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Note

Cassia grandis Linn. f. seed galactomannan: structural and crystallographical studies

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Received 9 January 2003; received in revised form 5 May 2003; accepted 21 May 2003

Abstract

Cassia grandis is a small or medium sized tree, found in abundance throughout India. The seeds contain about 50% endosperm gum and possess the characteristics of becoming a potential source of seed gum. The purified polysaccharide has been characterized as a pure galactomannan having a mannose–galactose ratio of 3.15; molecular weight (M_w) 80,200; polydispersity (M_w/M_n), 1.35 and intrinsic viscosity [η], 848 mL/g. Methylation, periodate oxidation, Smith degradation and ^{13}C NMR studies confirm that the polysaccharide has the basic structure of legume galactomannans consisting of a β-(1→4)-linked main mannan backbone to which galactose units are attached at O-6. The orthorhombic lattice constants of the hydrated gum are as follows: $a = 9.00$, $b = 24.81$, $c = 10.30$ Å. The crystallographic data establish that the probable space group symmetry of the unit cell is $P2_12_12$. The results are in contradiction to earlier reports (Indian J. Chem. 16B (1978) 966; J. Indian Chem. Soc. 55 (1978) 1216) in which a non-galactomannan polysaccharide structure has been assigned having a main chain of (1→4)-linked galactose and mannose units in the molar ratio 6:3, where 50% of the galactose units branched with two galactose and one mannose through 1→3 linkage.

Keywords: *Cassia grandis*; Galactomannan; Polysaccharide; Galactopyranose; Mannopyranose

Leguminous seeds are the potential source of seed gums[1,2] which are produced in large amounts for international consumption. These are used in a variety of industries[3] like paper, textile, pharmaceutical, cosmetics, mining, petroleum well-drilling. They are inexpensive, eco-friendly, non-toxic and considered as GRAS (Generally Recognised As Safe).[4,5] Current international trend demands the introduction of alternative source of seed gums. Our studies show that *Cassia grandis* seeds possess the characteristics of commercial gums and could be utilized in pharmaceutical[6] and food industries. It demonstrates the structural and crystallographical features of galactomannan and try to sort out some of the ambiguities regarding structure of the galactomannan. The structural and crystallographical aspects of the galactomannan have been undertaken for a better understanding of its properties and to clarify the uncertainty regarding the structural features of the polysaccharide.

Cassia grandis Linn. f. (Fam. Caesalpiniaceae) is a small or medium sized tree,[7] native of tropical and Central America and the West Indies, later introduced into India. It is planted as an ornamental tree in gardens and avenues, and for its shade. The seeds are dicotyledonous, light or dark brown, oval to round and medium sized (wt. of 100 seeds, ~42 gm). The seed contains endosperm (50–55%), protein (9–12%), pentosan (8–10%), water soluble gum (32–34%) and moisture (8–10%). The analysis of seed components is presented in Table 1 and the specification of the gum in Table 2 along with a comparison with the ISI code and US code of guar gum. The seeds have been identified as a potential source of commercial gums[8] and considered superior in terms of higher endosperm content (50–55%) and its easy mechanical separability due to bigger size as compared to existing Indian commercial sources like guar, dhaincha and cassia gums which contain 25–42% of endosperm.

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doi:10.1016/S0008-6215(03)00258-1

Table 1
Chemical analysis of the seed components of *C. grandis*

Seed parts	Protein (%)	Moisture (%)	Ether extract (%)	Ash (%)	Crude fibre (%)	Water soluble carbohydrates
Seed coat (25–30%)	16.1	5.2	2.2	3.8	35.5	Pentose
Endosperm (50–55%)	4.7	7.9	1.1	0.7	1.7	Galactomannan
Germ (19–23%)	36.9	4.6	8.6	4.9	27.8	Pentose, glucose

The purified galactomannan was a light creamish, homogeneous amorphous powder. Its molecular weight, polydispersity and intrinsic viscosity data are presented in Table 3. Its monosaccharide ratio as determined by HPLC and GLC–MS[9] of the corresponding alditol acetate derivatives (Table 4) indicated that the polysaccharide is a pure galactomannan having a mannose to galactose ratio of 3.15. This result was confirmed by ^{1}H and ^{13}C NMR spectroscopy taking in account the ratio of galactose and mannose, as determined by the relative areas of anomeric galactose and mannose (substituted and non-substituted). During the present studies, a relatively lower proportion of Man to Gal ratio was observed in ^{1}H NMR analysis, which might be due to an incomplete dissolution of some portion of the galactomannan in deuterium oxide (99.96% D). The findings are in accordance with other cassia seed galactomannans which also exhibited adequate range of Man–Gal ratio namely *C. spectabilis*[12] (2.65), *C. angustifolia*[13] (2.90), *C. nodosa*[14,15] (3.55), *C. siamea*[16] (2.55), *C. javanica*[17] (3.27).

Complete methylation of the purified polysaccharide was achieved through two successive Hakamori methylations[18] followed by two Purdie methylations.[19] Hydrolysis of the pre-methylated galactomannan yielded only three methylated sugars namely 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-mannose, 2,3-di-*O*-methyl-D-mannose in a molar ratio of 1.00:3.03:1.02 (GC–MS). Their characterisation indicates that the galactomannan possesses the basic structure of the legume galactomannans having a main chain of (1→4)-linked mannopyranosyl units to which single galactose stubs are attached as side chains at O-6. Like other Cassia seed galactomannans, it provides about 2/3 of unbranched mannose units necessary for synergistic interaction. The results are altogether different from those previously reported[10,11] for *C. grandis* seed polysaccharide with respect to sugar composition (Gal–Man–Xyl in a ratio of 7:5:1) and structural features. The presence of about 8% xylose in the polysaccharide might have been due to insufficient purification because no partially methylated derivatives of xylose have been obtained during methylation analysis. According to the previous reports, it is a non-galactomannan polysaccharide having a main chain of (1→4)-linked galactose and mannose units in a 2:1 molar ratio with 50% of the galactosyl units being branched with single substituent of two galactose and one mannose units at O-3.

On periodate oxidation, the polysaccharide consumed 1.20 mol of periodate with concomitant liberation of 0.21 mol of HCOOH per hexosyl unit. Theoretically, the proposed structure would consume 1.25 mol of the periodate and liberate 0.25 mol of HCOOH per hexosyl unit. Smith degradation of the polysaccharide yielded glycerol and erythritol in the molar ratio of 1.00:2.91. None of the sugar units resisted oxidation, which clearly

Table 2
Standard specifications of food grade *C. grandis* and guar gum

Components	Typical composition of *C. grandis* gum (%)	ISI code [a] for guar gum (%)	Food chemical specification (USA) for guar gum (%)
Galactomannan	81.5	75.0 (min)	66.0 (min)
Acid insoluble (Crude fibre)	1.7	7.0 (max)	7.0 (max)
Protein	4.7	5.0 (max)	10.0(max)
Moisture	7.9	13.0 (max)	
Ash	0.7		
Arsenic			3.0 ppm
Heavy metals like copper, chromium, cadmium, manganese, nickel and palladium	>2 ppm		10.0 ppm
Starch	Passes iodine test		Passes iodine test
pH of aq solution	6.0–7.0	5.5–8.0	
Viscosity (1%)	1760 cP	2500 cP	

[a] Indian specification of guar gum, ISI, New Delhi, 15: 3936-1981, Food Chemical Code (3rd ed.) 1981, National Academy Press, Washington, DC, 191.

Table 3
Preliminary analysis of *C. grandis* seed galactomannan

Average molecular Weight	Polydispersity	d*n*/d*c*	Intrinsic viscosity (mL/g)
$M_n = 5.95 \times 10^5$ $M_w = 8.02 \times 10^5$ $M_z = 3.76 \times 10^6$	$M_w/M_n = 1.35$	0.15	848

reveals that no (1→3) linkage exists in the galactomannan. The galactomannan was subjected to chromium trioxide oxidation which indicates that the D-mannopyranosyl units are β-linked (oxidizing more rapidly) and D-galactopyranosyl residues are α-linked. The anomeric configuration of the monomers was also confirmed by NMR analysis. The ^{1}H NMR spectrum of the galactomannan anomeric protons showed a doublet at δ (5.4 ppm) and a singlet at δ (5.1 ppm). These have been assigned to H-1 of galactopyranosyl and β-D-mannopyranosyl units, respectively. It indicates that these monomer units in the polymer would have the expected $^4C_1$ conformation.[20] The ^{13}C NMR spectrum showed fully resolved resonances of all carbon atoms (Table 5). The spectra are in close agreement with those reported for other legume galactomannans namely Guar, *C. angustifolia*, *C. spectabilis*, *C. siamea* and *C. nodosa*.

The nearest neighbour probabilities of D-galactosyl units along the D-mannosyl main chain has been evaluated through the resonances of C-4 of mannosyl units. The Man C-4 resonance splits into three signals (Fig. 1) corresponding to branching pattern A, B and C. Peak C is more prominent ($\sim$50%) followed by peak (B) at about 40% when compared to peak (A) suggesting a longer sequence of unbranched mannose units. Such pattern would be expected from a galactomannan having about three times more proportion of mannose than galactose.

The resonance for substituted mannopyranosyl residues at C-6 resulted in a triplet and is hereby interpreted in accordance with the results of Manzi and coworkers.[21] The peak at lowest field originated from the C-6 resonance of the intermediate unit from the groups of three contiguous substituted mannosyl units, as in triad (a) Scheme 1. The signals at higher field are due to a block of three contiguous mannosyl units, where only the intermediate residue is substituted (triad c) and the intermediate peak represents overlapping of signals from triads (b), where only two contiguous units are substituted. In the case of *C. grandis* seed galactomannan, triad b (about 80%) dominates the triad (a) and (c), which is due to the lower proportion of galactose in comparison to mannosyl units. The results provide limited information concerning the branching pattern in the galactomannan. A more detailed characterization and interpretation would require additional enzymatic and biosynthetic studies.[22,23]

A typical diffraction pattern of a well oriented and crystalline film of *C. grandis* gum extended to about 300% at 95% humidity is shown in Fig. 2. It possesses much similarity with the pattern of other legume seed galactomannans. The X-ray fibre diagram, based on the measurement of interplanar spacing *hkl* indices (Table 6), suggests an orthorhombic unit cell having $a = 9.00$, $b = 24.81$, $c = 10.30$ Å. It shows much resemblance to carob gum (Man–Gal = 3.13) with $a = 9.04$, $b = 30.61$, $c = 10.24$ Å and other legume seed gums. The lattice constants of *C. grandis* and previously reported galactomannans are presented in Table 7.

The X-ray diffraction studies showed a relative constancy of *a* and *c* dimensions, however the *b* dimension varies substantially in comparison to other gums (Table 7). The value[26] of the *b* dimension varies substantially from 11.6 to 33.2 Å depending upon the Man–Gal ratio, % relative humidity (RH) and extension of the gum film. In the pure mannan I, this value has been reported to be 7.21 Å. Chein and Winter[24] have already mentioned the sensitivity of the *b* dimension to the degree of substitution and have shown that it decreases with decreasing galactose content. According to Song and coworkers,[28] this dimension is also sensitive

Table 4
Sugar analysis of *C. grandis* seed galactomannan

Sugars	Sugar ratio				*m/z* (GC–MS)
	HPLC	GLC [a]	^{13}C NMR	^{1}H NMR	
D-Galactose	1.00	1.00	1.00	1.00	43, 73, 107, 115, 145, 217, 289
D-Mannose	3.20	3.15	3.13	3.10	43, 73, 107, 115, 145, 217, 289

[a] As corresponding alditol acetate derivate.

Table 5
Assignments of peaks in the ^{13}C NMR spectrum of *C. grandis* seed galactomannan

Type of unit	Chemical shifts (δ, ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Galactopyranosyl	99.68	69.39	70.44	72.24	72.01	62.04
β-D-Mannopyranosyl unbranched at O-6	100.88	70.93	72.36	77.21 77.44	75.91	61.54
β-D-Mannopyranosyl branched at O-6	100.76	70.93	72.36	77.44 77.71	74.31	67.63 67.49 67.33

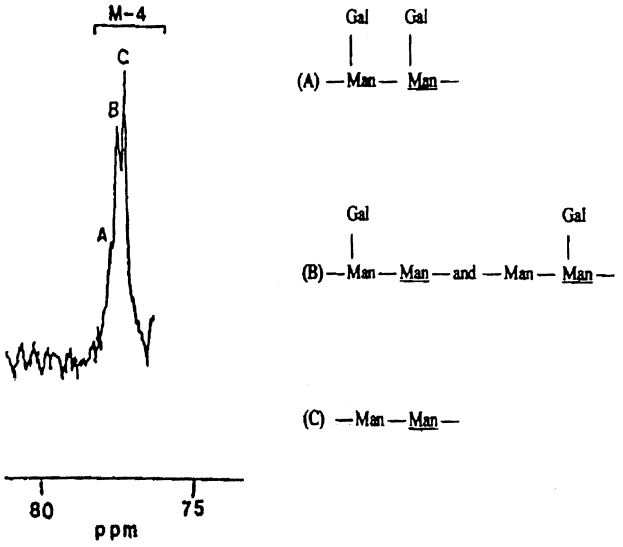
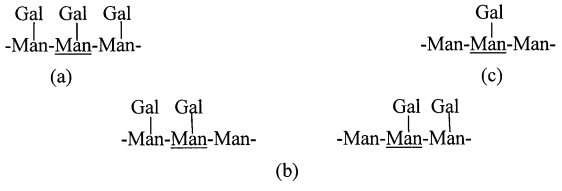


Fig. 1. ^{13}C NMR spectral region of C-4 (mannose); A, B and C are probable diad interpretation.



Scheme 1.

to hydration, and these authors found variation of 15% for samples maintained at constant RH values during X-ray experiments, compared to those recorded under vacuum.[25,29] It can be pointed out here that the X-ray diffraction of the sample was carried out under vacuum. According to these reports,[24,25,28,29] for the entire range of galactose substitution, from 0% in mannan I to 93% in fenugreek galactomannan, the structure is essentially the same and consists of an energetically preferred main-chain conformation together with an irregular lateral association. The near constancy of the fibre repeat (c) and of the lateral dimensions (a) suggest a packing

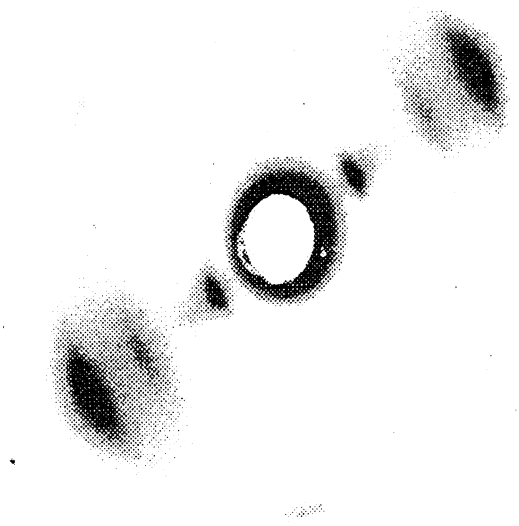


Fig. 2. X-ray diffraction in vacuo at room temperature of *Cassia grandis* seed galactomannan.

Table 6
Interplanar spacing (Å) of *C. grandis* seed galactomannan

hkl	Interplanar spacing (Å)
020	12.40
001	10.30
100	9.00
110	8.46
120	7.28
111	6.53
040	6.20
140	5.10
002	5.15
022	4.75
102	4.47
220	4.23
042	3.96
240	3.64
241	3.43
240	3.64
241	3.43

Table 7
Orthorhombic unit cell parameters from fibre diagrams for *C. grandis* and other seed galactomannans

Source	Man/Gal	Unit cell parameters (Å)		
		a	*b*	*c*
C. grandis	3.15	9.00	24.81	10.30
Tara[24]	3.00	8.91	24.17	10.46
Carob[25]	3.13	9.04	30.61	10.24
C. spectabilis[12]	2.51	9.12	25.63	10.28
C. didymobotrya[26]	3.04	9.00	24.62	10.30
Medicago sativa[27]	1.16	9.00	30.66	10.24
Mannan I[28]	0.00	8.92	7.21	10.27

model having mannose–mannose interaction and the third dimension (*b*) relates to the distance between the mannan sheets. Any gaps created by the absence of galactose substitution are expected to be filled with water at higher humidity.[28] The probable space group symmetry[28] of the unit cell is $P2_12_12$.

1. Experimental

1.1. Isolation and purification

The seeds of *C. grandis* were obtained from Pratap Nursery and Seed Store, Dehradun, India and identified at the Seed Herbarium of the Institute. The seeds were roasted for 5 min and subjected to the separation of the endosperm using a dry milling process with various mixer/grinder and different sieves. The endosperm was crushed to a 200 mesh-size using a high speed hammer mill. The crude gum thus obtained was purified by successive processes of fractional precipitation with EtOH, purification by Ba^{2+} complexing, filtration through membrane filters, dialysis in running distilled water and freeze drying.

1.2. Sugar analysis

The ratio of galactose and mannose in the galactomannan was determined by HPLC of the completely hydrolysed polysaccharide and GLC of the corresponding alditol acetate derivatives according to the previously described method.[12] The sugars were identified by GC–MS.

1.3. Molecular weight determination

The average molecular weight and polydispersity of the purified galactomannan were determined on a multi-angle laser light scattering apparatus (DAWN-DSP-F from WYATT Technology) online with a Waters 150-C ALC/GPC at 25 °C, using $\lambda = 632.8$ nm for 1 g/L initial solution after filtration through 3.0, 1.0, 0.5, and 0.2 μm Millipore filters. The system contained two Shodex columns of OH-PAC 804 and 805 series. The eluent used was 0.1 mol/dm^3 NH_4NO_3 with 0.5 g/L NaN_3 as preservative. The d*n*/d*c* was determined in the same solvent using a Brice-Phoenix Differential Refractometer.[12]

1.4. Methylation analysis

The dry polysaccharide (25 mg) was suspended in freshly distilled Me_2SO (15 mL) and extensively stirred for 28 h followed by mild sonication for 15 min. After stirring for 10 h, the partially dispersed residue was subjected to two successive Hakomori methylation.[18] The partially methylated product was again subjected to two successive Purdie methylation.[19] The polysaccharide thus obtained was completely methylated as it showed no IR –OH absorption at 3600–3400 cm^{-1}. The product was then subjected to hydrolysis and analysis of methylated fragments by GLC and GC–MS.[12]

1.5. Periodate oxidation analysis

The polysaccharide (150 mg) was dispersed in distilled water (30 mL) and the solution was cooled to 0 °C. A cold solution of sodium metaperiodate (0.36 M, 30 mL) was added to the solution and the volume was completed to 100 mL with cold water. The oxidation reaction was carried at 5 °C and the amount of periodate consumption and concomitant liberation of formic acid was estimated titrimetrically at fixed intervals of time. The oxidation was completed in 93 h.

1.6. Smith type degradation

The polysaccharide (125 mg) was subjected to periodate oxidation according to above mentioned method. The resulting oxo-polysaccharide was reduced with $NaBH_4$ (100 mg) at rt for 22 h followed by neutralisation and concentration. Glycerol and erythritol were estimated according to the chromotropic acid method.[30]

1.7. NMR studies

For ^{1}H NMR analysis, the polysaccharide soln (1 mg/mL) was exchanged in D_2O by repeated evaporation and finally dissolved in D_2O (99.96% D) by repeated evaporation. For ^{13}C NMR analysis, the polysaccharide (70 mg/L) was dissolved at 75 °C with continuous stirring for 10 h followed by mild sonication for 20 min and filtration. Both spectra were obtained according to the conditions described previously.[12,31]

1.8. X-ray diffraction studies

A 0.65% (w/v) polysaccharide soln was prepared with continuous stirring for 2 h at rt. The polysaccharide film was formed as coating on heated teflon plates by slow evaporation for 22 h at 25 °C. The film was cut in strip (1.5 × 7.5 mm) and hung with a load of 18 gm in a jar maintained at 95% RH. Elongation (300%) was achieved in 5 days. Crystallinity of the stretched films was further enhanced by annealing in a sealed high pressure bomb at 100 °C over aq $CuSO_4$ for 4 h. The resulting stretched films were mounted onto a 0.20 mm diameter, collimator and exposed in vacuo to CuK_α ($\lambda = 1.5418$) radiation from a Philips PW 4720 X-ray generator for 18–20 h using a Warhus flat film vacuum camera.

Acknowledgements

The authors are thankful to Dr P. Pushpangadan, Director, NBRI, Lucknow for his keen interest in the work. The authors are also thankful to Dr F.R. Taravel and Dr H. Chanzy, CERMAV-CNRS, France for NMR spectroscopy and X-ray diffraction studies, respectively. One of the authors (V.P.K.) is thankful to CSIR for providing an Emeritus Scientist Position and the other (H.J.) to the Department of Science and Technology, New Delhi, India for providing a Project Fellowship.

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