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SOME STRUCTURAL FEATURES OF THE D-GLUCAN FROM THE SEED OF *Mirabilis jalapa*

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ABSTRACT

The cotyledon of the seed of *Mirabilis jalapa* was found to contain a D-glucan Methylation, periodate oxidation, and graded and enzymic hydrolysis studies were conducted to elucidate its structure For every 38 D-glucosyl residues therein, 34 are (1→4)- and 3 are (1→3)-linked, the D-glucosyl unit at the branch point is linked through O-1, O-2, and O-4 In some places in the chain, there are at least three (1→3)-linked D-glucosyl residues in a sequence Both α- and β-D-glucosidic linkages are present in the polysaccharide, the former preponderating The D-glucan gave with iodine a faint blue color that had λ_{max} 420 nm

INTRODUCTION

An Indian plant, *Mirabilis jalapa*, family Nyctaginaceae, produces and stores a significant amount of a protein-free glucan[1] in the cotyledon in its seed The seeds and roots of the plant are used in Indian medicine[2] for curing syphilitic sores, subduing inflammation, and providing purgative action In this communication, structural elucidation of the D-glucan isolated from it is reported

EXPERIMENTAL

Descending paper-chromatography (p c) was performed on Whatman No 1 MM paper for qualitative separations, and Whatman No 3 MM paper for large amounts The following solvent systems (v/v) were used (*A*) 1-butanol–acetic acid–water (4 1 5, upper phase)[3], and (*B*) 9 2 2 ethyl acetate–acetic acid–water[4] Chromatograms were developed by using alkaline silver nitrate reagent[5] Evaporations were conducted in a rotary evaporator below 40° (bath temperature) Gel-filtration chromatography was performed with Sephadex LH-20, G-25, and G-100 Elutions were monitored both with a differential refractometer (Model R-403) and polarimetrically G l c was performed with a Hewlett–Packard Model 5713 A gas chromatograph fitted with an f i d detector and glass column (1 83 m × 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) Paper electrophoresis was conducted in a Shandon high-voltage apparatus. Model L 24 Optical rotations were measured with a Perkin–Elmer 141 polarimeter I r spectra were recorded with a Beckman IR-20-A spectrophotometer

Isolation of the polysaccharide — The seeds were freed from the coat, and were powdered The material (10 g) was dissolved in water (500 mL) and an equal volume of ethanol was added. to precipitate the polysaccharide material, this was freed of ethanol, and redissolved in water The precipitation was repeated, and the precipitate obtained was collected at the centrifuge. triturated with acetone, and dried over P_2O_5, yield 8 g The purified polysaccharide (20 mg) was dissolved in ammonium hydrogencarbonate buffer (pH 8 0), and the solution was applied to a column (80 × 2 5 cm) of Sephadex G-100 which was eluted with the same buffer The major portion of the material was eluted as a single fraction, and the solution was freeze-dried In high-voltage electrophoresis (40 V/cm) in borate buffer, pH 9 0, for 90 min, it moved towards the anode as a single component The purified polysaccharide was poorly soluble in cold water but moderately soluble in warm water and in 0 1M sodium hydroxide solution It had $[\alpha]_D^{26}$ +85° (*c* 0 2, water) and +91° (*c* 0 1, 0 1M NaOH solution), moisture content. 8 0% This material was used in further investigations

Hydrolysis of the polysaccharide — The polysaccharide was hydrolyzed with 0 5M sulfuric acid for 16 h at 100° in a sealed tube The acid was neutralized with barium carbonate, and the suspension was centrifuged The supernatant liquor was concentrated, and in p c (solvents *A* and *B*) gave only one spot, corresponding to glucose The polysaccharide (2 0 mg) was next hydrolyzed with 0 5M sulfuric acid with inositol (1.8 mg) as an internal standard, and the sugars were converted into their alditol acetates G l c gave a peak corresponding to that of glucose (besides that of inositol) The proportion of glucose was estimated to be 88 0% of the polysaccharide Glucose in the neutral hydrolyzate of the polysaccharide was also estimated, by using glucostat[6], to be 89 0%

Iodine complex of the D-*glucan* — The absorption spectrum of the iodine complex was measured under the conditions used by Peat and co-workers[7] An aqueous solution containing 0 02% of the polysaccharide was mixed with an equal volume of 0 4% iodine in aqueous potassium iodide The optical absorbance of the solution was measured against a blank containing 0 02% of iodine in 0 2% potassium iodide solution The absorption maximum was found to be at 420 nm The effect of salts, *viz*., $(NH_4)_2SO_4$, $MgSO_4$, $(NH_4)_3PO_4$, and $CaCl_2$, was measured by using the same solution, but containing 2% of the salt

Methylation of the glucan — Thoroughly dried polysaccharide (20 mg) was methylated twice by the Hakomori method[8] The final product was dialyzed, and the solution freeze-dried, yield 15 mg, $[\alpha]_D^{26}$ +50° (*c* 1 4, $CHCl_3$) The i r spectrum showed no absorption band at 3600–3300 cm^{-1}, indicating the absence of hydroxyl groups The fully methylated glucan was hydrolyzed by heating in a boiling-water bath with 90% formic acid, and then with 0 25M sulfuric acid[9] The methylated sugars

TABLE I

RESULTS OF THE METHYLATION ANALYSIS OF THE D-GLUCAN

Alditol acetate from	*Deduced structural unit*	*Retention time[a] (min)*		*Percent composition*	*Molar ratio*
		Column 1	*Column 2*		
2,3,4,6-Tetra-*O*-methyl-D-glucose	non-reducing end-group	1.00	1.00	3.1	1.1
2,4,6-Tri-*O*-methyl-D-glucose	(1→3)-linkage	1.96	1.83	7.7	3.1
2,3,6-Tri-*O*-methyl-D-glucose	(1→4)-linkage	2.50	2.32	84.4	33.1
3,6-Di-*O*-methyl-D-glucose	(1→2→4)-linkage	4.60	3.72	2.6	1.1

[a]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 on (*1*) ECNSS-M column at 170°, and (*2*) OV-225 column at 170

were converted into their alditol acetates[10], and these were analyzed by g.l.c. (columns *1* and *2*). The results are given in Table I.

Periodate oxidation — The D-glucan (4.0 mg) was dissolved in water (2.0 mL), and 0.04M sodium metaperiodate (2.0 mL) and water (2.0 mL) were added. The reaction was allowed to proceed in the dark at 4°. The consumption of the oxidant was monitored spectrophotometrically[11–13], and the formic acid liberated was estimated[14] by titrating with 0.01M NaOH, using Methyl Red as the indicator. The uptake of periodate and the liberation of formic acid became constant in 10 h, corresponding to 0.97 and 0.08 mol, respectively, per mol of hexosyl residue.

The D-glucan (40 mg) was oxidized with periodate for 10 h, the excess of the oxidant was decomposed with ethylene glycol, and the solution was dialyzed, and then freeze-dried. This material, in water, was reduced with sodium borohydride, and the solution was passed through a column of Dowex 50 (H^+) ion-exchange resin. Boric acid was removed by co-distillation with methanol, and the material was freeze-dried, yield 34 mg.

A part of the material (10 mg) was dissolved in 0.5M sulfuric acid, and the solution was kept for 24 h at room temperature. After the usual treatment, the hydrolyzate was examined by p.c., besides spots corresponding to polyhydric alcohols, glucose and an oligomer having R_{Glc} 0.38 (solvent *A*) were detected.

The periodate-oxidized material (2.0 mg) was completely hydrolyzed (along with an internal standard). The proportion of glucose resistant to periodate was estimated by g.l.c. to be 9.5%.

The periodate-oxidized, reduced, mildly acid-hydrolyzed[15] product (~60 mg) was separated on thick filter-paper. The zone containing the oligomer was cut out, and eluted with water. The solution was concentrated, and then lyophilized, to give a solid product, yield, 4.0 mg. It was homogeneous in paper chromatography, and had $[\alpha]_D^{26}$ +3° (*c* 0.1, water). The oligomer was methylated by the Hakomori

TABLE II

RESULTS OF METHYLATION STUDIES OF THE OLIGOMERS

Alditol acetate from	*Retention time[a] (min) in column* 1	2	*Mole ratio of methylated sugars in Graded-hydrolysis product* *Oligomer I*	*Oligomer II*	*Oligomer III*	*Oligomer IV*	*Oligomer V*	*Smith-degradation product*
2,3,4,6-Tetra-*O*-methyl-D-glucose	1 00	1 00	1 0	1 2	1 0	2 2	1 0	1 0
2,4,6-Tri-*O*-methyl-D-glucose	1 96	1 83					2 9	1 9
2,3,6-Tri-*O*-methyl-D-glucose	2 50	2 32	1 0	2 2	5 0	4 2	3 8	
3,6-Di-*O*-methyl-D-glucose	4 40	3 72				1 0		

[a]Retention times are given relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on (*1*) ECNSS-M column at 170°, and (*2*) OV-225 column at 170°

method[8], the permethylated product was hydrolyzed, and the methylated sugars were identified and estimated by g l c The results are given in Table II

Graded hydrolysis — The D-glucan (150 mg) was heated with 40% formic acid in a boiling-water bath for 1 25 h, and the formic acid was then removed *in vacuo* The hydrolyzate gave six spots in p c The mixture was separated into its components on 3 MM paper, using solvent *A* Each fraction isolated was found to be homogeneous

Fractions II–VI were designated as oligomers I–V These oligomers were separately methylated by the Hakomori method[8], and the product was isolated by extraction with chloroform The extract was washed with water, dried (anhydrous sodium sulfate), and evaporated, and the final product was dried over P_2O_5 *in vacuo* These methylated oligomers showed no hydroxyl-group absorption-band at 3600–3300 cm^{-1} in their i r spectra, indicating complete methylation The methylated oligomers were analyzed by the method described for methylation analysis of the glucan[9,10]

Fraction I (110 mg) In p c , it moved with glucose, $[\alpha]_D^{26}$ +53° (*c* 0 2, water) *Fraction II* (3 mg) It had $[\alpha]_D^{26}$ +115° (*c* 0 24), and in p c , it moved with maltose On complete hydrolysis followed by g l c , it gave glucose On hydrolysis, the fully methylated derivative yielded 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-glucose in the molar ratio of 1·1 *Fraction III* (3 mg) It had $[\alpha]_D^{26}$ +162° (*c* 0 24) Complete hydrolysis gave glucose The fully methylated derivative, on hydrolysis, yielded the 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl derivatives in the molar ratio of 1 2.1 *Fraction IV* (2 5 mg), $[\alpha]_D^{26}$ +125° (*c* 0 2) On hydrolysis, the permethylated derivative yielded 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-glucose in the molar ratio of 1·5 *Fraction V* (2.5 mg), $[\alpha]_D^{26}$ +93° (*c* 0 2) On hydrolysis, the permethylated derivative yielded 2,3,4,6-tetra-, 2,3,6-tri-, and 3,6-di-*O*-methyl-D-glucose in the molar ratios of 2 2·4 2 1

TABLE III

RESULTS OF OXIDATION OF ACETYLATED D-GLUCAN WITH Cr_2O_3

Time of oxidation (h)	D-*Glucose survived*[a] *(mg)*	D-*Glucose oxidized (%)*
0	10 0	0
1	7 2	28 2
2	5 5	44 6
3	3 6	63 9

[a]The amount of D-glucose that survived was measured by using *myo*-inositol (9 5 mg) as the internal standard

Fraction VI (4 mg), $[\alpha]_D^{26}$ −36° (*c* 0 33) On hydrolysis, the fully permethylated derivative yielded 2,3,4,6-tetra-, 2,4,6-tri- and 2,3,6-tri-*O*-methyl-D-glucose in the molar ratios of 1 2 97 3 9

Oxidation with chromium trioxide — To a mixture of the D-glucan (10 mg) and *myo*-inositol (9 5 mg) in formamide (1 0 mL) were added acetic anhydride (2 mL) and pyridine (2 5 mL), with stirring After stirring for 16 h at room temperature, the mixture was dissolved in chloroform (25 mL), washed with water (3 × 25 mL), dried (anhydrous sodium sulfate), and evaporated to dryness The material was re-acetylated in the same way The acetylation product was dissolved in glacial acetic acid (5 mL) and the solution kept at 50°, chromium trioxide (300 mg) was added, and aliquots were removed at intervals, and immediately diluted with water to stop the oxidation The mixture was extracted with chloroform and the extract was dried (anhydrous sodium sulfate), and evaporated to dryness The material so obtained was deacetylated with 0 2M sodium methoxide, and the solution passed through a column of Dowex 50 (H^+) ion-exchange resin The solution was evaporated to dryness, and the residue treated with 0 5M sulfuric acid for 16 h at 100° The sugars in the hydrolyzate were converted into their alditol acetates, and these were estimated by g l c The results are given in Table III

Enzymic hydrolysis — The D-glucan (20 mg) was dissolved in 20 mL of phosphate buffer, pH 5 5, and 1500 units (u) of alpha amylase from *Bacillus subtilis* (~1800 u/mg) were added After incubating the mixture for 12 h at 37°, an equal quantity of the enzyme was added, and the incubation was continued for 12 h Samples were removed at intervals of 4, 8, 12, and 24 h from the beginning of the reaction, heated for 30 min at 100°, centrifuged, and the supernatant liquor deionized by passage through columns of IR-45 and Dowex 50 (H^+) resins The solutions were concentrated to small volumes, and examined by p c (solvent *A*) In all cases, spots corresponding to glucose, maltose, and maltotriose (along with two unidentified components) were detected

In a separate experiment, the D-glucan (20 mg) in 20 mL of phosphate buffer, pH 5 5, was incubated for 24 h with 2 5 mg of beta amylase, obtained from sweet

potato. After adding a fresh amount of enzyme, the incubation was continued for another 24 h At the end of 48 h, the mixture was heated for 30 min at 100°, followed by centrifugation The supernatant liquor was de-ionized, and then concentrated to ~5 mL. Ethanol was added, and the white precipitate formed was separated at the centrifuge triturated with acetone and dried *in vacuo* over P_2O_5 yield 11 mg $[\alpha]_D^{26}$ +39° (c 0 2. water) The beta-amylase-treated material (7 mg) was oxidized for 10 h in the dark at 4° with 0 04M sodium metaperiodate The reaction was stopped by adding ethylene glycol. and the solution was dialyzed and then freeze-dried. yield 2 mg The percentage of glucose present in it was estimated by g l c to be 16 2%

RESULTS AND DISCUSSION

Cold-water extraction of the seeds of *Mirabilis jalapa* yielded a polysaccharide material which was repeatedly precipitated with ethanol It was further purified by gel filtration through a column of Sephadex G-100 The material obtained as the major component had $[\alpha]_D^{26}$ +85° contained 96% of glucose, and was paper-electrophoretically homogeneous The glucan gave a blue color with iodine solution[17], and the λ_{max} was at 420 nm (E_{max} 0 5) $(NH_4)_2SO_4$, $MgSO_4$, $(NH_4)_3PO_4$, and $CaCl_2$ had little effect on the λ_{max} value, but the E_{max} increased to 0 7 These results indicate the predominance in the polysaccharide of α-(1→4) linkages with short, exterior chains[16]

The D-glucan showed absorption bands at 1630 (br), 925, 891 (sh), 850, and 760 cm^{-1} in the i r. spectrum The bands at 850 and 891 cm^{-1} were, respectively, ascribed to α- and β-type glycosidic linkages in the polysaccharide[17] The bands at 850 and 925 cm^{-1} are characteristic of (1→4)-α-glucans The broad band at 1630 cm^{-1} was due to bound water[18] The i r spectrum, together with the positive specific rotation (which is much smaller than those of amylose and amylopectin), indicates the presence, in the D-glucan of α- and β-glucosidic linkages, the former being predominant

The D-glucan was converted into its fully methylated derivative by the Hakomori method[8] The methyl sugars obtained on hydrolysis thereof were converted into their alditol acetates, and these were examined by g l c The results are given in Table I Presence of 2.3,6- (84 4%) and 2,4,6-tri-*O*-methyl-D-glucose (7 7%) in the hydrolyzate of the fully methylated D-glucan indicates the existence of (1→4)- and (1→3)-linked D-glucosyl residues in the polysaccharide in the molar ratio of 11 . 1 For each 38 D-glucosyl units in the polysaccharide, there is one branch point, and the D-glucosyl residue at the branch point is 1,2,4-linked The 2,3,4,6-tetra-*O*-methyl-D-glucose obviously originated from the (nonreducing) end groups

On periodate oxidation, the D-glucan consumed 0 97 mol of the oxidant, liberating 0 08 mol of formic acid, per mol of D-glucosyl residue in 10 h The theoretical values for periodate consumption and formic acid liberation, calculated on the basis of the result of methylation studies, are 0 95 and 0 06 mol. respectively Thus,

the periodate-oxidation results are in close agreement with those of the methylation studies

On hydrolysis, the periodate-oxidized D-glucan yielded glycol, erythritol, and glucose Methylation studies showed that the total of (1→3)- and 1,2,4-linked D-glucose units in the polysaccharide was 10 3%, the D-glucosyl residues resistant to periodate oxidation were estimated to constitute 9 5% These results are in close agreement. Glycol and erythritol obviously originated from other portions of the polysaccharide The periodate-oxidized reduced product was subjected to mild, acid hydrolysis[15], and the hydrolyzate was found to contain glucose and an oligosaccharide (besides other polyhydric alcohols) The mixture was separated on thick filter-papers and the oligosaccharide was isolated in homogeneous state On methylation and hydrolysis, it gave 2,3 4,6-tetra- and 2,4 6-tri-*O*-methylglucose in the molar ratio of almost 1 2 This result showed that, at some places in the D-glucan three (1→3)-linked D-glucosyl residues are present in sequence

On graded hydrolysis, the D-glucan gave glucose and five oligomers which were isolated as homogeneous fractions They were characterized by methylation studies The results are given in Table II Oligomer I was found to be maltose, whereas oligomer II and oligomer III were maltotriose and maltohexaose respectively On hydrolysis, the methylated derivative of oligomer IV yielded 2,3,4,6-tetra- (2 mol), 2,3 6-tri- (4 mol), and 3,6-di-*O*-methyl-D-glucose (1 mol) This fragment obviously originated from that part of the polysaccharide which contained branch points This oligosaccharide contained seven D-glucosyl units, but the results of the present studies do not provide the exact distribution of D-glucosyl units in the two chains Oligosaccharide V had a negative specific rotation (−36°), and on methylation followed by hydrolysis, gave 2,3,4,6-tetra- (1 mol), 2 3 6-tri- (4 mol), and 2 4 6-tri-*O*-methyl-D-glucose (3 mol) This fragment contained both (1→4)- and (1→3)-linked D-glucosyl units and had some β-glucosidic linkages Smith degradation of the intact polysaccharide yielded a trimer containing (1→3)-linkages and the graded-hydrolysis studies supported this conclusion

The peracetate of the D-glucan was subjected to chromium trioxide oxidation[19] The results given in Table III show that some D-glucosyl residues were removed during the oxidation, but the rate was low As Cr_2O_3 oxidizes β- faster than α-linked sugar residues[20], it was concluded that the polysaccharide contains α- and β-D-glucosidic linkages, the former being preponderant Digestion of the D-glucan with alpha amylase yielded major quantities of D-glucose maltose, maltotriose, and a small proportion of D-gluco-oligosaccharides

The proportion of oligosaccharides decreased with increase in incubation time, indicating the presence of a majority of α-(1→4)-linkages in the polysaccharide On treatment with beta amylase, the glucan gave glucose maltose and an oligosaccharide The yield of the last was ~50% of the polysaccharide, and it had a specific rotation of +39° As beta amylase specifically cleaves α-(1→4)-linked D-glucosyl groups from non-reducing ends, it is concluded that the presence of (1→3)-linked D-glucosyl units or β-linkages, or both, in the polysaccharide chain does not allow the enzyme to

degrade the molecule completely into small fragments· this is further supported by the fact that periodate oxidation of beta-amylase-treated material yielded a material containing 16 2% of D-glucose. whereas the intact polysaccharide contained 9 5% of D-glucosyl units resistant to periodate

From these results, it is possible to outline the structural features of the D-glucan isolated from the seed of *Mirabilis jalapa* The polysaccharide is a D-glucan containing α-(1→4)- and (1→3)-linked (7 8%) residues, there are some β-D-glucosidic linkages present in the chain At certain places in the chain, (1→3)-linked trisaccharidic units are present The molecule is branched. and 2–3% of the D-glucose units in it are joined through O-1, O-2. and O-4 From these results, it is not possible to state the exact lengths of exterior and interior chains, or the distribution of (1→3)-linkages in the polysaccharide molecule

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