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A WATER-SOLUBLE GALACTOMANNAN FROM *SESBANIA AEGYPTIACA* SEEDS

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Abstract—A water-soluble galactomannan isolated from the seeds of *Sesbania aegyptiaca* contained D-galactose and D-mannose in the molar proportion of *ca* 1 1 67 Methylation analysis gave 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-mannose (mol ratio 3 2 3) and the result was corroborated by those from periodate oxidation followed by Smith degradation Controlled hydrolysis of the galactomannan afforded two disaccharides and two trisaccharides and their structures have been established Based on these results a structural formula for the galactomannan has been proposed The anomeric configurations of the sugar residues were determined on the basis of mild hydrolysis with dilute oxalic acid, reactions with several specific lectins and oxidation with chromium trioxide The number average degree of polymerization was found to be *ca* 35–38

INTRODUCTION

In view of the constantly increasing demand by industry for plant gums, we were prompted to undertake a chemical investigation of a galactomannan isolated from seeds of *Sesbania aegyptiaca*

RESULTS AND DISCUSSION

A polysaccharide was isolated from the aqueous extracts of the seeds of *Sesbania aegyptiaca* by repeated precipitation with ethanol On hydrolysis it gave D-galactose and D-mannose along with a trace of L-arabinose Purification of the polysaccharide via its copper complex [1] removed the arabinose-containing impurities as well as traces of uronides The purified material giving a single peak on gel filtration through a column of Sephadex G-100 [2], had $[\alpha]_{589.5}^{26} + 51.4°$, contained D-galactose and D-mannose in 1 1 66 (i e 3 5) molar ratio and was homogeneous on paper electrophoresis

To ascertain its structural features, the polysaccharide was fully methylated by the Hakomori method [5], followed by the Purdie method [6] The permethylated polysaccharide was hydrolysed with 85% formic acid, and then with 0 25 M sulphuric acid The alditol acetates [3] of the methylated hydrolysate were identified and their relative mol proportions determined, by GC (Table 1)

The polysaccharide contains D-galactose and D-mannose, both in the pyranose form The non-reducing ends are occupied by D-galactose units, as indicated by the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose and the backbone of the molecule is made up of 1 → 4-linked D-mannopyranose residues as proved by the presence of 2,3,6-tri-*O*-methyl-D-mannose in the hydrolysate of the methylated galactomannan The fact that 2,3-di-*O*-methyl-D-mannose is present in the hydrolysate indicates that the polysaccharide is branched and that at branch points the mannose residues are linked through C-1, C-4 and C-6

The results of periodate oxidation studies [7, 8] are in good agreement with the methylation analysis It was found that 1 mol of formic acid was liberated for every 2 67 hexose residues in 25 hr and that 1 39 mols of periodate reduced per hexose unit in 30 hr The theoretical value expected from the proposed structure is 1 38

The periodate-oxidized polysaccharide, on sodium borohydride reduction and hydrolysis, gave glycerol and erythritol in the mol ratio 1 1 6 (i e 3 5) The absence of mannose in the hydrolysate confirmed that the linkages at the branch points are through C-1, C-4 and C-6 as already mentioned above The length of the repeating unit (2 6), calculated from the amounts of glycerol and erythritol obtained, is in good agreement with the value obtained from methylation and periodate oxidation studies

To establish the stereochemistry of the linkages between the sugar units, the polysaccharide was subjected to graded hydrolysis (Table 2) After separation by CC and PC, the oligosaccharides were characterized as follows

Oligosaccharide I, $[\alpha]_{589.5}^{26} - 7.7°$, equivalent weight 332, on hydrolysis gave mannose only Fully methylated material on hydrolysis yielded 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-mannose in the mol proportion 1 1 The periodate oxidation of the disaccharide was carried out in acetate buffer (pH 3 8), 4 07 mols of periodate were reduced per mol of the disaccharide in 2 5 hr After borohydride reduction, hydrolysis and subsequent treatment, the oxidized product gave glycerol and erythriol, as identified and estimated by GC, in the mol proportion 1 1 This disaccharide was, therefore, assigned as 4-*O*-β-D-mannopyranosyl-D-mannose

Oligosaccharide II, $[\alpha]_{589.5}^{26} + 121°$, equivalent weight 330, on hydrolysis gave mannose and galactose in 1 1 mol ratio Sodium borohydride reduction and subsequent

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Table 1 Methylation analysis of the native polysaccharide (A) and the oligosaccharides (B)

Methyl sugars*	T† I	T† II	Mol proportion A	B I	B II	B III	B IV	Mode of linkage
2,3,4,6-Tetra-*O*-methyl-D-galactose	1 25	1 19	3	—	1	1	—	Gal_p-(1 →
2,3,4,6-Tetra-*O*-methyl-D-mannose	1 00	0 99	—	1	—	—	1	Man_p-(1 →
2,3,6-Tri-*O*-methyl-D-mannose	2 20	2 03	2	1	—	1	2	→ 4)-Man_p-(1 →
2,3,4-Tri-*O*-methyl-D-mannose	2 48	2 19	—	—	1	1	—	→ 6)-Man_p-(1 →
2,3-Di-*O*-methyl-D-mannose	4 83	3 69	3	—	—	—	—	→ 4,6)-Man_p-(1 →

*The methyl sugars were identified as the corresponding alditol acetates

†Retention times are relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D glucitol, on a column of (i) 3% of ECNSS-M, and (ii) 3% of OV-225

Table 2 Results of the graded hydrolysis studies

Spot	PC mobility*	Sugars
1	0 81	4-*O*-β-D-Mannopyrannosyl-D-mannose
2	0 67	6-*O*-α-D-Galactopyranosyl-D-mannose
3	0 42	6-*O*-α-D-Galactopyranosyl-4-*O*-β-D-mannopyranosyl-D-mannose
4	0 17	4-*O*-β-D-Mannopyranosyl-4-*O*-β-D-mannopyranosyl-D-mannose

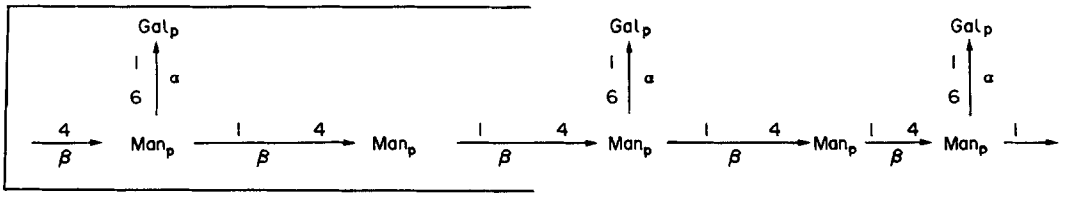
*Relative to D-galactose

hydrolysis gave galactose as reducing sugar Methylation analysis showed the presence of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,4-tri-*O*-methyl-D-mannose This indicates that galactose is glycosydically 1 → 6-linked to mannose with galactose at the non-reducing end The structure of the disaccharide is assigned as 6-*O*-α-D-galactopyranosyl-D-mannose

Oligosaccharide III, $[\alpha]_{589.5}^{26} + 37°$, equivalent weight 498, on hydrolysis gave galactose and mannose in the molar proportion of 1 2 Reduction and hydrolysis reduced 5 01 mols of periodate when oxidation was carried out with sodium metaperiodate in acetate buffer (pH 3 7) Methylation studies showed the presence of 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-mannose in 1 2 mol ratio

All these results indicated the structure of the trisaccharide to be 4-*O*-β-D-mannopyranosyl-4-*O*-β-D-mannopyranosyl-D-mannose

Based on the above results in can be concluded that the galactomannan has a structure of the following type



indicated the presence of galactose and mannose in 1 1 mol proportion with mannose at the reducing end A portion, oxidized with sodium metaperiodate in acetate buffer (pH 3 7), reduced 5 13 mols of the oxidant per mol of the sugar Methylation analysis showed the presence of equimolar proportions of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-mannose Thus the sugar seems to be a trisaccharide containing 1 mol of D-galactose and 2 mols of D-mannose residues, and the structure of the trisaccharide was established as 6-*O*-α-D-galactopyranosyl-4-*O*-β-D-mannopyranosyl-D-mannose

Oligosaccharide IV, $[\alpha]_{589.5}^{26} - 22°$, equivalent weight 494, showed the presence of mannose only on GC of its hydrolysate and on that of its reduction product obtained with sodium borohydride One mol of trisaccharide

The anomeric configurations of the sugar residues were determined by chromium trioxide oxidation [9] of the peracetylated product As chromium trioxide oxidizes β-linked sugar residues faster than those α-linked [10], it was concluded that the polysaccharide contained α- and β-D-glycosidic linkages, the former being preponderant From the results shown in Table 3 it appears that D-mannose was oxidized, and consequently its percentage decreased rapidly, but that the galactose was oxidized comparatively slowly, thereby showing that the galactose had the α-configuration, the rest having the β-configuration It was also noticed that only galactose residues were released when the polysaccharide was hydrolysed under very mild conditions with 0 02 N oxalic acid The resulting galactomannan after 4 hr had a galactose–mannose ratio of 1 2 The increased mannose

Table 3 Survival of sugars in chromium trioxide oxidation of acetylated galactomannan

Time of oxidation (hr)	Galactose	Mannose	Myo-inositol
0	3 1	4 98	10
1	2 26	0 91	10
2	1 17	0 24	10
3	0 97	0 13	10

content is attributed to the fact that the more labile galactose residues are α-linked The occurrence of α-D-galactose residues as branch points in the galactomannan from the seeds of *Sesbania aegyptiaca* was further confirmed by the fact that the α-galactosyl-specific lectins from the seeds of *Artocarpus integrifolia* and *Artocarpus lakoocha* [11] reacted with the galactomannan

Hypoiodite oxidation [4] of the polysaccharide indicated the degree of polymerization to be 35–38

EXPERIMENTAL

All solvent evaporations were conducted in a rotary evaporator below 40° (water bath temp) Specific rotations were recorded at 26° ± 1° and 589 6 nm PC (descending) Whatman No 1 and 3 MM Paper, solvent A, BuOH–pyridine–H_2O (6 4 3), solvent B, BuOH–EtOH–H_2O (4 1 5, upper layer) Aniline oxalate and alkaline $AgNO_3$ were used as sprays Gel-filtration chromatography Sephadex G-100 and G-25, Elutions were monitored by the PhOH–H_2SO_4 method GC-FID/TCD glass column (1 83 m × 6 mm) containing (a) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of sugars) and at 170° (for partially methylated alditol acetates), and (b) OV-225 at 155° A reporting integrator 3390A (Hewlett–Packard) was used to determine the mol ratios High-voltage electrophoresis (Shandon model L-24) buffer system (a) borate buffer, pH 9 5 and (b) Pi buffer, pH 6 5 The spots were developed with benzidine periodate

Isolation and purification of the polysaccharide An aq 1% suspension of powdered seed, made by adding 12 g of the powder to 1 2 l H_2O was kept on a boiling water bath for 1 hr and then agitated in an ultrasonic bath to produce a viscous soln The soln was filtered through a muslin cloth and the residue again treated with hot H_2O until all H_2O-soluble material was extracted The filtrate was cooled, and then centrifuged at 25–30 × 10^3 rpm The clear liquid was acidified to pH 4–5 with HOAc and added to twice its vol of EtOH drop-wise with constant stirring The pptated polysaccharide was allowed to settle overnight, separated by centrifugation, triturated (× 5) with EtOH and finally with Et_2O and dried *in vacuo* It was a white amorphous powder, yield, 7 2 g, ash 0 36%, pentosan 2 2%, uronic acid negligible, $[\alpha]_{589\,5}^{26}$ +49 5° (4% NaOH, *c* 0 5) D-galactose, D-mannose and traces of L-arabinose were obtained by PC using solvents A and B, and by GC after hydrolysis

The polysaccharide was purified by complexing with Fehling's soln [1] The material (5 g) was dissolved in 4% NaOH (250 ml) in an atmosphere of N_2, and freshly prepared Fehling's soln (125 ml) was added to it while it was stirred in a cold water bath (10°) The ppt was removed by centrifugation, washed with H_2O and stirred with 0 5 M HCl (350 ml) at 0° for 4 hr The resulting soln was poured into EtOH (700 ml) and the ppt was washed with 0 1 M HCl in Me_2CO–H_2O (3 2) The material was finally dried after washing with MeOH Yield, 2 8 g

The purified polysaccharide (30 mg) was dissolved in 0 05 M NH_4HCO_3 (3 ml, pH 8 0), and applied to a column (80 × 2 2 cm) of Sephadex G-100 The column was eluted with the same buffer, 3-ml fractions being collected The PhOH–H_2SO_4 method [2] was used to determine the carbohydrate content in each fraction, the elution pattern showed a single peak The recovery of the polysaccharide was 90%, $[\alpha]_{589\,5}^{26}$ +51 4° (4% NaOH, *c* 0 5) The material was electrophoretically homogeneous Hydrolysis of the polysaccharide gave D-galactose and D-mannose only

Estimation of sugars The polysaccharide (5 1 mg) was mixed with myo-inositol (2 5 mg, as int standard), and hydrolysed with 0 5 M H_2SO_4 for 16 hr at 100° in a sealed tube The acid was neutralized with $BaCO_3$ and the suspension centrifuged The supernatant liquor was passed through columns of Amberlite IR-120 (H^+) and Amberlite IR-45 (OH^-) After concn the components of the hydrolysate were converted into their alditol acetates [3] and analysed by GC (column a) It was found to contain galactose and mannose in 1 1 66 (i e 3 5) mol proportion The galactose content of the polysaccharide was estimated with Galactostat* to be 38%

Mild hydrolysis with oxalic acid To a soln of polysaccharide (500 mg) in H_2O (25 ml) 0 04 N oxalic acid (25 ml) was added and the mixture heated in a boiling water bath At intervals of 1 hr aliquots (10 ml) were withdrawn and pptated with EtOH (15 ml) The supernatant liquid on PC showed the presence of D-galactose only The pptated polysaccharide from the fourth stage was hydrolysed with 0 5 M H_2SO_4 The constituent sugars, as their alditol acetates, were estimated by GC using myo-inositol as int standard, D-galactose and D-mannose were present in the mol ratio of 1 2

Reducing end-group assay The number average degree of polymerization was found by hypoiodite oxidation[4] to be 35–38

Methylation of the polysaccharide The polysaccharide (20 mg, dried over P_2O_5) was dispersed by stirring for 1 hr in formamide (2 ml) To this dispersion was added Ac_2O (2 ml) and pyridine (2 5 ml) with stirring After stirring for 16 hr at room temp , H_2O (10 ml) was added drop-wise to the cooled soln,and the mixture was dialysed against H_2O Concn and purification were effected by passage through a column (30 × 1 5 cm) of Sephadex LH-20 which was irrigated with Me_2CO The eluant was monitored by polarimetry, the acetylated polysaccharide (17 mg) being eluted in the void vol

The acetylated polysaccharide was methylated by the Hakomori method [5] 10 mg dissolved in DMSO (5 ml) was treated with 2 M methylsulphinyl sodium (5 ml) under N_2 The gelatinous soln was agitated in an ultrasonic bath for 30 min and then kept at room temp overnight MeI (2 ml) was added drop-wise with cooling and the mixture was stirred for 2 hr The

*Procedure supplied by Worthington Biochemical Corporation, Freehold, New Jersey, along with 'Galactostat' enzyme

product was then dialysed, lyophilized and re-methylated by the Purdie method [6] The fully methylated sampled showed no OH bands in the IR Yield (9 3 mg), $[\alpha]_{589.5}^{26}$ +46° ($CHCl_3$, c 0 33) The fully methylated polysaccharide was hydrolysed by heating in a boiling water bath with 85% formic acid, and then with 0 25 M H_2SO_4 The methylated sugars were converted into their alditol acetates, and these were analysed by GC (columns a and b) The results are shown in Table 1

Periodate oxidation The polysaccharide was treated with 0 1 M sodium metaperiodate in the dark at 5° The liberated formic acid and the amount of periodate consumed during the reaction were estimated in the usual way [7, 8] at regular intervals The liberation of formic acid became constant in 25 hr, 2 67 hexose residues liberating 1 mol formic acid, and the periodate uptake became constant after 30 hr corresponding to 1 39 mol of the oxidant per hexose unit

In a separate expt, the polysaccharide (60 mg) in H_2O (30 ml) was oxidized with sodium metaperiodate soln (0 2 M, 30 ml) for 30 hr at 5° The periodate-oxidized product after neutralization with $Ba(OH)_2$ and centrifugation was reduced with $NaBH_4$ for 4 hr at room temp The resulting soln, after usual treatment, was examined by PC (solvent B), and by GC (as the alditol acetates) The ratio glycerol–erythritol was found to be 1 1 6, i e 3 5

Graded hydrolysis Based on the results of pilot expts, the galactomannan (2 0 g) was dispersed in H_2O (100 ml) and 0 2 M H_2SO_4 (100 ml) was added The mixture was heated in a boiling water-bath for 3 hr The soln after centrifugation was passed through columns of Dowex-50W X-8 (H^+) and IR-45 (OH^-), successively The resulting liquid was concd to a small vol (*ca* 50 ml) PC (system A) indicated the presence of D-galactose, D-mannose, two disaccharides and two trisaccharides The results are shown in Table 2

The separation of the oligosaccharides was conducted first by CC The sugar mixture was passed through three Sephadex G-25 columns (80 × 2 5 cm each) connected in series The columns were eluted with $CHCl_3$-satd H_2O, 5-ml fractions being collected The carbohydrate content in each fraction was determined by the PhOH–H_2SO_4 method On examination of the eluates two zones were found Each zone was further separated on Whatman No 3 MM filter papers using solvent system A Thus four oligosaccharides were obtained in a chromatographically pure form

The oligosaccharides were hydrolysed with 0 5 M H_2SO_4 for 10 hr in a boiling water-bath and, after the usual treatment, the sugars were identified and their amounts determined by PC and GC (as their alditol acetates) The reducing-end residues were determined by treatment with $NaBH_4$ followed by hydrolysis After usual treatment the sugars in the hydrolysate were characterized by PC using aniline oxalate as the spray reagent The linkages in the oligosaccharides were determined by methylation studies using the Hakomori procedure The methylated sugars obtained in each oligosaccharide were identified and estimated by GC after converting them into their alditol acetates The results are shown in Table 1

Oxidation of the polysaccharide with CrO_3 A mixture of polysaccharide (11 4 mg) and myo-inositol (8 6 mg) was well dispersed in formamide–pyridine (2 1) (6 ml), by stirring the suspension for 2–3 hr After addition of Ac_2O (2 ml) the mixture was stirred for 18 hr at room temp H_2O (20 ml) was added dropwise to the cooled soln and the mixture was dialysed against H_2O and lyophilized The product was reacetylated, to ensure complete acetylation, and purification was effected by passing it through a Sephadex LH-20 column (60 × 5 cm) irrigated with Me_2CO The eluant was monitored polarimetrically Powdered CrO_3 (400 mg) was added to the soln of acetylated polysaccharide (10 mg) in HOAc (5 ml) and the supension was agitated in an ultrasonic bath at 50° Aliquots were removed at intervals and diluted with H_2O immediately and extracted with $CHCl_3$ The extracts were then dried over Na_2SO_4 and evaporated to dryness The oxidized products were deacetylated with 0 2 M NaOMe, decationized with Dowex-50W X-8 (H^+) ion-exchange resin, and hydrolysed After the usual treatment, the hydrolysates were converted into their alditol acetates, and the mixtures analysed by GC (column a) (Table 3)

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