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Structure of a heteroxylan of gum exudate of the palm *Scheelea phalerata* (uricuri)

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Abstract

The polysaccharide isolated from the gum exudate of palm *Scheelea phalerata* (SPN) was water-insoluble and composed of Fuc, Ara, Xyl, and uronic acid moieties in a 5:34:54:7 molar ratio: 12% of phenolics were also present. A soluble polysaccharide (SPNa) was obtained after alkaline treatment, which contained Fuc, Ara, Xyl and uronic acid in a 7:44:42:7 molar ratio, with only 2% phenolics. SPNa had an $M_w \sim 1.04 \times 10^5$ g mol^{-1} and was almost monodisperse ($M_w/M_n : 1.25 \pm 0.22$). It had a branched structure with side chains of 2-*O*-substituted Xyl*p* (~8%) and 3-*O*-substituted Ara*f* (12%) units, and a large proportion of nonreducing end-units of Ara*f* (15%), Fuc*p* (10%), Xyl*p* (4%), and Ara*p* (6%). The (1 → 4)-linked β-Xyl*p* main-chain units were 3-*O*- (9%), 2-*O*- (13%), and 2,3-di-*O*- (13%) substituted. Its ^{13}C NMR spectrum contained at least 9 C-1 signals, those at δ 108.6 and 107.7 arising from α-Ara*f* units. Others were present at δ 175.4 from C-6 of α-Glc*p*A and δ 15.6 from C-6 of Fuc*p* units. The main chain of SPNa was confirmed by analysis of a Smith-degraded polysaccharide (SPDS): methylation analysis provided a 2,3-Me$_2$–Xyl (65%) derivative and its ^{13}C NMR spectrum showed five main signals typical of a (1 → 4)-linked β-Xyl*p* units. Methylation analysis of a carboxy-reduced polysaccharide (SPN-CR) revealed a 2,3,4,6-Me$_4$–Glc derivative (4%) arising from nonreducing end-units of Glc*p*A. α-Glc*p*A-(1 → 2)-αβ-Xyl*p* and α-Glc*p*A-(1 → 2)-β-Xyl*p*-(1 → 4)-αβ-Xyl*p* were obtained via partial acid hydrolysis of SPN, showing the structure of side-chain substituents on O-2 of the main-chain units.

Keywords: *Scheelea phalerata*; Arecaceae; Gum exudate; Heteroxylan

1. Introduction

Scheelea phalerata (Mart.) Burret (Brazilian common name: uricuri) is a palm tree (family Arecaceae) that produces a gum exudate that can form an aqueous gel. A preliminary examination of its polysaccharide showed it to be a "heavily substituted xylan" (Stephen, 1983), which is composed of a β-Xyl*p* (1 → 4)-linked main-chain totally *O*-substituted with complex side-chains. Some gum exudates have been shown to contain such polysaccharides, namely *Achras sapota* (Sapotaceae) (White, 1953a,b, 1954; Lambert et al., 1968), *Cercidium australe* (Fabaceae) (Cerezo et al., 1969), *Cercidium praecox* (Fabaceae) (Léon De Pinto et al., 1994) and the palms *Livistona chinensis* (Maurer-Menestrina et al., 2003) and *Syagrus romanzoffiana* (Simas et al., unpublished results), both of the Arecaceae family. The polysaccharides of gum exudates from these palms were shown to be complex, acidic heteroxylans containing fucosyl units, which had not been previously reported in plant gum exudate polysaccharides. This feature might be used as an aid to chemotaxonomy of palms: a structural analysis of the gum exudate polysaccharide from *Scheelea phalerata* is now carried out to show relationships between members of the Arecaceae family.

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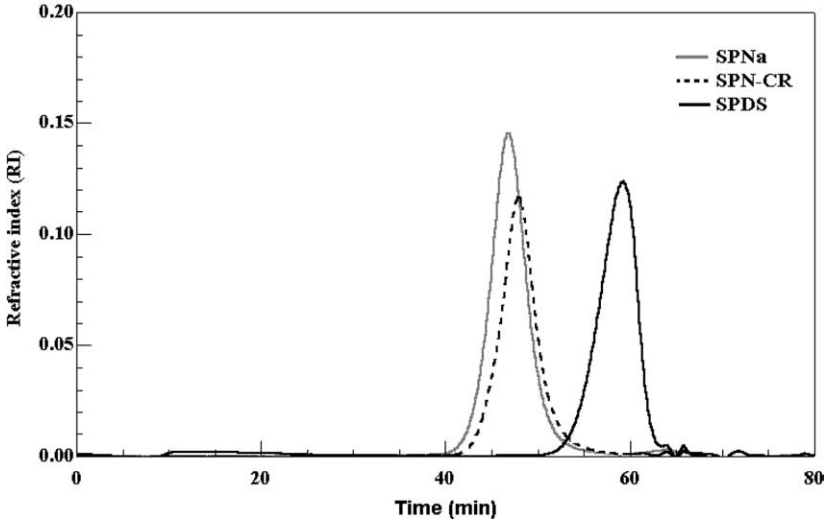

Fig. 1. Elution profile of SPNa, SPN-CR and SPDS at HPSEC-MALLS using a refractive index detector.

2. Results and discussion

The polysaccharide of *Scheelea phalerata* (SPN) was isolated by dispersing the gum in water, filtration of larger fragments through a fine cloth, and precipitation with ethanol. The resulting polysaccharide (SPN) was water-insoluble. A water-soluble product (SPNa) was obtained on treatment with 0.5 M NaOH at 25 °C, followed by neutralization and dialysis. SPN and SPNa contained Fuc, Ara, Xyl and uronic acid in 5:34:54:7 and 7:44:42:7 molar ratios, respectively (Table 1). HPSEC-MALLS analysis of SPNa showed it to have $M_w \sim 1.04 \times 10^5$ g mol^{-1} (10% error) (Fig. 1) and its polydispersity (M_w/M_n) of 1.25 ± 0.22 showed it to be near to monodisperse. The presence of uronic acid units was confirmed by GC-MS of a carboxy-reduced native polysaccharide (SPN-CR) that contained Glc and 4-Me–Glc (7:1 ratio), arising from Glc*p*A and 4-Me–Glc*p*A units, respectively.

Methylation GC-MS analysis was carried out using DB-225 and DB-210 capillary columns. The 2-*O*- and 3-*O*-Me–xylitol acetates were resolved using a the DB-210 column and 2,3,4-Me$_3$–Xyl, 2,3,4-Me$_3$–Ara, and the 2,3,4-Me$_3$–Fuc derivatives with a DB-225 column, although it was necessary to employ a lower temperature for the complete analysis. SPNa (Table 2) had a branched structure with a large proportion of nonreducing end-units (35%) of Ara*f* (15%), Fuc*p* (10%), Xyl*p* (4%), and Ara*p* (6%). The side-chains were composed of 2-*O*-linked Xyl*p* ($\sim$ 8%) and 3-*O*-linked Ara*f* (12%). The 2,3-Me$_2$–Xyl (10%) derivative arose from the 4-*O*-linked Xyl*p* of side chains or unsubstituted ones of the backbone. The 2-Me–Xyl (9%), 3-Me–Xyl (13%) and Xyl (13%) alditol acetates arose from the 3-*O*-, 2-*O*- and 2,3-*O*-linked units in the main-chain of $(1 \rightarrow 4)$-linked Xyl*p*, respectively. Methylation analysis of carboxy-reduced SPN-CR revealed a 2,3,4,6-Me$_4$–Glc derivative (4%) arising from Glc*p*A nonreducing end-units (Table 2).

The ^{13}C NMR spectrum of SPNa (Fig. 2(a)) had a complex anomeric region with at least 9 signals. Those at δ 108.6 and δ 107.7 were from 3-*O*-linked and nonreducing α-Ara*f* units, respectively (Ebringerová et al., 1990; Delgobo et al., 1998). The signals at δ 101.6 and δ 101.3 were from 4-*O*-linked and 2-*O*- and/or 3-*O*-linked β-Xyl*p* units, respectively (Gast et al., 1980; Bochicchio and Reicher, 2003), besides signals at δ 97.8–96.7, δ 102.6 and δ 103.1, which can be assigned to C-1 of nonreducing end units of α-Glc*p*A (Léon De Pinto et al., 1994), β-Xyl*p* (Gast et al., 1980), and α-Ara*p* (Gorin and

Table 1
Monosaccharide composition and colorimetric analysis of native polysaccharide of *Scheelea phalerata* gum exudate (SPN and SPNa), carboxy-reduced polysaccharide (SPN-CR) and Smith-degraded polysaccharide (SPDS)

Polysaccharide	Monosaccharide composition (mol%)[a]						Total sugar[c] (%)	Protein[d] (%)	Phenolics[e] (%)
	Fuc	Ara	Xyl	Glc	4-Me–Glc	Uronic acid[b]			
SPN	5	34	54	–	–	7	72	–	12
SPNa	7	44	42	–	–	7	76	–	2
SPN-CR	8	30	53	7	1	2	nd	nd	nd
SPDS	–	17	80	–	–	3	84	nd	nd

[a]Analyzed on a DB-225 column by GC-MS after total hydrolysis, reduction and acetylation. Determined by the colorimetric methods of [b]Filisetti-Cozzi and Carpita (1991). [c]Dubois et al. (1956). [d]Peterson (1977). [e]Bochicchio and Reicher (2003). nd, not determined.

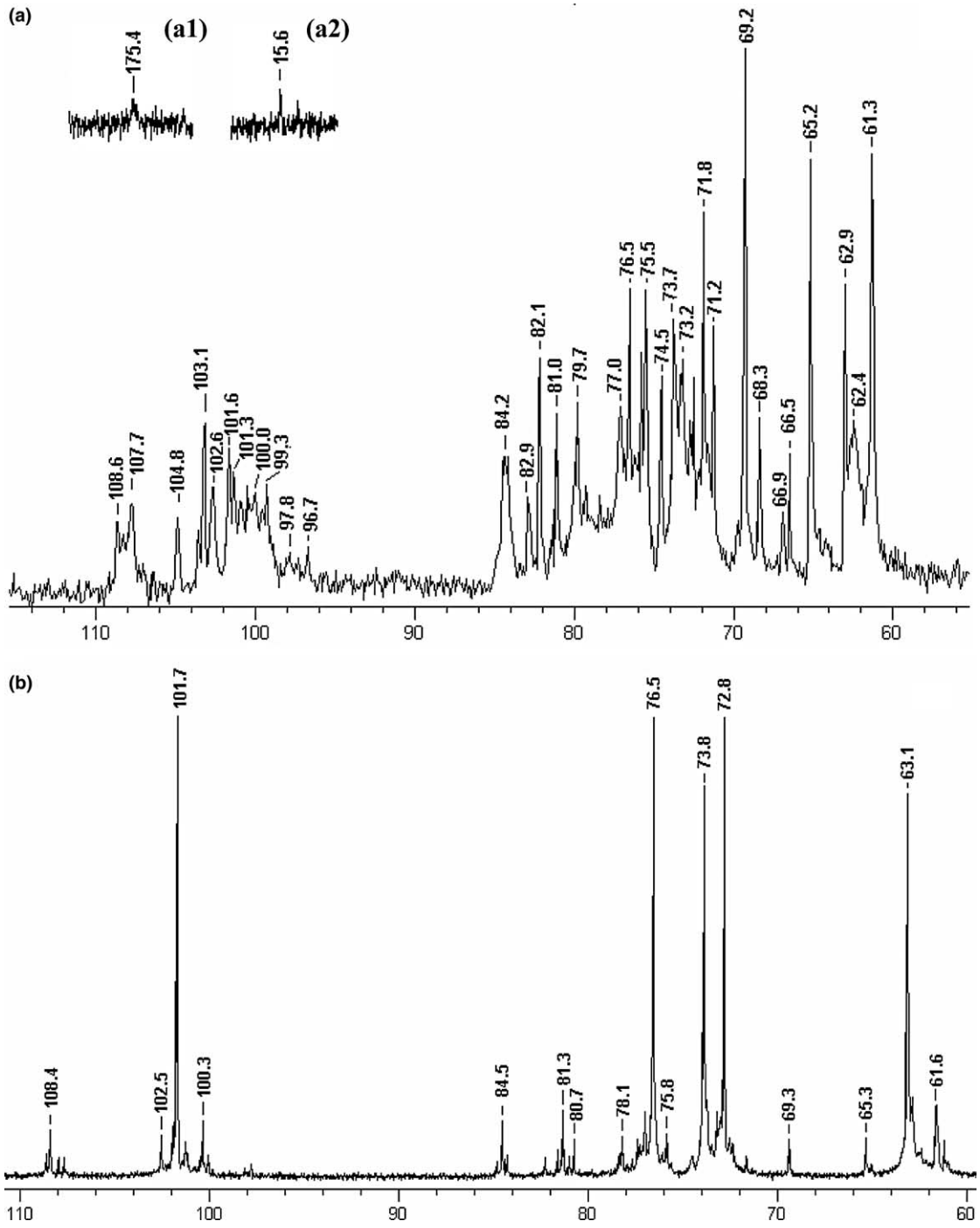

Fig. 2. ^{13}C NMR spectra of polysaccharide SPNa from *Scheelea phalerata* gum exudate (a) and polysaccharide obtained by controlled Smith degradation (SPDS) (b). Solvent D_2O at 30 °C (a) and 50 °C (b) with numerical values in δ (ppm). Insets: $\underline{C}O_2H$ (a1) and $\underline{C}H_3$ (a2) regions.

Mazurek, 1975), respectively. The resonance at δ 175.4 arose from C-6 of Glc*p*A (Delgobo et al., 1998) and that at δ 15.6 from CH_3 group of Fuc*p* units (Aspinall et al., 1991; Alquini et al., 2004). Signals at δ 84.2 and δ 61.3 are consistent with C-4 and C-5, respectively, of α-L-Ara*f* units (Delgobo et al., 1998). The complexity of the NMR and the methylation analysis indicated a complex, highly branched structure for SPNa.

A controlled Smith degradation revealed the core structure of SPNa: the product Smith-degraded polysaccharide (SPDS) (14% yield) gave rise to a homogeneous HPSEC-MALLS profile with a lower M_w than that of SPNa (Fig. 1). The M_w of SPDS could not be accurately calculated because it proved not to be completely monodisperse when a less quantitative light scattering detector was used (not shown). SPDS

Table 2
Partially *O*-methylalditol acetates formed on methylation analysis of native polysaccharide (SPNa), carboxy-reduced polysaccharide (SPN-CR) and Smith-degraded polysaccharide (SPDS)

Alditol acetate	Deduced linkages	R_t(min)[a]	% Fragment area		
			SPNa	SPN-CR	SPDS
2,3,5-Me_3–Ara	Ara*f*-(1→	7.83	15	4	13
2,3,4-Me_3–Ara[b]	Ara*p*-(1→	8.38	6	3	–
2,3,4-Me_3–Xyl[b]	Xyl*p*-(1→	8.46	4	1	4
2,3,4-Me_3–Fuc[b]	Fuc*p*-(1→	8.46	10	7	–
2,5-Me_2–Ara	→3)-Ara*f*-(1→	9.71	12	12	–
2,3,4,6-Me_4–Glc	Glc*p*-(1→	10.13	–	4	–
2,3-Me_2–Xyl[c]	→4)-Xyl*p*-(1→	10.91	∼10	∼10	65
3,4-Me_2–Xyl[c]	→2)-Xyl*p*-(1→	10.91	∼8	∼8	–
2-Me–Xyl[d]	→3,4)-Xyl*p*-(1→	14.04	9	13	5
3-Me–Xyl[d]	→2,4)-Xyl*p*-(1→	14.04	13	20	10
Xyl	→2,3,4)-Xyl*p*-(1→	18.19	13	18	3

[a] Retention times obtained with a DB-225 column at 215 °C.
[b] These were resolved with a DB-225 column at 180 °C with retention times of 2,3,4-Me_3–Ara, 2,3,4-Me_3–Xyl and 2,3,4-Me_3–Fuc of 17.91, 18.27 and 18.47 min, respectively.
[c] These are approximate values obtained by comparison of the heights of ions with *m*/*z* 118 and 129 (2,3-isomer) with those with *m*/*z* 117 and 130 (3,4-isomer).
[d] Resolved and quantified using a DB-210 column.

consisted of Ara:Xyl and uronic acid in a 17:80:3 molar ratio (Table 1) and its methylation analysis (Table 2) gave a 2,3-Me_2–Xyl (65%) derivative, corresponding to (1 → 4)-linked Xyl*p* main-chain units. Some of these units were substituted at O-3- (5%), O-2- (10%) and O-2,3 (3%) by non-reducing end units of Ara*f* (2,3,5-Me_3–Ara: 13%) and Xyl*p* (2,3,4-Me_3–Xyl: 4%). The ^{13}C NMR spectrum of SPDS (Fig. 2(b)) contained five prominent signals typical of (1 → 4)-linked β-Xyl*p* main-chain units (Ebringerová et al., 1990; Léon De Pinto

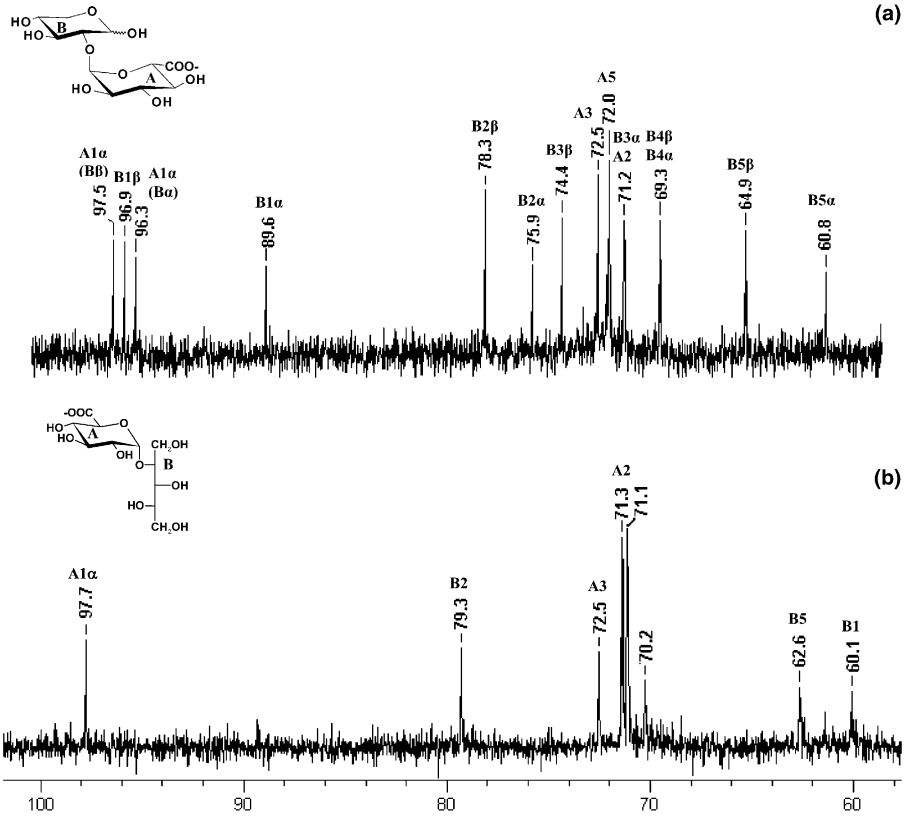

Fig. 3. ^{13}C NMR spectra of oligo-0.53 (a) and $NaBH_4$-reduced oligo-0.53 (b). The symbols (A) and (B) correspond to Glc*p*A and Xyl*p* residues, respectively; numbers 1–5 indicate the corresponding carbon nuclei. Solvent D_2O at 30 °C. Numerical values in δ (ppm).

Table 3
^{1}H and ^{13}C chemical shifts for oligo-0.53 and oligo-0.45, as determined by HMQC, COSY and TOCSY analysis

Oligo-0.53		α-Glc*p*A-(1 → (β-Xyl)	α-Glc*p*A-(1 → (α-Xyl)	→2)-β-Xyl	(→2)-α-Xyl
H-1/C-1	–	5.379/97.5	5.088/96.3	4.720/96.9	5.385/89.6
H-2/C-2	–	3.584/71.2	3.593/70.6	3.400/78.3[a]	3.648/75.9[a]
H-3/C-3	–	3.761/72.5	3.795/72.5	3.511/74.4	3.753/71.2
H-4/C-4	–	3.496/71.8	3.511/71.8	3.650/69.3	3.710/69.3
H-5/C-5	–	4.300/71.8	4.204/72.0	3.320 (ax.) 3.930 (eq.)/ 64.9	3.680 (ax.) 3.715 (eq.)/ 60.8
Oligo-0.45	→2)-β-Xyl-(1→	α-Glc*p*A-(1 → (β-Xyl)	α-Glc*p*A-(1 → (α-Xyl)	→4)-β-Xyl	→4)-α-Xyl
H-1/C-1	4.628–4.500/101.6	5.331/97.5	5.360/97.5	4.595/96.4	5.192/91.9
H-2/C-2	3.474/76.5[a]	3.560/71.0	3.579/71.0	3.258/73.9	3.552/na
H-3/C-3	3.511/74.4	3.759/72.6	3.759/72.6	3.587/73.9	3.783/na
H-4/C-4	3.674/69.3	3.492/71.9	3.492/71.9	3.800/76.1[a]	na
H-5/C-5	3.328 (ax.) 4.003 (eq.)/ 64.9	4.341/72.0	4.349/72.0	3.460 (ax.) 4.125 (eq.)/ 62.8	3.820 (ax.) 3.900 (eq.)/ 58.6

na, not assigned.
[a] Downfield from resonances of similar residues that are unsubstituted at indicated position. Values compared with standard shifts of methyl glycosides (Gorin and Mazurek, 1975).

et al., 1994; Maurer-Menestrina et al., 2003). The signal at δ 101.2 corresponded to C-1 of 2-*O*-substituted Xyl*p* main-chain units (Bochicchio and Reicher, 2003) that were not eliminated by the controlled Smith degradation. Those at δ 102.5, δ 69.3 and δ 65.3 can be assigned to C-1, C-4, and C-5, respectively, of remaining nonreducing end units of Xyl*p*, respectively (Gorin and Mazurek, 1975; Gast et al., 1980). The signals of α-L-Ara*f* non-reducing end units were at δ 108.4 (C-1), δ 80.7 (C-2), δ 78.1 (C-3), δ 84.5 (C-4) and 61.6 (C-5) (Ebringerová et al., 1990; Hirsch and Schraml, 1984). The resistance of Ara*f* units to periodate oxidation could be due to substitution at O-3 by fucosyl, arabinosyl, or xylosyl residues that occurred in SPNa. Taken together, the results suggest that SPDS is an arabinoxylan with a main chain of (1 → 4)-linked β-Xyl*p* units, which had substitutions at O-2 (10%) by Ara*f* nonreducing end units, at O-3 (5%) by Xyl*p* nonreducing end units, with a few units being di-*O*-substituted (3%). Although 3% of uronic acid was detected, no corresponding ^{13}C NMR spectroscopic signals were found. Its resistance to periodate oxidation could be due to 3-*O*-substitution, although partial hydrolysis did not give rise to a corresponding aldobiouronic acid, only that with an α-Glc*p*A-(1 → 2)-Xyl*p* structure (see below).

Further details on acidic side-chain components of SPNa were indicated by the structure of uronic acid-containing oligosaccharides liberated on mild acid hydrolysis. Two oligosaccharides were isolated with R_{Lact} 0.45 (oligo-0.45) and 0.53 (oligo-0.53). That with R_{Lact} 0.53 was characterized as a α-Glc*p*A-(1 → 2)-αβ- Xyl*p*. It ^{13}C NMR spectrum (Fig. 3(a)) showed four signals in C-1 region, which were consistent with α-Glc*p*A linked to α-Xyl*p* (δ 96.3), α-Glc*p*A linked to β-Xyl*p* (δ 97.5), α- and β-Xyl*p* reducing units (δ 89.6 and δ 96.9, respectively) (Maurer-Menestrina et al., 2003; Simas et al., unpublished results). The other ^{13}C NMR spectral signals were assigned by COSY, TOCSY and HMQC (Table 3). The C-2 shifts of α-Xyl*p* (δ 75.9) and β-Xyl*p* (δ 78.3) were consistent with 2-*O*-substituted Xyl*p* reducing end-units. The signals at δ 64.9 and δ 60.8 were from C-5 ($\underline{C}H_2$) of β-Xyl*p* and α-Xyl*p*, respectively. These were confirmed by inverted $\underline{C}H_2$ signals in the DEPT spectrum. After $NaBH_4$-reduction of oligo-0.53, ^{13}C NMR spectral analysis showed only a δ 97.7 signal (Fig. 3(b)) from C-1 of α-Glc*p*A terminal units and the C-1 signal of the reduced Xyl*p* residue appeared at δ 60.1, confirming the disaccharide structure. The signal at δ 62.6 was from C-5 of the reduced Xyl*p* units. Oligo-0.45 was submitted to methylation analysis and NMR spectroscopy. Methylation analysis involving a carboxy-reduction step incorporating NaB^2H_4 gave rise to acetates of 2,3-Me_2–Xyl-1-^2H (43%), 3,4-Me_2–Xyl-1-^2H (30%) and 2,3,4-Me_3–Glc-1-^2H, 6-$^2H_2$ (27%), arising from 4-*O*-, 2-*O*-substituted Xyl units and Glc*p*A non reducing end-units, respectively, consistent with α-Glc*p*A-(1 → 2)-β-Xyl*p*-(1 → 4)-αβ-Xyl*p* structure. In the ^{13}C NMR spectrum of the oligo-0.45 (Fig. 4(a)), the signals at δ 101.6 and δ 97.5 are from 2-*O*-substituted β-Xyl and α-Glc*p*A nonreducing end units, respectively (see Cavagna et al. (1984) for the 4-*O*-methyl derivative). Those at δ 96.4 and δ 91.9 were from 4-*O*-substituted Xyl*p* reducing end-units with respective β and α-configurations. The other ^{13}C signals were identified using COSY, TOCSY and HMQC analysis (Table 3), from which can be assigned a downfield C-2 signal (δ 76.5) of the middle β-Xyl*p* units, confirming the 2-*O*-substitution. The C-4 signal (δ 76.1) of Xyl*p* reducing end units also showed a downfield signal (α-shift + 7 ppm) that indicated substitution at O-4 of this unit. The signals at δ 64.9, δ 62.8 and δ 58.6 were from C-5 of 2-*O*-substituted Xyl*p*, β- and α-Xyl*p* reducing end units, respectively,

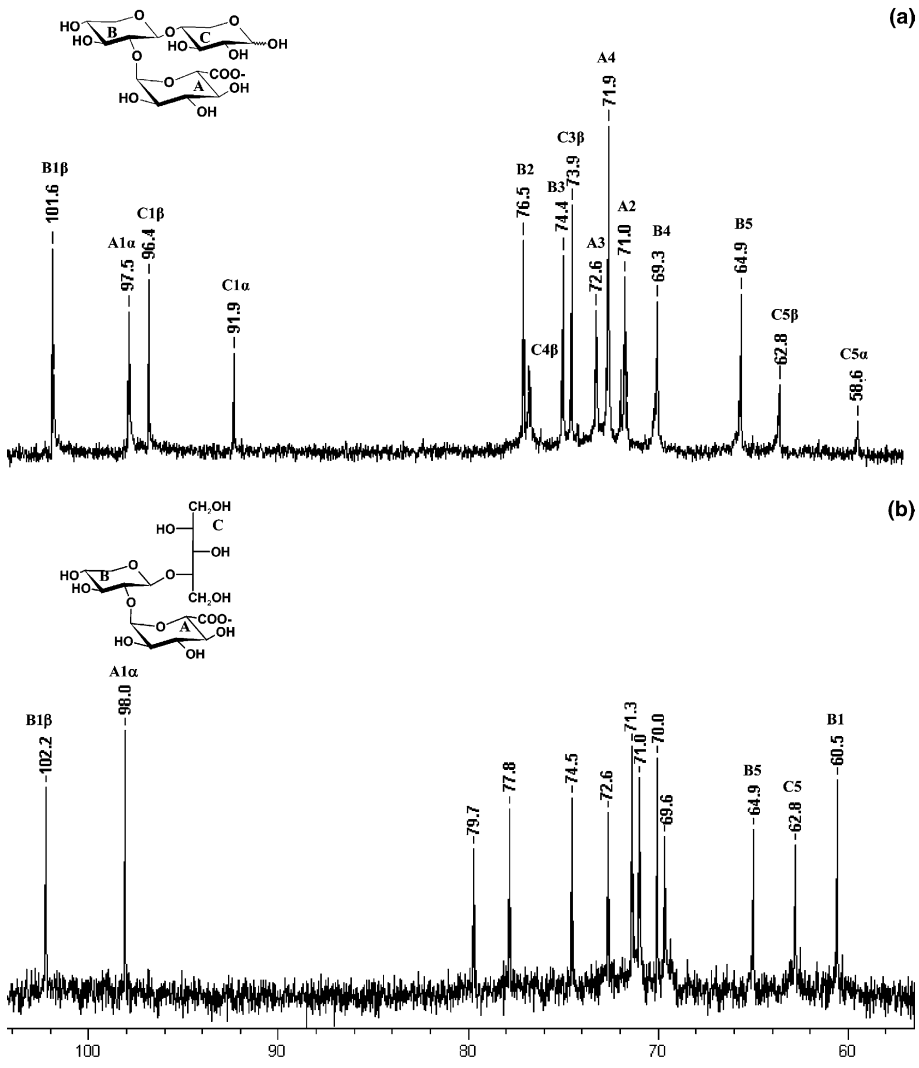

Fig. 4. ^{13}C NMR spectra of oligo-0.45 (a) and $NaBH_4$-reduced oligo-0.45 (b). The symbols (A), (B) and (C) correspond to Glc*p*A, middle Xyl*p*, and Xyl*p* reducing end-units, respectively; numbers 1–5 indicate the corresponding carbon nuclei. Solvent D_2O at 30 °C. Numerical values in δ (ppm).

which were confirmed by its inverted $\underline{C}H_2$ signals in the DEPT spectrum. Cavagna et al. (1984) and Maurer-Menestrina et al. (2003) found similar signals and assigned an aldotriouronic acid structure to a oligosaccharide obtained from birch wood and an oligosaccharide obtained from *L. chinensis* gum exudate, respectively, although their Glc*p*A units were 4-*O*-methylated. The ^{13}C NMR spectrum of $NaBH_4$-reduced oligo-0.45 (Fig. 4(b)) contained two signals in the anomeric region, that at δ 102.2 arising from the middle 2-*O*-substituted β-Xyl*p* unit and the other at δ 98.0 from α-Glc*p*A terminal units. The C-1 of reduced Xyl*p* was at δ 60.5 and its C-5 was at δ 62.8. This analysis confirmed the above trisaccharide structure.

The structures of oligo-0.45 and oligo-0.53 were in accord with their MALDI-TOF (+ve-ion mode) spectra, which contained molecular ions (Na^+ forms) with *m*/*z* 481 and *m*/*z* 349 corresponding to Glc*p*A–Xylp_2 and Glc*p*A–Xyl*p*, respectively. Partial acid hydrolysis of the polysaccharide from the palm *L. chinensis* (Maurer-Menestrina et al., 2003) gave rise to related oligosaccharides, except that 4-Me–Glc*p*A was present in a higher proportion than Glc*p*A. However, only Glc*p*A-containing oligosaccharides have now been characterized, although a trace of the 4-*O*-methyl derivative was present in SPNa.

The structure of the gum exudate polysaccharide of the palm *Scheelea phalerata* resembles closely those of the palms *L. chinensis* (Maurer-Menestrina et al., 2003) and *Syagrus romanzoffiana* (Simas et al., unpublished results). These polysaccharides have heteroxylan structures with similar monosaccharide compositions, but with differences in the proportions of Glc*p*A and it 4-*O*-methyl derivative. As previously mentioned, an interesting characteristic is the presence of fucosyl units as non-reducing end units in all three gums, which has not been found in other families of gum exudates. Further studies on other palm polysaccharides are necessary to

confirm if their presence can be a chemotaxonomic aid for members of Arecaceae family.

3. Experimental

3.1. Collection of gum exudate and isolation of its polysaccharide (SPN)

The gum exudate of *Scheelea phalerata* was collected from its trunk by Artur César Pinheiro Leite (SEPROF-Ac) in the State of Acre, Brazil. A sample (47 g) was stirred overnight in H_2O (~4 l), after which a dispersion was formed containing insoluble fragments, from which larger ones were removed by passage through a fine cloth. The filtrate was added to excess EtOH (3×), to give a precipitate, which was redispersed in H_2O, dialyzed against tap water (24 h), and then freeze-dried to give water-insoluble SPN (44% yield).

3.2. Preparation of SPNa

SPN (2.3 g) was solubilized in 0.5 M NaOH (300 ml) at 25 °C in the presence of a trace of $NaBH_4$, and the resulting solution was neutralized (HOAc) and then dialyzed (limit of exclusion mol. wt.16,000). It was freeze-dried to give a residual, water-soluble polysaccharide (2.0 g; SPNa).

3.3. Controlled Smith degradation of SPNa to give SPDS

SPNa (2 g) was dissolved in H_2O (200 ml) and oxidized with 0.1 M $NaIO_4$ (200 ml) for 72 h in the dark and ethylene glycol (35 ml) then added. The resulting solution was dialyzed (48 h) and reduced with $NaBH_4$ (until pH 10) overnight followed by neutralization (HOAc) and dialysis (48 h). The volume of solution was then reduced to ~20 ml with the pH adjusted to pH 2.0 with TFA and the polyalcohol partially hydrolyzed at ~96 °C for 40 min. The resulting solution was treated with 1 M NaOH to pH 5.0 and reduced in volume in vacuo to ~15 ml. The SPDS polysaccharide (14% yield) was obtained after precipitation with EtOH (100 ml).

3.4. Carboxy-reduction of SPNa to give SPN-CR

SPNa was carboxy-reduced using two successive cycles with carbodiimide–$NaBH_4$ according to Taylor and Conrad (1972) to give SPN-CR.

3.5. Colorimetric analysis

Total sugar was determined by phenol-sulfuric acid method (Dubois et al., 1956) using xylose as standard. Uronic acids were estimated using the improved *m*-hydroxybiphenyl method (Filisetti-Cozzi and Carpita, 1991) using glucuronic acid as standard, protein by the method of Peterson (1977) and phenolics as described by Bochicchio and Reicher (2003).

3.6. Monosaccharide analysis

Monosaccharide compositions were determined by hydrolysis with 1 M TFA at 100 °C for 8 h, the hydrolyzates evaporated to dryness, and the residues reduced with $NaBH_4$ and then acetylated with Ac_2O–pyridine at room temperature for 12 h (Wolfrom and Thompson, 1963a,b). The resulting alditol acetates was analyzed by GC-MS using a Varian model 3300 gas chromatograph coupled to a Finnigan Ion-Trap (model 810 R-12) mass spectrometer with a DB-225 capillary column (30 m × 0.25 mm i.d.) programmed from 50 °C (during injection) to 220 °C (constant temperature) at 40 °C min^{-1}. He was used as carrier gas. The products were identified by their typical retention times and electron impact profiles (Jansson et al., 1976).

3.7. Determination of homogeneity and molar mass (M_w) of SPNa, SPN-CR and SPDS fractions

HPSEC-MALLS analysis of fractions was carried out using a Waters size exclusion chromatography (SEC) apparatus coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering (MALLS) detector (data not shown). Four columns of Waters Ultrahydrogel 2000/500/250/120 were connected in series and coupled to a multidetection system. Refractive index increments were determined by using a Waters 2410 detector. The value of d*n*/d*c* (differential refractive index increment of the solvent–solute solution with respect to a change in solute concentration) was 0.139 for the SPNa fraction. Samples (1 mg/ml) were filtered through a 0.22 μm filter (Millipore) and then injected (250 μl loop). The eluent was 0.1 mol l^{-1} aq. $NaNO_2$ containing 200 ppm aq. NaN_3.

3.8. Methylation analysis

Polysaccharides (10 mg) were methylated according to Ciucanu and Kerek (1984), by dissolution in Me_2SO, followed by addition of powdered NaOH and MeI, the mixture being left for 18 h. Oligo-0.45 (2 mg) was methylated according to Tischer et al. (2002), by dissolving it in a drop of water before addition of Me_2SO, MeI was then added followed by NaOH. The resulting per-*O*-methylated derivatives were treated with 3% HCl–MeOH for 2 h at 80 °C and neutralized with Ag_2CO_3. In order to detect partially *O*-methylated fragments arising from Glc*p*A of oligo-0.45, its methanolysis product was carboxy-reduced with NaB^2H_4 in 0.1% NaOMe in MeOH at 70 °C for 2 h. The product

was then hydrolyzed with 1 M H_2SO_4 at 100 °C for 8 h. The resulting mixtures of partially *O*-methylated aldoses were reduced with NaB^2H_4 and acetylated as described above to give a mixture of partially *O*-methylated alditol acetates, which were analyzed by GC-MS. For this analysis, DB-225 and DB-210 capillary columns (30 m × 0.25 mm i.d.) were used, the former for overall identification of components at 50 to 215 °C (40 °C min^{-1}), with this temperature been maintained at 215 °C for 40 min. For resolution of alditol acetates of 2,3,4-Me_3–Fuc, 2,3,4-Me_3–Ara and 2,3,4-Me_3–Xyl, a temperature of 180 °C was necessary. The DB-210 column of similar dimensions, controlled at 215 °C, resolved acetates of 2-Me- and 3-Me–xylitol. The resulting partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (Jansson et al., 1976).

3.9. Production of uronic acid-containing oligosaccharides

SPN (3.5 g) was dissolved in H_2O (40 ml) overnight with TFA then added to give a 1 M concentration, and the solution then submitted to partial hydrolysis at ~96 °C for 2 h. After evaporation to dryness, the residue was dissolved in H_2O and fractionated on a charcoal–Celite column (Whistler and Durso, 1950; Whistler and BeMiller, 1962), which was eluted with H_2O followed by 40% aq. EtOH (Maurer-Menestrina et al., 2003). The fraction eluted with EtOH was purified on Whatman 3 MM filter paper (solvent: *n*-BuOH–pyridine–H_2O, 5:3:3 v/v) resulting in two components having R_{Lact} 0.53 and 0.45 in the same solvent and called oligo-0.53 and oligo-0.45, respectively.

3.10. Nuclear magnetic resonance spectroscopy

NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer with a 5 mm inverse probe. ^{13}C NMR (100.6 MHz) analyses were performed at 50 or 30 °C on D_2O solutions. Chemical shifts of the samples are expressed in ppm (δ) relative to internal standard of acetone (δ 30.2 and δ 2.22 for ^{13}C and ^{1}H signals, respectively). ^{1}H, ^{1}H COSY, TOCSY and ^{1}H (obs.) ^{13}C HMQC were carried out according to the Bruker Manual.

3.11. MALDI-TOF-MS of oligosaccharides

MALDI-TOF measurements were carried out using a Bruker BIFLEX III equipped with a 337 nm nitrogen laser, operating in a +ve-ion linear mode. The 2,5-dihydroxybenzoic acid matrix was prepared at 10 mg/ml in H_2O–CH_3CN (2:1). Samples were dissolved at concentrations between 100 and 200 pmol/μl in H_2O and mixed (5 μl) with the matrix solution (5 μl). A portion (1 μl) of the resulting mixture in 0.01 M NaCl was loaded on to the target, the solvent removed by aeration, and the sample transferred to the mass spectrometer. The standard used for calibration was Angiotensin II.

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