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Structure of a glycoglucuronomannan from the low-viscosity gum of *Vochysia lehmannii*

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Abstract

The polysaccharide (VSP) from the gum exudate of quaruba (*Vochysia lehmannii*) had two components of almost identical M_w centred at 24,800, as shown by HSPEC-MALLS. The presence of aggregates was shown since carboxy-reduction gave VSP-RED, which contained low molecular weight components with M_w 19,000 > 5800 and polydispersity ratios d*n*/d*c* 0.160 and 0.149, respectively. VSP formed low viscosity aqueous solutions and acid hydrolysis gave Man (30%), Ara (16%), Gal (10%), and Glc (14%). The latter arose partly from GlcA (30%). Methylation analysis revealed mainly neutral units of 2-*O*- (60%) and 2,3-di-*O*-substituted Man*p* (5%), and those of nonreducing ends (8%), 2-*O*- (3%),and 4-*O*-substituted Ara*p* and/or 5-*O*-substituted Ara*f* units (6%). VSP-RED contained Glc (45%), Man (35%), and Ara (13%) and methylation analysis indicated mainly 4-*O*-substituted Glc*p* (31%) and 2-*O*- (51%) and 2,3-di-*O*-substituted Man*p* units (5%). A predominant alternating structure for VSP was shown by its ^{13}C NMR spectrum, which contained 10 main signals and a small one of C-6 of Glc*p*A. This was confirmed by formation, on partial hydrolysis of VSP, of a tetrasaccharide, which was characterised by NMR spectroscopy and ESI-MS as β-Glc*p*A-(1 → 2)-α-Man*p*-(1 → 4)-β-Glc*p*A-(1 → 2)-Man, which arose from the main chain, thus confirming VSP to be a glycoglucuronomannan.

Keywords: *Vochysia lehmannii*; Gum polysaccharide; Aggregates; Low viscosity; Glycoglucuronomannan; Ara*f* side chains

1. Introduction

The present investigation is on the structure of the polysaccharide components of a gum exudate of a tree *Vochysia lehmannii* HERON, native to Brazil. This is a part of continuing studies involving gums of other species (Delgobo, Gorin, Jones, & Iacomini, 1998; Delgobo, Gorin, Tischer, & Iacomini, 1999; Menestrina, Iacomini, Jones, & Gorin, 1998), in order to determine if they have potential as replacements for imported gums (Sandford & Baird, 1983). We now find that the gum exudate of the tree, known locally as quaruba or quaruba branca, gave aqueous solutions of low viscosity. Campos-Santaella and Houghton (2002) indicated that it belongs to the family Vochysiaceae, which comprises approximately 200 species and 7 genera, in which are included *Vochysia* spp., distributed throughout tropical America. The better-known *Vochsyia thyrsoidea* Pohl forms a gum known locally as goma arábica de Lagoa Santa (State of Minas Gerais), by virtue of its similar formation of solutions of low viscosity (Almeida, Proença, Sano, & Ribeiro, 1998). It is common in savannah region of Brazil, at altitudes of more than 650 m. But any implied resemblance of the polysaccharide (VSP) obtained from the gum of *V. lehmannii* with that of gum arabic is superficial, as a preliminary study showed a much less complex C-1 portion of its ^{13}C NMR spectrum (Fig. 1A), than that of the polysaccharide of gum arabic (Fig. 1B) (Tischer, Gorin, & Iacomini, 2002a). VSP also contained high proportions of glucuronic acid (30%) and mannose (30%). Few glucuronomannan-based structures in gum polysaccharides have

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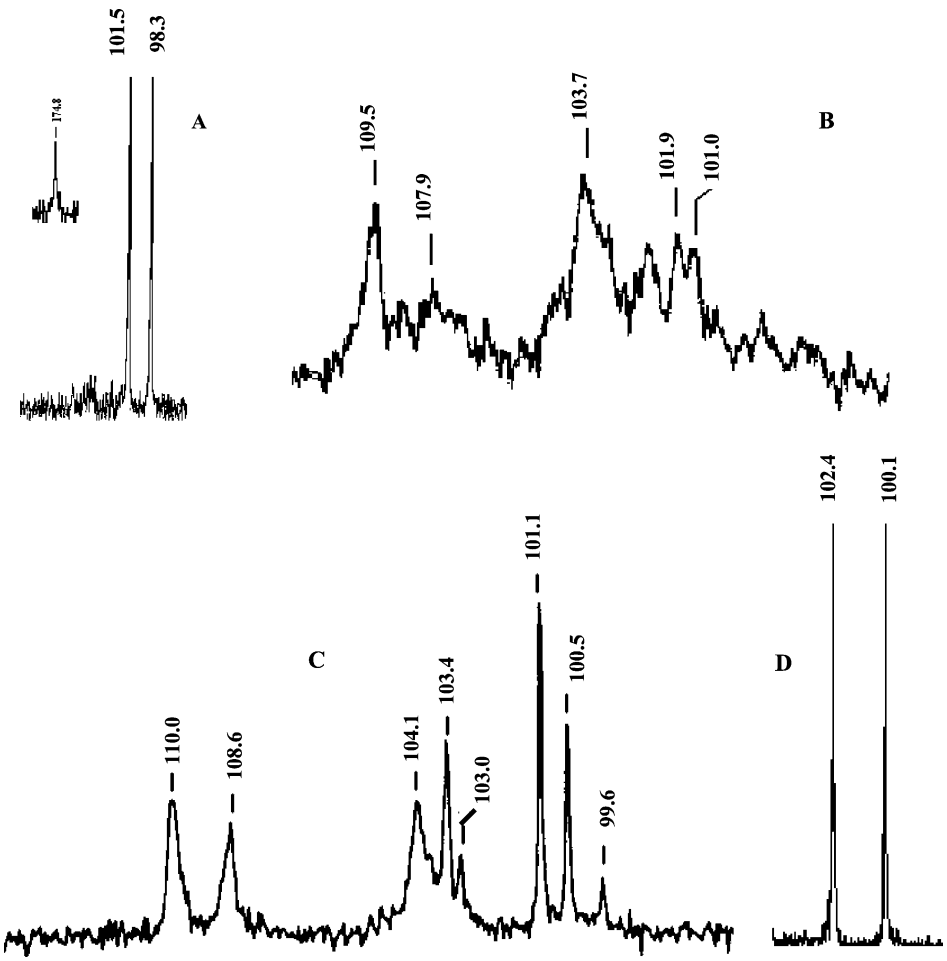


Fig. 1. Comparison of C-1 region of ^{13}C NMR spectrum of VSP and C-6 insert (A) and C-1 regions of polysaccharides from gum arabic (B) (D_2O solutions at 30 °C) and gum ghatti (C) (gel in Me_2SO-d_6). C-1 region of VSP-RED in 0.5 M NaOD–D_2O (D).

been reported, although one of the exceptions is from the high-viscosity gum ghatti, but when the C-1 region of the ^{13}C-NMR spectrum of VSP was compared with that of gum ghatti heteropolysaccharide, that of the latter (Fig. 1C) (Tischer, Iacomini, Wagner, & Gorin, 2002b) proved to be much more complex. Since these data pointed to a unique structure for VSP, it was further investigated.

2. Experimental

2.1. Collection of gum exudate

A sample of quaruba gum was collected from the trunk of a tree growing in the tropical region of Porto Acre, Municipality of Caquetá, State of Acre, Brazil and identified by one of us (N.M.P.).

2.2. Preparation of quaruba polysaccharide

The gum (37 g) was partly dissolved in H_2O (300 ml) and the remaining debris was removed by filtration, followed by centrifugation to give a supernatant, which was added to EtOH (3 vols) to give a precipitate (6.7 g) (low molecular weight carbohydrates were not present in the supernatant). A portion (2.0 g) was dissolved in H_2O (40 ml), which was frozen, thawed gently, and the resulting precipitate removed by centrifugation. Freeze drying of the supernatant provided polysaccharide VSP (1.6 g).

2.3. Cetavlon fractionation of quaruba polysaccharide

Following the method of Scott (1960), the polysaccharide (300 mg) in H_2O (50 ml) was added to a 3% solution of Cetavlon in H_2O at pH 7.0 (50 ml). The resulting precipitate was isolated by centrifugation, decomplexed with 4 M aq. NaCl and the solution added to excess EtOH. The precipitate was dialysed to give an acidic polysaccharide (240 mg). The supernatant of the Cetavlon precipitate was treated with aq. $Na_2B_4O_7$ at pH 8.5, which was then adjusted to pH 10.0, and then to 12.0, with aq. NaOH. No further precipitation took place.

2.4. Carboxy-reduction of VSP

This was carried out in two successive cycles according to Taylor and Conrad (1972) to give VSP-RED.

2.5. Monosaccharide composition of polysaccharides

VSP and VSP-RED were hydrolysed with 2 M TFA for 8 h at 100 °C and the products were successively reduced with $NaBD_4$, and acetylated with Ac_2O–pyridine. The resulting alditol acetates were examined using a capillary column of DB-225 (30 m × 0.25 mm i.d.), programmed from 50 °C (1 min) at 40 °C/min to 220 °C.

2.6. Uronic acid determination

Uronic acid contents were determined by an improved *m*-hydroxy-biphenyl colorimetric method (Filisetti-Cozzi & Carpita, 1991).

2.7. Paper chromatography

This was carried out on Whatman No. 1 for qualitative analysis and on Whatman 3 MM paper for the isolation of oligosaccharides, which were formed on partial hydrolysis (solvent: *n*-BuOH–pyridine–H_2O, 1:1:1 v/v). Products were detected by the $AgNO_3$–acetone dip method. (Trevelyan, Procter, & Harrison, 1950).

2.8. Partial hydrolysis of VSP

VSP (1.0 g) was partially hydrolysed in 1 M TFA (50 ml) at 100 °C for 3 h, which provided monosaccharides and two oligosaccharides with R_{Lact} 0.72 (Oligo-1) and 0.27 (Oligo-2) in the above 1:1:1 solvent. The oligosaccharides were isolated in 14 and 10% yield, respectively.

2.9. Methylation analysis of poly- and oligosaccharides

VSP and VSP-RED (5 mg of each) were methylated, after dissolution in Me_2SO, by the method of Ciucanu and Kerek (1983). After 18 h, the mixtures were acidified with aq. H_2SO_4 and were extracted with $CHCl_3$, which was washed twice with H_2O and evaporated. The products were fully *O*-methylated, since their IR spectra did not contain a band at $\sim 3400\ cm^{-1}$ corresponding to free hydroxyl groups.

In the case of the oligosaccharides (~1 mg), the procedure was identical, except that they were dissolved in a drop of water, followed by the sequential addition of Me_2SO, MeI, and NaOH, followed by vigorous agitation (Tischer et al., 2002a). Per-*O*-methylated material were methanolysed with 3% HCl in MeOH at 70 °C for 3 h and the product hydrolysed with 1 M H_2SO_4 at 100 °C for 18 h to give a mixture of partially *O*-methylated alditols. These were converted to partially *O*-methylated alditol acetates by successive treatments with $NaBD_4$ and Ac_2O–pyridine.

The methanolysates of the *O*-methylated oligosaccharides were treated with $NaBD_4$ in 0.1% NaOMe in MeOH at 70 °C for 3 h in an attempt to convert the CO_2H groups of uronic acid units to those of CH_2OH. This was unsuccessful as only the acetate of 3,4,6-tri-*O*-methylmannitol was detected by GC-MS (the conditions were identical to those described in Section 2.5, except that the final constant temperature was 210 °C).

2.10. HPSEC-MALLS analysis

The molecular weight distribution of VSP was determined using Wyatt Technology equipment incorporating columns of 2000, 500, 250, and 120 connected to a differential refractometer (model 2410, Waters) and a laser detector of light scattering at 632.8 ηm (Dawn DSP-F model), which provided readings of light scattering obtained at different intensities at different angles. The eluant was aq. 0.1 M $NaNO_2$ + 0.2 g/l NaN_3, at a flow rate of 0.6 ml/min.

VSP was dissolved in aq. $NaNO_2$ (1 mg/ml) and filtered through a cellulose membrane with an average pore diameter of 0.2 μm and a volume of 250 μl was injected into the apparatus. The results were provided directly with the aid of computer software ASTRA 4.70.07.

2.11. Fractionation of VSP-RED components by dialysis

VSP-RED (177 mg) was dialysed against distilled H_2O using membrane tubing (SPECTRA/POR) with a 16,000 mol. wt. cut-off. Retained was VSP-RED16R (134 mg) and dialysed was VSP-RED16E (43 mg). The ^{13}C NMR spectrum and monosaccharide composition of each fraction were determined.

2.12. ESI-MS analysis of oligosaccharides

Analyses were carried out using Quattro Ultima equipment in the positive- and negative-ion mode on samples (~1 ηg/μl) previously dissolved in H_2O, and CH_3CN added to give a 1:1 solvent ratio. Samples were applied using a manual loop injector (10 μl volume) on to a flow rate of 20 μl/min of the 1:1 solvent. The system was washed (6 ×) with the 1:1 solvent after each run.

2.13. NMR spectroscopy

NMR spectra were obtained with a Bruker 400 MHz DRX Avance spectrometer from solutions in 99.8% D_2O at 30 °C, except for VSP-RED, which only dissolved in 0.5 M NaOD in D_2O (shifts are expressed at δ in ppm, relative to external Me_4Si, δ = 0). ^1H, ^1H COSY, ^1H(obs.) ^{13}C HMQC, and ROESY determinations were carried out according to the Bruker Manual.

3. Results and discussion

An aqueous solution of ethanol-precipitated quaruba polysaccharide was frozen and on gentle thawing a trace of precipitate formed. The ^{13}C NMR spectrum of the material in the mother liquor showed it to be pure carbohydrate, whereas that of the precipitate showed the same signals of carbohydrate, along with those of 3% protein, determined separately. The polysaccharide in the supernatant (VSP) was thus further examined.

VSP was acidic, since on treatment with Cetavlon, precipitation occurred at pH 7.0 with no precipitation occurring on increasing the pH to 8.5, 10.0, and 12.0 in the presence of borate. Carbohydrate was not detected in the final mother liquor.

HPSEC-MALLS analysis of VSP (Fig. 2A), using a refractive index detector, showed a double peak centred at M_w ~24,800 and d*n*/d*c* 0.14. Light scattering detection showed a trace of high molecular weight material. As acidic polysaccharides can form aggregates (Banks & Greenwood, 1963), VSP was treated with 6 M aqueous urea in an attempt to dissociate any aggregates, but the HPSEC-MALLS profile remained unchanged.

However, aggregation was confirmed by HPSEC-MALLS examination of uronic acid-free VSP-RED, formed by carboxy-reduction of VSP with carbodiimide. The product also gave rise to two peaks on HPSEC-MALLS examination, although with a better resolution than found with VSP (Fig. 2B), and significantly lower M_w values and a low degree of polydispersity. The major component had M_w 19,000 g/mol (d*n*/d*c* 0.160) and the other 5800 g/mol (d*n*/d*c* 0.149). The components were fractionated using 16,000 cut-off dialysis tubing (Fig. 2C). Each component had the same monosaccharide ratio and ^{13}C NMR spectrum as those of VSP-RED (Fig. 1D).

VSP contained GlcA (30%), determined colorimetrically, and gave rise on acid hydrolysis to Man (30%), Ara (16%), Gal (10%), Glc (14%) (GC-MS of derived alditol acetates), and a trace of aldobiouronic acid (PC) (an iodine test on VSP showed that starch was absent). As shown in Table 1, methylation analysis of VSP showed structures of neutral units, namely nonreducing end units of Ara*f* (8%), Gal*p* (2%), and Glc*p* (2%), 2-*O*- (60%) and 2,3-di-*O*-subst. (5%) Man*p*, 4-*O*- (6%), 3,4-di-*O*-subst. Glc*p* (4%), 3-*O*-subst. Gal*p* units (4%), and Ara present as 2-*O*-subst. Ara*f* (3%) and 4-*O*-subst. Ara*p* and/or 5-*O*-subst. Ara*f* units (6%).

VSP-RED, in which Glc*p*A were reduced to neutral Glc*p* units, contained Glc (45%), with Man (35%), Ara (13%), and Gal (7%). Methylation analysis showed mainly (components of >1%), nonreducing end-units of Ara*f* (3%), 2-*O*- (51%) and 2,3-di-*O*-subst. Man*p* (5%), 3-*O*- (2%) and 3,4-di-*O*-subst. Gal*p* (3%), and 4-*O*- (31%) and 3,4-di-*O*-subst. Glc*p* (2%) units.

These data show 4-*O*-substituted Glc*p*A and 2-*O*-substituted Man*p* units to be present in the main chain of

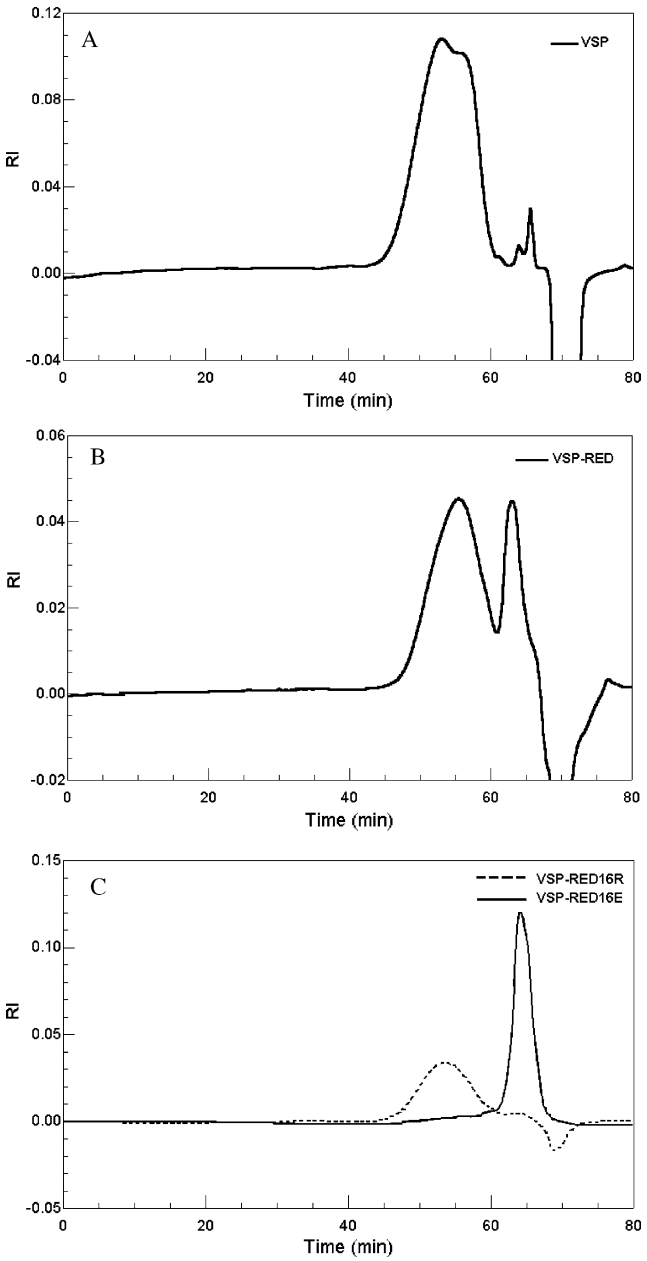


Fig. 2. HSPEC-MALLS profiles (refractive index detector) of VSP (A), VSP-RED (B), and retained VSP-RED16R and dialysed VSP-RED16E (C).

VSP. Its ^{13}C NMR spectrum (Fig. 1A) showed that they are arranged alternately as it contained 10 main signals and a small one of C-6 of Glc*p*A. This was confirmed by the spectrum of VSP-RED, which contained 11 main signals (Fig. 1D).

Despite the presence of considerable proportions of Gal and Ara, corresponding signals did not appear in Fig. 1A and B. The possibility of overlapping signals in the anomeric region was eliminated, since additional signals were not detected in HMQC ^{1}H(obs.) ^{13}C and ^{13}C, ^{1}H coupled spectra (not shown). The Gal and Ara units are thus present in a segment of the polysaccharides having restricted molecular motion.

Table 1
Partially *O*-methylated alditol acetates formed on methylation analysis of glycoglucuronomannan (VSP) and carboxy-reduced glycoglucuronomannan (VSP-RED)

Acetylated ald. ac (r.t.)[a]	VSP (%)	VSP-RED (%)	Acetylated ald. ac. (r.t.)[a]	VSP (%)	VSP-RED (%)
2,3,5-Me_3-Ara (7.16) 0.81	8	3	3,4,6-Me_3Man (10.99) 1.24	60	51
2,3,4-Me_3-Ara (7.56) 0.85	–	1	2,4,6-Me_3Gal (11.52) 1.30	4	2
3,5-Me_2-Ara (8.37) 0.94	3	1	2,3,6-Me_3Glc (12.06) 1.36	6	31
2,3,4,6-Me_4-Man (8.79) 0.99	2	1	4,6-Me_2Man (13.64) 1.54	5	5
2,3,4,6-Me_4Glc (8.89) 1.00	2	–	2,6-Me_2Gal (14.08) 1.58	4	3
2,3-Me_2-Ara (9.15) 1.03	6	–	2,6-Me_2Glc (14.46) 1.63	–	2
2,4-Me_2-Ara (9.26) 1.04	–	1			

[a] Retention time compared with that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

VSP was partially hydrolysed to form oligosaccharides with R_{Lact} 0.72 (Oligo-1) and 0.27 (Oligo-2), which were isolated by preparative PC. Methylation analysis of each, in which the per-*O*-methylated products were subjected to methanolysis, followed by $NaBD_4$ reduction and conversion to partially *O*-methylated alditol acetates, showed the presence of 2-*O*-substituted Man*p* units, but not those of glucitol from the uronic acid.

The ^{13}C NMR spectrum of Oligo-1 (Fig. 3A) shows a β-Glc*p*A-(1 → 2)-αβ-Man structure with the major α-anomer giving rise to signals at δ 101.6 (C-1′), 92.1 (C-1), and 78.3 (C-2) and the minor β-anomer to those at δ 103.7 (C-1′), 93.7 (C-1), and 80.7 (C-2). HMQC showed a β-configuration for the Glc*p*A units with high-field H-1′ signals of that linked to α-Man at δ 4.52 and a minor H-1′ signal of Glc*p*A linked to β-Man at δ 4.60. The HMQC-COSY approach showed the glycosidic substitution to be at O-2 of the α- and β-Man anomers with H-2/C-2 correlations at δ 4.08/78.3 and 4.15/80.7, respectively.

The ^{13}C NMR spectrum of Oligo-2 (Fig. 3B) contained signals corresponding to those of Oligo-1 at δ 103.6, 101.5, 93.7, 92.0, but with additional ones at δ 101.4

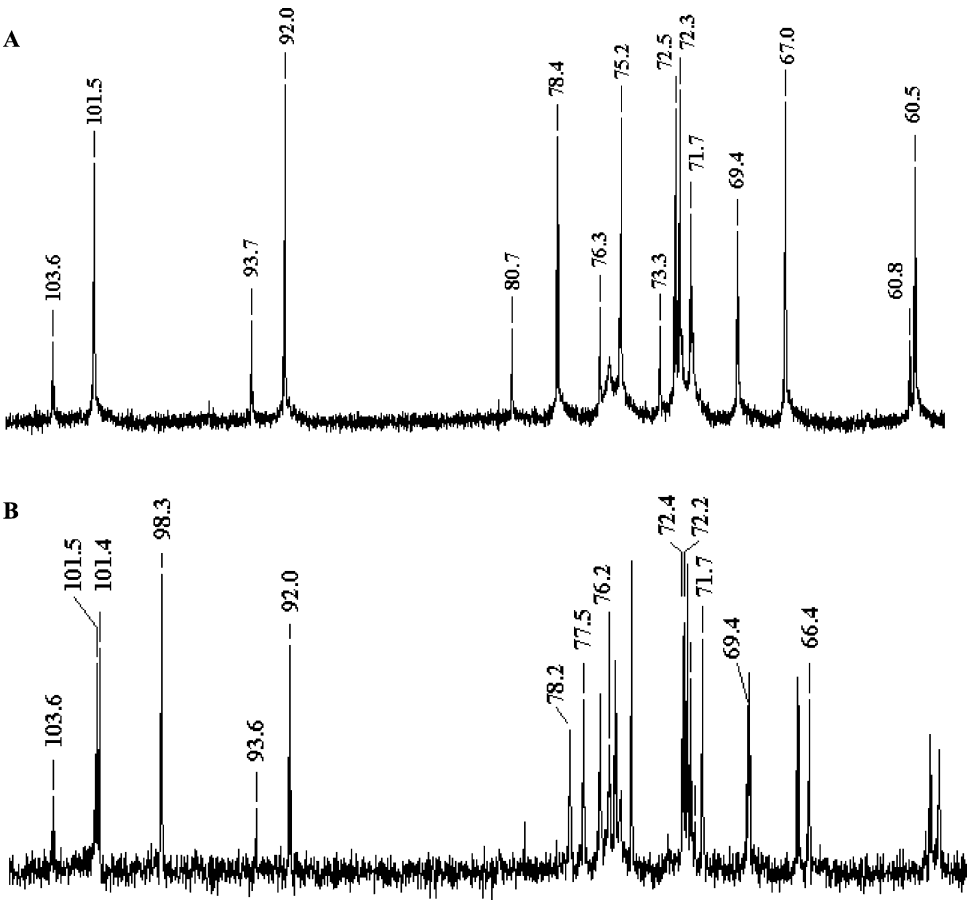


Fig. 3. ^{13}C NMR spectra of Oligo-1 (A) and Oligo-2 (B) in D_2O at 30 °C.

Table 2
^{1}H and ^{13}C NMR assignments for tetrasaccharide Oligo-2 (1)[a] based on COSY-HMQC approach

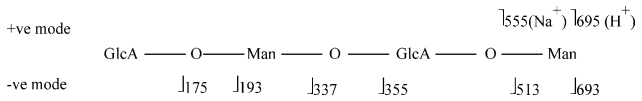
Unit	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4
β-Glc*p*A‴	4.48/101.5	3.38/72.25	3.71/67.0	3.52/71.7
→ 2)-α-Man*p*″-	5.27/92.0	4.03/78.2	–	–
→ 4)-β-Glc*p*A′-	4.46/101.4	3.36/72.4	3.67/66.4	3.78/76.25
→ 2)-α-Man*p*	5.40/98.3	4.18/77.5	–	–

[a] Shift values in δ ppm, solvent D_2O at 30 °C.

and 98.3. Although COSY examination only provided correlation up to H-2 for the α-Man*p* and H-4 for the β-Glc*p*A units, examination of its HMQC spectrum confirmed that the two Man*p* units were 2-*O*-substituted in the α-configuration and that one of the β-Glc*p*A units was 4-*O*-substituted (Table 2). On the basis of these proton data, the ROESY spectrum of Oligo-2 showed evidence of structure **1**. This structure with

β-Glc*p*A-(1→2)-α-Man*p*-(1→4)-*β*-Glc*p*A-(1→2)-α-Man*p* (1)

alternating repeating groups, each of GlcA to Man, was confirmed by ESI-MS in the -ve mode. A small molecular ion appeared at *m*/*z* 693 with a large daughter ion at *m*/*z* 513, corresponding to removal of the reducing end-unit. Others arose from successive cleavages occurring on both sides of other glycosidic bonds to form ions with groups at *m*/*z* 175 and 193, and at *m*/*z* 337 and 335. In the +ve mode, a prominent molecular ion was at *m*/*z* 695 (H^+ adduct) and only one daughter ion appeared at *m*/*z* 555 (Na^+ adduct) by removal of the Man reducing end.



The formation of a tetrasaccharide from VSP with alternating units of glucuronic acid and mannose, on partial acid hydrolysis, shows that it has the basic main-chain structure as found in the polysaccharide of gum ghatti (Stephen, 1983). This was based mainly on analyses by Aspinall, Auret, and Hirst (1958) and Aspinall, Bhavanandan, and Christensen (1965). However, an anomaly was reported by Aspinall and Christensen (1965), since on partial acetolysis, followed by deacetylation Glc*p*A-(1 → 2)-Man*p*-(1 → 2)-Man was apparently formed. A methylation, followed by a reduction step and a conversion to an methyl *O*-methyl glycosidic mixture was carried out and GC examination showed the presence of 3,4,6-Me_3-Man and 2,3,4-Me_3-Glc derivatives, although a trace of a di-*O*-methyl hexose was detected, which might have arisen from structure **1**.

Despite the similarity of the main chains of VSP and the polysaccharide from gum ghatti, the structure of VSP is much simpler as shown by the C-1 region of its ^{13}C NMR spectrum (Fig. 1A) when compared with that of gum ghatti (Fig. 1C; Tischer et al., 2002b). The viscosity of solutions of VSP are also much less than those of gum ghatti. The main chain of VSP is also similar to that of a glucuronomannoglycan another of the family Combretaceae (Myrtales), *Anogeissus leiocarpus* (Stephen, 1983).

The only resemblance found between VSP and a known polysaccharide is with an arabinoglucuronomannan isolated by Mori and Katō (1981) from suspension-cultures of *Nicotiana tabacum*. It gave rise to a C-1 region with similarities, except that a minor α-Ara*f* signal was present. Although there are differences in chemical shift values, with its main C-1 signals at δ 103.5 (β-Glc*p*A) and 100.3 (α-Man*p*), these were obtained using different spectral conditions, namely 0.9% NaCl in D_2O at 60 °C with 4,4-dimethyl-4-silapentane-1-sulphonate as internal standard. These, with a correction of approx. −1.8 ppm, correspond to ours for VSP, which were at δ 101.5 and 98.6, being obtained in D_2O at 30 °C, with Me_4Si as external standard.

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