Phytochemistry, 1976, Vol 15, pp 1111–1117. Pergamon Press. Printed in England.

GALACTOMANNANS AND A GALACTOGLUCOMANNAN IN LEGUME SEED ENDOSPERMS: STRUCTURAL REQUIREMENTS FOR β-MANNANASE HYDROLYSIS

B. V. McCLEARY, N. K. MATHESON and DARRYL M. SMALL
Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006 Australia

(*Received* 14 *January* 1976)

Key Word Index—*Cercis siliquastrum*; Leguminosae; seed endosperm; galactomannan; galactoglucomannan; β-mannanase.

Abstract—A series of galactomannans with varying degrees of galactose substitution have been extracted from the endosperms of legume seeds with water and alkali and the amount of substitution required for water solubility has been determined. Some were heterogeneous with respect to the degree of galactose substitution. The structural requirements for hydrolysis by plant β-mannanase have been studied using the relative rates and extents of hydrolysis of these galactomannans. A more detailed examination of the products of hydrolysis of carob galactomannan has been made. At least two contiguous anhydromannose units appear to be needed for scission. This is similar to the requirement for hydrolysis by microbial enzymes. Judas tree (*Cercis siliquastrum*) endosperm contained a polysaccharide with a unique composition for a legume seed reserve. Gel chromatography and electrophoresis on cellulose acetate indicated homogeneity. Hydrolysis with a mixture of β-mannanase and α-galactosidase gave a glucose-mannose disaccharide and acetolysis gave a galactose-mannose. These results, as well as the pattern of hydrolysis by β-mannanase were consistent with a galactoglucomannan structure.

INTRODUCTION

Galactomannans are found in the endosperm of many legume species. The proportion of galactose varies between species [1, 2]. Most can be extracted by water but sometimes (e.g. *Phoenix dactylifera* [3], with 10% galactose substitution) alkali must be used. Since mannans are only soluble in alkali, it would appear that the degree of galactose substitution determines water solubility. The reported MW for galactomannans from various sources, using physical techniques, has varied from 30000 to 300000 and by chemical end-group determination [4] values as low as 6000 have been reported.

β-Mannanase, one of the enzymes involved in galactomannan depletion on seed germination, is an endopolysaccharase that produces manno-oligosaccharides [5]. If the substrate is mannan then both bacterial and plant β-mannanases, if free of β-mannosidase, give mannobiose and mannotriose, although reversion products are also found. Galactomannans are hydrolysed to the same oligosaccharides if α-galactosidase (E.C.3.2.1.22) is also present. Mannotriose is not split and the rate of hydrolysis increases markedly at a $\overline{DP}$ of 5. The extent of hydrolysis of galactomannans by β-mannanase, free of α-galactosidase and β-mannosidase, is governed by the galactose content of the polysaccharide. With microbial β-mannanases, final hydrolysis percentages of 9, 2, 12 and 5 [6–9] have been reported for guaran (33% galactose) but higher values have been found for substrates with lower galactose contents; e.g. for caroban (23% galactose) 15% [7] and 39% [6]; for coffee bean galactomannan (2% galactose) 36% and 58% [8, 9]. Reese and Shibata [6] using a fungal β-mannanase, free of interfering glycosidase activities, found that the residue of high $\overline{DP}$, remaining after hydrolysis of guaran, had a galactose–mannose ratio of 1:2. On hydrolysis of caroban, an oligosaccharide was found with the suggested structure: Man–Man(Gal)–Man. It was proposed that two contiguous unsubstituted mannose residues were required for hydrolysis. Courtois and Le Dizet [7, 10] reported that a non-dialysable fraction produced by the action of *Bacillus subtilis* β-mannanase and preparations from lucerne and *Leucaena* spp. had mannose–galactose ratios approaching 1 and suggested that one unsubstituted mannose unit was required for hydrolysis by both fungal and plant β-mannanases.

In the present paper the extraction and properties of legume endosperm polysaccharides with a range of galactose contents have been studied and the action of a purified plant β-mannanase on galactomannans with varying galactose contents examined.

RESULTS AND DISCUSSION

Seeds were extracted exhaustively with cold water followed by NaOH–borate under N_2. Extracts with both solvents were purified via copper complexing. The fractions extracted with alkali were still contaminated with a xylan that co-precipitated on formation of the copper complex. The alkali soluble fractions of most seeds, after extraction, were soluble in hot water and remained in solution on cooling to room temperature, long enough to be chromatographed on DEAE-cellulose. The neutral galactomannan was not absorbed and the xylan fraction, which contained uronic acid, was bound. In those species in which all the galactomannan was extracted with cold water, the galactose content of the polysaccharide was greater than 23% (Table 1). When the galactomannan was only partly extracted with cold water, if this fraction

Table 1. Extraction of endosperm polysaccharides from legume seeds with cold water (CW) and alkali (A)

Species	Extraction solution	Amount %	Galactose content %
Cercis siliquastrum	CW	0	
	A	10	14
Cassia odorata	CW	1	16
	A	15	13
Cassia corymbosa	CW	12	26
	A	14	15
Cassia brewsteri	CW	27	18
	A	7	nd*
Cassia sophora	CW	6	28
	A	1	9
Cassia bicapsularis	CW	18	23
	A	3	9
Kennedia rubicunda	CW	13	23
	A	1	22
Gleditsia triacanthos	CW	18	23
	A	0	
Cyamopsis tetragonolobus	CW	21	33
	A	0	
Medicago sativa	CW	10	45
	A	0	

* nd—not determined.

was an appreciable proportion of total galactomannan, then the galactose content was above 20%, except for *Cassia brewsteri* where it was 18%. This extract was translucent and showed precipitation on storing at 2°. The alkali soluble fractions, when they were a significant proportion of the total, had lower galactose contents, in the range 9–15%. A number of the endosperms that contained polysaccharides with lower galactose contents gave both a water-soluble and an alkali-soluble extract, the galactose contents of which were very different: e.g. *Cassia corymbosa* produced approximately equal amounts of cold water and alkali soluble fractions, with values of 26 and 15%. The polysaccharides of *Cassia odorata* and *Cercis siliquastrum* were only effectively extracted by alkali. The results show that at least some galactomannans are heterogeneous with respect to galactose content and that about 20% galactose substitution is required for extraction by cold water.

All the polysaccharides, except that of the Judas tree (*Cercis siliquastrum*), after purification by copper complexing and DEAE cellulose chromatography, when necessary, contained only galactose and mannose. The composition of the polysaccharide from the Judas tree was unusual in that it also contained 7% glucose and this level remained constant during purification by Cu^{2+} complexing and DEAE cellulose chromatography. Galactomannan samples from lucerne, guar and carob, before purification via the copper complex contained 2–3% protein. This decreased to 0.7–1.2% after complex formation. Chromatography on DEAE-cellulose and gel filtration did not remove this remaining protein. All the samples were partly retarded on gel filtration on Merckogel SI 5000 Å (Table 2) and chromatographed as single peaks. There were differences in the degree of retardation but all the indicated MW values were much higher than those reported for galactomannans from chemical end group determination [4]. On electrophoresis on cellulose acetate, after dyeing with Procion red [11], they travelled as single sharp bands. The limiting viscosity numbers of the galactomannans were all high (490–1380 ml/g), consistent with their likely conformation in aqueous solution as an extended rod [12, 13]. There was a corre-

Table 2. Properties of cold water soluble galactomannans and Judas tree polysaccharide

Species	Elution vol. on Merckogel SI 5000 Å (ml*)	Electrophoretic mobility on cellulose acetate†	Limiting viscosity (ml/g)
Cercis siliquastrum	60	0.35	240
Cassia bicapsularis	54	nd	490
Cassia sophora	54	0.38	500
Kennedia rubicunda	51	0.50	650
Cassia corymbosa	54	nd	660
Cassia brewsteri	54	0.35	720
Carob	51	nd	1030
Lucerne	48	0.50	1250
Guar	49	0.45	1330
Honey locust	48	nd	1380

* Total volume 62 ml. † Relative to rate of movement of Procion dye.

Table 3. Hydrolysis of galactomannans by Honey locust β-mannanase B.

Source of polysaccharide	% Galactose	V_{max}*	% Hydrolysis
Bangalow palm (mannan)	0	23	36
Cassia corymbosa (alkali soluble fraction)	16	23	30
Cassia brewsteri (cold water soluble fraction)	18	23	29
Kennedia rubicunda (cold water soluble fraction)	23	23	nd
Carob	24	23	27
Cassia corymbosa (cold water soluble fraction)	26	23	22
Cassia sophora (cold water soluble fraction)	28	23	20
Guar	33	1.2	5.2
Lucerne	45	1.0	3.1

* Relative to a rate of 1 with lucerne galactomannan and determined using galactomannan solns of 0.01 to 0.2% (i.e. about 0.4–11 mM anhydromannose).

lation between elution volume and viscosity (correlation coefficient 0.92). The Judas tree polysaccharide had a lower viscosity and was highly retarded on Merckogel SI 5000 Å. Partial hydrolysis, enzymically and by acetolysis, indicated that it is most probably a galactoglucomannan with a mannan backbone in which some mannose units are replaced by glucose. β-Mannanase, free of α-galactosidase and β-mannosidase, hydrolysed it to 36% reducing sugar and the V_{max} was 23. These are similar values to those found for galactomannans with low galactose substitution (cf. Table 3). Hydrolysis with a mixture of β-mannanase and α-galactosidase (free of β-glucosidase, β-galactosidase, β-mannosidase and cellulase) gave oligosaccharides other than mannobiose and mannotriose. No residual polysaccharide was found, indicating that the polysaccharide was not a mixture of galactomannan and glucan. One of the fractions isolated had the same mobility on PC as a sample of the disaccharide Glc β1–4 Man [14] prepared by hydrolysis of orchid glucomannan. Mannobiose migrated at a different rate in the solvents used. Acidic hydrolysis of this disaccharide before and after borohydride reduction gave respectively, the reducing sugars glucose and mannose and glucose only. Hydrolysis by β-glucosidase, free of β-mannosidase, gave glucose and mannose consistent with this structure. Another hydrolysis fragment of the polysaccharide chromatographed with the mobility of a trisaccharide at a different rate to mannotriose. Acidic hydrolysis produced only glucose and mannose (1 part to 2). After borohydride reduction and acidic hydrolysis, equal parts of glucose and mannose were produced. Treatment with β-glucosidase gave products with the mobility on PC of glucose and mannobiose. The probable structure is thus: Glc β1–4 Man β1–4 Man.

Acetolysis produced three oligosaccharides that were separated by thick PC. Two, from their PC mobility and the detection of only mannitol hexaacetate on GLC after hydrolysis, reduction and acetylation, appeared to be mannobiose and mannotriose. The third fraction had the mobility on PC of a disaccharide and this mobility differed from that of the enzymically produced glucose-mannose disaccharide. GLC after hydrolysis gave mannose 53%, galactose 10% and glucose 37% indicating a mixture of two disaccharides of mannose and glucose and of galactose and mannose.

Galactoglucomannans occur widely as structural polysaccharides in the woody tissue of gymnosperms and angiosperms but the amounts are usually low [15]. They have been isolated from endosperms of seeds of Iridaceae and Liliaceae species [16], where the polysaccharide is present in much larger quantity, and would appear to be reserve material. Although galactoglucomannans have been found in small quantities in stem and leaf tissue of legumes (e.g. Townsville stylo [17] and red clover [18]), their presence in the endosperm of seeds of Leguminosae has not been reported. The isolation and characterization from Judas tree of a galactoglucomannan as more than 10% of seed weight is apparently unique. In other legume species where there is an endosperm reserve it has almost invariably been found to be galactomannan [1; cf. 19]. The ratio of monosaccharides (Gal–Glc–Man) is 2:1:11 and is quite different to that of the galactoglucomannans from the stems of legume species and the seeds of Iridaceae and Liliaceae (1:4:4–1:8:8) [14], the stems of an aquatic moss (1:1:3.7) [20], the stems of bracken fern [21] and the wood of gymnosperms (1:3:9 to 1:15:60) [15]. Seeds of another member of the tribe Cerceae, *Bauhinia purpurea* did not contain this or any other type of mannan reserve.

The hydrolysis by purified carob and lucerne β-mannanases of galactomannans with more than 23% of galactose substitution showed that the rate changed abruptly between 24 and 33% substitution and led to the suggestion that the structural requirement for effective hydrolysis is at least two contiguous unsubstituted anhydromannose units [12]. This would be similar to the requirement for fungal and bacterial β-mannanases free of interfering glycosidase activities [6], although studies with plant preparations [7] have indicated that only one mannose unit must be unsubstituted. To further study the effect of galactose substitution on rate of hydrolysis, some of the polysaccharides isolated were treated with a purified preparation of honey locust β-mannanase B [12] (Table 3). In the preparation of this β-mannanase, mannan dyed with Remazol–Brilliant Blue was found to be useful in the detection of activity in column fractions. The V_{max} remained at a constant, high value until the galactose content reached 28% and the percentage hydrolysis of the samples was inversely related to the degree of branching. After complete reaction, as judged from the

Table 4. Fractions from ethanol precipitation after β-mannanase hydrolysis of Carob galactomannan

Fraction	Total carbohydrate in the fraction as % of total carbohydrate in original polysaccharide	Galactose content as % of total Galactose in original polysaccharide	Galactose content as % of total carbohydrate in the fraction
Pptd by 2 vol EtOH	11	16	37
Pptd by 6 vol EtOH	43	47	29
Soluble in 6 vol EtOH	39	31	20
% Recovery	93	94	
Unfractionated polysaccharide hydrolysate	100	100	24

rate of change of reducing sugar values, the oligosaccharides present were examined by TLC in a solvent that allowed migration of oligosaccharides up to a $\overline{DP}$ of 15 [22]. The hydrolysate of Bangalow palm mannan was essentially free of oligosaccharides with a $\overline{DP} > 4$. Oligosaccharides with a $\overline{DP}$ between 4 and 15 and some immobile material were also present in the *Cassia corymbosa* (alkali soluble) hydrolysate. The amounts of these fractions progressively increased and mannobi- and triose decreased as the galactose content increased, until in *Cassia sophora* hydrolysate no mannobi- and tri-ose were found. No mobile oligosaccharides were detected in the guar and lucerne hydrolysates. Similar studies with three galactomannans [9] have been made but in another report [8] the percentage hydrolyses and sugars produced would indicate that other glycosidase activities such as α-galactosidase and β-mannosidase may have been present.

The oligosaccharides released on hydrolysis of carob galactomannan were examined in more detail. These were separated into three fractions (see Experimental), the first with $\overline{DP} > 15$, the second with $\overline{DP}$ 7 to at least 15 and the third with $\overline{DP}$ from 2 to 7. The total carbohydrate was determined by anthrone and the galactose contents with D-galactose dehydrogenase [12] and the results are shown in Table 4. In all three fractions many of the anhydromannoses are not substituted by galactose, in agreement with a requirement for two neighbouring unsubstituted mannoses for enzymic hydrolysis. The three fractions all contained significant amounts of galactose and the galactose: mannose ratios were significantly less than 1:1, consistent with the interpretation that the distribution of galactose along the mannan backbone in carob galactomannan is random. In a previous study [7, 10], the amounts of the constituent sugars in the non-dialysed fraction from enzymic hydrolysis of carob galactomannan were determined after PC separation and elution, by estimation of reducing activity. The values obtained (50% galactose) suggested that the galactose substitution in the original polysaccharide was in blocks. However, periodate oxidation of guaran has recently indicated a random substitution [23]. The oligomers remaining in solution after precipitation by 2 volumes of EtOH were fractionated by PC on thick paper using multiple irrigation (Table 5). A series of oligosaccharides were obtained that were not fractionated further on chromatography in three solvents. The oligomers with $\overline{DP} \leq 3$ contained no galactose. The galactose content and the $\overline{DP}$ indicated by the migration rate of the R_G 0.25 fraction suggested that it contained one galactose and three mannose units per molecule. α-Galactosidase produced galactose and mannotriose and β-mannosidase, which sequentially removes mannose units from the non-reducing end of manno-oligosaccharides and is blocked by substitution, gave mannose and an oligosaccharide of galactose and mannose (in ratio 1:2) with the PC mobility of a trisaccharide. These results indicate a structure of Man–Man(Gal)–Man for the original tetrasaccharide. During the preparation of honey locust β-mannosidase for these hydrolyses it was found that, on centrifugation, unlike α-galactosidase and β-mannanase, a half to a third of the activity remained in the pellet of insoluble cellular debris and was not recovered by washing. The R_G 0.13 fraction appeared from its mobility to be a pentasaccharide(s) and complete acidic and partial enzymic hydrolyses indicated that one galactose was attached to mannotetraose. The R_G 0.07 fraction appeared from the application of similar methods to be a heptasaccharide(s) with two galactoses attached to mannopentaose. The production of these structures further shows that a manno-glycosidic bond can only be hydrolysed by β-mannanase if neither of the anhydro-

Table 5. Oligosaccharide fractions separated by PC after β-mannanase hydrolysis of Carob galactomannan

Chromatographic mobility R_G	Amount % of total carbohydrate	Probable $\overline{DP}$	Galactose %
0.73	4	2	0
0.42	5	3	0
0.25	1	4	24
0.13	11	5	20
0.10	trace	6	nd
0.07	16	7	28
immobile	43		nd

mannose units involved in the bond are substituted by galactose, with the additional restriction, apparently common to endo-polysaccharases, that hydrolysis cannot readily occur at the end glycosidic bond of a mannan chain [5].

The effect of replacement of some anhydromannose units by glucose on the action of konjac β-mannanase has been reported [24]. When orchid glucomannan was treated with honey locust β-mannanase similar results were obtained. The oligosaccharides produced indicated that a Man β1–4 Glc linkage could be readily hydrolysed but not a Glc β1–4 Man linkage. Honey locust β-mannanase showed the same hydrolytic behaviour with manno-oligosaccharides and reduced manno-oligosaccharides as previously found [5]. In addition, extended incubation of mannotriose with high levels of enzyme gave mannobiose but no mannose. Under similar conditions mannotriitol gave no reaction. Reversion of two trisaccharide molecules to a hexasaccharide followed by hydrolysis to mannobiose and mannotetraose, which would be rapidly hydrolysed to mannobiose would explain this.

All the results obtained are consistent with the requirement for hydrolysis by honey locust β-mannanase being at least two contiguous unsubstituted mannose units. From the known mechanism of hydrolysis by endopolysaccharases, e.g. lysozyme, and the relative rates of hydrolysis of manno-oligomers by β-mannanase, the hydrolysis of galactomannan probably involves the recognition of 5 or 6 neighbouring anhydromannose units in the mannan chain. In the extended rod conformation that galactomannans probably adopt in aqueous solution [12, 13], galactose branches separated by none or an even number of mannose units would lie on opposite sides of the main chain and those separated by an odd number of mannose units would lie on the same side (Fig. 1). In a section as long as 5 or 6 anhydromannose units the disposition of any galactose substituents on either side of the mannan chain as well as the separation of substituents may affect the association of the chain and the enzyme.

EXPERIMENTAL

Details of some plant materials have been previously described [12, 25]. Seeds of the *Cassia* spp., *Kennedia rubicunda* and *Cercis siliquastrum* were obtained from or identified at the Royal Botanic Gardens, Sydney. Another sample of *Cercis siliquastrum* seed was supplied by Dr. M. A. Jermyn, C.S.I.R.O. Division of Protein Chemistry, Melbourne. Details of methods of viscosity determination, estimation of galactose by galactose dehydrogenase, glucose by glucose oxidase, total carbohydrate by anthrone, preparation of Bangalow palm mannan, honey locust α-galactosidase, β-mannanase, β-mannosidase, mannobiitol and mannotriitol have been described [12, 25]. Mannotetraose and mannopentaose were prepared by partial β-mannanase hydrolysis of Bangalow palm mannan and separation of oligosaccharides on thick PC. All solutions were concentrated below 40° under reduced pressure.

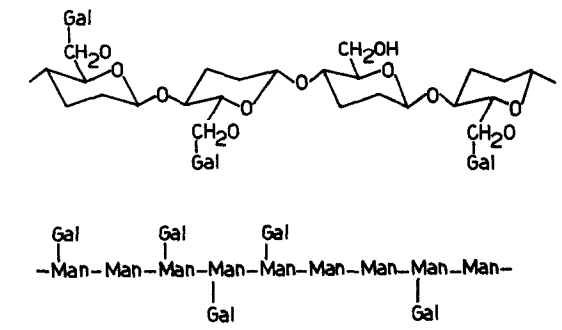

Fig. 1. Possible conformation of galactomannan in aqueous solution and arrangement of galactose side chains.

PC and TLC. Solvents used were A, *n*-BuOH–Py–H_2O–C_6H_6 (5:3:3:1); B, EtAc–HCOOH–HOAc–H_2O (18:3:1:4); C, EtOAc–Py–H_2O (2:1:2) and D, *n*-PrOH–$MeNO_2$–H_2O (5:2:3) [22]. On PC, sugars were developed with *p*-anisidine.HCl or $AgNO_3$. The TLC carrier was Si gel–kieselguhr 3:1, and sugars were developed with 5% H_2SO_4 in EtOH and heating (90–100° for 30 min and 140° for 5–10 min).

GLC. A glass column (2 m × 3.5 mm) was packed with 1.7% silicone oil (XF-1150) and 1.3% ethylene glycol succinate. The oven was operated isothermally at 170° with injection at 235°. The carrier gas was N_2 and *myo*-inositol hexa-acetate had a retention time of 60 min.

Extraction and purification of galactomannans. For water soluble galactomannans, extraction was carried out as previously described [25]. Then, the residue, after this extraction with Hg^{2+} solution, was added in 1% concentration to cold alkali solution (17% NaOH–3% H_3BO_3 free of O_2) and stirred under N_2 for 18 hr, homogenized under N_2 and centrifuged. Fehling's soln was added to the supernatant and the ppt. collected by centrifugation. If the ppt. was not blue, it was re-dissolved by neutralizing the chilled solution with cold HCl, centrifuging (20000 *g*, 15 min 25°) and adding Fehling's solution to the supernatant. The ppt. was re-dissolved in dil HCl, pptd in EtOH and washed and dried. If xylose was present in an acidic hydrolysis of this product, the polysaccharide was re-dissolved in hot KCl soln (10 mM), cooled and passed through a column of DEAE cellulose, when the non-bound fraction was collected and pptd in EtOH.

Extraction and purification of Judas tree polysaccharide. Dry seeds (40 g) were broken in a water-cooled mill and this allowed separation of most of the seed coat and cotyledon material from the leathery endosperms, which were extracted with boiling EtOH and dried. After soaking in cold 1 M NaOH for 2 hr the remainder of the seed coat could be removed without significant loss of mannose containing polysaccharide. Endosperms were well washed with water and a suspension dialyzed to remove NaOH. The dried product (11.6 g) was suspended in H_2O and finely macerated in an Ultra-Turrax. The soln was heated in a steam bath (2 hr), cooled and centrifuged. Residue was re-extracted 2× with hot H_2O. Fehling's soln was added to the supernatant and the product purified as described for galactomannan. The residue was extracted 3× with 17% NaOH–3% borate. After centrifugation, Fehling's soln was added to the supernatant and the polysaccharide purified as described for galactomannan. Hot H_2O extraction yielded 1.4 g, and alkali 2.3 g with a residue of 6.0 g. The residue on hydrolysis was shown to contain a similar sugar composition to the extracted fractions. Monosaccharide contents were determined with anthrone, galactose dehydrogenase and glucose oxidase [12, 26].

Chromatography on Merckogel. Polysaccharide soln (1 ml) (0.1–0.2% in 0.1 M NaCl, 0.5 mM EDTA) was applied to a column with a total vol of 62 ml. Eluted carbohydrate was measured by anthrone.

Electrophoresis on cellulose acetate. The method of Dudman and Bishop [11] was modified. Procion Brilliant Rot M-2B (1 ml, 1%) was added to a solution of polysaccharide (10 ml, 0.1%). NaCl (1 ml, 24%) was added after 5 min and after 30 min Na_2CO_3 (1.0 ml) to a final concentration of 0.1% and the solution kept overnight. Galactomannan was pptd as the Cu^{2+} complex, centrifuged and re-dissolved by careful addition of dil HCl and the dyed polymer separated from any unreacted dye on Sephadex G-25.

Protein content of galactomannans. The Folin–Lowry method was modified. To an aliquot (10 ml) of 0.1% galactomannan solution, Na_2CO_3 (0.4 g) and NaK tartrate

2%–$CuSO_4$ 1% soln (0.1 ml) were added, followed by Folin reagent (1.0 ml) and the soln stirred. After 15 min absorbance was read. The galactomannan precipitated from soln when the $CuSO_4$–tartrate reagent was added but re-dissolved on addition of Folin reagent.

Hydrolysis of galactomannans by β-mannanase. Percentage hydrolyses were determined by incubating a preparation of honey locust β-mannanase B (1.0 ml 0.1 U), that had no detectable β-mannosidase or α-galactosidase activity, with galactomannan solution (10 ml, 0.1%). Cold water insoluble polysaccharides were dissolved in NaOH and neutralized. Aliquots (0.1 ml) were removed at 0, 2, 4 and 8 hr, reaction stopped by boiling and reducing activity determined by ferricyanide (27). Parallel experiments in 10^{-5} M Hg^{2+} gave similar results. Carob galactomannan (1.0 g in 500 ml) was incubated with β-mannanase (20 U) for 24 hr at 30°, the solution deionized and concentrated until the carbohydrate content was 2%. Two vol of EtOH were added and the mixture kept at 2° for 30 min. The soln was centrifuged (20000 *g*, 10 min, 2°) and the ppt. washed with 66% chilled EtOH. After centrifugation, the supernatants were combined and the EtOH–H_2O ratio increased to 6:1. The ppt. was separated and the solvent in the supernatant soln distilled. The 3 fractions were dissolved in H_2O, evaporated to dryness to remove EtOH, redissolved in H_2O and the carbohydrate contents estimated with anthrone and the galactose contents with galactose dehydrogenase before and after acidic hydrolysis. Each fraction was incubated again with β-mannanase to ensure complete reaction. The oligosaccharides left in solution after pptn with 2 vol of EtOH were separated by thick PC (solvent A, 6×) which caused movement of fractions with a $\overline{DP}$ up to 5. The immobile material after elution was chromatographed (PC, solvent C 2×), when hexa- and heptasaccharide fractions were mobile. The total carbohydrate content was determined with anthrone and, after acidic hydrolysis, both galactose and total carbohydrate were estimated. After incubation with α-galactosidase the chromatographic mobilities of the oligosaccharide residues were compared with samples of manno-oligosaccharides prepared from Bangalow palm mannan. The fraction with an approximate $\overline{DP}$ of 4 was treated with β-mannosidase and the products of hydrolysis separated by thick PC and examined for galactose and total carbohydrate content and constituent sugars.

Preparation of Glcβ1–4 Man. Orchid tubers were macerated in 0.5 M KCl and the mixture stirred in a boiling water bath for 2 hr, filtered through 'Miracloth' and centrifuged (20000 *g*, 30 min, 25°). The glucomannan in soln was purified via the copper complex and the product contained only glucose and mannose. Glucomannan (0.5 g) was re-dissolved in alkali, the pH reduced to 5 and the solution incubated at 30° for 24 hr with β-mannanase (10 U). The solution was de-ionized, concentrated and the oligosaccharides separated by thick PC (solvent C). Significant fractions were mannobiose (37%), mannotriose (15%), Glcβ1–4 Man (9%), Glcβ1–4 Manβ1–4 Man (6%), unidentified oligomers of $\overline{DP} \leq 4$ (4%) and higher oligosaccharides (20%). The proportions of glucose and mannose were determined before and after borohydride reduction and their chromatographic mobility compared with published values [14, 28].

Characterization of oligosaccharides produced by hydrolysis of Judas tree endosperm polysaccharide with α-galactosidase plus β-mannanase. After hydrolysis with the enzymes, deionization and separation of oligosaccharides by thick PC (solvent C) gave, as well as mannobiose and mannotriose, a fraction (5%) that co-chromatographed with Glcβ1–4 Man and a fraction (4%) that co-chromatographed with Glcβ1–4 Manβ1–4 Man. These were separated and the sugar content and ratios determined before and after borohydride reduction, by anthrone, acidic hydrolysis and glucose oxidase and hydrolysis and PC to determine constituent sugars. The hydrolysis products from β-glucosidase treatment were also examined by PC. The β-glucosidase was prepared from germinated lucerne (3 days). It was separated from any β-mannosidase activity by DEAE cellulose chromatography and gel filtration on Sephadex G-100.

Acetolysis of Judas tree polysaccharide and isolation of oligosaccharides. Polysaccharide was hydrolysed by the method of Aspinall [29] with the following modifications. Acetylation was performed once. Acetolysis was carried out at 30° for 24 hr (conditions found to give maximum oligosaccharide yields). NaOMe (0.5 M) was used for deacetylation. After removal of cations and concentration, oligosaccharides were separated by PC (Whatman 3 MM, solvent A, 4×). The oligosaccharide fractions isolated were: R_G 0.20 (4% yield) which co-chromatographed with mannotriose; R_G 0.42 was isolated in 2% yield; R_G 0.60 (5% yield) which co-chromatographed with mannobiose.

Preparation of alditol acetates for GLC. Oligosaccharides were hydrolysed with 2 M F_3CCOOH at 100° for 2 hr. *myo*-Inositol was added as an internal standard. Reduction and acetylation were carried out by the method of Hansson [30], with the following modifications. $NaBH_4$ was dissolved in 0.5 M NH_4OH. Five MeOH distillations were followed by vacuum desiccation. Acetylation reagents were removed under vacuum and dried samples dissolved in CH_2Cl_2 for injection.

Acknowledgements—This work was supported by the University of Sydney Research Grant. Two of us (B.V.McC. and D.M.S.) wish to acknowledge Australian Government Postgraduate Research Awards and to thank the N.S.W. Department of Agriculture for leave. We are indebted to the Royal Botanic Gardens, Sydney for seeds.

REFERENCES

1. Bailey, R. W. (1971) in *Chemotaxonomy of the Leguminosae* eds. Harborne, J. B., Boulter, D. and Turner, B. L., p. 519. Academic Press, New York.
2. Whistler, R. L. and Smart, C. L. (1953) *Polysaccharide Chemistry* p. 292. Academic Press, New York.
3. Jindal, V. K. and Mukherjee, S. (1970) *Indian J. Chem.* **8**, 417.
4. Kooiman, P. (1971) *Carbohyd. Res.* **20**, 329.
5. Beaugiraud, S. C. and Percheron, F. (1968) *Bull. Soc. Chim. Biol.* **50**, 633.
6. Reese, E. T. and Shibata, Y. (1965) *Can. J. Microbiol.* **11**, 167.
7. Courtois, J. E. and Le Dizet, P. (1968) *Bull. Soc. Chim. Biol.* **50**, 1695.
8. Tsujisaka, Y., Hiyama, K., Takenishi, S. and Fukumoto, J. (1972) *J. Agr. Chem. Soc. Japan* **46**, 155.
9. Emi, S., Fukumoto, J. and Yamamoto, T. (1972) *Agr. Biol. Chem. (Japan)* **36**, 991.
10. Courtois, J. E. and Le Dizet, P. (1970) *Bull. Soc. Chim. Biol.* **52**, 15.
11. Dudman, W. F. and Bishop, C. T. (1968) *Can. J. Chem.* **46**, 3079.
12. McCleary, B. V. and Matheson, N. K. (1975) *Phytochemistry* **14**, 1187.
13. Sundararajan, P. R. and Rao, V. S. R. (1970) *Biopolymers* **9**, 1239.
14. Meier, H. (1960) *Acta Chem. Scand.* **14**, 749.
15. Timell, T. E. (1965) *Adv. Carbohyd. Chem.* **20**, 448.
16. Jakimow-Barras, N. (1973) *Phytochemistry* **12**, 1331.
17. Alam, M. and Richards, G. N. (1971) *Australian J. Chem.* **24**, 2411.
18. Buchala, A. J. and Meier, H. (1973) *Carbohyd. Res.* **31**, 87.
19. Varshney, S. C., Rizvi, S. A. I. and Gupta, P. C. (1973) *J. Agr. Food Chem.* **21**, 222.
20. Geddes, D. S. and Wilkie, K. C. B. (1972) *Carbohyd. Res.* **23**, 349.
21. Bremner, I. and Wilkie, K. C. B. (1971) *Carbohyd. Res.* **20**, 193.
22. Huber, C. N., Scobell, H. D., Tai, H. and Fisher, E. E. (1968) *Analyt. Chem.* **40**, 207.

23. Hoffman, J., Lindberg, B. and Painter, T. (1975) *Acta Chem. Scand.* B **29**, 137.
24. Shimahara, H., Suzuki, H., Sugiyama, N. and Nisizawa, K. (1975) *Agric. Biol. Chem. (Japan)* **39**, 293.
25. McCleary, B. V. and Matheson, N. K. (1974) *Phytochemistry* **13**, 1747.
26. Dahlqvist, A. (1966) *Methods in Enzymology*, (Colowick, S. P. and Kaplan, N. O., eds), Vol. 8, 584.
27. Park, J. T. and Johnson, M. J. (1949) *J. Biol. Chem.* **181**, 149.
28. Bouveng, H. O., Iwasaki, T., Lindberg, B. and Meier, H. (1963) *Acta Chem. Scand.* **17**, 1796.
29. Aspinall, G. O., Hunt, K. and Morrison, I. M. (1967) *J. Chem. Soc. C* 1080.
30. Hansson, J-A. and Hartler, N. (1969) *Svensk Papperstidning* **72**, 521.