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ENZYMIC HYDROLYSIS, FINE STRUCTURE, AND GELLING INTERACTION OF LEGUME-SEED D-GALACTO-D-MANNANS

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ABSTRACT

The fine structure of D-galacto-D-mannans was analyzed with purified α-D-galactosidases from germinated lucerne seed and from green coffee beans and with a commercial β-D-mannanase (Driselase) Each of the α-D-galactosidase enzymes removed D-galactosyl residues from the D-mannan backbone in a random fashion, α-D-galactosidase A from lucerne removed more than 90% of the D-galactosyl groups from each of the ten native D-galacto-D-mannans studied Mixtures of purified α-D-galactosidase C and β-D-mannanase B from lucerne hydrolyzed each of the ten D-galacto-D-mannans to D-galactose, β-D-mannobiose, and β-D-mannotriose, with trace amounts of D-mannose The degrees of hydrolysis by β-D-mannanase of native D-galacto-D-mannans, and of lucerne D-galacto-D-mannan pre-hydrolyzed by α-D-galactosidase, are dependent on the galactose contents The amounts of oligosaccharides produced from native D-galacto-D-mannans by Driselase β-D-mannanase parallel those obtained from lucerne D-galacto-D-mannan that had been pre-hydrolyzed to similar galactose levels with α-D-galactosidase, except for the D-galacto-D-mannans of *Leucaena leucocephala* and soybean, which are hydrolyzed with β-D-mannanase to a higher degree A direct correlation between the extent of hydrolysis of D-galacto-D-mannans by β-D-mannanase and the degree of gelling interaction with xanthan gum was observed, suggesting that interaction of D-galacto-D-mannans with xanthan does not absolutely require long sections of contiguous unsubstituted D-mannose residues but rather sections where all the galactosyl residues are located on one side of the main chain and may also serve as "junction zones"

INTRODUCTION

D-Galacto-D-mannans are reserve carbohydrates found in the endosperms of some legume seeds They consist of a β-D-(1→4)-mannan backbone to which are attached various amounts of single (1→6)-α-D-galactopyranosyl groups[1] On seed germination, these residues are rapidly split off by α-D-galactosidases[2–5] (EC3 2 1 22), β-D-mannanases[6–8] (EC 3 2 1 78), and β-D-mannosidases[3 5 7] (EC 3 2 1 25) Released sugars are taken up by the cotyledons and further metabolized[9 10]

The purification, properties, and modes of action of α-D-galactosidases and β-D-mannanases have been recently reviewed[11,12] The modes of action of a number of purified legume-seed, fungal, and bacterial β-D-mannanases on D-mannans, D-galacto-D-mannans, D-gluco-D-mannans, D-galacto-D-gluco-D-mannans, and D-manno-oligosaccharides have been reported[12–14] All these enzymes were shown to be *endo*-hydrolases, which cleave randomly within the D-mannan backbone The degrees of hydrolysis of D-galacto-D-mannans by β-D-mannanase, free of α-D-galactosidase and β-D-mannosidase, is governed by the D-galactose content of these polysaccharides[13]

Detailed studies on the mode of action of α-D-galactosidases on D-galacto-D-mannans, made by only one group of investigators[15,16], indicated that coffee-bean α-D-galactosidase does not hydrolyze the single D-galactopyranosyl groups at random along the D-galacto-D-mannan chain, but rather that it first acts on isolated groups and then on the extremities of sections highly substituted with these groups

The usefulness of α-D-galactosidase and β-D-mannanase in the analysis of the fine structures of D-galacto-D-mannans was first recognised by Courtois and Le Dizet[15,16], who concluded that the distribution of D-galactopyranosyl residues in D-galacto-D-mannans of low D-galactose content, (*e g* , carob) is not regular They suggested the existence of blocks of the D-mannan backbone that are totally (or almost totally) substituted with single D-galactopyranosyl groups, and of unsubstituted D-mannopyranose residues interspersed between the substituted D-mannopyranose residues to give a partial block structure This structure for carob D-galacto-D-mannan was supported by Baker and Whistler[17], who studied the distribution of D-galactopyranosyl groups by use of the known alkaline lability of 6-deoxy-6-*o*-*p*-tolylsulfonylhexopyranosides They proposed that the D-galactopyranosyl groups in guar D-galacto-D-mannan are distributed uniformly on every second D-mannose residue of the D-mannan chain, whereas in carob D-galacto-D-mannan these groups are arranged in blocks of consecutive D-mannopyranosyl units in the main chain The block-type disposition of the D-galactopyranosyl groups in carob D-galacto-D-mannan, rather than a random distribution, was considered better to explain the ability of the gum to interact with some polysaccharide systems possessing a high order of molecular association[18,19]

In contrast to these proposals, McCleary, Matheson, and Small[13] suggested that the distribution of D-galactopyranosyl groups in carob D-galacto-D-mannan is random This proposal was based on the structure of the oligosaccharides produced on hydrolysis of carob D-galacto-D-mannan with β-D-mannanase from honey-locust seeds These compounds included an array of D-galactose-containing D-manno-oligosaccharides and a fraction having a high degree of polymerization ($\overline{d\,p}$) and containing significantly less than 50% of D-galactose Furthermore, from the results of periodate oxidation of guaran (guar D-galacto-D-mannan), Hoffman *et al*[20] suggested that this polysaccharide also has a random distribution of D-galactopyranosyl groups.

The aim of the current work was to perform a detailed investigation of the

modes of action of three α-D-galactosidases and a β-D-mannanase from Driselase on a range of native and enzymically modified D-galacto-D-mannans, with a view to obtaining further information about the fine structures of these polysaccharides Attempts have been made to relate the proposed fine structures of these D-galacto-D-mannans to their gelling interaction with xanthan gum

EXPERIMENTAL

Plant materials — Seeds of *Hardenbergia violacea*, *Cassia corymbosa*, *Gleditsia triacanthos* (honey locust), *Cassia didymobotrya*, *Delonix regia*, *Dracena draco*, *Livistona australis*, and *Archontophoenix cunninghamiana* (bangalow palm) were obtained from the Royal Botanic Gardens, Sydney *Leucaena leucocephala* and *Medicago sativa* (lucerne) seeds were obtained from Wright Stephenson and Co , Australia Pty Ltd *Cyamopsis tetragonoloba* var. Groller seeds were obtained from Mr J Doughton, Department of Primary Industries, Darwin Northern Territory, and *Ceratonia siliqua* (carob) seeds from Mr R Thompson, Department of Agriculture, Cowra, N S W

Extraction and purification of D-*galacto*-D-*mannans* — All D-galacto-D-mannans studied were water-soluble and were extracted as previously described[4], except for soybean D-galacto-D-mannan, which was a generous gift from Prof G O Aspinall, York University, Ontario, Canada Yields of cold-water-soluble D-galacto-D-mannan from *Cassia didymobotrya*, *Leucaena leucocephala*, *Hardenbergia violacea*, and *Delonix regia* were 18, 14, 12, and 19%, respectively, and limiting viscosity numbers 920, 1040, 940, and 780, respectively Limiting viscosity numbers were determined with an Ubbelohde suspended-level viscometer[7] D-Mannans and D-gluco-D-mannans were extracted as previously described[7,21]

Chromatography — T l c was performed on Merck DC-Alufolien Kieselgel 60/Kieselgur F254 (0 2 mm) or DC-Alufolien Kieselgel 60 (0 2 mm) prepared plates, which were developed twice with 7 1 2 (v/v) 1-propanol–ethanol–water Spots were detected by spraying with 5% sulfuric acid in ethanol and heating to 110° Oligosaccharides were located by developing marker strips, and then relative amounts determined by scraping the appropriate sections into tubes, extracting with water, centrifuging, and determining the carbohydrate content by the anthrone method[22]

For g l c determination of carbohydrate components, D-galacto-D-mannan or oligosaccharide samples[4] were hydrolyzed with acid, neutralized with barium carbonate, and samples (5 mg) reduced with sodium borohydride, and acetylated[23], g l c was performed as previously described[13] The galactose to mannose ratios of some D-galacto-D-mannans as determined by this technique were slightly different from the values previously obtained by a method employing D-galactose dehydrogenase for D-galactose and anthrone for total carbohydrate determination[4] Determination by g l c is considered to be more reliable and accurate

Gelling interaction of D-*galacto*-D-*mannans with xanthan* — (*a*) *Determination with Brabender Amylograph* (*Duisburg*) The D-galacto-D-mannan solution (200 mL,

0 02–0 2% w/v) was added to the xanthan solution (200 mL, 0 2% w/v), heated to 60°, blended with an Ultra-turrax instrument, cooled to 10°, and then re-equilibrated to 20° The entire solution was added to the Amylograph bowl, and experiments were performed with the 350-cmg cartridge at 80 r p m and 20° over a time span of 30 min

(*b*) *Determination with Brookfield Synchro-Lectric viscometer* The solutions were prepared as for the Amylograph studies The values were determined at 15° and 20 r p m at 30-sec intervals over a period of 5 min After 1 min, readings were relatively constant and consequently the values reported are those obtained at 1 min after switching on the instrument The solutions used in both techniques were salt free and contained 0 1% (v/v) of formaldehyde as preservative

(*c*) *Determination by the test tube technique* The D-galacto-D-mannan solutions (5 mL, 0 01–0 1%, w/v) were added to xanthan solutions (5 mL, 0 02–0 1% w/v), each in 0 5M potassium chloride, heated to 60°, and mixed The mixtures were cooled to 2°, shaken vigorously, and centrifuged (27 000*g*, 20 min) in pre-weighed tubes The gels were washed twice by shaking with 0 5M potassium chloride and recovered by centrifugation The volume occupied by the gel, after the third centrifugation, was determined by weighing, and the gel was resuspended in water and degraded with β-D-mannanase, and the carbohydrate content determined by the anthrone method[22] Based on these values, the concentration of carbohydrate per mL of gel volume was calculated

Isoelectric focusing of proteins — Isoelectric focusing was performed with the LKB Multiphor. Ampholines were prepared by Mr A B Blakeney, N S W Dept Agriculture, Research Centre, Yanco, Australia, using a slight modification of the technique of Vinogradov *et al*[24] Isoelectric focusing gels were prepared according to Karlsson *et al*[25]

Staining for α-D-galactosidase activity in polyacrylamide gels — The substrate solution was prepared by dissolving 1-naphthyl α-D-galactopyranoside (250 mg) (Koch-Light Laboratories Ltd , Colnbrook, SL3 OBZ Buckinghamshire, England) in acetone (12 5 mL) with addition of 0 5M acetate buffer (pH 5, 12 5 mL), and it was stored at below 0° between uses The enzyme stain was prepared immediately before use by adding Fast Blue BB salt (20 mg) to a solution containing the substrate solution (0 6 mL), 0 5M acetate buffer (pH 5, 5 mL), and water (15 mL) The gels were incubated in the enzyme stain solution at 40°, and bands appeared within 10 min

Enzyme Units — One unit (kat) of β-D-mannanase activity is defined as the amount of enzyme that will release 1 mol of mannose reducing-sugar equivalent[26] from soluble D-mannan at pH 5 and 40° in 1 s One unit (kat) of α-D-galactosidase is the amount of enzyme that will release 1 mol of *p*-nitrophenol from *p*-nitrophenyl α-D-galactopyranoside, or, 1 mol of galactose reducing-sugar from lucerne D-galacto-D-mannan, at pH 5 and 40° in 1 s

Assay of β-D-mannanase activity — β-D-Mannanase was routinely assayed with RBB-carob galactomannan as substrate[27].

Purification of Driselase β-D-mannanase — Driselase (Kyowa, Hakko Kogyo Co Ltd , Japan) is a commercial product prepared from culture solutions of *Basidio-*

mycetes sp β-D-Mannanase was extracted by suspending the commercial flour (10 g) in 0 1M Tris (200 mL, pH 8), the suspension was centrifuged (20 000*g*, 15 min), and the supernatant solution was chilled to 2° and dialyzed against 10mM Tris (5 L, pH 8) for 16 h The dialysis was performed under these conditions to avoid digestion of the dialysis sac by cellulase activity This solution was chromatographed on DEAE-cellulose (2 5 × 15 cm, 2°) with an elution gradient of 0–200mM potassium chloride in 10mM Tris (pH 8) The active fractions were concentrated by dialysis against Poly(ethyleneglycol) 4000 and then dialyzed against 0 1M acetate buffer (pH 5) plus 0 5M potassium chloride Aliquots of the β-D-mannanase preparation (5 mL, 2 μkat) were applied to glucomannan–AH-Sepharose affinity columns (1 7 × 15 cm) and chromatographed as previously described[21] Columns of these dimensions effectively handled 2 μkat of enzyme activity and have been reused up to 8 times The active fractions eluted from the affinity columns were concentrated, dialyzed against distilled water, and stored frozen Aliquots were lyophilized and redissolved in a minimum quantity of water for the determination of isoelectric points by isoelectric focusing and for the determination of mol wts by SDS–polyacrylamide gel disc-electrophoresis[28]

Purification of α-D-galactosidases — α-D-Galactosidase A from lucerne was purified as previously described[4,27] α-D-Galactosidase C was purified by chromatography on DEAE- and CM-cellulose and gel filtration on Sephadex G-100 The preparation was further purified by thin-layer gel isoelectric-focusing The gel was stained for α-D-galactosidase activity, and the appropriate band removed, crushed, and extracted Last traces of β-D-mannanase were removed by chromatography on D-gluco-D-mannan–AH-Sepharose Coffee-bean α-D-galactosidase was a commercial preparation obtained from Boehringer (Mannheim, Germany) This preparation was essentially devoid of β-D-mannanase and β-D-mannosidase activities

Hydrolysis of D-galacto-D-mannans by α-D-galactosidase — For near-complete removal of D-galactosyl groups from D-galacto-D-mannans, D-galacto-D-mannan (4 mL, 0 1% w/v) was incubated with lucerne α-D-galactosidase A (0 2 mL, 20 nkat/mL on D-galacto-D-mannan) for 0 to 20 h Aliquots (0 2 mL in duplicate) were removed for the determination of reducing activity and of released galactose, by the galactose dehydrogenase method[4] The enzyme gave no increase in reducing activity on incubation with mannan Lucerne galactomannan with a range of galactose content was obtained by incubating this polysaccharide (5 mL, 0 5%) with either α-D-galactosidase A or C from lucerne or coffee-bean α-D-galactosidase (0 1–10 nkat on lucerne galactomannan) for 16 h

Hydrolysis of D-galacto-D-mannans by β-D-mannanase — Standard conditions involved the incubation of unbuffered galactomannan (20 mL, 0 5%, w/v) with β-D-mannanase (2 mL, 0 2 μkat/mL on soluble mannan) for 0 to 18 h Aliquots (20–100 μL) were removed for measurement of reducing activity[26] Samples (2 mL) were heated to denature β-D-mannanase and lyophilized, and the volume readjusted to a 2% (w/v) concentration of carbohydrate Aliquots (20–30 μL) were applied to t l c plates In some experiments, the high-d p material was precipitated by the

addition of 2 vol of ethanol to this solution The precipitate was washed with 66% (v/v) aqueous ethanol, and the washings were added to the ethanol-soluble material

For the determination of K_m and V_{max}, galactomannan, or insoluble or soluble mannan (1 0 mL, 0 01–0 4%, w/v) in acetate buffer (pH 5, 0 1M) was incubated at 40° with β-D-mannanase (0 3 nkat on soluble mannan) for 0, 2, 5, and 10 min The reaction was stopped by addition of *p*-hydroxybenzohydrazide reagent solution[26] (5 mL), and the reducing activity determined

Determination of the degrees of hydrolysis — The degree of hydrolysis of galactomannans by β-D-mannanase was calculated as the amount of mannose reducing sugar equivalent per mL (*p*-hydroxybenzohydrazidemethod[26]) divided by the total carbohydrate content per mL (anthrone method[22]) expressed as a percentage The degree of hydrolysis of galactomannans by α-D-galactosidase was calculated as the amount of galactose reducing sugar equivalent per mL (*p*-hydroxybenzohydrazide method[26]) divided by the total carbohydrate per mL (anthrone[22]) expressed as a percentage Absorbance values of equal amounts of galactose or mannose with the anthrone technique were the same

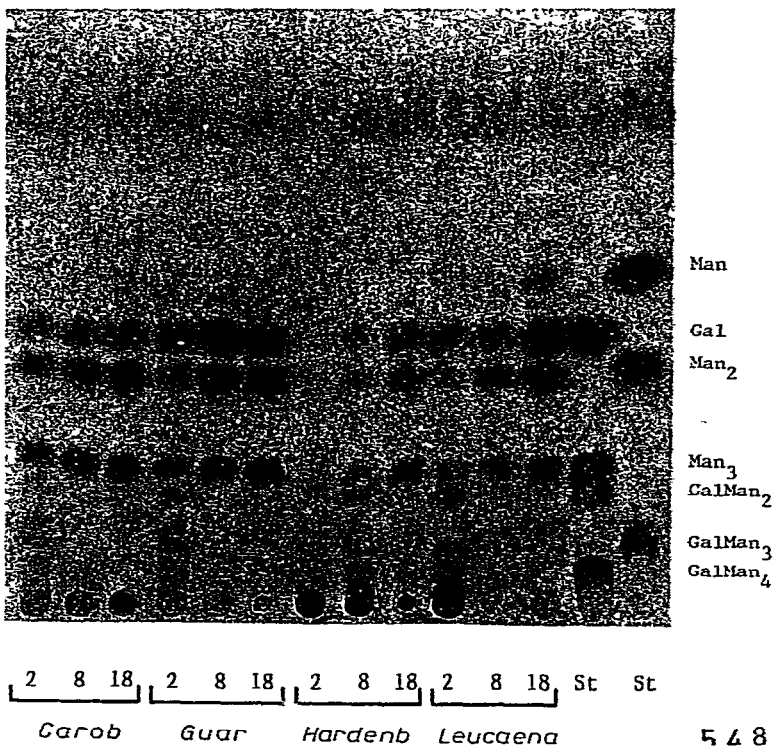


Fig 1 T l c of the hydrolysis products of galactomannans by α-D-galactosidase plus β-D-mannanase mixtures Aliquots (2 mL) of a solution containing lucerne α-D-galactosidase C (0 2 μkat/mL on *p*-nitrophenyl α-D-galactopyranoside) and β-D-mannanase B (0 1 μkat/mL on soluble mannan) were incubated with galactomannans from carob, guar, *Hardenbergia*, and *Leucaena* (2 mL, 0 5%) Aliquots were removed at 2, 8, and 18 h for chromatography

RESULTS

Hydrolysis of D-*galacto*-D-*mannans by mixtures of* α-D-*galactosidase plus* β-D-*mannanase* — To obtain information on the comparative structural features of the galactomannans studied, samples were incubated with an excess of α-D-galactosidase C plus β-D-mannanase B from lucerne[4,7] Oligosaccharides produced on hydrolysis

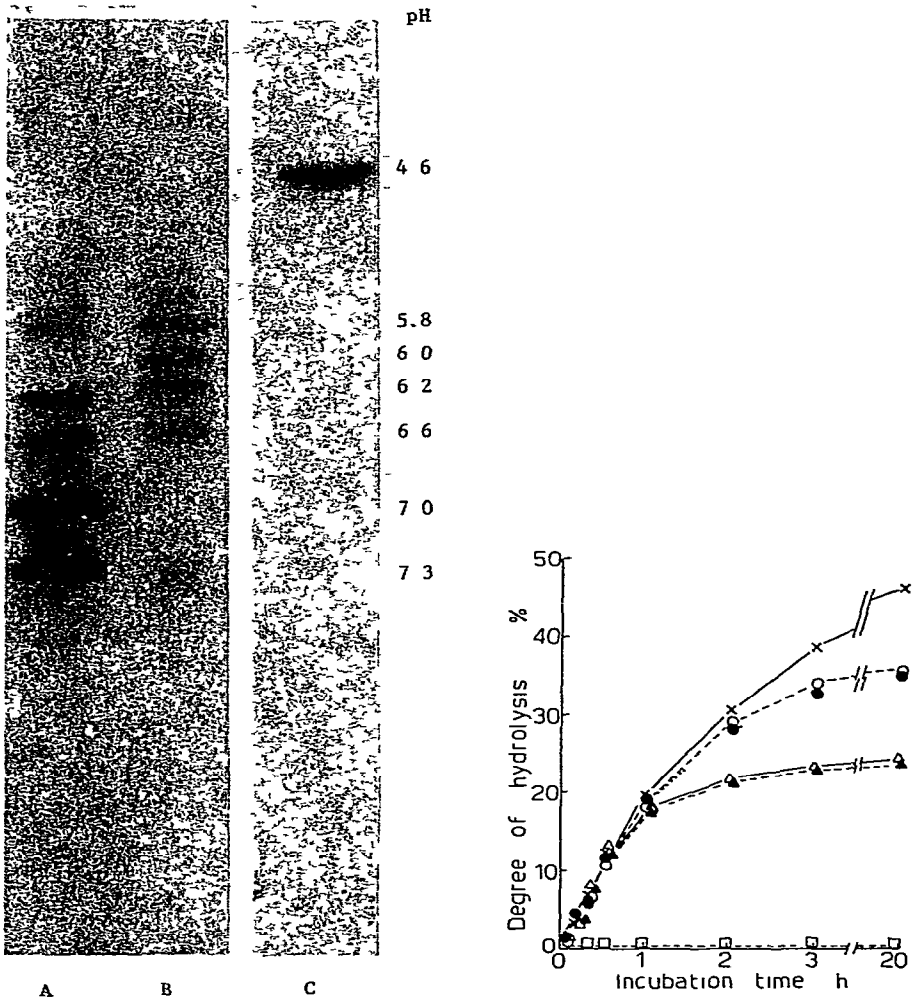


Fig 2 Patterns obtained in thin-layer polyacrylamide gel isoelectric focusing of α-D-galactosidases A and C from lucerne and coffee-bean α-D-galactosidase The activity was detected by staining with a solution of 1-naphthyl α-D-galactopyranoside–Fast Blue BB (A) Coffee-bean α-D-galactosidase, (B) lucerne α-D-galactosidase A, and (C) lucerne α-D-galactosidase C

Fig 3 Hydrolysis of galactomannans by α-D-galactosidase A from lucerne Galactomannan (4 mL, 0 1 %, w/v) was incubated with lucerne α-D-galactosidase A (4 nkat) on galactomannan for 0 to 20 h. The galactomannans hydrolyzed are those from lucerne (×), *Leucaena leucocephala* (○), guar (●), *C corymbosa* (△), carob (▲), and bangalow palm (mannan) (□)

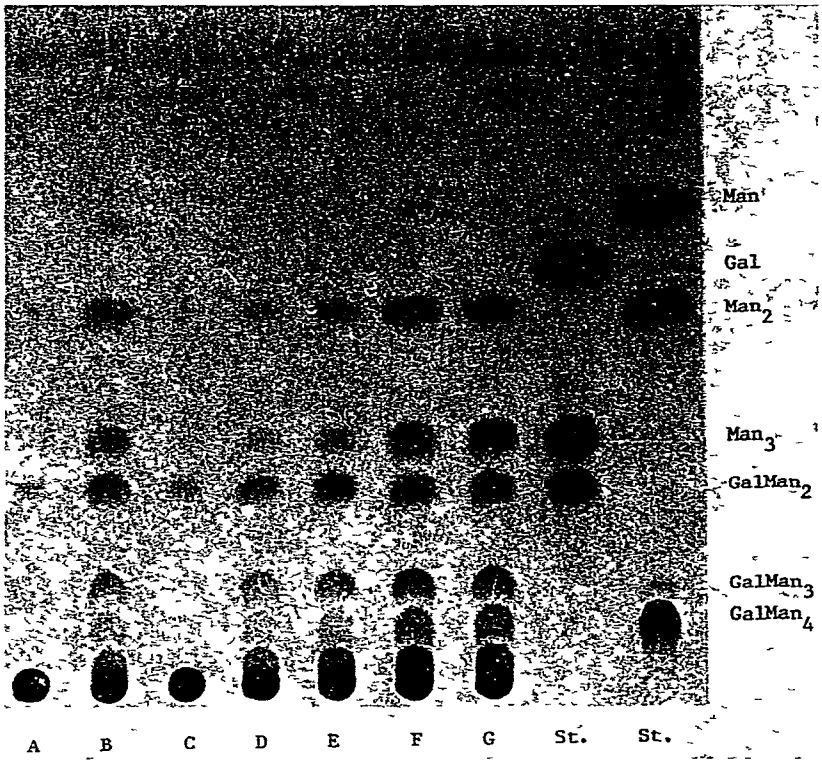


Fig 4 T l c of oligosaccharides produced on hydrolysis of lucerne galactomannan, pre-hydrolyzed with coffee-bean and lucerne α-D-galactosidase A, by Driselase β-D-mannanase Galactomannan (2mL, 0 5% w/v) was incubated with Driselase β-D-mannanase (40 nkat) for 60 min, and aliquots were removed for chromatography (A) and (B), pre-treated with coffee-bean α-D-galactosidase, (C) to (G), pretreated with lucerne α-D-galactosidase A The galactose to mannose ratios of pre-treated lucerne galactomannan were (A) 44 56, (B) 30 70, (C) 43 57, (D) 37 63, (E) 33 67, (F) 21 79, and (G) 19 81, (St) standard sugars

of four galactomannans by these enzymes are shown in Fig 1 On extended incubation, this enzyme mixture, which was devoid of β-D-galactosidase and β-D-mannosidase, completely reduced each of the ten native galactomannans studied to galactose, mannobiose, and mannotriose with only traces of mannose.

Hydrolysis of D-*galacto*-D-*mannans by* α-D-*galactosidases* — Of the three enzyme preparations used, *i e* , α-D-galactosidases A and C from lucerne[4] and coffee-bean α-D-galactosidase, only α-D-galactosidase C from lucerne appeared as a single band of activity on polyacrylamide gel isoelectric focusing (Fig 2) The relative rates and degrees of hydrolysis of five galactomannans by lucerne-seed α-D-galactosidase A are shown in Fig 3 The initial rates of hydrolysis of these and of galactomannans from five other legume sources (*cf* Experimental section), were similar In all cases, at least 90% of the D-galactosyl groups present in the galactomannan could be removed, and in most cases more than 95% was removed In each case, this resulted in the formation of a white precipitate of insoluble mannan-type material.

The mode of action of α-D-galactosidases on galactomannans was studied by incubating the particular α-D-galactosidase with a galactomannan highly substituted with D-galactosyl groups (*i e*, lucerne galactomannan, 47% of D-galactose), to give various degrees of hydrolysis The α-D-galactosidase was then denatured, and free D-galactose removed by ethanol precipitation of the remaining polysaccharide These polysaccharide fractions were hydrolyzed by Driselase β-D-mannanase, and the resulting oligosaccharides quantitatively determined and characterized The oligosaccharides produced when pre-hydrolysis was performed with coffee-bean α-D-galactosidase and lucerne α-D-galactosidase A are shown in Fig 4 When pre-hydrolysis was performed with α-D-galactosidase C from lucerne, similar hydrolysis products were obtained on incubation of the resulting polysaccharides with β-D-mannanase The oligosaccharides produced were preparatively separated by paper chromatography, and then characterized by enzymic hydrolysis with α-D-galactosidase A from lucerne[4] and with β-D-mannanase from Driselase preparation The monosaccharide constituents were identified and quantitatively determined by g l c of the alditol acetates[13] The oligosaccharides characterized included mannobiose (Man_2), mannotriose (Man_3), a trisaccharide with a Gal to Man ratio of 1 2 ($GalMan_2$), mannotetraose (Man_4), a tetrasaccharide with a Gal to Man ratio of 1 3 ($GalMan_3$), and a pentasaccharide with a Gal to Man ratio of 1 4 ($GalMan_4$)

Hydrolysis of D-*galacto*-D-*mannans by Driselase* β-D-*mannanase* — (*a*) *Purification and properties of* β-D-*mannanase* Driselase β-D-mannanase was purified 120-fold from the crude extract with a final specific activity on soluble mannan of 504 nkat/mg, with an overall recovery of 45% (Table I) This preparation was completely devoid of α-D-galactosidase, β-D-mannosidase, β-D-glucosidase, and cellulase activities It did not bind irreversibly to the glucomannan–AH-Sepharose column[21], but rather was retarded to various degrees, depending on both the amount of enzyme added and the prior use of the column (Fig 5)

TABLE I

PURIFICATION OF DRISELASE β-D-MANNANASE[a]

Stage of purification	*Protein (mg)*	*β-D-Mannanase (μkat)*[b]	*Spec activ (nkat/mg)*	*Recovery*		*Purification (-fold)*
				Per step (%)	*Overall (%)*	
Crude	3120	13 0	4 2			1 0
Dialyzed (pH 8)	2000	13 0	6 5	100	100	1 6
DEAE-cellulose (pH 8)	272	6 2	22 7	48	48	5 4
Affinity chromatography[c] on glucomannan–AH-Sepharose	11 5	5 8	504 3	94	45	120 0

[a]From 10 g of commercial powder [b]With soluble mannan as substrate [c]Enzyme chromatographed in three separate lots of = 2 μkat through the glucomannan–AH-Sepharose affinity column (1 7 × 15 cm, pH 5, 2°)

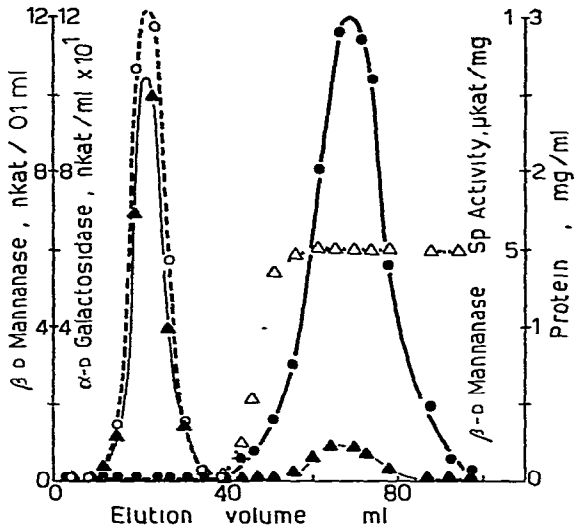


Fig 5 Substrate-affinity chromatography of Driselase β-D-mannanase on glucomannan–AH-Sepharose Driselase β-D-mannanase (4 mL, 2*u*kat) in 0 5M KCl plus 0 1M acetate buffer (pH 5) was applied to a column (1 7 × 15 cm) and eluted with the same solution β-D-mannanase (●), α-D-galactosidase (○), protein (▲), and specific activity (△)

Driselase β-D-mannanase recovered from an affinity-chromatography column gave two major and a few very minor protein bands on isoelectric focusing All bands appeared to have enzyme activity The two major bands with pI values of 5 0 and 5 5 were quantitatively extracted from gel slabs following separation, and were found to have properties identical with those of the enzyme preparation before isoelectric focusing The optimal pH for activity was pH 3 0, with half maximal activities at pH 2 5 and 6 0 On storage for 18 h at 4°, the enzymes showed no loss in activity in the pH range 4–10, but lost 50% of activity at pH 2 5 On storage for 18 h at 40°, both enzymes were stable in the pH range 4–6, but showed a sharp drop in activity at pH values above and below this range On incubation for 30 min at pH 5, the enzymes were stable over the temperature range 2–60° The temperature causing a 50% loss of maximal activity was 64° With an incubation time of 5 min, the temperature for maximal activity was 65° and the energy of activation was 95 4 kJ/mol in the temperature range 20–30° In the temperature range 35–55°, the energy of activation was 52 8 kJ/mol Both enzymes had a mol wt, as determined by sodium dodecyl sulfate–polyacrylamide gel disc-electrophoresis[28], of 53 000 On incubation of the two separated β-D-mannanases (pI 5 0 and 5 5) and the whole preparation (before isoelectric focusing) with a range of galactomannans, each enzyme fraction was found to give the same degree of hydrolysis and products of hydrolysis, and to have the same K_m for a given galactomannan For this reason, all the current studies on the mode of action of Driselase β-D-mannanase on galactomannans employed the whole enzyme preparation recovered from an affinity-chromatography column of glucomannan–AH-Sepharose

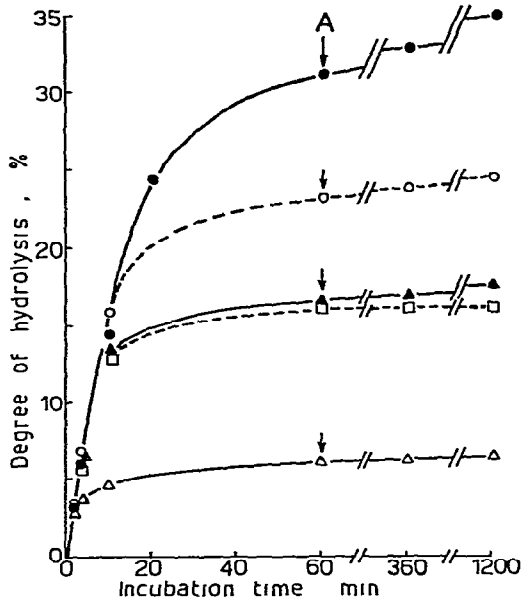


Fig 6 Hydrolysis of galactomannans by Driselase β-D-mannanase Galactomannan (20mL, 0 5%, w/v, unbuffered) was incubated with β-D-mannanase (0 4 μkat on soluble mannan) The galactomannans hydrolyzed are those from carob (●), soybean (○), *Hardenbergia violacea* (▲), *Leucaena leucocephala* (□), and guar (△)

(*b*) *Mode of action* Driselase β-mannanase is an *endo*-hydrolase, which gives a rapid decrease in the viscosity of galactomannan solutions with only a slow increase in reducing sugar level The enzyme hydrolyzes bangalow-palm mannan to D-mannobiose and D-mannotriose Initially, only trace amounts of D-mannose are detectable, but with extended incubation, D-mannotriose is hydrolyzed to D-mannobiose plus D-mannose Hydrolysis of D-galacto-D-mannans is limited by the degree of substitution of the mannan backbone with D-galactopyranosyl groups Rates and degrees of hydrolysis of some galactomannans by this enzyme are shown in Fig 6

Some parameters controlling the kinetics of hydrolysis by Driselase β-D-mannanase of a number of native galactomannans, and lucerne galactomannan prehydrolyzed by α-D-galactosidase, are presented in Table II As the galactose content of the polysaccharides increases to 32%, no significant change in the K_m or relative V_{max} values is observed However, as the galactose content approaches 34–38%, the K_m values double, and the relative V_{max} values decrease by 10–20% In general, the percent of hydrolysis by Driselase β-D-mannanase increases as the galactose contents of the galactomannans decrease The two exceptions are for the galactomannans from soybean and *Leucaena leucocephala*, which are hydrolyzed to a greater degree than other galactomannans with similar galactose contents

The amounts of the various oligosaccharides produced on extended incubation of carob galactomannan with Driselase β-D-mannanase have been determined after t l c separation, and are presented in Fig 7 Incubation of Driselase β-D-mannanase

TABLE II

PROPERTIES OF DRISELASE β-D-MANNANASE[a]

Source of substrate	*Gal/Man ratio*	K_m		*Relative* V_{max} *(nkat/mg)*	*Degree of hydrolysis*[c] *(%)*
		(%, w/v)	mM[b]		
Mannan					
Livistona australis					
Soluble	0/100	0 005	0 3	500	44
Insoluble	0/100	0 11	6 5	500	44
Galactomannans					
Carob	23/77	0 03	1 4	987	31
Delonix regia	22/78	0 03	1 4	987	32
Cassia didymobotrya	22/78	0 03	1 4	987	32
Cassia corymbosa	23/77	0 03	1 4	987	31
Honey locust	27/73	0 03	1 4	987	24
Soybean	32/68	0 03	1 4	987	23
Hardenbergia violacea	34/66	0 06	2 4	901	17
Leucaena leucocephala	38/62	0 07	2 7	868	16
Guar	38/62	0 15	5 7	818	6
Lucerne galactomannan, pre-treated with α-D-galactosidase[d]	43/57	0 08	2 8	868	7
	37/63	0 06	1 6	919	10
	33/67	0 03	1 2	987	19
	21/79	0 02	1 0	987	32
	19/81	0 01	0 5	987	34
	5/95	0 005	0 3	987	43

[a]Activity determined on a range of galactomannans and on a mannan [b]Expressed as mM of D-mannose residues (mol wt 162) in the polysaccharide [c]On incubation of galactomannan (20 mL, 0 5%, w/v) with β-D-mannanase (0 4 μkat) for 60 min Calculated as the amount of mannose reducing-sugar equiv divided by the total carbohydrate content determined by the anthrone reaction, expressed as a percentage [d]α-D-Galactosidase A from lucerne

with carob galactomannan was marked by a transient increase and then decrease of the oligosaccharides Man_4, $GalMan_4$, and Man_3 Mannose, Man_2, and $GalMan_2$ steadily increased in amount with time of incubation The sugars produced on incubation of each of these oligosaccharides with excess β-D-mannanase are shown in Fig 8 Mannotriose was hydrolyzed to Man_2 and Man, $GalMan_4$ to Man_2 and $GalMan_2$, and $GalMan_3$ to $GalMan_2$ and Man_2 Mannobiose and $GalMan_2$ were resistant to further hydrolysis The mechanism by which $GalMan_3$ was hydrolyzed to $GalMan_2$, Man_2, and a trace amount of D-mannose cannot be adequately explained at this stage The oligosaccharides $GalMan_3$, $GalMan_4$ and Man_3, when reduced by sodium borohydride, were resistant to further hydrolysis by β-D-mannanase The proportion of the high $\overline{d\ p}$ fraction, *i e*, that precipitated by 2 vol of ethanol, decreased rapidly from 100% to 8% in the first 60 min of incubation, but then remained relatively constant (Fig 7) Changes in the galactose content of these fractions from carob

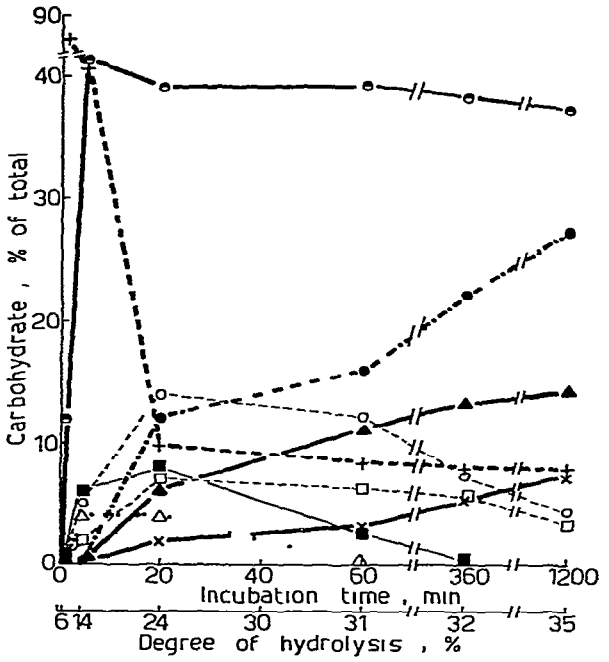


Fig 7 Oligosaccharides produced on extended incubation of carob galactomannan with Driselase β-D-mannanase Galactomannan (20 mL, 0 5%, w/v, unbuffered) was incubated with β-D-mannanase (0 4 μkat on soluble mannan) Fractions separated and quantified were carbohydrate precipitated by 2 vol of ethanol (+), material immobile in t l c (◓), $GalMan_4$ (■), $GalMan_3$ (□), Man_4 (△), $GalMan_2$ (▲), Man_3 (○), Man_2 (●), and Man (×)

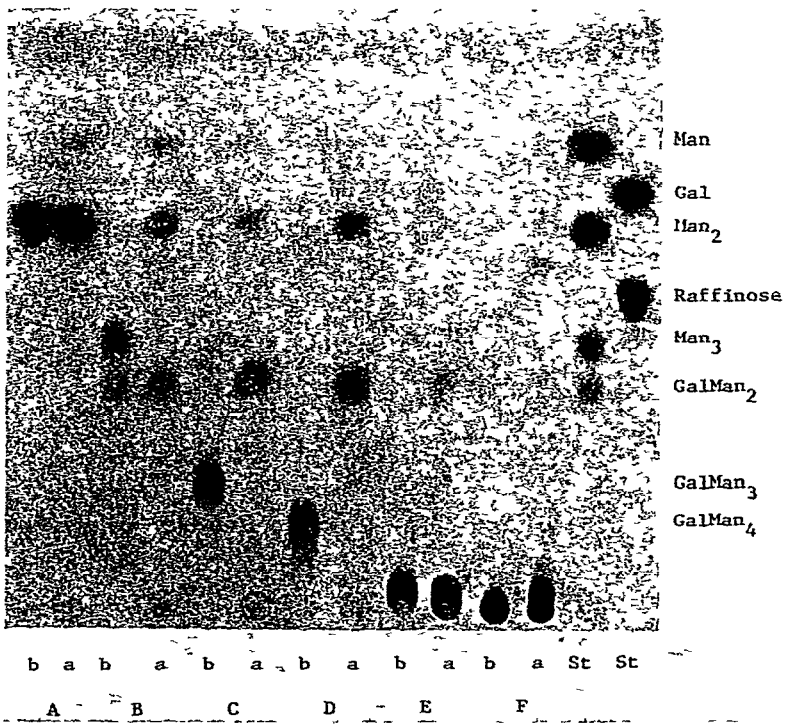


Fig 8. T l c of the hydrolysis products of oligosaccharides by Driselase β-D-mannanase The oligosaccharide (0 1 mL, 2%, w/v) was incubated with Driselase β-D-mannanase (40 nkat) for 18 h at 40° (A) Man_2, (B) Man_3 plus $GalMan_2$, (C) $GalMan_3$, (D) $GalMan_4$, (E) Gal_2Man_5, and (F) material immobile, (St) standard sugars, (b) before hydrolysis, (a) after hydrolysis

TABLE III

FRACTIONATION, BY ETHANOL PRECIPITATION, OF THE PRODUCTS OF HYDROLYSIS OF CAROB AND GUAR GALACTOMANNANS BY DRISELASE β-D-MANNANASE[a]

Galactomannan	*Incub time (min)*	*Degree of hydrolysis[b] (%)*	*Carbohydr soluble[c] (%)*	*Carbohydr precipitated[d]*	
				%	*Gal/Man ratio*
Carob	0	0	0	100	23/77
	1	6	12	88	25/75
	5	14	59	41	27/73
	20	24	90	10	36/64
	60	31	92	8	36/64
	360	32	93	7	36/64
	1200	35	93	7	36/64
Guar	0	0	0	100	38/62
	5	4	11	89	38/62
	30	5	12	88	38/62
	180	6	13	87	38/62
	1200	7	13	87	38/62
	2600	7	13	87	38/62

[a]The galactomannan (20 mL, 0 5% w/v) was incubated with Driselase β-D-mannanase (0 4 μkat) for 0 to 2600 min at 40° [b]See footnote *c*, Table II [c]In 2 vol of ethanol [d]By 2 vol of ethanol

TABLE IV

FRACTIONATION, BY ETHANOL PRECIPITATION, OF THE PRODUCTS OF HYDROLYSIS OF GALACTOMANNANS WITH DRISELASE β-D-MANNANASE[a]

Source of galactomannan	*Native polysaccharide*		*Ethanol-precipitatad*		*Ethanol-soluble*	
	Gal/Man ratio	*Hydrol[b] (%)*	*(%)*	*Gal/Man ratio*	*(%)*	*Gal/Man ratio*
Lucerne	47/53	1	97	47/53	3	[c]
Guar	38/62	7	87	38/62	13	31/69
Leucaena leucocephela	38/62	16	50	42/58	50	32/68
Hardenbergia violacea	34/66	17	58	38/62	42	26/74
Soybean	32/68	23	12	42/58	88	21/79
Honey locust	27/73	21	17	35/65	83	24/76
Cassia corymbosa	23/77	34	11	34/66	89	21/79
Carob	23/77	34	9	36/64	91	21/79
Cassia didymobotrya	22/78	35	4	40/60	96	21/79
Delonix regia	22/78	35	6	35/65	94	21/79
Lucerne, prehydrolyzed with α-D-galactosidase[d]	43/57	7	86	45/55	14	33/67
	37/63	10	80	42/58	20	29/71
	33/67	19	33	41/59	67	31/69
	21/79	32	6	42/58	94	18/82
	19/81	34	1	42/58	99	19/81

[a]Incubations as described in footnote to Table III for 2600 min [b]See footnote *c*, Table II [c]Not determined [d]α-D-Galactosidase A from lucerne

galactomannan and guar galactomannan are shown in Table III On extended incubation, the D-galactose to D-mannose ratio of this high-$\overline{d\ p}$ fraction from carob galactomannan hydrolyzate increased from 23 77 to 36 64, whereas in guar galactomannan hydrolyzate, it remained relatively constant at 38 62 Thus, in each case, a high-$\overline{d\ p}$ fraction resistant to β-D-mannanase and containing less than 50 % of galactose persisted These studies were extended to include a number of native galactomannans and lucerne galactomannan prehydrolyzed to various degrees by α-D-galactosidase The D-galactose to D-mannose ratios of the fractions precipitated by 2 vol of ethanol following extended incubation of these galactomannans with Driselase β-D-mannanase are shown in Table IV With the exception of lucerne galactomannan, which has a high initial galactose content, in no case was the D-galactose to D-mannose ratio of any of these fractions found to increase to ~1 1, as was previously found for similar fractions produced on hydrolysis of a range of galactomannans by a β-D-mannanase enzyme from *Bacillus subtilis*[15]

Analysis of the fine structure of D-*galacto*-D-*mannans* The fine structure of galactomannans has been studied by characterizing and determining quantitatively the oligosaccharides produced on hydrolysis of these polysaccharides by β-D-mannanase Since the array of oligosaccharides produced is a function of the ratio of enzyme to carbohydrate, of the time of incubation, and of the incubation conditions, these factors were carefully controlled For the analysis of the arrays of amounts of oligosaccharides produced, all galactomannans were hydrolyzed to a degree equal to

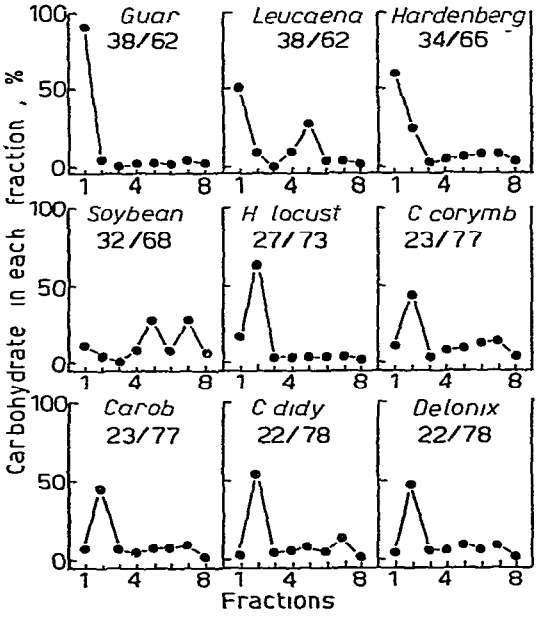


Fig 9 Oligosaccharides produced on hydrolysis of a range of native galactomannans by Driselase β-D-mannanase Galactomannan (20 mL, 0 5%, w/v, unbuffered) was incubated with β-D-mannanase (0 4 μkat) for 60 min The fractions are (1) Ethanol precipitate, (2) immobile in t l c , (3) $GalMan_4$, (4) $GalMan_3$, (5) $GalMan_2$, (6) Man_3, (7) Man_2, and (8) Man

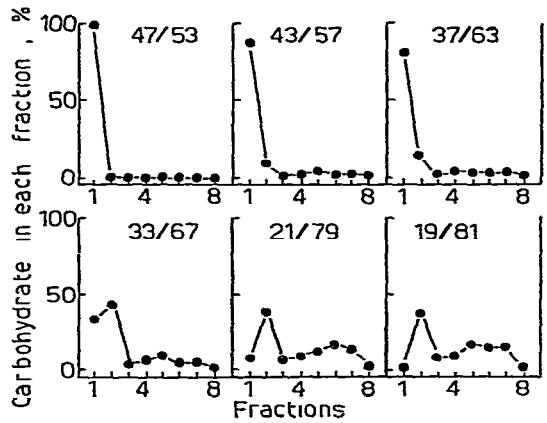


Fig 10 Oligosaccharides produced by Driselase β-D-mannanase, hydrolysis of lucerne galactomannan, prehydrolyzed to various galactose contents with α-D-galactosidase Galactomannan (2 mL, 0 5%, w/v, unbuffered) was incubated with β-D-mannanase (40 nkat) for 60 min The fractions are (1) Ethanol precipitate, (2) material immobile in t l c , (3) $GalMan_4$, (4) $GalMan_3$, (5) $GalMan_2$, (6) Man_3, (7) Man_2, and (8) Man

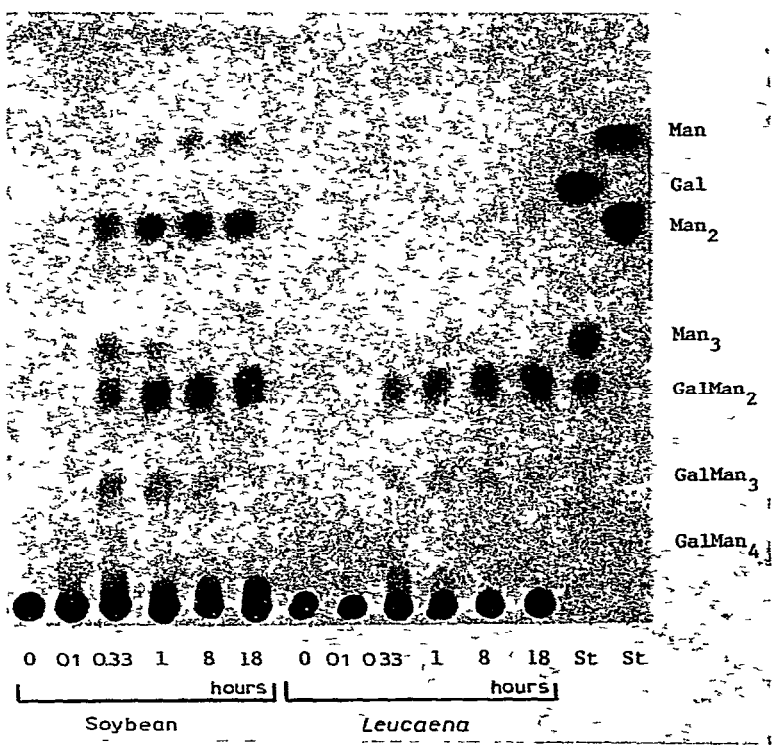


Fig 11 T l c of the hydrolysis products of soybean and *Leucaena leucocephala* galactomannans by Driselase β-D-mannanase Galactomannan (20 mL, 0 5%, w/v) was incubated with Driselase β-D-mannanase (0 4 μkat), and aliquots were removed at 0–18 h for chromatography, (St), standard sugars

point "A" in Fig 6, and the oligosaccharides were quantitatively determined after the separation The patterns of oligosaccharides produced on hydrolysis of ten native galactomannans and of lucerne galactomannan, prehydrolyzed to various degrees with α-D-galactosidase (lucerne α-D-galactosidase A), are shown in Figs 9 and 10 As the D-galactose content of the galactomannans decreases, an increased proportion of low-$\overline{d\ p}$ oligosaccharides is observed In general, the patterns of amounts of oligosaccharides produced on hydrolysis of native galactomannans are similar to those produced from lucerne galactomannan, prehydrolyzed to similar galactose content by α-D-galactosidase The low-$\overline{d\ p}$ oligosaccharides include, in addition to mannobiose and mannotriose, significant quantities of the D-galactose-containing manno-oligosaccharides $GalMan_2$, $GalMan_3$, and $GalMan_4$

The patterns for the hydrolyzates of galactomannans from *Leucaena leucocephala* and soybean seeds are different from those of other galactomannans with similar galactose contents Soybean-galactomannan hydrolyzate contains high levels of Man_2 and $GalMan_2$, whereas the major low-$\overline{d\ p}$ oligosaccharide present in the hydrolyzate of *Leucaena leucocephala* galactomannan is $GalMan_2$ Changes in the proportions of these oligosaccharides on extended incubation with β-D-mannanase are shown in Fig 11 At all stages of hydrolysis of *Leucaena leucocephala* galactomannan, $GalMan_2$ was the major, low-$\overline{d\ p}$ oligosaccharide product

The elution patterns of the carbohydrate components, obtained on chromatography of these hydrolyzates on Sephadex G-25, are shown in Fig 12 The hydroly-

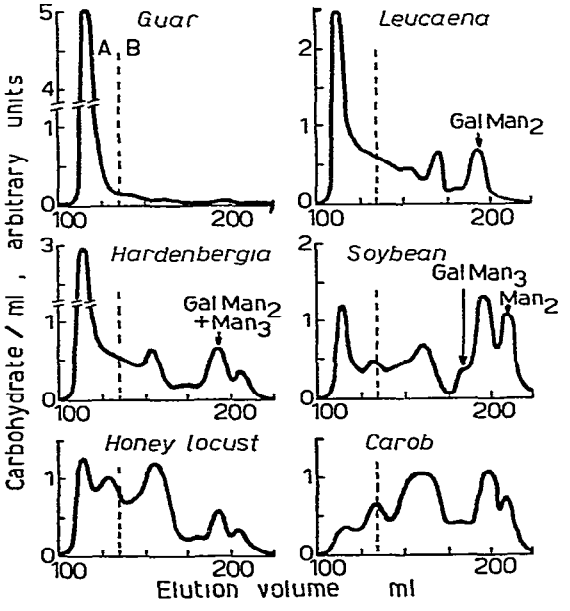


Fig 12 Gel filtration on Sephadex G-25 of products of hydrolysis of galactomannans by Driselase β-D-mannanase Galactomannan (20 mL, 0 5%, w/v, unbuffered) was incubated with Driselase β-D-mannanase (0 4 μkat) for 60 min Aliquots [0 4 mL, 2% (w/v) of carbohydrate] were applied to the column

TABLE V

GELLING INTERACTION OF GALACTOMANNANS WITH XANTHAN[a]

Source of galactomannan	*Final conc (%)*		*Carbohydrate (%)*		*Carbohydr content (mg/mL of gel)*
	Galacto-mannan	*Xanthan*	*As gel*	*Soluble*	
Carob	0 05	0 01	0	100	0 0
	0 05	0 02	54	46	2 7
	0 05	0 03	62	38	3 5
	0 05	0 04	76	24	4 9
	0 05	0 05	82	18	5 4
	0 04	0 05	84	16	5 3
	0 03	0 05	86	14	5 2
	0 02	0 05	90	10	5 1
	0 01	0 05	89	11	2 9
	0 005	0 05	70	30	1 8
Leucaena leucocephala	0 05	0 05	62	38	2 3
	0 04	0 05	68	32	2 0
	0 03	0 05	67	33	1 7
	0 02	0 05	72	28	1 6
	0 01	0 05	64	36	1 2
Cassia corymbosa	0 05	0 05	80	20	5 3
Cassia didymobotrya	0 05	0 05	73	27	5 1
Honey locust	0 05	0 05	66	34	3 7
Hardenbergia violacea	0 05	0 05	68	32	3.6
Guar	0 05	0 05	0	100	0 0
Lucerne	0 05	0 05	0	100	0 0

[a]Galactomannan solutions (0 01–0 1%, w/v, 5 mL) were added to xanthan solutions (0 02–0 1%, w/v, 5 mL), each in 0 5M KCl, heated to 60°, and mixed These were cooled to 2°, shaken vigorously, and centrifuged (25 000*g*, 20 min) The gels were washed twice with 0 5M KCl and recovered by centrifugation The carbohydrate content per mL of gel was determined following centrifugation

TABLE VI

VISCOSITY OF GALACTOMANNANS AND GALACTOMANNAN–XANTHAN SOLUTIONS[a]

Source of galactomannan	*Final conc (%, w/v)*		*Viscosity (mPa s)*
	Galactomannan	*Xanthan*	
Carob	0 0	0 2	330
	0 2	0 0	23
	0 1	0 1	4100
	0 05	0 1	4610
	0 04	0 1	4435
	0 02	0 1	3900
	0 01	0 1	1600
Guar	0 2	0 0	50
	0 1	0 1	1400
Leucaena leucocephala	0 2	0 0	25
	0 1	0 1	3800

[a]Values determined with a Brookfield Synchro-Lectric viscometer at 15°, 20 r p m and at 1 min after switching on The values were relatively constant with time, up to 5 min The solutions were salt-free and contained 0 1% (v/v) of formaldehyde as preservative

zates of galactomannans from *Cassia corymbosa*, *Cassia didymobotrya*, and *Delonix regia* showed chromatographic patterns very similar to that of carob galactomannan hydrolyzate. The fractions represented by "A" (Fig 12) are approximately equal to the material precipitated on the addition of 2 vol of ethanol

Gelling interactions of D-*galacto*-D-*mannans with xanthan*[29 30] The gelling interaction between galactomannans and xanthan gum was studied by use of three techniques Brookfield viscometry, Amylograph viscometry, and a technique that is based on the observation that, in the presence of salt, galactomannan–xanthan gels break down into "gel-islands"[31] which can be recovered by high-speed centrifugation, and into a nongelling polysaccharide which is removed by washing the gels with 0 5M potassium chloride The results obtained with the third technique on a number of galactomannans at various concentrations and xanthan at 0 05% (w/v) are shown in Table V In general, as the D-galactose contents of galactomannans

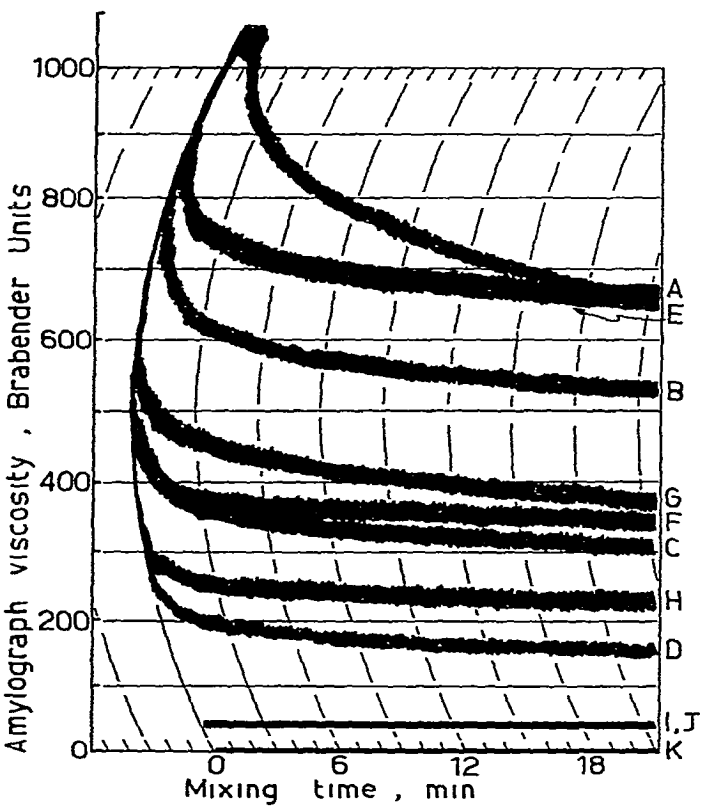


Fig 13 Gelling interactions of galactomannans with xanthan as determined with a Brabender (Duisburg) Amylograph Samples are (A) Carob GM (0 1 or 0 075%, w/v) + xanthan (0 1%, w/v), (B) carob GM (0 05%, w/v) + xanthan (0 1%, w/v), (C) carob GM (0 03%, w/v) + xanthan (0 1%, w/v), (D) carob GM (0 01%, w/v) + xanthan (0 1%, w/v), (E) honey-locust GM (0 1%, w/v) + xanthan (0 1%, w/v) (F) honey-locust GM (0 05%, w/v) + xanthan (0 1%, w/v), (G) *Leucaena* GM (0 1%, w/v) + xanthan (0 1%, w/v), (H) guar GM (0 1%, w/v) + xanthan (0 1%, w/v), (I) lucerne GM (0 1%, w/v) + xanthan (0 1%, w/v), (J) xanthan (0 1%, w/v), and (K) galactomannans (0 1%, w/v) and baseline

increase, the amount of polysaccharide in the gel complex decreases slightly, until a D-galactose to D-mannose ratio of 1·9 : 3·1 (guar) is reached. There is also a decreased concentration of carbohydrate per volume of gel as the D-galactose content increases. With guar galactomannan, trace quantities of gel could be detected but could not be recovered by centrifugation. Decrease in the concentration of carbohydrate per volume of gel on the interaction of xanthan with galactomannans of increasing D-galactose content was paralleled by the changes observed with decreasing concentrations of carob galactomannan over the range 0·03–0·005% (w/v) in such mixtures.

Increases in viscosity and gelling interaction on mixing galactomannan and xanthan solutions (in the absence of added salt) were also studied by use of the Brookfield Synchro-Lectric Viscometer, and the results are shown in Table VI. Although this technique distinguished between the interaction of various galactomannans with xanthan, the viscosities were not directly related to the amount of galactomannan present in such mixtures. Thus, mixtures containing carob galactomannan (0·02%, w/v) plus xanthan (0·1%, w/v) gave viscosity readings similar to those of mixtures containing carob galactomannan (0·1%, w/v) plus xanthan (0·1%, w/v).

The most useful technique for studying the interaction was found to be one based on the Brabender Amylograph. The results obtained with a range of galactomannans and xanthan are shown in Fig. 13. All mixtures contained 0·1% (w/v) of xanthan and 0·1% (w/v) or less of galactomannan. Xanthan alone, at a concentration of 0·1% (w/v), gave a low Amylograph viscosity, and galactomannans alone at the same concentration gave no reading. However, mixtures of carob galactomannan and xanthan gave very high values. As the concentration of carob galactomannan in galactomannan–xanthan mixtures decreased from 0·075 to 0·01% (w/v), a proportionate decrease in Amylograph viscosity was observed. An inverse relationship between the D-galactose content of most galactomannans and their interaction with xanthan gum was also apparent. Thus, as the D-galactose content increased from 23% (carob) to 48% (lucerne), the degree of interaction decreased until no apparent gelling-interaction was observed with lucerne galactomannan. The one exception was the galactomannan from *Leucaena leucocephala*, which interacted to a much greater degree with xanthan than would be expected on the basis of its D-galactose content (*cf.* guar galactomannan with the same D-galactose content). This greater interaction of *Leucaena leucocephala* galactomannan with xanthan gum correlates with the greater degree of hydrolysis of the galactomannan by Driselase β-D-mannanase.

DISCUSSION

The complete hydrolysis of the galactomannans to D-galactose, D-mannobiose, and D-mannotriose by a mixture of purified lucerne-seed α-D-galactosidase C and β-D-mannanase B (Fig. 1) indicates that all the D-galactopyranosyl groups are α-linked and that the D-mannopyranose residues in the mannan backbone are essenti-

ally β-(1→4) linked The presence of odd linkages[32] in the mannan backbone might be expected to result in the production of higher-$\overline{d\ p}$ manno-oligosaccharides, which were not in fact observed when a ratio of enzyme to galactomannan high enough to ensure the completion of these enzymic reaction was used These results are consistent with the structures proposed for guar, carob, and lucerne galactomannans, based on results obtained by chemical techniques[33]. *Leucaena leucocephala* galactomannan has been reported[32] to contain some major branch-points in the molecule, however, such a proposal is not consistent with the production of only galactose, mannobiose, and mannotriose on hydrolysis of this galactomannan by α-D-galactosidase plus β-D-mannanase (Fig 1)

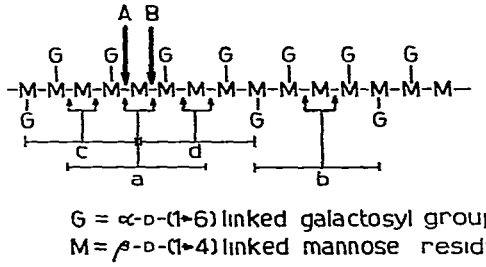
It has been suggested[15 16] that coffee-bean α-D-galactosidase removes α-D-galactopyranosyl groups from the mannan backbone of galactomannans that are highly substituted with galactosyl groups, *i e*, lucerne galactomannan (Gal to Man ratio of 47 53), in a sequential, "zipper-like" fashion For galactomannans with lower degrees of galactose substitution, it was proposed[15 16] that the enzyme first removes isolated α-D-galactopyranosyl groups, *i e*, those adjacent to unsubstituted D-mannose residues, and then acts at the extremities of sections highly substituted with galactosyl groups If such a proposition were correct, it might be expected that the polysaccharides recovered from such treatment would produce, on hydrolysis with β-D-mannanase, mostly mannobiose and mannotriose from the sections unsubstituted with galactosyl groups, and a high-$\overline{d\ p}$, β-D-mannanase-resistant fraction containing approximately equal quantities of galactose and mannose from the highly substituted sections of the galactomannan Little, or no low-$\overline{d\ p}$, galactose-containing manno-oligosaccharides would be produced However, in the current investigations the products released on β-D-mannanase hydrolysis of lucerne galactomannan, prehydrolyzed to various degrees by α-D-galactosidases from both lucerne and coffee-bean seeds, were found to include significant quantities of the galactose-containing manno-oligosaccharides $GalMan_2$, $GalMan_3$ and $GalMan_4$ (Figs 4 and 10) This result suggests that all three α-D-galactosidases remove galactosyl groups in a random manner from the mannan backbones of galactomannans In support of this, the high-$\overline{d\ p}$, β-D-mannanase-resistant fractions from these hydrolyzates did not contain approximately equal quantities of galactose and mannose, values for the galactose to mannose ratios were closer to 41 59–42 58 (Table IV)

Mannans and galactomannans with low degrees of galactose substitution were rapidly depolymerized by Driselase β-D-mannanase The enzyme had a much greater affinity for soluble (K_m 0 005%, w/v) than for insoluble mannan (K_m 0 11%, w/v) (Table II), however, at saturating levels of substrate V_{max} values were the same The reason for the V_{max} values on both soluble and insoluble mannan to be only about half of those on galactomannans of low galactose content is not immediately apparent It may possibly be due to the lower-$\overline{d\ p}$ of mannans compared to that of galactomannans

On hydrolysis of *Leucaena leucocephala*, carob, and a number of other galactomannans by Driselase β-D-mannanase, the oligosaccharide $GalMan_2$ was produced in

significant quantities (Figs 7, 9, 10, and 11), therefore, this enzyme must be able to act at some points of single unsubstituted mannose residues in the galactomannan backbone However, since the enzyme did not produce a high-$\overline{d\ p}$ fraction in which all mannose residues were substituted with galactosyl groups (Tables III and IV), it was concluded that the enzyme could not hydrolyze at all points of single, unsubstituted mannose residues Consequently, the requirements for hydrolysis of galactomannans by this enzyme are not the same as those proposed for other β-D-mannanases[13 15 34]. In general, β-D-mannanases readily hydrolyze mannohexaose and mannopentaose[6 12 35], but not mannotetraose or mannotriose, thus, it has been suggested[13] that the hydrolysis of galactomannans by β-D-mannanase probably involves the recognition of at least 5 or 6 neighboring mannose residues in the mannan chain In a section of this length, the disposition of any galactosyl group on either side of the mannan chain, as well as the distance between the groups, may affect the association between the chain and the enzyme In the extended conformation that galactomannans probably adopt in aqueous solution[7,36], galactosyl groups separated by no, or by an even number of, mannose residues would lie on opposite sides of the main chain, and those separated by an odd number of mannose residues would lie on the same side[36] Thus, it is suggested that Driselase β-D-mannanase can hydrolyze at points of single, unsubstituted mannose residues if, and only if, all galactosyl groups in the vicinity (*i e*, 2–3 mannose residues on either side) of the bond to be hydrolyzed are positioned on only one side of the mannan chain Thus, this enzyme can readily act at point "a" (Scheme 1), but not act at points "b", "c", or "d", owing to steric hindrance associated with the disposition of galactosyl groups At present, it is not known whether this enzyme preferentially hydrolyzes at point A or B in Scheme 1, although from structural considerations it might be proposed that hydrolysis at point B would be favored

In a previous communication[13], it was reported that a β-D-mannanase from honey-locust seeds requires at least two contiguous, unsubstituted mannose residues at the point of scission for hydrolysis of galactomannan This proposal was based on the absence of the galactose-containing trisaccharide, $GalMan_2$, in β-D-mannanase



Scheme 1 Proposed structural requirements for hydrolysis at single unsubstituted D-mannose residues by Driselase β-D-mannanase Hydrolysis occurs only at point "a" Steric hindrance due to the disposition of D-galactopyranosyl groups prevents or hinders hydrolysis at points "b", "c", and d" It is not known whether the enzyme preferentially hydrolyzes at point A or B

hydrolyzates of carob galactomannan, as well as by the persistence of a high-$\overline{d\ p}$ fraction that contained significantly less than 50% of galactose This requirement for hydrolysis of galactomannans by honey-locust β-D-mannanase differs from that now proposed for Driselase β-D-mannanase The greater ability of Driselase β-D-mannanase to hydrolyze at points of single, unsubstituted mannose residues in galactomannans is paralleled by its kinetics of hydrolysis of highly substituted galactomannans For example, Driselase β-D-mannanase hydrolyzes guar galactomannan (38% of Gal) at 83% of the rate (V_{max}) of carob galactomannan (23% of Gal), whereas honey-locust β-D-mannanase[13] hydrolyzes guar galactomannan at only 5% of the rate of carob galactomannan

The usefulness of β-D-mannanase in the analysis of the fine structures of galactomannans has been discussed by numerous researchers, but such studies have been performed by only one group[15,16] The major reason for this has been the difficulties associated with obtaining β-D-mannanases devoid of interfering α-D-galactosidase and β-D-mannosidase These difficulties have recently been overcome by the development of a simple and specific assay for β-D-mannanase employing carob galactomannan dyed with Remazol Brilliant Blue R[27] and the introduction of substrate-affinity chromatography for the purification of this enzyme[21] The commercial Driselase preparation is a convenient source of β-D-mannanase containing 1 3 μkat of activity on soluble β-D-mannan per g of crude powder.

In a study employing β-D-mannanases from *Bacillus subtilis* and legume seeds, it has been suggested[15,16] that the galactosyl groups in carob galactomannan occur in blocks along the mannan backbone However, in the present investigation, it has been found that the oligosaccharides produced by hydrolysis of carob and other galactomannans of similar galactose to mannose ratios with Driselase β-D-mannanase include significant quantities of low-$\overline{d\ p}$, galactose-containing manno-oligosaccharides This indicates that in fact, the galactosyl groups are randomly distributed This proposal is supported by the finding that in no instance did the galactose to mannose ratio of the high-$\overline{d\ p}$, β-D-mannanase-resistant fraction from galactomannans of low initial galactose content increases to ~1 1 (Table IV)

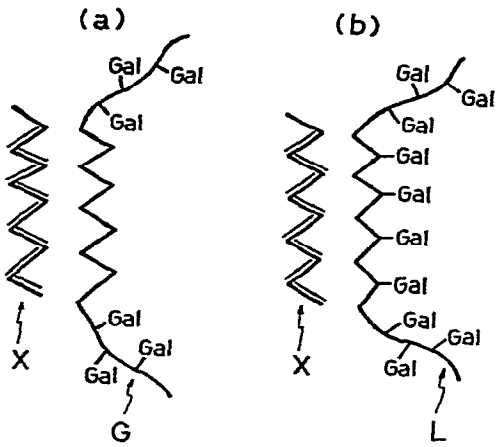
The two galactomannans from soybean and *Leucaena leucocephala* appear, from the amounts of oligosaccharides produced on β-D-mannanase hydrolysis, to have fine structures different from those of other galactomannans Both galactomannans are hydrolyzed by β-D-mannanase to a degree greater than that of other galactomannans of similar galactose content Soybean-galactomannan hydrolyzate contains relatively large quantities of the oligosaccharides Man_2 and $GalMan_2$, with lesser amounts of Man_3 *Leucaena leucocephala* galactomannan hydrolyzate contains $GalMan_2$ as the major, low-$\overline{d\ p.}$ product This oligosaccharide represents at least 25% of the total carbohydrates of the hydrolyzate Thus, it is concluded that large sections of *Leucaena leucocephala* galactomannan consist of the repeating unit $[(Gal\rightarrow)Man\rightarrow Man]_n$ and consequently, since there is unlikely to be any rotation about the mannosyl→mannosyl glycosidic bond due to steric hindrance[36], that all galactosyl groups in these sections would be located on one side of the mannan

chain This inference, in view of the previously proposed requirements for hydrolysis of galactomannans at single unsubstituted mannose residues by Driselase β-D-mannanase, is consistent with the high degree of hydrolysis of *Leucaena leucocephala* galactomannan, as compared to guar galactomannan, which has the same galactose content (*i e*, 38%) (Table IV)

It has been proposed[17 37] that guar galactomannan has a uniform distribution of galactosyl groups along the mannan backbone, being composed almost entirely of the repeating unit $[(Gal\rightarrow)Man\rightarrow Man]_n$ However, this galactomannan is hydrolyzed by Driselase β-D-mannanase to a degree of only 6%, whereas similar sections in *Leucaena leucocephala* galactomannan appear to be quite susceptible to hydrolysis, releasing $GalMan_2$ as the major low-$\overline{d\,p}$ oligosaccharide (Fig 11) Thus, it is suggested that guar galactomannan has a galactose distribution that is irregular to random This proposal is supported by the observation that low, but detectable, levels of each of the oligosaccharides Man_2, $GalMan_2$, Man_3, and $GalMan_3$ are produced on incubation of this galactomannan with β-D-mannanase (Fig 9)

Galactomannans interact strongly with a number of polysaccharides[18,19], one of these being an extracellular polysaccharide from *Xanthomonas campestris*, termed xanthan[29 30] Mixtures of xanthan and galactomannans with low galactose contents, *e g*, carob galactomannan, form gels at total carbohydrate concentrations as low as 0 2% However, galactomannans with higher galactose contents, *e g*, guar galactomannan, show only a slight degree of interaction with xanthan Thus, it has been proposed[18 19] that long sections of contiguous. unsubstituted mannose residues at the "junction zones" are required for interaction of galactomannans with xanthan and other polysaccharides

In the current investigations, the results obtained are in agreement with those previously reported[18 29 30]. However, the degree of interaction of *Leucaena leucocephala* galactomannan with xanthan gum is greater than would be expected on the basis on its galactose content (Tables V and VI, Fig 13) Some correlation between the relative degrees of interaction of *Leucaena leucocephala* and carob galactomannans with xanthan gum (Fig 13) and the relative degrees of hydrolysis by Driselase β-D-mannanase seems apparent Thus, *Leucaena* galactomannan at 0 1% (w/v) interacts with xanthan gum to a degree approximately equal to that of carob galactomannan at 0 04% (w/v) (*i e*, 40% as effective, Fig 13), and the ratio of the degrees of hydrolysis of *Leucaena* and carob galactomannans by Driselase β-D-mannanase is approximately 16 31 (Table IV) From the oligosaccharides produced by β-D-mannanase hydrolysis of *Leucaena leucocephala* galactomannan, *i e*, low levels of mannobiose and mannotriose and high levels of $GalMan_2$, it is apparent that this galactomannan does not contain long sections of contiguous unsubstituted mannose residues In fact, it would seem that a significant proportion of the chain (≐ 25%) consists of the repeating unit $[(Gal\rightarrow)Man\rightarrow Man]_n$ with the galactosyl groups in these sections positioned on one side of the mannan backbone Thus, it is suggested that no long sections of contiguous, unsubstituted mannose residues at the "junction-



Scheme 2 Proposed structural requirements in D-galacto-D-mannans for gelling interaction with other polysaccharides (a) A model proposed for the interaction between chains of xanthan (X) and galactomannan (G) by Morris *et al* [18] (b) The current model proposed for the interaction between chains of xanthan (X) and *Leucaena leucocephala* galactomannan (L)

zones", as previously proposed[18 31], are required for interaction of galactomannans with xanthan gum Rather, it is considered that xanthan gum may also interact with sections of the galactomannan where galactosyl groups are positioned on only one side of the mannan backbone (Scheme 2) Such a theory better explains the lower, but significant, interaction of xanthan gum with guar galactomannan[31] Guar galactomannan does not contain long sections of contiguous, unsubstituted mannose residues, as shown by the very low levels of mannobiose and mannotriose released on β-D-mannanase hydrolysis

Driselase β-D-mannanase causes a rapid drop in Amylograph viscosity of carob galactomannan–xanthan gum solutions, indicating that the chain length of the galactomannan is critical in the formation of the three-dimensional gel network Furthermore, carob galactomannan (0 1 %, w/v) in the presence and absence of xanthan gum (0 1 %, w/v) is hydrolyzed to apparently the same degree by β-D-mannanase, possibly suggesting that only a very small proportion of the unsubstituted sections of carob galactomannan is involved in interaction with xanthan gum at the "junction-zones"

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