22 Model FTIR Spectrometer on 2 mg of freeze-dried GP-F_1 in 300 mg of KBr.[19] Cellulose (4 mg) and starch (4 mg) in 300 mg KBr were used as standards. Scans were conducted in the range 1800–500/cm at a resolution of 2/cm.

3.10. ^{13}C NMR

^{13}C NMR spectra were obtained on a Bruker DRX-400 NMR Spectrometer with polysaccharide solutions (15 mg/mL GP-F_1, and 5 mg/mL periodate-oxidised polysaccharide) in Me_2SO at 400 MHz (30 °C). Chemical shifts were referred to tetramethylsilane.[38]

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