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STRUCTURE OF GALACTOMANNAN FROM SEEDS OF *Crotalaria alata*

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A galactomannan of MW 540 kDa was isolated from seeds of Crotalaria alata. *Chemical and spectral (PMR and ^{13}C NMR) methods established that the main chain of the galactomannan consisted of* β*-1,4-mannan with* α*-1,6-D-galactopyranose side chains.*

Keywords: polysaccharide, galactomannan, methylation, periodate oxidation, PMR and ^{13}C NMR spectroscopy.

We reported previously that seeds of *Crotalaria alata* (Fabaceae) represent a source of a water-soluble galactomannan polysaccharide [1, 2]. The isolation and structure elucidation of galactomannans from various species of the genus *Crotalaria* were also reported [3–6]. Their contents varied from 6.6 to 20% with different monomer ratios. However, galactomannans from *C. alata* introduced into Uzbekistan have not been investigated. The chain of galactomannans isolated from seeds of various *Crotalaria* species consisted mainly of *β*-1,4 bonded mannans in which several mannose residues were replaced by C-6 *α*-D-galactopyranose residues although individual galactose residues in *C. medicaginea* Lam. galactomannan were connected to the main chain through an *α*-1,3-bond [6].

The goals of the present work were to isolate and elucidate the structure of galactomannan from *C. alata* seeds that was obtained by extraction with cold water followed by purification using Fehling's solution. The monosaccharide composition of the purified polysaccharide included galactose and mannose in a 1:3 ratio. The polysaccharide was heterogeneous according to gel chromatography and was fractionated by EtOH in order to produce four homogeneous fractions in yields of 15.0% (fr. 1), 65.0% (fr. 2), 13.0% (fr. 3), and 3.0% (fr. 4). Total acid hydrolysis showed that all fractions consisted of galactose and mannose and differed only in the quantitative monosaccharide contents and molecular weights (MW) (Table 1).

Table 1 shows that fr. 2 had the greatest yield, was homogeneous according to gel filtration, and was designated GMS by us.

GMS was an amorphous white powder with $[\alpha]_D^{22}$ +20° (*c* 0.1%, H_2O) and dissolved in H_2O to form viscous solutions (η_{rel} 49.86, *c* 0.75%). Its MW was calculated as 540 kDa using the sedimentation constant and the Doublier method [7]. The IR spectrum of GMS contained characteristic absorption bands at 815 cm^{-1} (pyranose ring), 872 (*β*-glycoside bond), and 725 (*α*-glycoside bond).

Structural features of GMS were elucidated using chromic and periodate oxidation and methylation. Smith oxidation [8] of GMS consumed 0.93 mol of oxidant per mol of anhydro unit and released 0.11 mol of formic acid. Paper chromatography (PC) of the hydrolysis products of the polyalcohol detected mainly erythritol, which was indicative of a 1,4-bond between hexose residues in GMS, and glycerol, which provided evidence of a high degree of polymerization and the presence of 1,6-bonds between hexose residues. PC also identified traces of mannose, which showed that the GMS chain was slightly branched.

Methylation of GMS by the Hakomori method [9] produced the completely methylated product in 63% yield with $[\alpha]_D$ +27° (*c* 0.1%, $CHCl_3$). The completeness of the methylation was confirmed by IR spectroscopy from the lack of absorption bands at 3200–3600 cm^{-1} (OH). The permethylate of GMS was subjected sequentially to formolysis and hydrolysis. GC of the hydrolysis products with known markers identified 2,3,4,6-*O*-Me_4-D-Gal (14.8%), 2,3,4,6-*O*-Me_4-D-Man (2.06%), 2,3,6-*O*-Me_3-D-Man (43.9%), and 2,3-*O*-Me_2-D-Man (11.5%). The presence of 2,3,6-*O*-Me_3-D-Man as the principal product was consistent with 1,4-bonds between mannose residues in the main chain. The identification of 2,3,4,6-*O*-Me_4-D-Gal and di-*O*-methyl-D-mannose confirmed the periodate-oxidation data regarding branching in the macromolecule chain where the second galactose component was bonded to the main chain through a 1,6-bond. The formation of 2,3,4,6-*O*-Me_4-D-Man showed that the GMS chain had mannopyranose residues on the non-reducing end.

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TABLE 1. Principal Characteristics of *C. alata* Galactomannan Fractions

Fraction	Precipitant volume, mL	Fraction yield, %	Gal–Man ratio	η_{rel} (*c* 0.5%; H_2O)	MW, kDa
1	50	15.0	1:3.2	55.4	658
2	100	65.0	1:3.0	49.8	540
3	150	13.0	1:2.8	31.2	230
4	200	3.0	–	–	–

TABLE 2. Characteristics of GMS and GMSD

Parameter	Galactomannans	
	GMS	GMSD
MW, kDa	540	25
Gal–Man ratio	1.0:3.0	1.0:2.7
$[\alpha]_D^{22}$ (*c* 0.1%; H_2O)	+20°	+24°
η_{rel} (*c* 0.75%; H_2O)	49.8	25.6
IR spectra, cm^{-1}	815, 872, 725	814, 872, 720

TABLE 3. Chemical Shifts in PMR and ^{13}C NMR Spectra of *C. alata* Galactomannan (D_2O, TSP Standard, δ_C 1.6 ppm, δ_H 0.0 ppm)

Residue	C-1 H-1	C-2 H-2	C-3 H-3	C-4 H-4	C-5 H-5	C-6 H-6, 6′
α-Gal*p*-(1	99.97	69.60	70.45	70.55	72.44	62.39
↓	5.02	3.82	3.94	3.99	3.89	3.74
						3.74
6)	101.16,	71.07	72.52	77.93	74.52	67.69
→4)-*β*-D-Man*p*-(1→	101.33[a]	71.16[a]	3.80	78.05	74.44[b]	3.97
	4.75	4.12		78.20[b]	3.89	3.78
	4.74[a]			3.87		
→4)-*β*-D-Man*p*-(1→	101.16	71.07	72.62	77.70	76.21	61.72
	101.33[a]	71.16[a]	3.80	3.82	3.55	3.91
	4.75	4.12				3.74
	4.74[a]					

[a]Depending on the presence or absence of *α*-Gal*p* in the glycosylated residue; [b]depending on the through-space environment of the residue.

The positive specific rotation of GMS permethylate and an absorption band at 725 cm^{-1} in the IR spectrum were indicative of an *α*-glycoside bond for D-galactopyranose. This was confirmed by oxidation of fully acetylated GMS by chromic anhydride [10].

The structure of galactomannan GMS was also elucidated using NMR spectroscopy. The spectrum of destroyed GMS was also recorded.

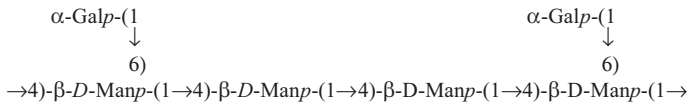
As shown above, GMS formed a very viscous aqueous solution even at low concentrations. This degraded significantly the resolution of the ^{13}C NMR spectrum. The destroyed polysaccharide (GMSD) was a fragment of lower MW and the same monosaccharide ratio [11] and was used to obtain well resolved PMR and ^{13}C NMR spectra.

GMSD was an amorphous white powder with $[\alpha]_D^{22}$ +24° and MW 25 kDa. It dissolved completely in H_2O and had η_{rel} 25.6. The hydrolysate of GMSD contained galactose and mannose in a 1:2.7 ratio. The principal parameters of GMSD agreed with those of GMS. Differences were observed in the MW and viscosity (Table 2).

Anomeric atoms showed two main resonances in the PMR spectrum (Table 3) with chemical shifts δ 5.02 and 4.74 ppm and a 1:2.3 ratio of integrated intensities. Resonances of other protons were located at δ 3.4–4.2 ppm. The ^{13}C NMR spectrum (Table 3) had strong resonances for anomeric C atoms at δ 101.33, 101.16, and 99.97 ppm in addition to minor resonances at δ 95.01 and 94.88 ppm. Resonances of C atoms bonded to a single O atom were found at δ 61–79 ppm.

Resonances in PMR and ^{13}C NMR spectra were assigned using 2D homonuclear ^{1}H–^{1}H COSY, TOCSY, and ROESY and heteronuclear ^{1}H–^{13}C HSQC and HMBC. An analysis of COSY, TOCSY, and ROESY spectra revealed β-mannopyranose (β-Man*p*) and α-galactopyranose (α-Gal*p*) residues in the polymer. The HSQC spectrum showed that all α-Gal*p* residues were terminal whereas all β-Man*p* residues were substituted in the 4-positions and a part of them also in the 6-positions (Table 3). ROESY and HMBC spectra exhibited *trans*-glycoside correlation peaks for H-1 (α-Gal*p*)/C-6 (β-Man*p*) and H-1 (β-Man*p*)/C-4 (β-Man*p*). This indicated unambiguously that the polymer chain included →4)-β-Man*p*-(1→ moieties where some of the residues were substituted at the C-6 hydroxyl by α-Gal*p* residues.

Splitting of C-4 resonances of disubstituted β-Man*p* residues indicated indirectly that residues unsubstituted and substituted at C-6 were statistically (not block) distributed. This could be explained by the effect of the through-space environment, i.e., different combinations of C-6-substituted and unsubstituted residues as a glycosylating and glycosylated pyranose for each actual residue.



The minor resonances at δ_H 5.02 and 4.74 ppm and δ_C 95.01 and 94.88 ppm, which were characteristic of α- and β-Man*p* residues at the reducing end of the chain, allowed the average chain length →4)-β-Man*p*-(1→ to be determined as approximately 30 units.

The chemical and spectral investigations found that galactomannan from seeds of *C. alata* consisted of β-1,4-bonded D-mannopyranose residues, a part of which was substituted in the *O*-6-position by individual α-D-galactopyranose residues. A comparison of these data with the literature on *Crotalaria* plant galactomannans enabled the structure of the *C. alata* polysaccharide to be identified as the most common type of galactomannan (mannan) in the family Fabaceae. However, *C. alata* GMS differed from those of other *Crotalaria* species with respect to content, viscosity, specific rotation, ratio of monosaccharide residues, molecular weight, and degree of polymerization. The mannopyranose chain structure had a statistical distribution of terminal galactopyranose residues.

EXPERIMENTAL

We studied seeds of *C. alata* that were collected in F. N. Rusanov Botanical Garden during ripening (Oct. 15, 2011). TLC was carried out on Silufol UV_{254} and KSK silica gel (LS-5/40 mm) plates using solvent systems (v/v) $CHCl_3$–MeOH (9:1, 1), $CHCl_3$–Me_2CO (30:1, 2), and MEK–NH_4OH (1%) (30:1, 3). PC used Filtrak FN 18 paper and 1-BuOH–Py–H_2O (6:4:3, 1). Spots were identified by anilinium biphthalate (1) and KIO_4–$KMnO_4$–benzidine (2).

Specific rotation of polysaccharides and their methyl derivatives was measured at 20–23°C on a Zeiss polarimeter in 1-dm tubes of volume 10 mL and in 0.5-dm tubes of volume 1 mL. IR spectra of galactomannans in pressed KBr pellets were recorded on a PerkinElmer Model 2000 IR-Fourier spectrometer using 100 scans. GC analysis was performed on a Chrom-5 chromatograph with a flame-ionization detector and a stainless-steel column (200 × 0.3 cm) of XE-60 Silicone (5%) on Chromaton NAW (0.200–0.250 mm) at 210°C with N_2 carrier gas at flow rate 60 mL/min. Molecular weights of the galactomannans were determined as before [7].

PMR and ^{13}C NMR spectra were taken in D_2O at 313 K on a Bruker AV-600 instrument using sodium 3-(trimethylsilyl)propionate (TSP) with δ_H 0.0 ppm and δ_C 1.6 ppm as a standard. 2D spectra were taken and processed using standard Bruker methods.

The monosaccharide composition of the galactomannans was determined by total acid hydrolysis in H_2SO_4 (1 N) at 100°C for 8 h. The hydrolysates were neutralized by $BaCO_3$, de-ionized by KU-2 (H^+) cation exchanger, and evaporated. Monosaccharides were identified by PC (system 1, detector 1) and GC (as aldononitrile acetates) [12].

Isolation of Water-soluble Polysaccharide (WSPS). Seeds (100 g) of *C. alata* were grounded and treated with refluxing $CHCl_3$:MeOH (1:1) in a 1:3 ratio on a water bath for 1 h. WSPS were extracted three times by H_2O (1:5 ratio) with stirring at room temperature. Pulp was separated from the aqueous extract by centrifugation. The aqueous extracts were combined and precipitated by EtOH in a 1:3 ratio. The resulting precipitate was separated, rinsed with EtOH of increasing concentration, and dried. Then, the precipitate was dissolved in H_2O and purified by adding Fehling's reagent according to the literature method [8]. The final yield of purified GMS was 15% of the air-dried raw material.

Fractionation of Polysaccharides. WSPS (1 g) from *C. alata* were dissolved in H_2O (100 mL), stirred vigorously, and treated dropwise with EtOH (50 mL, 96°). The resulting white precipitate (fraction 1) was separated by centrifugation, rinsed with EtOH, and dehydrated with Me_2CO. Yield, 0.15 g. The supernatant liquid was treated with another portion of EtOH (50 mL). The resulting finely dispersed precipitate was separated and worked up analogously. Yield of fraction 2 (GMS), 0.65 g. Fraction 3 was obtained by adding another portion of EtOH (50 mL) to the supernatant. The yield was 0.13 g. The supernatant solution was evaporated to half the volume and treated with another portion of EtOH (50 mL). Yield of fraction 4, 0.03 g.

Periodate Oxidation of GMS. GMS (0.05 g) were dissolved in H_2O (24.9 mL), treated with $NaIO_4$ solution (5.1 mL, 0.25 M), and left at +5°C. Aliquots (1 mL) were taken every day and titrated with sodium thiosulfate solution (0.01 N). The consumption of $NaIO_4$ was 0.93 mol after 15 d. The yield of HCOOH was 0.11 mol. The oxidized product was dialyzed (after destroying periodate with ethyleneglycol), treated with $NaBH_4$ (0.1 g), and left overnight. The solution was treated with KU-2 (H^+) cation exchanger. The filtrate was evaporated with MeOH. The dry solid was hydrolyzed by H_2SO_4 (5 mL, 1 N) for 8 h at 100°C. PC (system 1, detectors 1 and 2) of the hydrolysis products detected mainly erythritol and glycerol.

Methylation of GMS. Galactomannan (0.1 g) was methylated by the Hakomori method [9]. The yield of GMS permethylate was 0.063 g. GMS permethylate (0.05 g) was subjected to formolysis by HCOOH (2 mL, 85%) for 1 h, cooled, and evaporated. Then, the precipitate was hydrolyzed by H_2SO_4 (0.5 N) for 8 h at 100°C. The hydrolysate was worked up as usual. TLC (systems 1-3, detector 1) detected 2,3,4,6-tetra-*O*-Me-D-mannose, 2,3,4,6-tetra-*O*-Me-D-galactose, 2,3,6-tri-*O*-Me-D-mannose, and di-*O*-Me-D-hexose.

Oxidation of GMS by Chromic Anhydride [10]. GMS (0.05 g) was dissolved in formamide (5 mL), treated with anhydrous Py (2.5 mL) and dropwise with acetic anhydride (2.5 mL), and heated for 1 h on a water bath at 50°C. GMS acetate was precipitated by icy distilled H_2O. The precipitate was separated and rinsed with MeOH and Me_2CO to afford GMS peracetate (0.056 g). The IR spectrum lacked an OH absorption band (3200–3600 cm^{-1}) and showed clearly resolved absorption bands at 1720 and 1240 cm^{-1}.

GMS peracetate (0.05 g) was treated with CrO_3 (0.02 g) in glacial AcOH (2 mL), heated for 3 h at 50°C, diluted with H_2O, and extracted with $CHCl_3$. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness. The solid was dissolved in H_2SO_4 solution (1 mL, 1 N) and hydrolyzed for 8 h on a boiling-water bath. The hydrolysate was worked up as usual. PC (system 1, detector 1) detected galactose.

Depolymerization of GMS. GMS (0.5 g) was dissolved in TFA (50 mL, 0.1 M) and hydrolyzed at 100°C on a water bath for 45 min. The hydrolysate was cooled to room temperature and precipitated by EtOH (150 mL). The resulting precipitate was separated by centrifugation. The solid was rinsed with EtOH and dehydrated by Me_2CO. Yield of depolymerized GMS, 0.28 g.

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