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STRUCTURAL STUDIES OF AN ACIDIC POLYSACCHARIDE FROM THE SEEDS OF *Acacia auriculaeformis* A. Cunn.

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ABSTRACT

An acidic polysaccharide isolated from the defatted seeds of *Acacia auriculaeformis* contained L-arabinose, D-xylose, D-galactose, D-glucose, and D-glucuronic acid in the molar ratios 1.5:2:2.2:1:3. Autohydrolysis of an aqueous solution of the polysaccharide yielded an electrophoretically homogeneous, degraded polysaccharide consisting of D-xylose, D-galactose, D-glucose, and D-glucuronic acid in the molar ratios 1:3.9:1.9:6.7. Methylation analysis, periodate oxidation, and Smith degradation of the native and degraded polysaccharide and their carboxyl-reduced derivatives indicated that the polysaccharide is branched with 3,6-linked galactosyl and 2,4-linked glucosyl residues at the branch points to which are attached chains comprised of arabinose, xylose, glucose, galactose, and glucuronic acid residues *via* different linkages. Oxidation of the acetylated polysaccharide with chromium trioxide indicated that most of the glucose and arabinose residues were α, while xylose, galactose, and glucuronic acid were mostly β. A d.p. of ~89 was established for the polysaccharide by end-group analysis.

INTRODUCTION

Acacia auriculaeformis A. Cunn. (Leguminosae), a medium-sized evergreen plant, grows abundantly in India, and its seeds have nutritional value[1]. There has been considerable work[2–5] on the seeds of *A. auriculaeformis*, but the structure of its seed polysaccharide has not been reported. We now report on an acidic polysaccharide isolated from the seeds.

RESULTS AND DISCUSSION

An acidic polysaccharide was isolated by precipitation with 3:1 ethanol–acetone from an aqueous 10% trichloroacetic acid extract of the defatted seeds of *A. auriculaeformis*. After passing through a column of DEAE-cellulose, the major

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portion of the material, obtained as a single component, was decationised with Amberlite IR-120 (H^+) resin. The homogeneity of the polysaccharide, which had $[\alpha]_{589.6}^{26}$ −9° (water), was checked by (*a*) high-voltage paper electrophoresis and (*b*) gel permeation through Sephadex G-100 using borate buffer. Acid hydrolysis of the original and the carboxyl-reduced[6] polysaccharide showed the average repeating-unit to consist of arabinose, xylose, galactose, glucose, and glucuronic acid (Table I). The enhanced proportion of glucose in the carboxyl-reduced derivative confirmed the original presence of glucuronic acid. The contents of carbohydrate[7] and uronic acid[8] of the purified polysaccharide were 99% and 35%, respectively. The $[\alpha]_D$ values of the sugars isolated accorded with L-arabinose, D-xylose, D-galactose, D-glucose, and D-glucuronic acid. Autohydrolysis of an aqueous solution of the original polysaccharide for 55 h at 100° gave the degraded polysaccharide, which was isolated by precipitation with ethanol–acetone, had $[\alpha]_{589.6}^{26}$ −15° (water), contained xylose, galactose, glucose, and glucuronic acid (Table I), and was electrophoretically homogeneous.

Hydrolysis of the original polysaccharide with 0.3M sulphuric acid (5 h, 100°) gave an aldobiouronic acid which was identified conventionally as 3-*O*-(D-glucopyranosyluronic acid)-D-galactose.

The degraded polysaccharide, the native polysaccharide, and their carboxyl-reduced products were methylated by the Hakomori[9] and Purdie[10] methods and then hydrolysed; the products were converted into the alditol acetates and analysed by g.l.c. The results are given in Table II.

Hydrolysis of the methylated degraded polysaccharide (*B*) and its carboxyl-reduced derivative (*D*) gave 2,3,4-tri-*O*-methyl-D-xylose (2 mol), 2,3,4,6-tetra-*O*-methyl-D-glucose (2 mol), and 2,3,4,6-tetra-*O*-methyl-D-galactose (1 mol), indicating the non-reducing ends to be D-xylopyranosyl, D-glucopyranosyl, and D-galactopyranosyl units. Only 2 mol of 2,3,6-tri-*O*-methyl-D-glucose were detected in the hydrolysate of the methylated degraded polysaccharide, whereas its carboxyl-reduced derivative yielded 17 mol, indicating that all the D-glucuronic acid residues were present in the chain and were 4-linked. The presence of 2,4-di-*O*-methyl-D-galactose (5 mol) in the hydrolysate of the methylated degraded polysaccharide

TABLE I

COMPOSITION OF MONOSACCHARIDES IN ORIGINAL (*A*), DEGRADED (*B*), CARBOXYL-REDUCED *A* (*C*), AND CARBOXYL-REDUCED *B* (*D*) POLYSACCHARIDES FROM *Acacia auriculaeformis*

Monosaccharide (%)	A	B	C	D
L-Arabinose	13.5		14.0	
D-Xylose	18.0	6.0	18.0	7.0
D-Galactose	23.0	28.0	24.0	29.0
D-Glucose	10.5	14.0	44.0	64.0
D-Glucuronic acid	35.0[a]	52.0[a]		

[a]Determined by the carbazole–sulphuric acid method[8].

TABLE II

METHYLATED PRODUCTS FROM ORIGINAL (*A*), DEGRADED (*B*), CARBOXYL-REDUCED *A* (*C*), AND CARBOXYL-REDUCED *B* (*D*) POLYSACCHARIDES OF *Acacia auriculaeformis*[a]

Methylated sugars[b]	T[c]		*Mole proportions*				*Linkage pattern*
	(a)	(b)	A	B	C	D	
2,3,5-Ara	0.48	0.41	5		5		Ara*f*-(1→
2,3,4-Xyl	0.68	0.54	5	2	5	2	Xyl*p*-(1→
2,5-Ara	1.10	0.84	2		2		→3)-Ara*f*-(1→
2,4-Xyl	1.34	1.10	1		1		→3)-Xyl*p*-(1→
2,3-Xyl and/or 3,4-Xyl	1.54	1.19	3		3		→4)-Xyl*p*-(1→, and/or →2)-Xyl*p*-(1→, and/or →5)-Xyl*f*-(1→
2,3,4,6-Glc	1.00	1.00		2		2	Glc*p*-(1→
2,3,4,6-Gal	1.25	1.19		1		1	Gal*p*-(1→
2,4,6-Gal	2.28	2.03	4	3	4	3	→3)-Gal*p*-(1→
2,3,6-Glc	2.50	2.32		2	15	17	→4)-Glc*p*-(1→
3,6-Glc	4.40	3.73	4		4		→2,4)-Glc*p*-(1→
2,4-Gal	6.35	5.1	5	5	6	5	→3,6)-Gal*p*-(1→

[a]See Experimental for details. [b]2,3,5-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol, *etc.* [c]Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity, on a column of (*a*) 3% of ECNSS-M, and (*b*) 3% of OV-225.

and its carboxyl-reduced derivative indicated the polysaccharide to be highly branched with 3,6-linked D-galactopyranosyl residues at the branch points. Characterisation of 2,4,6-tri-*O*-methyl-D-galactose indicated that the degraded polysaccharide contained, *inter alia*, 3-linked D-galactosyl residues. The results of methylation studies of the original polysaccharide agreed with the mode of linkage proposed for the degraded polysaccharide. The additional formation of 3,6-di-*O*-methyl-D-glucose (4 mol) indicated the presence of 2,4-linked glucose residues. Characterisation of 2,3,4-tri-*O*-methyl-D-xylose (5 mol) and 2,3,5-tri-*O*-methyl-L-arabinose (5 mol) indicated that the non-reducing ends of the original polysaccharide were D-xylopyranosyl and L-arabinofuranosyl units. The formation of 2,5-di-*O*-methyl-L-arabinose (2 mol), 2,4-di-*O*-methyl-D-xylose (1 mol), and 2,3-di-*O*-methyl-D-xylose and/or 3,4-di-*O*-methyl-D-xylose (3 mol) [not distinguishable by g.l.c. of their alditol acetates[11]] indicated that the original polysaccharide contained, *inter alia*, 3-linked L-arabinofuranosyl, 3-linked D-xylopyranosyl, and 4-, and/or 2- and/or 5-linked D-xylosyl residues.

The results of periodate-oxidation studies[12,13] supported the above linkages in the original and the degraded polysaccharide. The original and the degraded polysaccharide consumed 0.77 and 0.92 mol of periodate per hexosyl residue in 14 and 11 h, respectively. The observed values accorded with the theoretical values calculated on the basis of methylation analysis. Hydrolysis of the periodate-oxidised and borohydride-reduced original polysaccharide yielded arabinose (11%), xylose (5%), galactose (59%), and glucose (25%), whereas the degraded polysaccharide

TABLE III

OXIDATION OF ACETYLATED POLYSACCHARIDE (*A*) AND CARBOXYL-REDUCED, ACETYLATED POLYSACCHARIDE (*B*) WITH CrO_3

Material	*Time of oxidation (h)*	*Sugar analysis*[a]			
		Glucose	*Galactose*	*Xylose*	*Arabinose*
	0	2.4	4.1	4.4	3.3
A	0.5	1.7	0.8	1.5	1.1
	1.5	0.8	0.4	0.7	0.4
	0	5.1	2.8	2.2	2.0
B	0.5	2.0	0.6	0.8	0.8
	1.5	0.8	0.3	0.4	0.3

[a]In molar proportions.

gave only galactose. The periodate-oxidised degraded polysaccharide, on Smith degradation, yielded 6-*O*-(D-galactopyranosyl)-D-galactose, the structure of which was established by hydrolysis and methylation analysis. Thus, the degraded polysaccharide contains the structure.

$$\rightarrow 3)\text{-Gal-}(1\rightarrow 6)\text{-Gal-}(1\rightarrow$$
$$\qquad\qquad\qquad\qquad\quad 3$$
$$\qquad\qquad\qquad\qquad\quad \uparrow$$

In order to ascertain the anomeric configurations of the different sugars residues, the peracetylated polysaccharide and its carboxyl-reduced derivative were oxidised with chromium trioxide[14,15]. The results are shown in Table III. The rate of disappearance of glucose, galactose, and xylose during the oxidation indicated that most of the glucose residues were α, and that the other two units were β. The $[\alpha]_D$ value of the aldobiouronic acid and the rate of disappearance of glucose from the carboxyl-reduced polysaccharide indicated that most of the glucuronic acid residues were β. Since acetylated furanoses are non-specifically oxidised[14] by CrO_3, the anomeric configuration of arabinose cannot be specified. However, the rate at which the majority of the arabinose residues were removed during the mild treatment with acid indicated most of them to be α.

End-group analysis[16] gave a value of ~89 for the average d.p.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer model 241 MC spectropolarimeter at 25 ±1° and 589.6 nm. I.r. spectra were recorded with an Acculab 10 Beckman instrument for KBr pellets. U.v. and visible spectra were recorded with a Beckman Model 26 spectrophotometer. High-voltage

paper electrophoresis was conducted with a Labor Model No.OE 201 instrument, on Whatman No. 1 filter paper, using borate buffer (pH 9.5.). Analytical and preparative p.c. were performed on Whatman No. 1 and 3MM paper, respectively, using *A*, the upper phase of 4:1:5 1-butanol–acetic acid–water; *B*, 8:2:1 ethyl acetate–pyridine–water; *C*, 4:1:5 1-butanol–ethanol–water; and *D*, 18:3:1:4 ethyl acetate–acetic acid–formic acid–water; and detection with alkaline silver nitrate and aniline oxalate. T.l.c. was carried out on Silica Gel G (100–200 mesh) with solvents *B* and *C*, and detection with aniline–diphenylamine–phosphate and sulphosalicylic acid[17]. Gel filtration was performed on a column (75 × 2.5 cm) of Sephadex G-100 with borate buffer (pH 9.5). Evaporations were performed under diminished pressure at ≤45° (bath) and small volumes of aqueous solutions were lyophilised. G.l.c. of the monosaccharides as their alditol acetates and partially methylated alditol acetates was conducted on glass columns containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) and (*b*) 3% of OV-225 on Gas Chrom Q (100–120 mesh), with nitrogen as the carrier gas, using Packard Model 419 and Hewlett–Packard Model 5713 A gas chromatographs each equipped with a flame-ionization detector.

Isolation and purification of the polysaccharide. — Ground seeds of *Acacia auriculaeformis* were extracted with acetone (Soxhlet) for 48 h and then air-dried. The defatted seed powder (200 g) was stirred with aqueous 10% trichloroacetic acid (1 L) for 4 h. The insoluble materials were removed, and the filtrate was centrifuged for 15 min at 18,000*g* and then stirred into 3:1 ethanol–acetone (3 L). The yellowish precipitate was collected by centrifugation and washed several times with 3:1 methanol–chloroform, and a solution in aqueous 10% trichloroacetic acid was diluted with ethanol–acetone. The precipitate was collected and washed with methanol–chloroform. This procedure was repeated thrice. Finally, an aqueous solution of the polysaccharide was dialysed and then lyophilised to yield 9 g of product.

The polysaccharide (100 mg) was eluted from a column (47 × 2.5 cm) of DEAE-cellulose successively with water, and 0.1, 0.2, and 0.3M borax solutions (500 mL each). The fractionation was monitored by the phenol–sulphuric acid method[7]. The eluate containing the major polysaccharide fraction was dialysed and then lyophilised. A solution of the polysaccharide (89 mg) thus obtained in water (100 mL) was passed through a column (79 × 2.5 cm) of Amberlite IR-120 (H^+) resin. The eluate (145 mL) was concentrated and the polysaccharide (83 mg) was precipitated with ethanol–acetone. The homogeneity of this polysaccharide, $[\alpha]_{589.6}^{26}$ −9° (*c* 0.03, water), was tested by gel filtration through Sephadex G-100 and by high-voltage paper electrophoresis using borate buffer (pH 9.5) in each experiment. The carbohydrate content[7] of the polysaccharide was 99%.

Hydrolysis of the polysaccharide. — The polysaccharide (15 mg) was hydrolysed with 10 mL of 0.5M sulphuric acid at 100° for 16 h. The hydrolysate was neutralised with barium carbonate, centrifuged, decationised with Amberlite IR-120 (H^+) resin, and concentrated. P.c. (solvents *A*–*C*) and t.l.c. (solvents *B* and *C*) revealed L-arabinose, D-xylose, D-galactose, D-glucose, and D-glucuronic acid, the

configurations of which were established by isolation through preparative p.c. (solvent *A*) and measurements of optical rotations.

Preparation of the degraded polysaccharide. — Pilot experiments with dilute mineral acids, formic acid, and water indicated that autohydrolysis for 55 h at 100° was best suited for the preparation of the degraded polysaccharide. Thus, a solution of the polysaccharide (6 g) in water (500 mL) was heated on a boiling water bath for 55 h, then concentrated to 100 mL, dialysed against distilled water for 96 h, and poured, with stirring, into 3:1 ethanol–acetone (500 mL). The precipitate was collected by centrifugation, washed several times with ethanol, and dried *in vacuo*. The product (3.8 g) had $[\alpha]_{589.6}^{26}$ −15° (*c* 0.02, water) and was electrophoretically homogeneous. Hydrolysis of the degraded polysaccharide with 0.5M sulphuric acid (5 h, 100°) gave [p.c. (solvents *A* and *B*), t.l.c. (solvents *B* and *C*)] xylose, galactose, glucose, and glucuronic acid.

Carboxyl-reduction[6]. — To a stirred solution of the polysaccharide (original polysaccharide, 40 mg in 30 mL of water; degraded polysaccharide, 42 mg in 30 mL of water) was added 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate (1 g), and the pH was kept at ~4.75 by the addition of 0.01M hydrochloric acid. After 2 h, 2M sodium borohydride (65 mL) was added dropwise during 1 h and the pH was kept at ~7 by simultaneous addition of 4M hydrochloric acid. The solution was stirred for 1 h, then dialysed against distilled water for 96 h, and lyophilised. The process was repeated twice, to ensure complete reduction.

Determination of the component sugars. — The proportions (35% and 52%, respectively) of glucuronic acid in the original and the degraded polysaccharide were determined by the carbazole–sulphuric acid method[8], using D-glucuronic acid as the standard. The original polysaccharide, the degraded polysaccharide, and their carboxyl-reduced derivatives were mixed separately with *myo*-inositol and hydrolysed with 0.5M sulphuric acid as described above. The neutral sugars present in the hydrolysates were converted into their alditol acetates and analysed by g.l.c. The results are given in Table I (columns *a* and *b*).

The original polysaccharide (360 mg) was hydrolysed with 0.3M sulphuric acid for 5 h at 100°. The hydrolysate was neutralised with barium carbonate, centrifuged, concentrated, and added to the top of a column (28 × 2.5 cm) of De-Acidite FF ($HCOO^-$) resin. The column was irrigated exhaustively with distilled water and then with aqueous 10% formic acid. The eluate containing acidic sugars was freed from formic acid by codistillation with water under diminished pressure. P.c. (solvent *D*) of the acid sugars revealed glucuronic acid and a slower moving fraction, R_{Gal} 0.28, which was isolated by preparative p.c. (solvent *D*). The latter fraction had $[\alpha]_{589.6}^{25}$ +11.5° (*c* 0.3, water) and hydrolysis with 0.5M sulphuric acid (11 h, 100°) gave [p.c. (solvents *A* and *B*)] galactose and glucuronic acid. Borohydride reduction followed by acid hydrolysis gave glucuronic acid but no galactose. The fraction was methylated by the Hakomori method[9], and a portion of the product was reduced with lithium aluminium hydride[18] and then remethylated by the Purdie method[10]. Each product was hydrolysed with 0.5M sulphuric acid for 10 h at

100° and the products were converted into the alditol acetates. G.l.c. [columns (*a*), (*b*)] indicated the formation of 2,4,6-tri-*O*-methylgalactose from the methylated compound, and 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose in the molar ratio 1:1 from the methylated, reduced, and remethylated compound.

Methylation analysis. — The original polysaccharide, the degraded polysaccharide, and their carboxyl-reduced derivatives (15 mg each) were dispersed in methyl sulphoxide (15 mL each) and methylated by one Hakomori[9] treatment followed by two consecutive Purdie methylations[10], to yield products that showed no i.r. absorption for hydroxyl.

The methylated products were hydrolysed with aqueous 90% formic acid for 2 h at 100°, the formic acid was removed by codistillation with water, and the products were heated with 0.5M sulphuric acid for 12 h at 100°. The neutral sugars present in the hydrolysates were converted into the alditol acetates and analysed by g.l.c. [columns (*a*) and (*b*)]. The results are shown in Table II.

Periodate and Smith-degradation studies. — (*a*) The original and degraded polysaccharide were treated with 0.1M sodium metaperiodate in the dark at 5°. The consumptions of oxidant were monitored spectrophotometrically and were constant within 14 and 11 h, respectively, at 0.77 and 0.92 mol per hexosyl residue.

(*b*) The original (50 mg) and the degraded (500 mg) polysaccharide were each treated with 0.1M sodium metaperiodate (25 mL and 200 mL, respectively) for 14 and 11 h, respectively, at 5° in the dark. The solutions were then treated with ethylene glycol (2 and 15 mL, respectively), kept overnight, dialysed, and concentrated, and the products were reduced conventionally with sodium borohydride (0.1 and 1 g, respectively) at room temperature. Part (10 mg) of each product (42 and 395 mg, respectively) was hydrolysed with 0.5M sulphuric acid for 16 h at 100°. The products were examined by p.c. (solvent *A*) and g.l.c. (as the alditol acetates). Besides lower polyhydric aldehydes and alcohols, the original polysaccharide yielded arabinose (11%), xylose (5%), galactose (59%), and glucose (25%), whereas the degraded polysaccharide gave galactose only.

(*c*) A solution of a portion (380 mg) of the product in (*b*) in 0.5M sulphuric acid (40 mL) was kept for 30 h at room temperature. P.c. (solvent *A*) of the products revealed galactose and a product with R_{Gal} 0.2. The latter product, isolated by preparative p.c. (solvent *A*), had $[\alpha]^{25}_{589.6}$ +29° (*c* 0.2, water), and hydrolysis by 0.5M sulphuric acid for 8 h at 100° gave [p.c. examination (solvents *A* and *D*)] galactose. The product was methylated (Hakomori) and then hydrolysed to give 2,3,4,6-tetra-*O*-methylgalactose and 2,3,4-tri-*O*-methylgalactose in the ratio 1:1.

Oxidation with chromium trioxide. — To a mixture containing carboxyl-reduced polysaccharide (15.1 mg) and *myo*-inositol (7.2 mg) in formamide (4 mL) were added acetic anhydride (4 mL) and pyridine (5 mL). The mixture was stirred for 16 h at room temperature, then dissolved in chloroform, washed with water, dried (Na_2SO_4), and concentrated to dryness. The process was repeated in order to ensure complete acetylation. To a solution of the residue in glacial acetic acid (8 mL) was added powdered chromium trioxide (300 mg) with stirring at 50°. Aliquots

were removed at intervals, diluted immediately with water, and extracted with chloroform, and the extract was dried (Na_2SO_4) and concentrated to dryness. The products were deacetylated with methanolic 0.2M sodium methoxide, decationised with Amberlite IR-120 (H^+) resin, and hydrolysed with M sulphuric acid for 8 h at 100°. The products were converted into the alditol acetates and analysed by g.l.c. A similar experiment was performed using 8.7 mg of *myo*-inositol and 10 mg of native polysaccharide. The results are shown in Table III.

End-group analysis. — To a solution of the native polysaccharide (47.5 mg) in 0.05M sodium hydroxide (7 mL) was added sodium borohydride (50 mg), and the mixture was kept for 48 h at room temperature. The excess of borohydride was decomposed with dilute acetic acid, and the pH of the solution was adjusted nearly to 6. The solution was cooled to 5°, 0.5M sodium metaperiodate solution (8 mL) was added, and the volume of the mixture was brought to 25 mL. The oxidation was allowed to proceed in the dark, and, periodically, aliquots (2 mL) were treated with saturated aqueous lead acetate (3 mL) and the formaldehyde was determined by the chromotropic acid procedure. A similar set of experiments was performed with D-mannitol.

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