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GALACTOMANNAN CHANGES IN DEVELOPING *GLEDITSIA TRIACANTHOS* SEEDS

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Key Word Index—*Gleditsia triacanthos*; Leguminosae; seeds; honey locust; galactomannan; polysaccharide biosynthesis; legume endosperm.

Abstract—Galactomannan has been extracted from the endosperm of seeds of *Gleditsia triacanthos* (honey locust) at different stages of development, when the seed was accumulating storage material. Properties of the different samples have been studied. The molecular size distribution became more disperse as galactomannan accumulated and the galactose: mannose ratio decreased slightly. Some possible reasons for these changes are discussed.

INTRODUCTION

Galactomannans are found in the endosperm of a number of legume seeds [1–5], serving as a reserve polysaccharide and also probably maintaining a moist environment for the germinating seed. They consist of a $(1 \rightarrow 4)$-β-D-mannan chain, substituted on the 6-hydroxyl by single α-D-galactosyl groups. In mature seeds the galactose content varies according to species and can be as low as 15% or as high as *ca* 50%, indicating in the latter case an almost complete substitution of all the mannosyl units in the β-D-mannan chain. These values are a mean of the values of individual molecules but this mean shows consistency for mature seed samples of the same species from different sources [6, 7]. A spectrum of values is found for fractions prepared by fractional ethanol precipitation, or different fractions can be prepared by cold and hot water extraction of galactomannans with a galactose content near 20%.

The galactose to mannose ratio of the galactomannan from *Gleditsia ferox* seed was examined [8] when the seed was young and mature and from *Trigonella foenum-graecum* (fenugreek) [9] at a number of stages of development; in neither case were significant differences detected. For *G. ferox* these values were estimated from the optical rotation of acidic hydrolysates and for the fenugreek samples from colorimetric estimation after PC separation.

Unlike fenugreek galactomannan, in which nearly all the mannosyl units are substituted, many other galactomannans at seed maturity are not fully substituted and one possible pattern of biosynthesis is that the molecules are initially formed fully substituted but that later some galactosyl groups are removed. Another hypothesis is that they are synthesized with a constant ratio.

Aspects of the structure and properties of the galactomannan from mature honey locust have been examined on several occasions [10–13]. The galactose content was found to be 22–24% by densitometric determination after PC of an acid hydrolysate and from methylation analysis [10], and to be 23% by estimation of D-galactose in an acid hydrolysate with D-galactose dehydrogenase (EC 1.1.1.48) and total carbohydrate by anthrone-H_2SO_4 [11]. The yield from mature seed was *ca* 20% and limiting viscosity numbers of 44 [10] and 13.8 dl/g [11] have been recorded. Gel chromatography on porous glass (Merckogel SI 5000 Å) [11] indicated a large size in solution; it was included, showing a broad range of size, with a single peak at an elution volume of 48 ml in a column with a total volume of 62 ml. The elution volumes of other galactomannans ranged from 48 to 51 ml. In conjunction with the behaviour on ultracentrifugation (when the sedimentation constant showed high concentration dependence and boundary hyperfine sharpening), the high viscosity [11] and the theoretical calculation of the favoured conformation [14], it was suggested [12] that an extended rod conformation contributed to the structure in a poor solvent, such as neutral salt solutions of low concentration. Chromatography of guar galactomannan on porous silica in neutral salt solution has also indicated a high molecular size in aqueous solution [*11, 15, 16*].

The theoretical conformation of the $(1 \rightarrow 4)$-β-D-mannan chain is ribbon-like with a two-fold screw axis, which places neighbouring galactosyl units, joined to the hydroxymethyl groups that are attached to pyranose rings, on opposite edges of the ribbon [12, 14, 17, 18].

This paper describes the essentially quantitative recovery, without apparent degradation, of galactomannan from honey locust seeds at different stages of seed filling, its purification and various properties that describe aspects of structural change that accompany its accumulation.

RESULTS AND DISCUSSION

Seeds were collected from a single tree and the sampling times refer to the period after anthesis (Tables 1–3). Prior to 9 weeks, the endosperm was too small to isolate. Seeds were also collected at 24 weeks when pods and seeds had dried fully. Throughout growth the relative size of endosperm and cotyledons remained similar but the condition of the endosperm depended on age. At weeks 9, 10 and 12 it was gelatinous and readily separated from the testa. At weeks 14 and 24 there was a close association

Table 1. Residual fraction after extraction of *Gleditsia triacanthos* endosperm

Sampling time (wk)	Average weight of endosperm (mg)	Galactomannan extracted per endosperm (mg)	Residue after extraction per endosperm (mg)	Fraction of residue solubilized by β-mannanase (%)	Galactomannan in residue (mg per endosperm)	Fraction of total galactomannan not extracted (%)
9	17	8.6	2.2	11.8	0.26	2.9
10	20	11.7	2.8	10.5	0.29	2.5
12	40	31	3.9	11.5	0.45	1.4
14	85	62	9.8	16.7	1.6	2.6
24	87	64	11.2	16.0	1.8	2.8

Table 2. Gel chromatography and limiting viscosity numbers of galactomannans from endosperms of *Gleditsia triacanthos* at different stages of development

Sampling time (wk)	Limiting viscosity number (dl/g ± 0.2)	K_{av}*
9 A	11.8	0.32
9 B	—	0.32
10	12.1	0.31
12	12.0	0.29
14 A	11.5	0,0.31
14 B	—	0,0.31
Mature	13.5	0,0.31

$$*K_{av} = \frac{\text{elution volume} - \text{void volume}}{\text{total volume} - \text{void volume}}.$$

with the testa and both were hard; they could be separated after boiling in ethanol. The average weight of an endosperm increased from 9 weeks after anthesis to 24 weeks (Table 1), with the main increase occurring between 10 and 12 weeks.

Precautions were taken to stop hydrolytic enzymic activity on collection by freezing in dry ice and then destroying activity by boiling in ethanol. α-Galactosidase activity is detectable in mature seeds and any extraction procedure that does not eliminate this, such as cold water extraction of flour, can lead to modification to the galactose content and possibly incomplete extraction of galactomannan; depolymerization of the mannan chain would also be possible if β-mannanase were present. Essentially complete extraction of the flours was achieved by a combination of repeated fine grinding with an Ultra-Turrax type homogenizer and an all-glass Tenn-Broeck grinder. Efficient extraction of galactomannan into aqueous solution without degradation requires that the endosperm be well-swollen and ground finely after the denaturation of hydrolytic enzymes. All extraction solutions were less than 0.25% w/v with respect to endosperm flour.

The percentages of residual endosperm after two cold water and one hot water extraction were all between 10 and 14% (Table 1). A further hot water extraction and an alkaline extraction yielded insignificant amounts of product on precipitation with acidified ethanol.

The residues after two cold water and one hot water extraction were incubated with β-(1 → 4)-D-mannanase (EC 3.2.1.78) to estimate residual galactomannan; the percentages of the residues solubilized were between 10% and 17%, indicating that the fraction of galactomannan remaining was always less than 3% of the total galactomannan (Table 1). The material solubilized by β-D-mannanase may not be galactomannan but another, structural polymer based on the β-(1 → 4)-D-mannan backbone such as galactoglucomannan; however, the galactose:mannose ratio of this material was very similar to that of the extracted galactomannan.

The solubilized galactomannan was purified via its copper complex, and after this treatment, hydrolysis and

Table 3. Galactose contents and degrees of hydrolysis by β-D-mannanase of *Gleditsia triacanthos* galactomannan of different stages of endosperm development

Sampling time (wk)	Galactose content* Method A	Method B	Method C	Degree of hydrolysis by β-D-mannanase (%)
9	34	—	29	7.5
10	34	30	30	8.1
12	32	—	30	8.6
14	30	27	28	10.7
Mature	28	24	26	15.0

*Method A: α-D-galactosidase plus β-D-mannanase then D-galactose dehydrogenase: total sugar by anthrone. B: Acid hydrolysis then D-galactose dehydrogenase: total sugar by anthrone. C: Acid hydrolysis and GC of alditol acetates.

GC showed that more than 97% of carbohydrate was accounted for by galactose and mannose. The percentage of galactomannan in the endosperm increased from 9 to 12 weeks (51–78%) and then remained relatively constant, although the amount of galactomannan per seed increased rapidly from 9 to 14 weeks (Table 1). Endosperms at 24 weeks yielded 73% of galactomannan and 13% of residue. Values of 68.3 and 18.5 have recently been reported [13].

The limiting viscosity numbers of the samples (Table 2) were all high and did not change significantly in weeks 9 to 14, but there was some increase at 24 weeks. This may have reflected the greater amount of β-(1 → 4)-mannan chain per unit weight of carbohydrate, due to the slight decrease in galactose content, in combination with a somewhat greater molecular size. Gel chromatography, using medium pressure on Fractogel HW-75(F) with 0.1 M NaCl as solvent (Fig. 1, Table 2), gave evidence of differences. Elution of the 9, 10 and 12 week samples showed a single relatively broad peak with a K_{av} of 0.32, which would result from a polydisperse population of molecules with a high molecular size in solution. When the 14 week and 24 week samples were examined the K_{av} of each peak was similar to the earlier samples but the polydispersity was greater. Material that eluted at the void volume was obtained and the included peak was even more disperse. As galactomannan accumulated with time of seed development the range of molecular sizes increased, particularly at the high end. The elution pattern of waxy maize starch and glucose is shown in Fig. 1 and dextran T-2000 in Fig. 2.

An aspect of the high molecular size of galactomannans, as determined by gel chromatography, is the possibility that this is due to aggregation. The 11 week sample was chromatographed in 0.1 M KOH and 0.05 M H_2SO_4 without change in the elution volume or shape of the elution curve. Another possibility is that aggregation can occur in part of the sample or in parts of individual molecules with low galactose content or sections with the galactosyl substitution isolated to one edge of the ribbon-

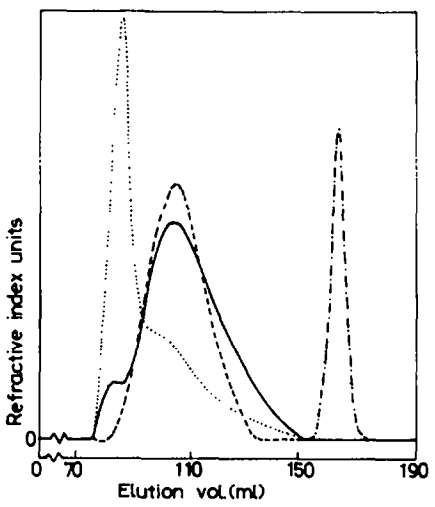

Fig. 1. Gel chromatography on Fractogel TSK HW-75(F) of honey locust galactomannan from the seed endosperm, isolated at 9 weeks (- - - -) and 14 weeks (————) after anthesis, and of waxy rice starch (. . . . .) and glucose (· — ·—).

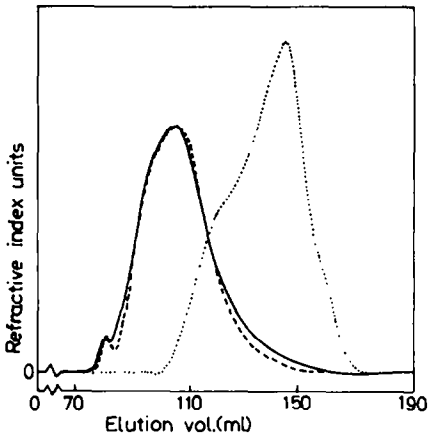

Fig. 2. Gel chromatography on Fractogel TSK HW-75(F) of guar galactomannan with galactose:mannose ratios of 39:61 (————) and 19:81 (- - - -) and of dextran T-2000 (. . . . .).

like conformation [19, 20]. Samples of guar galactomannan from which some of the D-galactosyl groups had been removed [21] with α-D-galactosidase (EC 3.2.1.22) were chromatographed. No significant change in the elution curve and the K_{av} of the maximum of the curve was observed in the range of galactose to mannose ratios in the samples from 39:61 to 16:84. In Fig. 2, the elution profiles on Fractogel TSK 75(F) for two of the samples, with ratios of 39:61 and 19:81, are shown, together with dextran T-2000. Samples with ratios of 34:66, 29:71 and 16:84 were also chromatographed. The K_{av} values of the main peaks were all in the range 0.29–0.30 and the areas of the peaks at the void volume all between 2.4 and 3.3% of the total area. The samples with the two lowest galactose contents (19:81 and 16:84) would associate heterogeneously with polysaccharides like agarose and carrageenan [19]. The results support the model [12, 14, 17, 18] that the high molecular size in solution, as determined by gel chromatography, and also the high solution viscosity, is due to molecules having a high proportion of rod-like extended sections in water and also in dilute alkali, which would indicate that it too is not a good solvent.

The galactose contents of the honey locust polysaccharides were estimated by three methods. In the first the galactomannan was depolymerized by a mixture of β-D-mannanase and α-D-galactosidase [7], the D-galactose released estimated using D-galactose dehydrogenase [22] and total carbohydrate estimated by anthrone–sulphuric acid [23]. In the second method D-galactose was measured with D-galactose dehydrogenase [22] after acidic hydrolysis, and total sugar with anthrone–sulphuric acid whilst and in the third, both sugars were estimated as alditol acetates by GC after acidic hydrolysis, reduction and acetylation. The galactose content decreased slightly with time of development (Table 3). The second method gave lower galactose contents but this is probably due to acidic hydrolysis causing some sugar decomposition, whereas anthrone would measure total carbohydrate.

Consistent with the decrease in galactose content, the degree of hydrolysis by β-(1 → 4)-D-mannanase increased with time of development (Table 3). The degree of hydro-

lysis of galactomannans is inversely proportional to the galactose content [20, 24].

The oligosaccharide products of β-D-mannanase digestion were chromatographed on Bio-Gel P-2 and the results for the first five sampling times are shown in Fig. 3 and Table 4; the mannose residue at the reducing end is italicized in the table. The numbers over the peaks give the degree of polymerization (d.p.) and the products contributing to these peaks in the β-mannanase hydrolysate of hot water-soluble carob galactomannan [25] are also shown in Table 4. The increase in the amounts of products of lower d.p. 2,3,4 and 7 with stage of development reflects the increased hydrolysis by β-D-mannanase (Table 3) which is a consequence of the decreasing galactose content. The high level of the fraction of d.p. 7 relative to the fraction of d.p. 3 is consistent with a high component of segments of neighbouring galactosyl substituents as in Fig. 4(a), as opposed to single substituents separated by an odd number of unsubstituted mannosyl residues [Fig. 4(b)] which results in substitution on the same edge of the β-mannan ribbon [12, 17, 18].

It has been proposed [4, 24] that the different levels of substitution of mannosyl units by galactosyl groups in the biosynthesis of galactomannans of different species is a consequence of steric and conformational effects in the synthesizing complex of membrane, galactomannan, the two polysaccharide synthases [(1 → 4)-β-D-mannosyl transferase and (1 → 6)-D-galactosyl transferase] and the two nucleoside diphosphosugar substrates. The endosperm of legume seeds, like locust bean, is a thickened secondary wall. Non-cellulosic cell-wall polysaccharides

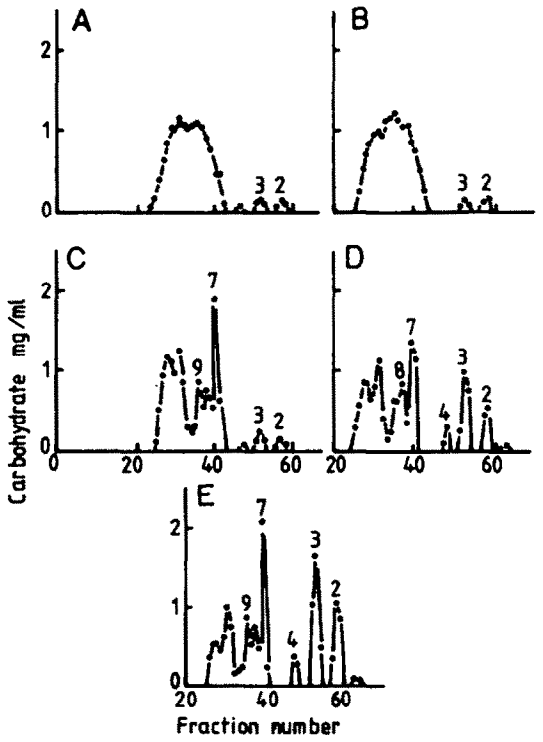

Fig. 3. Chromatography on Bio-Gel P-2 of hydrolysates of locust bean galactomannans by β-mannanase: A, week 9; B, week 10; C, week 12; D, week 14; E, mature.

are synthesized in Golgi bodies or membranes of endoplasmic reticulum prior to movement in Golgi bodies to the plasmalemma, for release to the outside of the cell. The synthesis and secretion of galactomannan involves the rough endoplasmic reticulum [26]. The biosynthesis of a structurally related polysaccharide, xyloglucan, requires concurrent linking of the glucosyl and xylosyl residues [27]; the insolubility of the (1 → 4)-β-D-mannan chain makes prior synthesis of a completed mannan chain followed by galactosyl substitution unlikely.

The extended, rod-like structure of the galactomannan chain means that the recent galactosyl group that has been attached, in conjunction with the membrane shape and the associated enzymic proteins, can sterically affect the ease of substitution of newly added mannosyl units. In polysaccharides like those from lucerne and fenugreek, with nearly complete substitution of mannosyl units, no steric hindrance to further substitution occurs, but in those like carob and honey locust, with fewer galactosyl substituents, there would be a reduced probability of substitution until a sufficient length of unsubstituted mannan chain had appeared [24]. The favoured conformation of the β-D-(1 → 4)-mannan chain, a two-fold screw axis with an extended rod-like structure, means that substitution on either edge of this ribbon-like chain is affected differently by previous galactosyl substituents.

One explanation for the decreasing D-galactosyl substitution that accompanies rapid galactomannan deposition could be that the conformation of the synthesizing complex undergoes some conformational change. The membrane may change its shape slightly. A second hexose transferase may appear at this stage of rapid accumulation. Another possibility, that α-D-galactosidase, present at very low levels at maturity, causes limited removal of galactosyl groups, seems less likely. This process occurs with preferential removal of galactosyl groups from one edge of the ribbon-like conformation [21, 24], leading to a galactosyl distribution as shown in Fig. 4(b), whereas the oligosaccharides produced by depolymerization with β-D-mannanase indicate a structure with a high level of structural units of the type shown in Fig. 4(a), i.e. neighbouring pairs of substituted mannosyl units. Also, the high viscosity and molecular size, as indicated by gel chromatography of the 24 week sample shows that there has been no depolymerization of the main chain by β-mannanase.

EXPERIMENTAL

Plant sampling. Seed pods were collected from a single tree. The initial sampling was at 9 weeks after anthesis. Samples collected at 9–14 weeks were frozen immediately in dry ice. Mature seeds were collected at 24 weeks.

Endosperm isolation. Mature seeds were notched with a scalpel in the hilum region and boiled in EtOH for 10 min. They were then split longitudinally along the median axis. The split seeds were boiled in EtOH for 10 min and the endosperm separated from the seed coat and cotyledons and stored in EtOH.

Seeds were removed from frozen 14 week pods, split and boiled in EtOH for 10 min. The endosperms were dissected and stored in EtOH.

Week 12 pods were separated into seeds and pods while frozen, the seeds incised at the end opposite to the hilum and the endosperm and cotyledons squeezed through this incision. After boiling in EtOH for 10 min, the endosperm and cotyledons were separated.

Table 4. Oligosaccharide components of Bio-Gel P-2 fractions

Degree of polymerization	Oligosaccharides present*
2	M–*M*
3	Ga | M–M–*M* ; M–*M*
4	Ga | M–M–*M*
7	Ga | M–M–M–M–*M* | Ga
8	Ga | M–M–M–M–M–*M* | Ga ; Ga | M–M–M–M–M–*M* | Ga ; Ga | M–M–M–M–*M* | Ga Ga
9	Ga Ga | M–M–M–M–M–*M* | Ga ; Ga Ga | M–M–M–M–M–*M* | Ga ; Ga Ga | M–M–M–M–M–*M* | Ga ; Ga | M–M–M–M–M–M–*M* | Ga

*M– = Manβ1–, *M* = –4Man and Ga = Gal –M– = –4Manβ1–.
α1
|
6

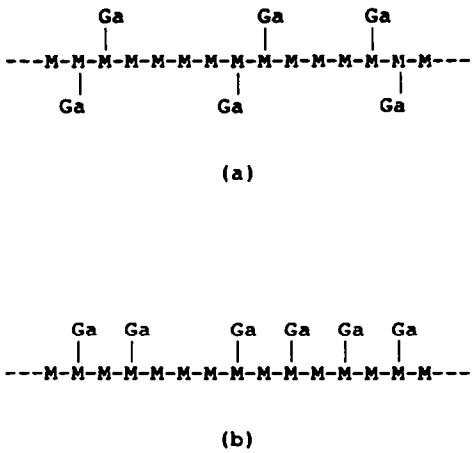

Fig. 4. Types of segments in galactomannan leading to fragments of d.p. 7 (a), and d.p. 3 (b), after β-D-mannanase hydrolysis.

Week 10 seeds had a very thin seed coat, so it was necessary for the seeds to remain in the pod structure and to manipulate the pods while frozen. They were broken to expose the tips of the seeds, these were then incised and the endosperm and cotyledons extruded. These were boiled in EtOH for 10 min and dissected into endosperm and cotyledons and the former stored in EtOH.

Week 9 pods were boiled in EtOH for 10 min prior to applying the procedure adopted for 10 week seeds. This was required to distinguish between pod and seed material. Endosperms were dried by solvent exchange.

Preparation of endosperm flour. The isolated endosperms were ground in a H_2O-cooled mill to a fine flour which was boiled in EtOH for 10 min. The cooled suspension was macerated in an Ultra-Turrax homogenizer and the flour collected by centrifugation (6000 *g* × 20 min), washed with EtOH, Me_2CO and Et_2O and dried under vacuum.

Extraction of galactomannan. Endosperm flour (0.5 g) was dispersed in EtOH (5–10 ml) in a centrifuge bottle (190 ml). 0.5% NaCl (50 ml) was added, the flour swollen overnight at 4° and the mixture warmed to room temp. and macerated with an Ultra-Turrax homogenizer. The mixture was then ground in portions in

a glass Tenn-Broeck grinder. This manipulation was performed × 3 giving a final solvent vol. of 150 ml. After centrifugation (6000 *g* × 20 min), the supernatant was poured into 3 vols of EtOH, to give a white fibrous mass that was stored. The residue from extraction was suspended in 0.5% NaCl (50 ml) and re-extracted by the same procedure. The two fibrous ppts formed on EtOH pptn were combined. The residue was again macerated and ground by the same procedure and then heated at 90° for 10 min. The soln was cooled, centrifuged and the supernatant treated with 3 vols of EtOH. The combined galactomannan ppts were centrifuged, washed with EtOH, Me_2CO and Et_2O and dried under vacuum. The residue from extraction was treated similarly.

Purification of galactomannan. Crude galactomannan was dissolved in 0.5% NaCl (340 ml/g original endosperm flour) and Fehling's soln (15 ml/g) added. The ppt was collected by centrifugation, re-suspended in 0.5% NH_4OAc (280 ml/g flour) and the Cu complex decomposed with a few drops of 1M HCl. The polysaccharide was pptd by adding 3 vols of EtOH, centrifuged and re-dissolved in 0.5% NH_4OAc (340 ml/g flour), pptd in EtOH (3 vols) and washed with EtOH, Me_2CO and Et_2O and dried under vacuum.

Limiting viscosity numbers. These were determined in an Ubbelohde suspended-level viscometer at 25° in 0.5 M KCl with *in situ* dilution. The range of galactomannan concn was 0.025–0.060%.

Gel chromatography. Waxy rice starch granules were first dispersed in DMSO, pptd by the addition of EtOH, washed with EtOH, Me_2CO and Et_2O and dried under vacuum. Polysaccharide samples were prepared at a concn of 0.06% in 0.1 M NaCl, 0.001 M Na_2EDTA, 0.02% NaN_3 by shaking occasionally over 18 hr and applied from a 1 ml loop to a column of Fractogel TSK HW-75(F) (Merck) measuring 1.6 × 88 cm. The solvent was 0.1 M NaCl and 0.001 M Na_2EDTA; the pressure was maintained at 50 psi. Polysaccharide was detected with a refractive index detector. In comparing the chromatographic behaviour in 0.1 M KOH, 0.05 M H_2SO_4 and 0.1 M NaCl, azide was omitted, the 1 ml fraction loaded manually and carbohydrate in eluant fractions measured with PhOH–H_2SO_4.

Determination of galactose:mannose ratios by acid hydrolysis and D-galactose dehydrogenase. Galactomannan (25 mg) was hydrolysed with 0.75 M H_2SO_4 (2.5 ml) at 100° for 3 hr, cooled, neutralized with 1.5 M NaOH, diluted to 25 ml with 0.1 M Tris–HCl buffer (pH 8.5) and centrifuged. Galactose content was estimated [22] by diluting this neutralized hydrolysate 10-fold with buffer and an aliquot (2.5 ml) (containing *ca* 60 μg galactose) mixed with 13 M NAD^+ (0.1 ml) and D-galactose dehydrogenase (50 μl–0.25 U) and the *A* 340 nm compared with a standard curve of D-galactose. Total carbohydrate was determined with anthrone–H_2SO_4 [23] using a mixture of mannose and galactose (3:1) for calibration.

Determination of galactose:mannose ratios by α-D-galactosidase and β-D-mannanase hydrolysis and D-galactose dehydrogenase. Aliquots (0.1 ml) of a soln (~1 mg/ml) of galactomannan were used for determination of total carbohydrate by the anthrone procedure [23]. Other aliquots (0.2 ml) were treated [6] with a mixture (20 μl) of purified guar-seed α-D-galactosidase II (2U) plus purified *Aspergillus niger* β-D-mannanase (1U) and 0.1 M NaOAc buffer (0.2 ml, pH 4.5) at 40° for 1 hr. The pH was adjusted by adding 0.2 M Tris–HCl buffer (2.5 ml) and D-galactose determined with D-galactose dehydrogenase [22].

GC of alditol acetates. Galactomannan (10 mg) was hydrolysed in 2 M TFA (2 ml) at 120°. After $NaBH_4$ redn, MeOH evapn, acetylation with Ac_2O and 1-methylimidazole, and extraction with CH_2Cl_2, GC was performed; injection temp. 250°, column temp. 200°, support 3% Silar 10C on 80-100 WHP, flow rate 50 ml N_2/min, detection by FID using *myo*-inositol as int. std.

β-Mannanase treatment of residues after extraction of galactomannan. Dry residue (20 mg) was suspended in 50 mM NaOAc buffer (4.8 ml, pH 4.5) and treated with purified *A. niger* β-mannanase (10 U) at 40° for 4 hr, with occasional gentle shaking. After centrifugation (1000 *g* × 10 min), aliquots (0.1 ml) were used for estimation of total carbohydrate by the anthrone method [23]. Separate aliquots were incubated with α-D-galactosidase plus β-D-mannanase and D-galactose released estimated with D-galactose dehydrogenase [22].

Hydrolysis of galactomannans with β-mannanase and chromatography on Bio-Gel P-2 of released oligosaccharides. This was performed as described previously [17, 25]; the oligosaccharides were characterized by a combination of physical, chemical and enzymic techniques [25].

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