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STRUCTURE OF THE β-D-GALACTAN ISOLATED FROM THE PODS OF *Dolichos lablab* LINN.

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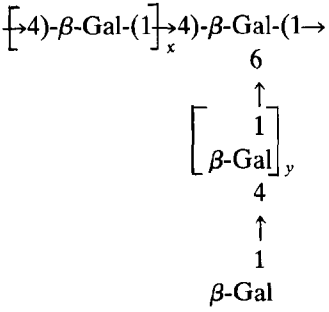
ABSTRACT

Hot-water extraction of the chlorophyll-free pods of *Dolichos lablab* Linn. furnished a mixture of polysaccharides containing mainly GalA and Gal, along with smaller proportions of Glc, Ara, and Rha. The mixture was fractionated by treatment with calcium chloride, followed by chromatography through a column of DEAE-cellulose (phosphate), to yield a β-D-galactan (**1**) containing Gal (95.6%) and a trace of GalA. Methylation analysis of **1** furnished 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3-di-*O*-methyl-D-galactose in the molar ratios of 1:28:0.88. On periodate oxidation, **1** reduced 0.91 mol of the oxidant per hexosyl residue, and liberated one mol of formic acid per 29.4 mol of galactosyl units. Smith degradation of **1** furnished mainly threitol. From these results, and from enzymic reactions and spectroscopic data, a structure is suggested for the repeating unit of **1**. The $\bar{M}_n$ of permethylated **1** was determined to be 1.206×10^4.



INTRODUCTION

The young, thick pods of *Dolichos lablab* Linn., mostly available during the winter, are widely used as an edible, green vegetable in India. In addition, the pods and other parts of the creeper have been reported to have various medicinal uses[1(a),(b)]. An investigation was, therefore, undertaken to determine the carbohydrate composition of the pods, and isolation and characterization of a β-D-galactan component from this source are reported herein.

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RESULTS AND DISCUSSION

The thick, young pods of *Dolichos lablab* Linn. (collected from the same creeper) were opened up, to remove the seeds (~4–6), and cut into small pieces. These were suspended in 95% ethanol, homogenized with fresh 95% ethanol, and the suspension filtered through a Nylon cloth; this treatment removed the green color. The resulting pulp was extracted with hot water, and the extract centrifuged. The material in the clear, supernatant liquor was precipitated with ethanol at pH 4.5. This furnished a crude polysaccharide* (Ps-I) in 0.23% yield (with respect to the weight of the green pods). Ps-I had $[\alpha]_{589.5}$ +197.36°, and contained 64.9% of GalA, and the neutral sugars Gal, Ara, Rha, and Glc in the molar ratios of 5.18:1:1.35:0.81 (see Table I). Because of the high content of uronic acid, the proportion of hexose in Ps-I could not be determined at this stage.

The i.r. spectrum of Ps-I had characteristic bands at 1735 (s), 1620 (m), 1260 (m), 890 (m), and 833 (w) cm^{-1}. The bands at 1735 and 1260 cm^{-1} were evidently due to some proportion of ester linkages[2(a),(b)], and that at 1620 cm^{-1} was, in all probability, due to some carboxylate anions[2(a)]. These findings, and the carbohydrate composition, indicated that Ps-I was a typical, pectic substance having galactose as the second major monosaccharide component.

An aqueous solution of Ps-I gave a light-violet color with iodine. This fact,

TABLE I

COMPOSITION OF POLYSACCHARIDES Ps-I, Ps-II, Ps-III, Ps-IV, Ps-N_1, Ps-N_2, AND Ps-A

Methods of isolation	*Ps fractions*	$[\alpha]_{589.5}$ *(degrees)*[a]	*Composition*		*Mole ratio*[b]			
			GalA (%)	*Total hexose (%)*	*Gal*	*Ara*	*Rha*	*Glc*
Hot-water extraction	Ps-I	+197.36 (0.228)	64.9	—[c]	5.18	1	1.35	0.81
Diastase treatment	Ps-II	+196.78 (0.249)	57.4	19.7	9.04	1	1.03	trace
Alpha-amylase treatment	Ps-III	+190.12 (0.547)	56.8	19.2	7.76	1	1.68	
$CaCl_2$ treatment (0.1%)	Ps-IV	+80 (0.25)	18.9	67.2	8.07	1	0.52	
Column chromatography of Ps-IV with 25mM NaH_2PO_4	Ps-N_1	+51.3 (0.20)		95.6				
0.1M NaH_2PO_4	Ps-N_2	[d]	22.7	56.3	5.71	1	0.55	
0.1M–0.5M NaCl gradient	Ps-A	[d]	59.3	—[c]	3.13	1	0.65	

[a]In water; concentration in parentheses. [b]From g.l.c. [c]—, could not be determined. [d]Not done.

*Ps = polysaccharide.

and the presence of glucose in Ps-I, indicated that the latter had a starch- or amylose-type of component. This was further corroborated by the fact that subsequent treatment of Ps-I, first with diastase, and then with alpha-amylase (or the reverse order) furnished Ps-III; this retained all of the other monosaccharides almost intact, except glucose (see Table I).

To obtain a hexose-enriched fraction, Ps-III was repeatedly fractionated by treatment with calcium chloride. This yielded Ps-IV, which had $[\alpha]_{589.5}$ +80°, GalA 18.9%, and total hexose[3,4] 67.2%. The neutral monosaccharides, *viz.*, Gal, Ara, and Rha, of Ps-IV were present in the molar ratios of 8.07:1:0.52 (see Table I).

Further fractionation of Ps-IV was effected by chromatography through a column of DEAE-cellulose (phosphate), using 25mM NaH_2PO_4 buffer, pH 6, as the eluant. Fractions responding positively to the phenol–sulfuric acid test[4] responded very weakly to the carbazole test[5] also. These fractions were pooled, recycled through a fresh column of DEAE-cellulose (phosphate), and processed, to yield Ps-N_1 which had $[\alpha]_{589.5}$ +51.3°, and contained Gal 95.6%, and a trace of GalA.

Other polysaccharide fractions, Ps-N_2, and Ps-A, obtained by subsequent elution of the first DEAE-cellulose column with a stronger phosphate buffer (0.1M), and with sodium chloride (0.1–0.5M gradient), were comparatively lower in hexose content (see Table I), and were not further investigated.

Although Ps-N_1 retained a trace of GalA, the gradual elimination of the latter during the isolation process indicated that these GalA residues originated from the pectic acid contaminant, and the absence of any aldobiouronic acid in the hydrolyzates of the different polysaccharide preparations also supported this conclusion. Ps-N_1 was, therefore, considered to be essentially a homogalactan. Its i.r. spectrum retained the original absorption bands at 1260 (s) and 890 (m) cm^{-1}, whereas the other, previously mentioned, bands were either very weak (1735 cm^{-1}), or absent.

The band at 1260 cm^{-1} was obviously due to C–O stretching, and that at 890 cm^{-1} clearly indicated[6] a β-D linkage for the galactan Ps-N_1, and this was further supported by the absence of bands in the region 839–810 cm^{-1}. Also, in the case of Ps-I, the presence of a weak i.r. band at 833 cm^{-1}, and the absence of any characteristic band in the region 905–876 cm^{-1} could now be assigned to possible association of an α-D-glucan component with Ps-I.

Ps-N_1 was permethylated (ensured by the absence of an OH band in the i.r. spectrum) by one Hakomori[7] and two Purdie[8] treatments. The product was fractionally precipitated from chloroform by means of an increasing volume of petroleum ether. This furnished essentially one fraction, having $[\alpha]_{589.5}$ −19.2°. The permethylated product was hydrolyzed, and the partially methylated monosaccharides were characterized by p.c., and g.l.c., using authentic samples as references. This revealed the presence of 2,3,4,6-tetra-, 2,3,6-tri-, and 2,3-di-*O*-methylgalactose, in the molar ratios of 1:28:0.88. G.l.c.–m.s. analysis of the mixture of alditol acetates

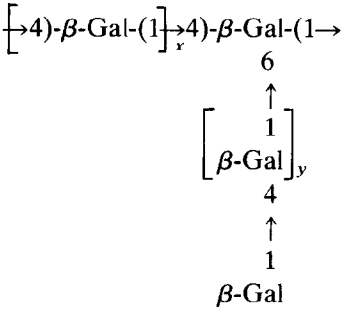
TABLE II

G L C.–M.S AND G.L.C OF ALDITOL ACETATES DERIVED FROM METHYLATED Ps-N_1

Components (as alditol acetates of galactose)	*RRT[a] (column II)*	*Mole ratio*	*Major mass fragments*	*Nature of linkage*
2,3,4,6-Me_4	0.797	1	43, 45, 87, 101, 117, 129, 145, 205	Gal*p*-(1→
2,3,6-Me_3	**1.000**	28	43, 45, 87, 117, 233	→4)-Gal*p*-(1→
3,6-Me_2	1.137	trace	43, 45, 87, 129, 189, 233	→2,4)-Gal*p*-(1→
2,3-Me_2	1.277	0.88	43, 117, 127, 261	→4,6)-Gal*p*-(1→

[a]Relative retention time with respect to that of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol as unity.

(see Table II) corroborated this finding. The trace of 3,6-di-*O*-methylgalactose, indicated only by g.l.c.–m.s. (see Table II), probably originated from partial demethylation of some tri- or tetra-*O*-methylgalactose, or both, during hydrolysis, and does not seem to have any structural significance. Hence, it may be concluded that the D-galactosyl residues of the D-galactan are in the pyranoid form and that the main linkage is (1→4), with branching from O-6 of some of the residues. Structure **1** ($x + y = 28$) depicted for the repeating unit of the β-D-galactan would be consistent with these data.



1

On periodate oxidation, the galatan (Ps-N_1) reduced 0.91 mol of the oxidant per hexosyl residue, and liberated one mol of formic acid per 29.4 mol of galactosyl residues. Smith degradation of the galactan furnished threitol (major), glycerol (trace), and glycolaldehyde. Also, when these degradation products (as alditol acetates) were examined by g.l.c., the result corroborated this finding, and no galactose could be detected. A galactosyl residue linked through O-1, O-2, and O-4 (giving a 3,6-di-*O*-methylgalactose) should have survived periodate oxidation. Therefore, these results are also consistent with the proposed formula **1**.

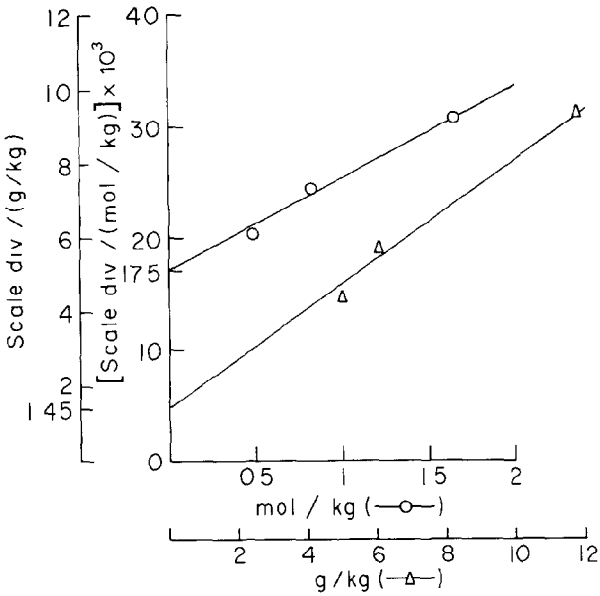


Fig. 1. Determination of $\bar{M}_n$ of the permethylated D-galactan. [—O—, standard polystyrene; —Δ—, permethylated sample. Intercepts: $K_{calib} = 17.5 \times 10^3$, $K_{meas} = 1.45$.]

The spectral finding of β-D linkages in the galactan was corroborated by the results of a study of the action of β-D-galactosidase thereon. Also, in an Ouchterlony test against anti-β-D-Gal-specific lectin[9], the galactan responded positively. The low specific rotation of the galactan, and the negative specific rotation of the permethylated product contributed additional support to this conclusion.

The molecular weight of the permethylated galactan was determined by vapor-pressure osmometry[10] in chloroform solution at 45°. Polystyrene of standard molecular weight ($\bar{M}_n$ 9000) was used for calibration. From the results (see the Experimental section, and Fig. 1), the $\bar{M}_n$ for the permethylated galactan was calculated to be 1.206×10^4.

Mention may be made of the fact that the water-soluble carbohydrate materials isolated from the seed hulls and endosperm of the field-bean variety (a different source) of *Dolichos lablab* Linn. were shown to be essentially arabinogalactans[11]. Also, other such materials subsequently isolated from the same source display very little similarity to our findings, except (probably) one of their glucan[12] components.

EXPERIMENTAL

Materials and methods. — Whatman No. 1 papers were used for partition chromatography (p.c.), using the following solvent-systems (v/v): (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) 5:5:1:3 ethyl acetate–pyridine–acetic acid–water, (*C*)

3:1:1 1-butanol–acetic acid–water, and (*D*) 40:11:19 1-butanol–ethanol–water. Staining reagents were (*a*) alkaline silver nitrate, (*b*) benzidine periodate, and (*c*) aniline hydrogenoxalate. G.l.c. was performed with a glass column (*I*; 6 mm × 1.83 m) containing 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of neutral sugars), and at 170° (for alditol acetates of partially methylated, neutral sugars), using the instrument reported[13] earlier. G.l.c.–m.s. was conducted with an Automated Finnigen Mat (Model 1020) instrument fitted with a CP-Sil-5 column (*II*, 0.25 mm × 10 m), using helium as the carrier gas.

Diastase was obtained from A. Constantino C. Favria, Italy, alpha-amylase from Hanku Kyoei Bussan Co., Japan (specific activity 5 units/mg), and β-D-galactosidase (β-D-galactoside galactohydrolase from *Aspergillus niger*, EC 3.2.1.23) from Sigma, U.S.A.

Total hexose was estimated by the L-cysteine–sulfuric acid method[3] or the phenol–sulfuric acid method[4], and uronic acid was estimated by the carbazole method[5].

Molecular weight was determined with the help of a Knauer vapor-pressure osmometer, No. 11.00, using the universal probe No. 12.02.00. Standard polystyrene ($\bar{M}_n$ 9000, Lot No. 80314) was purchased from Pressure Chemical Company, Pittsburgh, PA 15201, U.S.A.

All evaporations were conducted under diminished pressure at a temperature not exceeding 40°. Specific rotations were recorded, at equilibrium, with a Perkin–Elmer polarimeter, Model 241, at ~25° (average room temperature, r.t.). Spectral data were recorded with a Perkin–Elmer model 297 and 337 (for i.r.), a Beckman DB (for colorimetric assay), and a Pye Unicon SP-500 spectrometer (for u.v. absorption).

Isolation of the crude polysaccharide. — The seed-free, green pods (average weight, ~9 g per piece) of *D. lablab* Linn. (300 g) were cut into small pieces, and suspended in 95% ethanol (400 mL) for 24 h. The alcohol was squeezed out (this removed most of the green color), and the residue was homogenized for 3–5 min with fresh 95% ethanol (3 × 250 mL). The suspension was filtered through Nylon cloth, and the residue was squeezed, and air-dried, to yield almost colorless pulp (14.6 g). The pulp was extracted with hot water (2 × 250 mL) by stirring (2 × 6 h) on a boiling-water bath. The slurry was cooled to r.t., filtered through a Nylon cloth, and the opaque filtrate centrifuged at 6000 r.p.m. for 45 min at 15°. The clear, supernatant liquor was acidified (pH 4.5) with cold acetic acid, and the polysaccharide was precipitated with ethanol (3 vol.). The mixture was kept overnight at 4°; then, the gelatinous precipitate was collected by centrifugation, and dried by solvent exchange, to yield a white, amorphous powder (Ps-I, ~690 mg). An aqueous solution of Ps-I gave a violet color with iodine solution, and the compound had the composition given in Table I.

Identification of the components. — Ps-I (12 mg) was hydrolyzed with 0.5M H_2SO_4 (4 mL) for 16 h on a boiling-water bath and the hydrolyzate was made neutral with prewashed $BaCO_3$, and decationized with Dowex-50W X-8 (H^+) resin.

The resulting solution was evaporated to a syrup, and this was examined by p.c. (solvent *A*, staining reagent *a*), which indicated the presence of GalA, Gal, Glc, Ara, and some unidentified spots. The syrup was diluted to ~1.5 mL with water, and the solution applied to a column (1 × 15 cm) of Dowex-1 X-4 (formate) resin which was eluted with water (200 mL) until the effluent responded negatively to the Molisch test[14]. The effluent was evaporated to dryness, and the mixture of neutral monosaccharides was converted[15] into their alditol acetates in the usual way, and identified by g.l.c. (see Table I).

The column of Dowex-1 X-4 (formate) resin was eluted with 0.3M formic acid (100 mL), and the effluent evaporated thrice with water. P.c. (solvents *A*, and *C*; staining reagent *a*) of the residue indicated the presence of only GalA in it. The acid was converted into its methyl ester methyl glycoside[16], and this was reduced. The resulting, neutral sugar was identified as galactose by p.c. (solvent *A*, staining reagent *a*), and by g.l.c.

Treatment of Ps-I with diastase. — Ps-I (660 mg) was dispersed in water (660 mL) by stirring for 1 h on a hot-water bath. The suspension was cooled, and centrifuged at 17,000 r.p.m. for 1 h at 4°. The residue was discarded, and the supernatant liquor was converted into a 0.2M acetate buffer, pH 4.6, to which was added diastase (25.5 mg), and the mixture incubated, under a drop of toluene, for 24 h at 37°. The enzyme was deactivated by heating for 20 min at 80°, and the mixture was cooled to r.t., and centrifuged. The supernatant liquor was extensively dialyzed, the dialyzate concentrated, and the concentrate examined by p.c. (solvent *A*, staining reagent *a*), which revealed the presence of Glc only. The bag contents were processed, to yield solid Ps-II (~610 mg); it retained a trace of Glc (see Table I).

Treatment of Ps-II with alpha-amylase. — Ps-II (~600 mg) was dispersed in water (600 mL). The solution was made into a 0.2M acetate buffer, pH 5.5, treated with alpha-amylase (17 mg), and processed as described for the diastase treatment. As before, Glc appeared in the p.c. of the dialyzate. The bag contents were processed, to yield Ps-III (~580 mg) having the composition given in Table I. This composition of Ps-III remained unchanged when the order of treatment of Ps-I with the enzymes was reversed. Ps-III did not give any color with iodine solution.

Fractionation of Ps-III with calcium chloride. — A clear, aqueous solution (0.1%) of Ps-III (570 mg) was twice fractionated with calcium chloride, as described[17], to yield Ps-IV (~340 mg; for composition, see Table I). No further enrichment in the hexose content of Ps-IV could be achieved by repeating the process.

Chromatography of Ps-IV on a column of DEAE-cellulose (phosphate). — Ps-IV (320 mg; containing hexose 67.2%, and uronic acid 18.9%) was dispersed in 25mM NaH_2PO_4 buffer, pH 6 (8 mL) by stirring for 2 h at 80°. The mixture was cooled, and centrifuged, and the clear solution was fractionated (in 5-mL portions, at the rate of 20 mL/h) through a column (2.5 × 60 cm) of DEAE-cellulose (phosphate), using the same buffer. Fractions 26–44, which responded positively (mainly) to the phenol–sulfuric acid test, also responded very faintly to the car-

bazole test. These were pooled, dialyzed, and processed, to yield Ps-N_1 (~57 mg). Ps-N_1 (56 mg) was recycled through a fresh column of DEAE-cellulose (phosphate), but the composition remained practically unchanged. Subsequent lots of Ps-N_1 were isolated in the same way.

Next, the column was eluted with 0.1M NaH_2PO_4 buffer, pH 6, and fractions 78–98 (which responded positively to both of the aforementioned color reactions) were processed, to yield Ps-N_2 (4.7 mg).

Following this, a sodium chloride gradient (0.1–0.5M) was applied to the column, and the same monitoring system was used. This procedure yielded Ps-A (68.1 mg) from fractions 140–160. The compositions of fractions Ps-N_1, Ps-N_2, and Ps-A are given in Table I.

Methylation analysis of Ps-N_1. — Ps-N_1 (11.4 mg) was subjected to permethylation, as reported earlier[17]. The product (13.5 mg) had a weak OH band in the i.r. spectrum, and so was subjected to two further methylations by refluxing methyl iodide and silver oxide (Purdie). The resulting, permethylated product (light-yellow flakes; yield 12.8 mg; $[\alpha]_{589.5}$ −19°) had no OH band in its i.r. spectrum. It was dissolved in chloroform (1 mL), and fractionally precipitated with petroleum ether (4 vol.). The first turbidity (permethylated Ps-N_1) was processed, to afford a solid (~11 mg; $[\alpha]_{589.5}$ −19.2°), and the second (~1 mg), obtained by evaporation, was not further investigated.

The permethylated Ps-N_1 (2 mg) was treated with formic acid, and then hydrolyzed as described earlier[17]. P.c. (solvent *D*, staining reagent *c*) of the hydrolyzate furnished practically one spot, corresponding to tri-*O*-methylgalactose. The sugars in the rest of the hydrolyzate were converted[18] into the alditol acetates, and identified by g.l.c., and by g.l.c.–m.s., using authentic samples or literature values[19,20], or both, for comparison. The results are given in Table II.

Treatment of Ps-N_1 with β-D-galactosidase. — A solution of Ps-N_1 (2 mg) in 0.01M citrate buffer, pH 4.0 (0.2 mL) was treated with β-D-galactosidase (0.4 unit in 0.2 mL) under the conditions described earlier[17]. Control experiments (*i.e.*, polysaccharide blank and enzyme blank) were also set up under identical conditions. Only the enzyme-containing reaction-mixture released Gal, in ~40 h.

Periodate oxidation of Ps-N_1. — Ps-N_1 (4 mg) in water (2 mL), in duplicate, was oxidized with 0.04M sodium metaperiodate (2 mL) in the dark at 4°. The periodate uptake, monitored spectrophotometrically[21] at intervals, became constant in ~50 h, corresponding to reduction of 0.91 mol (average value) of the oxidant per hexosyl residue. The rest of this reaction mixture and the duplicate were pooled, and the excess of periodate in a 5-mL aliquot of the mixture was reduced with ethylene glycol (0.3 mL). After 15 min, formic acid in the resulting solution was titrated with standard 5mM NaOH. The liberation of one mol of formic acid corresponded to the oxidation of 29.4 hexosyl residues. Following the determination of formic acid, the same solution was extensively dialyzed, and then concentrated to ~2 mL by lyophilization. The material in the resulting solution was reduced with KBH_4 (20 mg), and, after the usual processing, was hydrolyzed with M trifluoroace-

tic acid (1 mL) for 10 h at 100°. The acid was evaporated with water (2 × 2 mL), and the residue examined by p.c. (solvent *A*, staining reagent *b*). This revealed the presence of glycolaldehyde (trace), glycerol (trace), and threitol (major). Part of the residue, in water (1 mL), was reduced with KBH_4 (10 mg). After the usual processing this was converted into the alditol acetates, and these were subjected to g.l.c. (column *I*). The column temperature was programmed from 80° (initial stay, 2 min) to 180° (final stay, 30 min) at the rate of 8° per min. Components of the mixture (*viz.*, ethylene glycol, glycerol, and threitol, having retention times of 5, 12.5, and 19 min, respectively) were identified by comparing their retention times with those of authentic samples.

Determination of molecular weight of the permethylated galactan. — The experiment was conducted at 45°, using three different concentrations of the standard (polystyrene, $\bar{M}_n$ 9000), or the sample, in chloroform solution. The curves of the plot of scale division/mol (or g for the sample)/kg of chloroform *vs.* mol(or g)/kg of chloroform for both the standard and the sample were extrapolated to zero concentration. The intercepts on the *y* axis (see Fig. 1) corresponding to K_{calib} (*i.e.*, standard), and that for K_{meas} (*i.e.*, sample) amounted to 17.5×10^3 and 1.45, respectively. From this, the $\bar{M}_n$ (K_{calib}/K_{meas}) for the permethylated D-galactan was found to be 1.206×10^4.

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