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Note

Structural studies of a polysaccharide from the seeds of *Nelumbo nucifera*

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The medicinal plant *Nelumbo nucifera* (Nymphaeaceae), a handsome aquatic herb with a stout creeping rhizome, grows abundantly throughout India, and its leaves and rhizomes are widely used to treat diarrhoea, dysentry, and dyspepsia[1]. While there has been considerable work carried out in studying various parts of the plant[1–9] the structure of its seed polysaccharide has not yet been reported. We now report on a neutral polysaccharide isolated from the seed of *N. nucifera*. Extraction of the powdered and defatted seeds of *N. nucifera* with cold water furnished a crude polysaccharide that was further purified through fractionation over columns of Sephadex G-100 and G-150. The purified polysaccharide was found to be neutral and had $[\alpha]^{26}_{589.6}$ −19.6° (*c* 0.23, water). It appeared to be honmogeneous by high-voltage paper electrophoresis. Hydrolysis of the polysaccharide produced arabinose, galactose, mannose, and glucose in the

TABLE I

Products of acid hydrolysis of the polysaccharide from Nelumbo nucifera[a]

Sugars	$[\alpha]_D^{22}$ *(Water)*	R_{Gal}[b]		*T*[c]	*Molar ratio*
		Solvent A	*Solvent B*		
D-Galactose	+79° (lit. +80°)[d]	1.00	1.00	0.87	3.9
L-Arabinose	+103° (lit. +105°)[d]	1.58	1.60	0.28	3.0
D-Mannose	+13.5° (lit. +14°)[d]	1.42	1.40	0.74	1.0
D-Glucose	+52° (lit. +53°)[d]	1.14	1.13	1.00	2.1

[a]See Experimental section for details. [b]R_F Values of sugars, relative to that of D-galactose as 1.00. [c]Retention times of the corresponding alditol acetates, relative to that of hexa-*O*-acetyl-D glucitol as 1.00. [d]Ref. 21.

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TABLE II

Methylation studies of the polysaccharide from *Nelumbo nucifera*

Methylated sugars[a] (as alditol acetates)	T^b		*Mole Proportion*	*Mode of linkage*
	1	*2*		
2,3,5-Ara	0.48	0.44	14	Ara*f*-(1→
2,3,4,6-Glc	1.00	1.00	0.85	Glc*p*-(1→
2,3,4,6-Man	1.00	0.99	6	Man*p*-(1→
2,4,6-Gal	2.29	2.03	7	→3)-Gal*p*-(1→
2,3,6-Man	2.20	2.02	4	→4)-Man*p*-(1→
2,3,6Glc	2.50	2.31	17	→4)-Glc*p*-(1→
2,6-Gal	3.65	3.14	32	→3,4)-Gal*p*-(1→
2,4,6-Glc	1.96	1.82	3	→3)-Glc*p*-(1→
2,3,4-Ara	0.73	0.54	16	Ara*p*-(1→

[a] 2,3,5-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-L-arabinitol, *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 1.00 on a column of (1) 3% ECNSS-M and (2) 3% OV-225.

molar ratios of 3.0:3.9:1.0:2.1 (see Table I). The configurations of galactose, mannose, and glucose as D and arabinose as L were assigned from optical rotation measurements. Complete methylation of the polysaccharide by the Hakomori method[10] and hydrolysis of the derived permethylated polysaccharide yielded the sugars as shown in Table II. These data reveal that both L-arabinofuranosyl and L-arabinopyranosyl units are present at the non-reducing ends. A small number of the non-reducing termini are occupied by D-glucopyranosyl and D-mannopyranosyl units. The formation of 2,4,6-tri-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, 2,3,6-tri-*O*-methyl-D-glucose, and 2,4,6-tri-*O*-methyl-D-glucose indicated the polysaccharide contained *inter alia*, 3-linked D-galactopyranosyl, 4-linked D-mannopyranosyl, 4-linked-D-glucopyranosyl, and 3-linked D-glucopyranosyl residues. Finally the presence of a large molar proportion of 2,6-di-*O*-methyl-D-galactose indicated the polysaccharide to be highly branched with 3,4-linked D-galactopyranosyl units at the branch points. Upon oxidation until consumption of periodate ion ceased (44 h), the polysaccharide consumed 0.75 mole of sodium metaperiodate per mole of sugar residue, with the liberation of 0.2 mole of formic acid per hexosyl unit. Hydrolysis of the periodate-oxidized and borohydride-reduced polysaccharide gave D-galactose (98%) and D-glucose (13%). None of the monomer units in the polysaccharide survived a second Smith degradation. Mild acid hydrolysis of the polysaccharide removed some of the arabinosyl units providing further evidence that these arabinosyl units were furanoid.

EXPERIMENTAL

General methods. — Evaporations were performed under diminished pressure at ~ 45° (bath) and small volumes of aqueous solutions were lyophilized. Paper chroma-

tography (descending) for detection and preparative isolation of the sugars was performed on Whatman No. 1 and 3 MM papers respectively using solvent mixtures (v/v) A, (8:3:1 ethyl acetate–pyridine–water); B, (the upper layer of 4:1:5 butanol–acetic acid–water); and C, (upper layer of 4:1:5 butanol–ethanol–water). Visualising reagents were (a) ammoniacal silver nitrate[11] and (b) saturated aqueous aniline hydrogen oxalate[12]. Optical rotations were determined with a Perkin–Elmer Model 241 MC spectropolarimeter. I.r. spectra (KBr pellets) were recorded with a Beckman Acculab 10 instrument. U.v. and vis. spectra were recorded with a Beckman Model 26 spectrometer. G.l.c.of the monosaccharide as their alditol acetates[13] and partially methylated alditol acetates[14] was conducted on glass columns containing (1) 3% of ECNSS-M on GasChrom Q (100–120 mesh) and (2) 3% of OV-225 on GasChrom Q (100–120 mesh), with nitrogen as the carrier gas, using a Packard Model 419 and a Hewlett–Packard Model 5713A gas chromatographs each equipped with a flame ionization detector.

Isolation and purification. — Air-dried seeds (600 g) of *N. nucifera*, following solvent extraction and cold water extraction as previously described[15], yielded 20 g of polysaccharide. A portion (0.25 g) of the polysaccharide was purified through fractionation over column of Sephadex G-100 (2.5 × 100 cm), using sodium phosphate buffer (pH 7.4). The major polysaccharide fraction that had no appreciable u.v. absorption was freeze dried after exhaustive dialysis. As high-voltage paper electrophoresis revealed the fraction to be heterogeneous, it was again fractionated on a column of Sephadex G-150 (2.5 × 100 cm) with the same phosphate buffer as the eluant. The major portion of the material was eluted as a single peak, along with two other small ones. The resulting major polysaccharide (carbohydrate content[16] 98.8%) was again passed through a Sephadex G-100 column using the same phosphate buffer. Fractions containing phenol–sulphuric acid positive materials were collected, dialyzed against distilled water, and lyophilized to give 122 mg of product. This polymer was shown to be free from uronic acid[17], halogen, nitrogen, and sulfur[18], and was used for further analysis.

Homogeneity of the polysaccharide. — The homogeneity of the polysaccharide was checked by high-voltage paper electrophoresis on a strip of Whatman No. 1 paper (20 × 18 cm) with an applied voltage of 600 V and 15 mA, using borate buffer pH 10.4 at 2° in a Shandon Model L-24 instrument. Detection was carried out as previously described[15].

Acid hydrolysis. — The polysaccharide (45 mg) was hydrolysed with 25 mL of M sulfuric acid using *myo*-inositol (5 mg) as the internal standard for 18 h at 100°. The hydrolyzate was neutralized with barium carbonate, centrifuged, the centrifugate deionized with Amberlite IR-120 (H^+) resin, and then concentrated. P.c. (Solvents A and B) of the hydrolyzate revealed arabinose, galactose, mannose, and glucose, the identities of which were further confirmed by g.l.c. (column 1) of the derived alditol acetates[13]. Their complete identification as L-arabinose, D-galactose, D-mannose, and D-glucose was then achieved by isolation through preparative p.c. (solvent C) and measurements of optical rotations.

Methylation analysis. — The polysaccharide (25 mg) was twice sequentially

methylated by the method of Hakomori[10] using Me_2SO (15 mL) and methyl sulphinyl sodium (15 mL, 30 mmol) to give a product (29 mg) that showed no i.r. absorption for hydroxyl. The fully methylated polymer was treated with aqueous 90% formic acid (15 mL) for 4 h at 100°, the formic acid was removed by codistillation with water, and the product was hydrolyzed with 0.5M sulfuric acid (10 mL) for 14 h at 100°. The hydrolyzate was neutralised with $BaCO_3$, centrifuged, the supernatant solution was deionized with Amberlite IR-120 (H^+) resin, and evaporated *in vacuo*. The residue was repeatedly extracted with hot chloroform (4 × 5 mL), and the combined extracts were evaporated to dryness, redissolved in water (5 mL), and then reduced with $NaBH_4$ (3.12 mmol). After decomposition of the excess borohydride with acetic acid (0.5 mL), the solution was deionized with Amberlite IR-120 (H^+) resin, freed from boric acid by repeated codistillation with methanol *in vacuo*. The alditols were then acetylated with 1:1 pyridine–acetic anhydride (5 mL, 26.5 mmol) for 25 min at 100°. After repeated evaporation of the solvents with dry toluene (4 × 5 mL), the product was dried *in vacuo* over solid NaOH. The partially methylated alditol acetate mixture was then analyzed by g.l.c. (columns 1 and 2).

Periodate oxidation and Smith degradation. — The polysaccharide (5 mg) was dissolved in water (5 mL) and treated with 0.04M sodium metaperiodate (20 mL) at 4° in the dark. Consumption of the oxidant became constant (monitored spectrophotometrically[19] at 225 nm) within 44 h, and the amount of periodate consumed was 0.75 mole per mole of hexosyl unit. The amount of formic acid liberated, as estimated by the method of Hirst and coworkers[20], was 0.2 mole per mole of hexosyl residue.

In a separate experiment the polysaccharide (15 mg) was oxidized with 0.04M sodium metaperiodate (20 mL) in the dark for 44 h at 4°. The excess of periodate was decomposed by adding ethylene glycol (1.5 mL). The solution was then dialyzed, the dialyzate was concentrated and treated with Amberlite IR-120 (H^+) resin, boric acid was removed by coevaporation with methanol and dried to yield 14 mg of product. A part (2 mg) of the product was hydrolyzed (along with *myo*-inositol) with 0.5M sulfuric acid (5 mL) for 18 h at 100°. After usual processing the product was examined by p.c. (solvent B) and g.l.c. (as the alditol acetates), and it was determined that galactose and glucose were the surviving monosaccharides. The remaining part of the product (12 mg) was treated with 0.5M H_2SO_4 for 24 h at room temperature, the acid neutralized with $BaCO_3$, centrifuged, the supernatant solution was deionized with Amberlite IR-120 (H^+) resin, and concentrated. The residue was subjected to a second Smith degradation, and no monosaccharide was detected after acid hydrolysis of the oxidized solution.

Mild selective hydrolysis. — The polysaccharide (8 mg) was hydrolyzed with 0.1M trifluoroacetic acid (2 mL) for 20 min at 95°, and the product was dialyzed against distilled water, affording non-dialyzable polymeric material and a dialyzate. The presence of only arabinose in the dialyzable fraction was shown by p.c. in solvent A and also by g.l.c. as its alditol acetate[13] using column 1.

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