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STRUCTURAL STUDIES OF AN ARABINOXYLAN ISOLATED FROM THE LEAVES OF *Neolitsea cassia*

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ABSTRACT

A water-soluble arabinoxylan giving highly viscous solutions was isolated from the leaves of *Neolitsea cassia* (L.). The molar ratio of D-xylose to L-arabinose was 1:2.7, but small proportions of other sugars were also present. Linkage analysis, partial acid hydrolysis, and ^{1}H- and ^{13}C-n.m.r. spectroscopy revealed that the polysaccharide was highly branched with a backbone of (1→4)-linked β-D-xylopyranosyl residues each of which was substituted at both O-2 and O-3 with short side-chains composed of terminal α- and β-L-arabinofuranosyl groups and (1→3)-linked α-L-arabinofuranosyl residues.

INTRODUCTION

The tree *Neolitsea cassia* (Family – Lauraceae) grows in Sri-Lanka, India, and Malaya. Its bark and leaves are used for the treatment of fractures. The highly viscous, aqueous extract of the leaves is mixed with flour for the preparation of certain sweetmeats, and the powdered leaf is used for dusting the cut surface of coconut inflorescences in the process of toddy tapping[1].

We now report on the water-soluble polysaccharides of the leaf mucilage of *Neolitsea cassia*.

EXPERIMENTAL

Analysis of leaves. — Leaves from *Neolitsea cassia* (L.) were collected near Peradeniya (Sri-Lanka), freeze-dried, and ground (Wiley mill) to pass a 1-mm screen, and the contents of dry matter (d.m.), ash, and crude protein were analysed by standard methods[2]. The ground leaves were extracted with aqueous 80% ethanol and chloroform in a Soxhlet apparatus[3]. The residue was analysed for starch[4], Klason lignin[5], and non-starch polysaccharide residues[5].

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Isolation and purification of the polysaccharide. — Fresh leaves (200 g, 59.1% d.m.) were homogenised and extracted with methanol (2 L) at room temperature. The air-dried residue was further extracted with water (2 L) at room temperature. The viscous aqueous extract was squeezed through a voile cloth, dialysed for 3 days against distilled water, and deproteinised by partitioning using chloroform and 1-butanol[6]. The aqueous phase was concentrated under reduced pressure at <40°. Polymeric material, precipitated by the addition of 4 vol. of ethanol, was isolated by centrifugation, and a dispersion in water was dialysed for 2 days against distilled water and then freeze-dried to yield the crude polysaccharide (0.63% of dry leaves).

Gel-permeation chromatography. — The crude polysaccharide (25 mg) was suspended in water (15 mL), and insoluble material (12 mg) was removed by centrifugation. The supernatant solution was applied to a column (2.6 × 100 cm) of Sepharose CL-4B (Pharmacia) and eluted with water. The absorbance (280 nm) of the effluent was monitored (UVICORD SII, LKB). Fractions (6 mL) were collected and their contents of carbohydates were analysed by the phenol–sulfuric acid method[7].

A suspension of the crude polysaccharide (25 mg) in 0.1M phosphate buffer (15 mL, pH 7.5) was incubated with protease (1 mg, Type VIII from *Bacillus subtilis*; No. P-5380, Sigma) for 4 h at 60°. After removing the insoluble material (8 mg) by centrifugation, the supernatant solution was fractionated on Sepharose CL-4B as described above.

Partial acid hydrolysis. — Crude polysaccharide (50 mg) was hydrolysed with 0.125M sulfuric acid (5 mL) at 80° for 40, 80, or 160 min. Each hydrolysate was neutralised ($BaCO_3$), the supernatant solution was isolated by centrifugation[8], and polymeric material was precipitated with ethanol (4 vol.), isolated by centrifugation, washed with ethanol, and dried under vacuum.

Sugar analysis. — The content of neutral sugar residues in the isolated polysaccharides was determined by g.l.c. of the alditol acetates after hydrolysis with 2M trifluoroacetic acid for 2 h at 121°, reduction with KBH_4, and acetylation[9]. The content of glycosyluronic acid residues was determined by a decarboxylation method[5].

The absolute configuration of the main sugar residues in the crude polysaccharide (5 mg) was determined by the method of Gerwig *et al.*[10].

Linkage analysis. — Isolated polysaccharides (2 mg) were dried under vacuum, and solutions in dry methyl sulfoxide (1 mL) were methylated by a modified Hakomori method[11]. The methylated polysaccharides were hydrolysed with 2M trifluoroacetic acid for 1 h at 121° and the products were reduced (KBH_4) and acetylated[11]. The identities of the *O*-acetylated, *O*-methylated alditols were established by their g.l.c. retention times on a DB-1 capillary column[12], and by their e.i.-mass spectra[13] (70 eV, Finnigan 4021 mass spectrometer, Incos 2000 data system).

N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra (90 MHz) were recorded for solutions of dried polysaccharide fractions (~5 mg) in D_2O (0.5 mL, internal sodium

TABLE I

CHEMICAL COMPOSITION OF THE LEAVES OF *Neolitsea cassia* AND OF A CRUDE POLYSACCHARIDE ISOLATED FROM A DEPROTEINIZED WATER EXTRACT OF THE LEAVES (% OF DRY LEAVES)

Component	*Leaves*	*Crude polysaccharide*
Aqueous 80% ethanol- and chloroform-soluble extracts	29.6	
Crude protein (N × 6.25)	8.9	0.04
Starch	6.1	
Non-starch polysaccharides	39.4	0.54
Rha[a]	0.3	0.01
Ara[a]	13.0	0.40
Xyl[a]	10.1	0.13
Man[a]	0.2	0.01
Gal[a]	0.9	0.02
Glc[a]	8.7	0.02
Uronic acid[a]	4.0	0.00
Klason lignin	17.9	
Ash	2.5	

[a]Given as "anhydro" sugars.

3-trimethylsilyltetradeuteriopropionate) at 90° with a Jeol Fx-90Q spectrometer. ^{13}C-N.m.r. spectra (22.5 MHz) were recorded for solutions of dried polysaccharide (15 mg) in D_2O (1.4 mL, external Me_4Si) at 35°.

RESULTS AND DISCUSSION

The leaves from *Neolitsea cassia* (Table I) contained 39% of non-starch polysaccharides, with arabinose, xylose, and glucose as the main constituents.

The crude polysaccharide, isolated from the aqueous extract, contained mainly arabinose and xylose together with small proportions of other sugars (Table I). Protein was also present in small proportion in the crude polysaccharide, but no uronic acids could be detected. However, the arabinose and xylose residues in the crude polysaccharide constituted only 2% of the total amount of these

TABLE II

GLYCOSYL COMPOSITION[a] OF THE CRUDE POLYSACCHARIDE AND OF FRACTIONS I–IV (FIG. 1)

Residue	*Crude polysaccharide*	*I*	*II*	*III*	*IV*
Rha	1	3	2	4	4
Ara	67	64	64	63	62
Xyl	25	23	25	23	22
Man	1	2	2	2	2
Gal	3	4	3	4	5
Glc	3	4	3	5	5

[a]Relative percentage calculated on the sum of the residues determined.

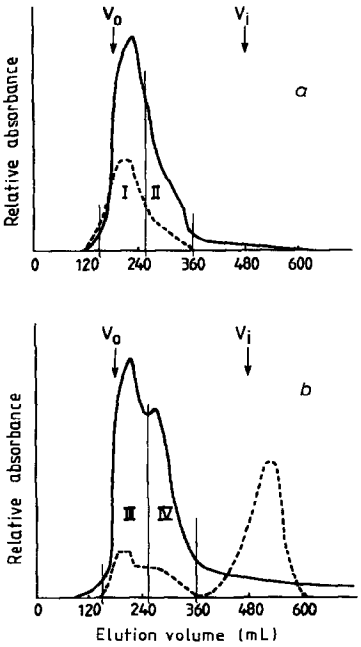

Fig. 1. Gel-permeation chromatography of (*a*) the crude polysaccharide and (*b*) the protease-treated crude polysaccharide on a Sepharose CL-4B column. The void (V_0) and included (V_i) volumes of the column are indicated. The fractions were analysed for total carbohydrate by the phenol–sulfuric acid method (——) and for protein by the absorbance at 280 nm (---).

residues present in the leaves. The molar ratio of arabinose and xylose in the crude polysaccharide was 2.7:1 (Table II).

Only 52% of the freeze-dried, crude polysaccharide was soluble in water. Gel-permeation chromatography of the water-soluble part on Sepharose CL-4B gave a broad peak containing both carbohydrates and protein (Fig. 1a), which was separated into fractions I and II. The composition of these fractions (Table II) was similar to that of the crude polysaccharide. The water-insoluble part of the crude polysaccharide contained arabinose and xylose in the ratio 2.8:1 with small proportions of other sugars.

On treatment of the crude polysaccharide with protease in phosphate buffer, 68% dissolved. Gel-permeation chromatography of the soluble part gave a broad peak (Fig. 1b). However, compared to the crude product (Fig. 1a), more low-molecular-weight material was eluted and the u.v. profile showed protein degradation. The higher protein content of this fraction may be attributed to the added protease. The compositions (Table II) of fractions III and IV in Fig. 1b were similar to those of the crude polysaccharide and to those of fractions I and II in Fig. 1a.

Since the fractionations did not significantly change the glycosyl composition of the polysaccharide, the crude polysaccharide (*A*) was used for most of the

TABLE III

LINKAGE ANALYSIS (MOL %) OF THE ARABINOXYLAN (*A*) AND THE PARTIALLY HYDROLYSED ARABINOXYLANS (*B–D*)[a]

Residue	*Positions of O-methyl groups*	T[b]	*Sample*				*Structural unit deduced*
			A	*B*	*C*	*D*	
Ara	2,3,5	339	32	26	19	4	Ara*f*-(1→
Xyl	2,3,4	381	—	—	—	4	Xyl*p*-(1→
Ara	2,5	445	37	34	22	4	→3)-Ara*f*-(1→
Ara	2,3	488	3	4	—	—	
Xyl	2,3	496	—	4	34	82	→4)-Xyl*p*-(1→
Xyl	2	609	—	10	20	5[c]	→3,4)-Xyl*p*-(1→
Xyl	3	609	—				→2,4)-Xyl*p*-(1→
Xyl	—	707	28	21	5	1	→2,3,4)-Xyl*p*-(1→

[a]*B–D* obtained after partial hydrolysis of *A* for 40, 80, and 160 min, respectively, with 0.125M sulfuric acid at 80°. [b]Retention time (× 1000) of the corresponding alditol acetate relative to that of *myo*-inositol hexa-acetate on a DB-1 capillary column (2 min at 150°, 150 → 225° at 3°/min, and 225° until the last peak eluted). [c]Mainly 2,4-linked xylosyl residues (m.s. data).

structural analysis. The arabinosyl residues were found to be L and the xylosyl residues D.

Linkage analysis revealed terminal arabinofuranosyl groups, 3-linked arabinofuranosyl residues, and 2,3,4-linked xylopyranosyl residues in the molar ratios 1.2:1.3:1.0 (Table III). The pyranosidic form of the xylosyl residues could not be established in this analysis and is discussed below. Methylated/acetylated derivatives of terminal arabinosyl groups are known to be very volatile which may explain the low yield compared to that of branched xylosyl residues. Small amounts of 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylarabinitol were also detected, which may or may not originate from a constituent of the arabinoxylan.

Partial hydrolysis of *A* with acid for 40, 80, and 160 min gave polysaccharides *B–D*. Linkage analysis of *B* revealed (Table III) 4-, 2,4-, and 3,4-linked xylopyranosyl residues. The 2,4- and 3,4-linked residues could not be separated by g.l.c., but were identified by g.l.c.–m.s. A significant decrease of the arabinose–xylose molar ratio was observed. These results indicated that the acid-labile side chains of arabinofuranosyl residues had been attached to O-2 and O-3 of the doubly branched xylosyl residues. The absence of terminal xylosyl groups suggested a backbone of acid-stable xylopyranosyl residues.

Polysaccharide *C* contained the same constituents as *B* (Table III), but a lower content of arabinose and a higher content of 4-, 2,4-, and 3,4-linked xylopyranosyl residues.

4-Linked xylopyranosyl residues constituted 82% of polysaccharide *D*. Small amounts of terminal xylopyranosyl groups revealed some hydrolysis of the backbone. The absence of terminal xylofuranosyl groups indicated that all xylosyl residues were pyranosidic.

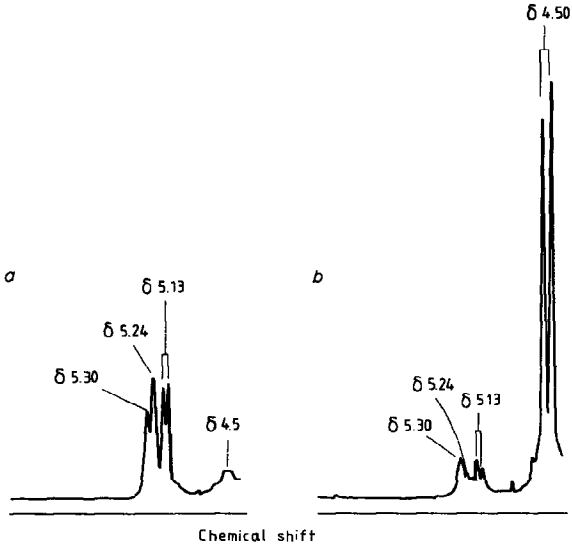

Fig. 2. ^{1}H-N.m.r. spectra (anomeric region) of (*a*) the arabinoxylan (*A*), and (*b*) the partially hydrolysed arabinoxylan (*D*). The chemical shifts were assigned relative to internal $Me_3SiCD_2CD_2CO_2Na$.

The results of these partial hydrolyses suggested that the arabinoxylan had a backbone of 4-linked xylopyranosyl residues, each of which was substituted at both O-2 and O-3 with side chains of arabinofuranosyl residues.

Polysaccharide *A* gave (Fig. 2a) ^{1}H-n.m.r. signals for anomeric protons at δ 5.24 ($J_{1,2}$ <2 Hz) and 5.30 ($J_{1,2}$ <2 Hz) assigned to α-L-arabinofuranosyl residues, a doublet at δ 5.13 ($J_{1,2}$ 4.0 Hz) assigned to β-L-arabinofuranosyl residues, and a broad peak at δ 4.5 assigned to β-D-xylopyranosyl residues by comparison with data in the literature[14] (Table IV). Decoupling near H-2 of the arabinofuranosyl residues caused a collapse of the doublet at δ 5.13. The broad peak from the β-D-xylopyranosyl residues in the backbone may be due either to the highly substituted nature of these residues or to the attachment of different side chains.

The same signals were observed in the ^{1}H-n.m.r. spectra of polysaccharides *B–D* (Table IV). However, for *D*, there was an increase in the sharpness and relative intensity of the signal at δ 4.50 ($J_{1,2}$ 7.2 Hz) from the β-D-xylopyranosyl residues (see Fig. 2b). The residues associated with the resonance at δ 5.24 were more susceptible to acid hydrolysis than those associated with the resonances at δ 5.13 and 5.30, which showed similar relative intensities in all four samples. The residues corresponding to the last two signals were linked mainly to O-2 of the xylopyranosyl residues in the backbone, as shown in the linkage analysis (Table III). An additional signal of low intensity and close to those from the α-L-arabinofuranosyl residues, which could also be detected in the partially hydrolysed samples (Table IV), may be assigned to new terminal α-L-arabinofuranosyl groups formed during the partial acid hydrolysis.

TABLE IV

^{1}H-N.M.R. DATA FOR THE ANOMERIC PROTONS OF THE NATIVE (*A*) AND PARTIALLY HYDROLYSED ARABINOXYLANS (*B–D*)[a]

Arabinoxylan	*(δ)*[b]	$J_{1,2}$ *(Hz)*	*Assigned configuration*[c]
A	~4.50 (b)		β-Xyl
	5.13 (d)	4.0	β-Ara
	5.24 (s)	<2	α-Ara
	5.30 (s)	<2	α-Ara
B	~4.50 (b)		β-Xyl
	5.13 (d)	3.9	β-Ara
	5.24 (s)	<2	α-Ara
	5.30 (s)	<2	α-Ara
	5.38 (s)	<2	α-Ara
C	4.50 (d)	7.2	β-Xyl
	5.13 (d)	4.2	β-Ara
	5.24 (s)	<2	α-Ara
	5.30 (s)	<2	α-Ara
	5.38 (s)	<2	α-Ara
D	4.50 (d)	7.2	β-Xyl
	5.13 (d)	4.2	β-Ara
	5.24 (s)	<2	α-Ara
	5.30 (s)	<2	α-Ara

[a]See Table III. [b]Relative to internal $Me_3SiCD_2CD_2CO_2Na$. [c]Xyl*p* and Ara*f*.

From the relative intensities of the signals from the anomeric protons and their change during acid hydrolysis, it is suggested that both terminal α- and β-L-arabinofuranosyl groups occur, with the latter being more acid-stable. It is also suggested that the 3-linked L-arabinofuranosyl residues (δ 5.30) have the α configuration.

The ^{13}C-n.m.r. results supported the ^{1}H-n.m.r. assignments. The C-1 signals at δ 110.95 (lit.[15] 110.96), 110.30 (lit.[15] 110.35), and 103.25 (lit.[16] 101.84) were assigned to α- and β-L-arabinofuranosyl, and β-D-xylopyranosyl residues, respectively. The difference in chemical shift between the observed and reported signals for the β-D-xylopyranosyl residues was probably due to the highly substituted nature of these residues[17].

It is concluded that the water-soluble arabinoxylan isolated from the leaves of *Neolitsea cassia* contains a backbone of 4-linked β-D-xylopyranosyl residues which is fully substituted with short side chains of L-arabinofuranosyl residues. Our results indicate single α-L-arabinofuranosyl groups attached mainly at positions 3 and short chains of 3-linked α-L-arabinofuranosyl residues terminated with a β-L-arabinofuranosyl group attached mainly at O-2. Further experiments are necessary to determine the exact chemical structure of the side chains.

Previous studies of polysaccharides of related species from the same family have been reported. Arabinoxylans have been isolated from the bark of *Cinnamomum iners*[8] and *Persea macrantha*[16], and the leaves of *Litsea polyantha*[18], which have backbones of 4-linked xylopyranosyl residues, but different side chains. Those of the first two arabinoxylans contain both arabinofuranosyl and α-D-xylopyranosyl residues, whereas those of the last contain terminal, 3-linked, and 3,5-linked arabinofuranosyl residues. The structure of the arabinoxylan from *Neolitsea cassia* is quite different from those of the above polysaccharides.

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