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SOME STRUCTURAL FEATURES OF THE POLYSACCHARIDE OF MAHUA (*Madhuca indica*) FLOWERS

NUPUR SARKAR AND BISHNU P. CHATTERJEE*
Department of Macromolecules, Indian Association for the Cultivation of Science, Jadavpur, Calcutta 700 032 (India)

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ABSTRACT

On gel filtration through Sephadex G-150, the water-soluble polysaccharide from Mahua flowers gave two homogeneous fractions. The first fraction was found to contain D-galactose, L-arabinose, L-rhamnose, D-xylose, and D-glucuronic acid in the molar ratios of ~21:5:1:1:6. Methylation analyses were conducted on the polysaccharide and its carboxyl-reduced derivative, and the results were corroborated by those from periodate oxidation followed by Smith degradation. The anomeric configurations of the different sugar residues in the polysaccharide were determined by chromium trioxide oxidation of the acetylated polysaccharide.

INTRODUCTION

The flowers of Mahua (*Madhuca indica*) constitute a rich source of sugars, and are used, next in importance to cane molasses, in distilleries as raw material, as well as being eaten raw or cooked. Mahua flowers are regarded as cooling, tonic, and demulcent, and are used in alleviating coughs, colds, and bronchitis[1]. The total sugars reported therein amount to 73%, and the sugars identified are sucrose, maltose, glucose, fructose, arabinose, and rhamnose[2]. Despite having such versatility, no structural investigation of the polysaccharide of Mahua flowers has as yet been made. In the present communication, some structural features of one of the polysaccharide fractions are reported.

EXPERIMENTAL

General methods. — All evaporations were conducted in a rotary evaporator at 40°, unless stated otherwise. Descending paper-chromatography (p.c.) was performed on Whatman Nos. 1 and 3 MM papers, using as solvent systems (v/v): (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) 1-butanol–acetic acid–water (4:1:5, upper layer), and (*C*) 9:2:2 ethyl acetate–acetic acid–water. The spray reagent used was

*To whom correspondence should be addressed.

alkaline silver nitrate. Optical rotations were measured with a Perkin-Elmer Model 241 MC spectropolarimeter at 23 ± 1° and 589.6 nm. The homogeneity of the polysaccharides was determined by high-voltage paper-electrophoresis conducted in a Shandon high-voltage electrophoresis instrument Model L-24. Infrared spectra were recorded with a Beckman IR-20 A spectrophotometer, and ultraviolet and visible spectra with a Yanaco SP-I spectrophotometer. Gas–liquid chromatography (g.l.c.) was performed with a Hewlett–Packard model 5730 A gas chromatograph fitted with a flame-ionization detector and glass columns (1.83 × 6 mm) packed with (*1*) 3% of ECNSS-M on Gas-Chrom Q (100–120 mesh) and (*2*) 3% of OV-225 on Gas-Chrom Q (100–120 mesh). All g.l.c. analyses were conducted (at 185° for unmethylated sugars, and at 165° for methylated sugars) by converting the sugars into their alditol acetates[3].

Source of Mahua flowers. — The flowers of Mahua (*Madhuca indica* L.) were collected at Jhajha in Bihar, by courtesy of Mrs. N. Roy, in April, 1980.

Extraction of polysaccharides from Mahua flowers. — Mahua flowers (100 g) were crushed with ethanol (300 mL) in a blender, and the slurry was stirred overnight, squeezed through a piece of Nylon cloth, and the solid air-dried (yield, 40 g). The dry material was then stirred overnight with cold water (600 mL) at room temperature, and the slurry was again squeezed through a piece of Nylon cloth. The polysaccharides were isolated both from the residue and the filtrate by the following procedures.

The filtrate was centrifuged at 15,000 r.p.m. in a Sorvall RC-5B refrigerated centrifuge for 1 h. To the cold, clear liquid was added cold ethanol (2.5 vol.), and a whitish precipitate separated out. The precipitate was collected by centrifugation, washed thrice with dry methanol, triturated with petroleum ether, and dried. The polysaccharide was dissolved in water (400 mL), reprecipitated with ethanol (1 L), and the precipitate collected by centrifugation. The process of dissolution in water and precipitation with ethanol was repeated twice more, until a fairly white precipitate was obtained. It was dried over phosphorus pentaoxide; yield 250 mg, $[\alpha]_{589.6}^{23}$ −55° (*c* 0.5, water), and designated PS-A.

The residue obtained by filtration of the slurry through Nylon cloth was suspended in water (600 mL) and heated for 4 h in a boiling-water bath. The polysaccharide was obtained by employing the procedure just described. This polysaccharide was designated PS-B; yield, 162 mg; $[\alpha]_{589.6}^{23}$ +40° (*c* 0.6, water).

Purification of polysaccharide PS-A. — The polysaccharide PS-A (80 mg) was purified by passing an aqueous solution of it through a column (95 × 1.8 cm) of Sephadex G-150. The column was eluted with water, 5-mL fractions being collected. The fractions were automatically monitored with a Waters Associates' Differential Refractometer Model 403 fitted with a recorder. Two peaks, one comprising fractions 17–28, and the other, fractions 32–50, were obtained (see Fig. 1). The fractions respectively containing each of these polysaccharides were combined, separately dialyzed against distilled water, and freeze-dried. The polysaccharide obtained from the fractions constituting the first peak was termed PS-A1: yield, 25 mg, $[\alpha]_{589.6}^{23}$

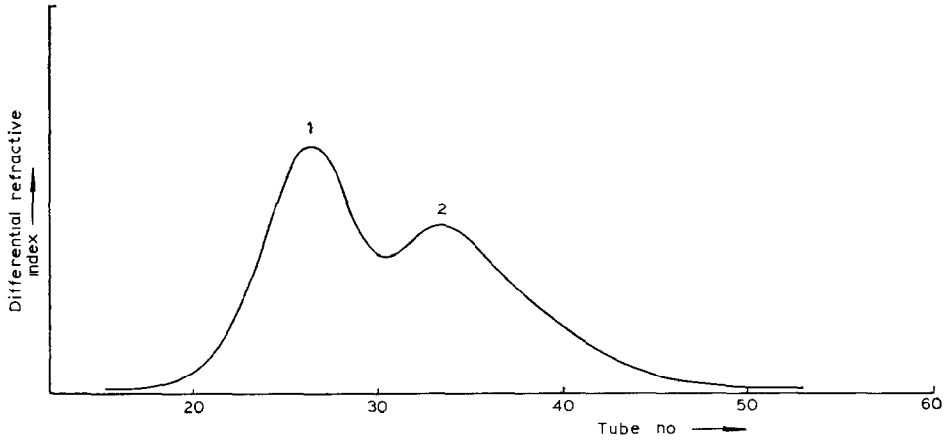

Fig. 1. Gel chromatography of polysaccharide PS-A from Mahua flowers by elution with water, and monitoring with a differential refractometer. (Assignment of peaks: 1, Fraction I; 2, Fraction II.)

$-9.3°$ (*c* 0.4, water), and that obtained from the fractions in the second peak, PS-AII. The yield was 50 mg, and it had $[\alpha]^{23}_{589.6}$ $-19.5°$ (*c* 0.4, water). Both PS-AI and PS-AII moved as a single component when electrophoresed (37 V/cm) for 90 min in phosphate buffer, pH 6.9.

Hydrolysis and sugar analysis of the polysaccharides PS-AI and PS-AII. — The polysaccharides PS-AI and PS-AII (2 mg each) were separately hydrolyzed with 0.5M sulfuric acid for 20 h at 100°. The acid was neutralized with barium carbonate, the slurry was filtered through a Celite bed, and the filtrate was decationized with Dowex-50W X8 (H^+) resin, and concentrated to a small volume. Parts of the filtrate were examined by paper chromatography using solvents *A*, *B*, and *C*. The polysaccharide PS-AI gave spots corresponding to galactose, arabinose, xylose, rhamnose, and glucuronic acid, and a faint spot near the base line, whereas PS-AII, in addition

TABLE I

COMPOSITION[a] OF MONOSACCHARIDES IN THE ORIGINAL POLYSACCHARIDE (PS-AI) FROM MAHUA FLOWERS, AND ITS CARBOXYL-REDUCED DERIVATIVE

Monosaccharide	*Original polysaccharide, PS-AI* (%)	*Carboxyl-reduced polysaccharide, PS-AI* (%)
D-Galactose	71.7	59.5
L-Arabinose	21.3	16.2
L-Rhamnose	3.8	3.0
D-Xylose	2.5	2.0
D-Glucose	—	19.6

[a]Percentage values were obtained by g.l.c. analysis.

to all of the sugars present in PS-AI, gave a spot for glucose. The other parts of the respective filtrate were converted into their alditol acetates, and these were analyzed by g.l.c. (column *I*). When estimation of sugars was desired, a known amount of *myo*-inositol (accurately weighed) was added to the polysaccharide before hydrolysis by the foregoing procedure. The results are given in Table I.

Preparation of carboxyl-reduced PS-AI[4] To a solution of polysaccharide PS-AI (40 mg) in water (40 mL) was added 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC, 1 g), with stirring, and the pH was kept at 4.75 during the reaction by the addition of 0.01M hydrochloric acid. After 2 h, 2M aqueous sodium borohydride (20 mL) was added dropwise for 1 h, and the pH of the solution was kept at 7 by the simultaneous addition of 4M hydrochloric acid. After 1 h, the solution was dialyzed against distilled water for 24 h, and freeze-dried. The procedure was repeated once, to ensure complete reduction of the carboxyl groups. The yield was 35 mg.

The carboxyl-reduced PS-AI (2 mg) was mixed with *myo*-inositol (0.2 mg, as the internal standard), and hydrolyzed with 0.5M sulfuric acid as previously described. The alditol acetates of the sugars were estimated by g.l.c. using column *I* (see Table I). The presence of glucose in the hydrolyzate confirmed that the uronic acid was glucuronic acid, as glucose was absent from the hydrolyzate of the original polysaccharide PS-AI. The uronic acid in PS-AI was estimated by the carbazole sulfuric acid method[5], with D-glucuronic acid as the standard, and the content was found to be 20%.

The carboxyl-reduced PS-AI was prepared in several batches, and hydrolyzed as described earlier. The sugars were resolved, and isolated by preparative paper-chromatography using solvent *A*. The specific rotations of these sugars were determined: galactose, +78° (lit.[6a] +79° for D-galactose); glucose, +51 (lit.[6b] +52.5 for D-glucose); arabinose, +100° (lit.[6c] +105 for L-arabinose); rhamnose, +8 (lit.[6d] +9.18 for L-rhamnose); and xylose, +18° (lit.[6e] +19° for D-xylose).

Methylation analysis of PS-AI and carboxyl-reduced PS-AI The polysaccharide PS-AI (8.0 mg) and its carboxyl-reduced product (4.0 mg) were each dissolved in dimethyl sulfoxide (8 mL and 4 mL, respectively) in separate vials, by ultrasonication, and treated with 2M methylsulfinyl sodium[7] (4 mL and 2 mL, respectively) under a nitrogen atmosphere. The solutions were stirred overnight, methyl iodide (4 mL and 2 mL, respectively) was added dropwise, with external cooling, to the vials, and the mixtures were stirred for 2 h. The products in the vials were flushed with a stream of nitrogen, dialyzed, and lyophilized. The material was remethylated with methyl iodide and silver oxide by the Kuhn procedure[8]. The i.r. spectra of the methylation products showed no hydroxyl absorption band at 3600–3300 cm^{-1}, indicating the absence of free hydroxyl groups. The methylated PS-AI (6.0 mg) had $[\alpha]_{589}^{23}$ -8.82° (*c* 0.3, chloroform). A portion of the methylated PS-AI and methylated, carboxyl-reduced PS-AI were separately hydrolyzed, first with 90% formic acid for 1 h in a boiling-water bath, and then with 0.5M sulfuric acid for 16 h at 100°. The hydrolyzates were made neutral with barium carbonate, and the alditol

TABLE II

METHYLATION ANALYSIS OF THE POLYSACCHARIDE (PS-AI) FROM MAHUA FLOWERS, AND OF ITS CARBOXYL-REDUCED PRODUCT

Sugars[a]	T[b]		*Approximate mol. proportions*		*Mode of linkage*
	1	2	*Original PS-AI*	*Carboxyl-reduced PS-AI*	
2,3,5-Ara	0.47	0.43	5	4	Ara*f*-(1→
2,4-Ara	1.41	1.08	2	1	→3)-Ara*f*-(1→
2,3,4-Xyl	0.67	0.54	1	1	Xyl*p*-(1→
3,4-Rha	0.91	0.86	1	1	→2)-Rha*p*-(1→
2,3,4,6-Gal	1.24	1.19	3	2	Gal*p*-(1→
2,3,6-Gal	2.41	2.23	5	5	→4)-Gal*p*-(1→
2,3,4-Gal	3.39	2.89	4	3	→6)-Gal*p*-(1→
2,3-Gal	5.67	4.69	3	3	→4,6)-Gal*p*-(1→
2,4-Gal	6.40	5.11	8	8	→3,6)-Gal*p*-(1→
2,3,4,6-Glc	**1.00**	**1.00**	—	6	Glc*p*A-(1→

[a]2,3,5-Ara = 2,3,5-tri-*O*-methyl-L-arabinose, etc. [b]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 (1) a 3% ECNSS-M column at 165°, and (2) a 3% OV-225 column at 165°.

acetates were prepared as usual and examined by g.l.c. (columns *1* and *2*). The results are summarized in Table II.

Periodate oxidation and Smith degradation of PS-AI. — The polysaccharide PS-AI was treated with 0.1M sodium metaperiodate in the dark at 5°. Consumption of the oxidant, monitored spectrophotometrically[9], became constant in 72 h, corresponding to a consumption of 1.10 mol per mol of hexosyl residue.

In a separate experiment, polysaccharide PS-AI (40 mg) was treated with 0.1M sodium metaperiodate (40 mL) in the dark for 72 h at 5°. The excess of the periodate was decomposed by adding ethylene glycol (5 mL). The solution was dialyzed, concentrated to ~10 mL, and the product reduced with sodium borohydride[10] (250 mg) overnight at room temperature. The mixture was made neutral with acetic acid, dialyzed, and freeze-dried. The yield was 32 mg. A part of the reduced, periodate-oxidized material (2 mg) was hydrolyzed with 0.5M sulfuric acid for 20 h at 100°. A portion of the hydrolyzate, after neutralization, was examined by paper chromatography (solvent *A*), and another portion was converted into the alditol acetates, and these were analyzed by g.l.c. (column *1*). Besides lower polyhydric alcohols and aldehydes, galactose and arabinose were detected. Another part of the reduced, periodate-oxidized PS-AI (10 mg) was kept with 0.5M sulfuric acid (1 mL) for 24 h at room temperature. After the usual treatment, paper-chromatographic examination using solvent *A* revealed spots corresponding to galactose (faint), arabinose, and three oligomers whose R_{Lac} values were 0.98, 1.3, and 1.73. The material was subjected to a

TABLE III

SURVIVAL OF SUGARS[a] IN THE OXIDATION OF ACETYLATED, CARBOXYL-REDUCED PS-AI WITH CHROMIUM TRIOXIDE

Time (h)	myo-*Inositol*	*Galactose*	*Glucose*	*Arabinose*	*Rhamnose*	*Xylose*
0	10	15.30	11.10	6.20	1.30	0.48
1	10	0.38	0.54	1.10	0.35	-
2	10	0.14	0.54	0.55	0.17	

[a]The sugars were analyzed, and estimated, by g.l.c., using column *I* at 185°.

second periodate oxidation at 5° in the dark. After the usual treatment, the product was isolated by lyophilization. On complete hydrolysis of the material, followed by the usual processing, and g.l.c. analysis, only one peak, corresponding to galactose, was detected.

Oxidation of carboxyl-reduced PS-AI with chromium trioxide[11]. — A mixture of carboxyl-reduced PS-AI (6 mg) and *myo*-inositol (1 mg) was dissolved in formamide (0.5 mL). To the solution were added pyridine (3 mL) and acetic anhydride (2.5 mL), and the mixture was stirred for 16 h at room temperature. The formamide was removed under diminished pressure at 80°, and the product was reacetylated, to ensure complete acetylation. Powdered CrO_3 (300 mg) was added to a solution of the acetylation product in glacial acetic acid (3 mL), and the mixture was stirred at 50°. Aliquots were removed at intervals of 0, 1, and 2 h, and each was immediately diluted with water to stop further oxidation. The solution was extracted with chloroform (3 × 20 mL), and the extracts were combined, washed with water (3 × 20 mL), dried (anhydrous sodium sulfate), and evaporated to dryness. The product was deacetylated with 0.2M sodium methoxide (1 mL) for 4 h, decationized with Dowex-50W X8 (H^+) resin, and the material hydrolyzed with 0.5M H_2SO_4 for 16 h at 100°. After the usual treatment, the hydrolyzate was analyzed by g.l.c. (column *I*). The results are shown in Table III.

RESULTS AND DISCUSSION

After removal of free sugars by ethanol treatment, the Mahua flowers were extracted with cold water overnight, and the polysaccharide was isolated from the aqueous filtrate by repeated precipitation with ethanol. The polysaccharide was designated PS-A, and had $[\alpha]_{589.6}^{23}$ −55°. The residue was extracted with hot water for 4 h, and a second polysaccharide was obtained from the clear, hot-water extract by ethanol precipitation. This polysaccharide, $[\alpha]_{589.0}^{23}$ +40°, was termed PS-B. On gel filtration through a column of Sephadex G-150, polysaccharide PS-A was separated into two fractions (monitored by a differential refractometer), and each was separately lyophilized. The two fractions of polysaccharides were designated PS-AI and PS-AII,

respectively. Both polysaccharides were found homogeneous by high-voltage electrophoresis; they had $[\alpha]_{589.6}^{23}$ $-9.3°$ and $-19.5°$, respectively. The polysaccharides PS-AI and PS-AII were separately hydrolyzed with 0.5M sulfuric acid for 20 h at 100°. On paper-chromatographic examination using different solvents, the hydrolyzate of PS-AI gave spots corresponding to galactose, arabinose rhamnose xylose, and glucuronic acid (and a faint spot near the base line), whereas that of PS-AII gave a spot corresponding to glucose (besides all of the monosaccharides present in PS-AI). G.l.c. analysis of the alditol acetates from the components of the hydrolyzate of PS-AI gave peaks corresponding to galactose, arabinose, rhamnose, and xylose.

The sugar components in the polysaccharide having been identified, it was necessary to estimate them (see Table I). The uronic acid was determined spectrophotometrically by the carbazole–sulfuric acid method[5], and its proportion was found to be 20%. The carboxyl groups in polysaccharide PS-AI were reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate[4]. The alditol acetates obtained from the hydrolyzate of the carboxyl-reduced PS-AI showed the presence of galactose (59.5), arabinose (16.2), rhamnose (3.0), xylose (2.0), and glucose (19.6%). The presence of glucose in the hydrolyzate of the reduced polysaccharide confirmed the presence of glucuronic acid. The sugar components were isolated by preparative paper-chromatography, and their configurations, as determined by their specific rotations, were found to be D-galactose, L-arabinose, L-rhamnose, D-xylose, and D-glucuronic acid.

The polysaccharide PS-AI and its carboxyl-reduced product were methylated, first by the Hakomori method[7] and then by the Kuhn method[8]. The i.r. spectra of the permethylated products showed no hydroxyl stretching at 3600–3300 cm^{-1}, indicating that the methylation was complete. A portion of the permethylated polysaccharide was hydrolyzed, first with formic acid for 2 h, and then with 0.5M sulfuric acid for 20 h. The alditol acetates of the partially methylated sugars in each hydrolyzate were identified, and their relative molar proportions were determined by g.l.c. The results are summarized in Table II. From these results, some structural features of Mahua polysaccharide PS-AI may be developed. In the hydrolyzate of methylated PS-AI and its carboxyl-reduced product, L-arabinose was found to be present as its 2,3,5-tri- and 2,4-di-*O*-methyl derivatives, which indicated that the arabinose residues are present both in the furanose and the pyranose form; the former resulted from nonreducing groups, whereas the latter were formed from residues present in the interior part of the polysaccharide and joined in (1→3) linkage. Similarly, L-rhamnose and D-xylose are both present in the pyranose form; the former, having (1→2) linkages, is in the interior part of the molecule, as 3,4-di-*O*-methylrhamnose was obtained, whereas the latter is present as nonreducing groups, as revealed by the formation of 2,3,4-tri-*O*-methylxylose.

The only glucose derivative that could be characterized was 2,3,4,6-tetra-*O*-methylglucose, and this was detected only as nonreducing end-groups. Characterization of large proportions of 2,4-di-*O*-methylgalactose and 2,3-di-*O*-methylgalactose in these two polysaccharides indicated that the molecule is highly branched, and that

the branch points originate at O-1, O-3, and O-6, and O-1, O-4, and O-6 of D-galactosyl residues. The rest of the galactosyl residues in the chain are (1→4)- and (1→6)-linked, as 2,3,6-tri- and 2,3,4-tri-*O*-methylgalactose were obtained. A point still not explained is that the sum of the nonreducing ends [*viz.*, tetra-*O*-methyl-galactose and -glucose (carboxyl reduced PS-AI) and tri-*O*-methyl-arabinose and -xylose] exceeds the branch points, *viz.*, the di-*O*-methylgalactose.

The polysaccharide PS-AI was oxidized with sodium metaperiodate, and the consumption of periodate was monitored spectrophotometrically[9]. The consumption of periodate became constant in 72 h, and corresponded to 1.10 mol of periodate per mol of hexosyl residue. The observed value of periodate uptake was in good agreement with the theoretical amount required for the linkages proposed. On complete hydrolysis, the Smith-degraded material gave galactose (24%) and arabinose (3%), besides glycerol and other polyhydric alcohols, and aldehydes. The proportion of sugars resistant to periodate oxidation was in good agreement with the theoretical values expected from the methylation studies. When the material was hydrolyzed under mildly acidic conditions, traces of arabinose and galactose were detected. In addition to these sugars, spots corresponding to three oligomers having R_{Lac} values of 0.98, 1.3, and 1.73 were obtained, which indicated that arabinose residues had been released during the mild treatment with acid. This hydrolysis was found suitable for release of the three oligosaccharides. When subjected to a second periodate oxidation, followed by reduction with $NaBH_4$, the Smith-degraded material gave a product containing galactose. Identification of this product after the second periodate oxidation suggests the possibility of a backbone of (1→3)-linked galactopyranosyl residues in the polysaccharide

In order to ascertain the anomeric configurations of the different sugar residues, the acetylated derivative of the carboxyl-reduced PS-AI was subjected to oxidation with chromium trioxide[11,12] in acetic acid for different time-intervals at 50°, using *myo*-inositol as the internal standard. After deacetylation with sodium methoxide, the products were hydrolyzed, and the alditol acetates of the different sugars were estimated by g.l.c. The results are shown in Table III. From these results, it appears that the amounts of all of the sugar components decreased very rapidly with time. This is due to abstraction of an axially oriented 1-proton to yield a 5-hexulosonic acid, thus leading to the disappearance of the sugar residues having β-glycosidic linkages. Hence, it may be concluded that all of the monosaccharides present in Mahua polysaccharide PS-AI are β-linked.

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