Carbohydrate Research, 139 (1985) 237–260
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE FINE STRUCTURES OF CAROB AND GUAR GALACTOMANNANS

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(Received May 1st, 1984; accepted for publication, October 11th, 1984)

ABSTRACT

The distribution of D-galactosyl groups along the D-mannan backbone (fine structure) of carob and guar galactomannans has been studied by a computer analysis of the amounts and structures of oligosaccharides released on hydrolysis of the polymers with two highly purified β-D-mannanases isolated from germinated guar seed and from *Aspergillus niger* cultures. Computer programmes were developed which accounted for the specific subsite-binding requirements of the β-D-mannanases and which simulated the synthesis of galactomannan by processes in which the D-galactosyl groups were transferred to the growing D-mannan chain in either a statistically random manner or as influenced by nearest-neighbour/second-nearest-neighbour substitution. Such a model was chosen as it is consistent with the known pattern of synthesis of similar polysaccharides, for example, xyloglucan; also, addition to a preformed mannan chain would be unlikely, due to the insoluble nature of such polymers. The D-galactose distribution in carob galactomannan and in the hot- and cold-water-soluble fractions of carob galactomannan has been shown to be non-regular, with a high proportion of substituted couplets, lesser amounts of triplets, and an absence of blocks of substitution. The probability of sequences in which alternate D-mannosyl residues are substituted is low. The probability distribution of block sizes for unsubstituted D-mannosyl residues indicates that there is a higher proportion of blocks of intermediate size than would be present in a galactomannan with a statistically random D-galactose distribution. Based on the almost identical patterns of amounts of oligosaccharides produced on hydrolysis with β-D-mannanase, it appears that galactomannans from seed of a wide range of carob varieties have the same fine-structure. The D-galactose distribution in guar-seed galactomannan also appears to be non-regular, and galactomannans from different guar-seed varieties appear to have the same fine-structure.

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0008-6215/85/$ 03.30

INTRODUCTION

Several techniques have been employed in studies of the distribution of D-galactosyl groups along the D-mannan chain of plant galactomannans, *i.e.*, the "fine-structure"[1]. Most legume-seed galactomannans consist of linear chains of (1→4)-linked β-D-mannopyranosyl residues, to which are attached varying proportions of (1→6)-linked α-D-galactopyranosyl groups[2] as single-unit side-chains. However, exceptions have been reported (*e.g.*, refs. 3 and 4).

On the basis of X-ray data from stretched films of guar galactomannan, Palmer and Ballantyne[5] concluded that the polymer consists of an alternating arrangement of substituted and unsubstituted D-mannosyl residues, a structure which was supported by the results of the chemical studies of Baker and Whistler[6]. Such an arrangement requires that the galactomannan has a galactose–mannose ratio of 1:2, but, as noted by Painter[1], most guar galactomannan samples contain significantly more than 33% of D-galactose (usually 38 ±2%). Increasing the D-galactose content from 33 to 38% has a dramatic effect on the number of D-mannosyl residues substituted. Furthermore, recent X-ray diffraction studies[7] of films of several galactomannans showed little change with D-galactose content in the range 21–34%, indicating that the unit cell can apparently accommodate widely different proportions of side-groups with only minor structural changes. Thus, X-ray diffraction has so far led to no definite conclusions about the fine-structure of galactomannans.

In contrast to the chemical studies of Baker and Whistler[6], a chemical procedure developed by Hoffman and Svensson[8] indicated that the D-galactosyl groups in guar galactomannan are arranged mainly in pairs and triplets, whereas that developed by Hall and Yalpani[9] indicated that they are distributed in blocks. These conflicting results are possibly due, in part, to the large number of steps involved and the incompleteness of each chemical reaction. This was particularly so for the latter procedure[9] in which, for example, the oxidation of D-galactose by D-galactose oxidase proceeded to only 60–70% of completion.

A detailed theoretical analysis of the kinetics of the periodate oxidation of guar and carob galactomannans, together with results of methylation analysis[10], led Painter *et al.*[11] to conclude that the distribution of D-galactosyl groups in guar galactomannan was similar to that proposed by Hoffman and Svensson[8]. In contrast, carob galactomannan was reported to consist of long blocks of contiguous unsubstituted D-mannosyl residues, long blocks in which every second D-mannosyl residue is substituted with D-galactose, and shorter blocks in which there is a high density of D-galactosyl groups.

Good ^{1}H- and ^{13}C-n.m.r. spectra were obtained after partial depolymerisation of several galactomannans[12]. The determined diad frequencies of substituted and unsubstituted D-mannosyl residues were roughly consistent with a random arrangement of D-galactosyl groups in all the galactomannans studied. More recently[1], this proposal has been refined, with the conclusion that the results obtained

rule out strictly regular and simple two-block structures but do not oppose a more complex type of block structure, such as that proposed by Courtois and Le Dizet[13,14] and Painter *et al.*[11] for carob galactomannan, or the paired and triplet distribution of D-galactose substitution proposed for guar galactomannan[8].

An alternative approach to the analysis of galactomannan fine-structure has employed the galactomannan-degrading enzymes, α-D-galactosidase and β-D-mannanase. β-D-Mannanase is of particular value since it cleaves the (1→4)-linked β-D-mannan main-chain in regions unsubstituted, or lighly substituted, by D-galactose. Until recently, the major restriction on the use of this enzyme was the limited data available on the precise action pattern and the substrate-binding requirements. Pioneering studies, employing a purified β-D-mannanase from *Bacillus subtilis*, gave results which indicated that the D-galactosyl groups in carob galactomannan are distributed in a partial block-type pattern[13]. However, the significance of this conclusion was diminished by the limited accuracy of the procedures employed to measure the D-galactose–D-mannose ratios of the recovered fractions. More recently, the use of purified β-D-mannanases from seeds of *Gleditsia triacanthos*[15] and from *Irpex lacteus* culture filtrates[16] showed that the β-D-mannanase-resistant material of high d.p. present in the hydrolysates of several galactomannans was only moderately enriched in D-galactose. From this result and from a detailed chromatographic study of the oligosaccharides of low d.p., it was concluded that, in most cases, the distribution of D-galactosyl groups along the chain is neither strictly regular nor block-like. Furthermore, comparison of the oligosaccharides of low d.p. produced by β-D-mannanase digestion of galactomannans having identical degrees of galactose substitution indicated significant variation in the distribution of D-galactose along the mannan chain.

Early studies[14,17] with α-D-galactosidase indicated that some D-galactosyl groups in galactomannans were fully accessible to the enzyme, whereas, apparently, others were not. It was also reported[18] that galactomannans having the same initial D-galactose content were hydrolysed at different rates and to different extents by a particular α-D-galactosidase, which was thought to indicate different distributions of D-galactosyl groups in these galactomannans. However, more recent studies with several α-D-galactosidases[16,19] showed that the relative initial rates of hydrolysis and the extent of hydrolysis of a range of galactomannans having quite different D-galactose contents and fine-structures, as shown by β-D-mannanase digestion, were very similar. This finding indicates that α-D-galactosidases have limited use in the analysis of the fine-structure of galactomannans.

Recently[20], the substrate specificity and subsite-binding requirements of several highly purified β-D-mannanases have been studied in detail, using well characterised oligosaccharide substrates and by a detailed characterisation of the oligosaccharides released on hydrolysis of galactomannans and glucomannan. β-D-Mannanases with different subsite-binding requirements have been identified[21] and characterised[20]. In the current study, two of these enzymes, isolated from germinating guar-seed and from culture filtrates of *Aspergillus niger*, have been employed in

an analysis of the fine-structures of carob and guar galactomannans. A computer programme which simulated galactomannan synthesis and accounted for the specific subsite-binding requirements of the β-D-mannanases was also employed.

EXPERIMENTAL

Materials. — Samples of the certified carob *(Ceratonia siliqua)* varieties Tylliria, Apostolika, Koumbota, and Koundourka were kindly provided by Dr. P. J. Orphanos (Ministry of Agriculture and Natural Resources, Nicosia, Cyprus). Other carob varieties, including Casuda, Tylliria, Arnele, Clifford, SFAX, and Santa Fe, were provided by Dr. H. Esbenshade (International Tree Crops Institute USA Inc., Winters, California, U.S.A.). Commercial samples of carob seeds (varieties unknown; originating from Turkey, Crete, Greece, Italy, Sicily, Spain, Portugal, Morocco, Algeria, and Egypt) and seed of *Caesalpinia spinosa* were provided by Mr. G. Feldman (Unipektin AG, Zurich, Switzerland). The commercially available carob-flours Indal (Industries de Alfarrova, Portugal), Vihep Merinos (Vihep and Merinos SA, Athens, Greece), Margel (Mar-Gel Food Products, Surrey, U.K.), and Cerasol (Cerasol Products Ltd., Knutsford, Cheshire, U.K.) were also examined. Seed of the guar varieties[22] *Pusa naubahar*, Brooks, CP39 Type 2, CP66-Type 1, *Pusa mosami* Type 1, IC 9203, and Katherine local was obtained from Mr. K. Jackson (Department of Primary Industries, Biloela, Queensland, Australia) and Mr. J. Doughton (Department of Primary Industries, Wheat Research Institute, Queensland, Australia). Seed of *Caesalpinia pulcherima, Sophora japonica, Cassia fistula,* and *Parkinsonia aculatea* was obtained from Flamingo Seeds Pty. Ltd. (East Nowra, N.S.W. Australia); *Caesalpinia vesicaria* from the Fairchild Tropical Gardens (Miami, Florida, U.S.A.); *Sesbania cannabina* from Dr. I. Wood (C.S.I.R.O., Cunningham Laboratories, Brisbane, Queensland, Australia); *Leucaena leucocephala, Medicago sativa,* and *Trigonella foenum-graecum* from a commercial supplier.

Chromatography. — T.l.c. and Bio-Gel P-2 chromatography were performed as previously described[23,24]. Column chromatography on Bio-Gel P-6 (2.5 × 70 cm) and Sephadex G-100 (2.5 × 80 cm) was performed at room temperature with distilled water, and the eluates were analysed for carbohydrate (anthrone)[25] and D-galactose (α-D-galactosidase/β-D-galactose dehydrogenase)[22].

Enzymes. — Guar-seed and *A. niger* β-D-mannanases were purified, as previously described by affinity chromatography on mannan–AH-Sepharose[26].

Substrates. — Hot-water-soluble, cold-water-soluble, and total carob galactomannan, and guar galactomannan were extracted and purified as previously described[24]. Other galactomannans were extracted by a similar procedure. Galactose-depleted fenugreek galactomannan (Gal/Man = 48:52) was prepared by incubating 1% solutions (50 mL) of galactomannan in 20mM acetate buffer (pH 5) with guar-seed α-D-galactosidase II[27] (53–211 nkat on *p*-nitrophenyl α-D-galactopyranoside) at 35° for 20 h and then at 100° for 10 min. D-Galactose was removed by dialysis and the solutions of polysaccharides were adjusted to 0.4%.

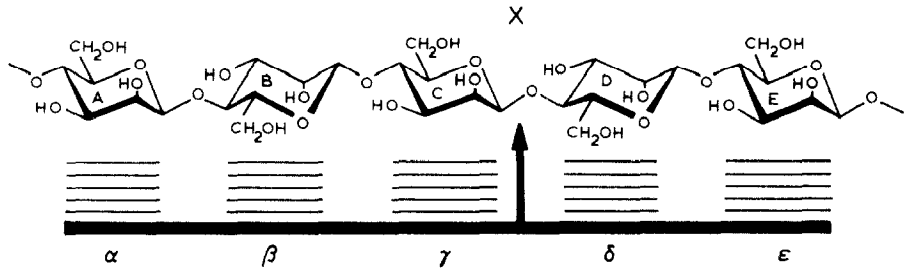
Hydrolysis of galactomannan by β-D-mannanase. — Solutions of native or galactose-depleted galactomannan (50 mL, 0.4%) in 20mM acetate buffer (pH 4.5) were incubated with either *A. niger* or guar-seed β-D-mannanase (80 nkat on carob galactomannan) at 40° for 20 h and then at 100° for 10 min. The solutions were concentrated, adjusted to 1%, and centrifuged (20,000*g*, 10 min), aliquots were removed for determination of reducing sugar (Nelson–Somogyi)[28] and total carbohydrate (anthrone)[25], and the degree of β-D-mannanase hydrolysis was determined[20]. Aliquots (5 mL) were fractionated by chromatography on Bio-Gel P-2 and the recovered oligosaccharides characterised as previously described[24].

Galactose–mannose ratios. — Galactose–mannose ratios were determined by g.l.c. of the alditol acetates, essentially according to the procedure of Albersheim *et al.*[29]. The ratios were also determined by an enzymic procedure[22] and very similar values were obtained.

Computer studies. — Computer programmes were developed which simulated the synthesis of galactomannan by a chain-extending sequence. Such a programme was chosen in preference to one in which D-galactose was added to a pre-formed mannan chain, as the former mechanism is more likely to simulate galactomannan synthesis *in vivo*. Little information is available on the synthesis of galactomannan, but studies[30] of the structurally similar polysaccharide, xyloglucan, have shown that synthesis proceeds with transfer of the branch units to the growing (1→4)-β-D-glucan chain.

The simulated galactomannans had either a random distribution of D-galactosyl groups or a distribution biased towards a particular pattern, in response to nearest-neighbour/second-nearest-neighbour interactions. In each programme, the length of the simulated mannan backbone was 1,000 residues. For the programme that simulated a galactomannan with a random distribution of D-galactose in the growing mannan chain, the probability of a given D-mannosyl residue being substituted was totally independent of the nature of substitution of the rest of the chain. A single probability factor was involved and this was equal to the proportion of D-mannosyl residues substituted by D-galactose. In contrast, in the nearest-neighbour/second-nearest-neighbour model for the computer-simulated synthesis of galactomannan, the probability, in the growing mannan chain, of a given D-mannosyl residue being substituted by D-galactose is dependent on the nature of substitution of the previous two residues. Four probabilities are involved, namely, P_{00}, P_{01}, P_{10}, and P_{11} (where 0 and 1 represent unsubstituted and substituted D-mannosyl residues, respectively). These probabilities were optimised, in turn, through a minimisation of the sum of squared differences between a range of supplied experimental data and the corresponding computed values. The supplied experimental data comprised the D-galactose content of the galactomannan, the degree of hydrolysis of the galactomannan by the *A. niger* and the guar-seed β-D-mannanases, and the amounts and structures of the oligosaccharides of d.p. 2–9 released by the *A. niger* enzyme and those of d.p. 2–7 released by guar-seed β-D-mannanase. The computed values for the amounts and structures of oligosaccharides were obtained

by subjecting the simulated galactomannan to "attack" by the computer-simulated β-D-mannanases. The β-D-mannanase selected a point "X" at random on the mannan chain and then examined the residues on the left-hand side and right-hand side of that point (Scheme 1). With the simulated guar-seed β-D-mannanase, cleavage occurred at X only if there were at least three residues on the left-hand side and two on the right-hand side of X and if residues B, C, and D were unsubstituted. Residues A and E could be unsubstituted (0) or substituted (1). With *A. niger* β-D-mannanase, cleavage at X occurred only if there were at least two residues on both the left-hand side and the right-hand side of X and residues B and D were unsubstituted. Residues C and E could be unsubstituted or substituted. The simulated galactomannan was subjected to 100 waves of 100 random attacks and the number of successful encounters per wave of attacks was recorded as well as the points of cleavage. Cleavage was essentially complete after 40–50 waves of attacks; after 100, the programme recorded the total number of fragments as well as the structures and weight-percentages of the individual fragments. To reduce variability, the whole programme of galactomannan synthesis (by either the random or the non-random process) and degradation was repeated 100 times and averages were taken.



Scheme 1. Representation of subsite binding between β-D-mannanase and the (1→4)-β-D-mannan chain.

The statistical treatment of data in the current model is essentially the same as that employed by Painter, Gonzalez, and Hemmer[11] in the analysis of periodate-oxidation data. In both, a second-order Markov process was employed. The probabilities in the current treatment relate to those in the Gonzalez treatment as follows: $P_{00} \equiv P_{221} = 1 - P_{222}$; $P_{10} \equiv P_{211} = 1 - P_{212}$; $P_{01} \equiv P_{121} = 1 - P_{122}$; $P_{11} \equiv P_{111} = 1 - P_{112}$. Gonzalez also introduced probabilities P_1, P_{11}, P_{12}, P_{21}, and P_{22}; P_1 is the degree of substitution of the chain. The relationships governing the above probabilities are: $P_{11} = P_{211}/(P_{112} + P_{211})$; $P_{12} = P_{112}/(P_{112} + P_{211})$; $P_{21} = P_{221}/(P_{122} + P_{221})$; and $P_{22} = P_{122}/(P_{122} + P_{221})$; also, $P_1 = P_{21}/(P_{12} + P_{21})$ and $P_2 = P_{12}/(P_{12} + P_{21})$. Gonzalez fitted data using P_1, P_{11}, P_{111}, and P_{222} as variables, whereas, in the current model, P_{221} (P_{00}), P_{211} (P_{10}), P_{121} (P_{01}), and P_{111} (P_{11}) were employed, but this should have no significant effect.

RESULTS AND DISCUSSION

General properties of carob and guar-seed galactomannans. — Some properties of galactomannans derived from different carob varieties are shown in Table I. Properties of the seed galactomannans from different guar varieties have been reported[22]. The total galactomannan content of carob seed ranged from 18 to 31% of the dry weight of the whole-seed flour. Extraction of finely milled carob flour with cold water and then hot water reproducibly gave two quite distinct fractions. Further extraction with 10% aqueous sodium hydroxide gave insignificant amounts of galactomannan. The ratio of the cold-water-soluble (CWS) and hot-water-soluble (HWS) fractions varied, depending on the carob flour being extracted. Whether this variation was a varietal effect, or simply due to varying climatic conditions at the time of seed maturation, is not known. In general, there were insignificant differences between HWS carob galactomannan fractions derived from all the materials analysed. The content of D-galactose was 18 ±2%, the limiting viscosity was 12 ±2 dL/g, and the degree of hydrolysis by *A. niger* β-D-mannanase was 26 ±1%. The CWS fraction had properties quite different from those of the HWS material, but the variation in properties of CWS carob galactomannan fractions was minimal: the D-galactose percentage was 25 ±1%, the limiting viscosity was 10.0 ±1.0 dL/g, and the degree of hydrolysis by *A. niger* β-D-mannanase was 20 ±2%. It has been shown[19] that the viscosity of dilute solutions of galactomannans is totally dependent on the nature of the (1→4)-linked β-D-mannan chain, with the proviso that there is a requirement for sufficient D-galactose substitution to maintain solubility. It has also been shown[31], at least for guar galactomannan, that there is a direct relationship between the limiting viscosity number of galactomannan and molecular weight. It is thus concluded that the difference in the intrinsic viscosities of CWS and HWS carob galactomannans is due solely to the different proportions of D-mannan chain. If the theoretical intrinsic viscosities are calculated for the D-mannan chains of the two polysaccharides[19], a value of 15 ±2 dL/g is obtained for both. These results contrast with published data[17] which indicate that the two polysaccharide fractions have markedly different molecular weights.

In contrast to carob, the galactomannan from freshly milled guar-seed could be extracted, essentially quantitatively, with cold water (25°) using an Ultraturrax blender. Purified guar galactomannans differed little in their D-galactose content (38 ±2%), degree of hydrolysis by β-D-mannanase (5 ±1%)[22], or specific viscosities (15 ±2 dL/g).

Hydrolysis of galactomannan by β-D-mannanase. — The extent of hydrolysis and the products of hydrolysis of carob galactomannan fractions and guar galactomannan by β-D-mannanases are a function of the D-galactose content of the polysaccharide and of the particular β-D-mannanase employed. In these studies, the β-D-mannanases from *A niger* and germinating guar-seed were used. Details of the action patterns and subsite-binding requirements of these two enzymes have been reported[20]. With each enzyme, subsites β and δ bind to the hydroxymethyl

TABLE I

SOME PROPERTIES OF GALACTOMANNANS DERIVED FROM DIFFERENT CAROB-SEED VARIETIES AND COMMERCIAL CAROB FLOURS

Carob variety or source of commercial flour	*Fraction analysed and yield (%)*	*Gal:Man[c] ratio*	*L.v n.[d] (dL/g)*	*Degree of hydrolysis (%)*
Ceratonia siliqua *(carob)*				
var. Tylliria[a]	Total (30)	22:78	14.3	23
Koumbota	Total (31)	21:79	14 3	22
Koundourka	Total (30)	22:78	14.1	24
Apostolika	Total (29)	22·78	12.7	23
Casuda[b]	HWS (19)	19·81	10.9	26
	CWS (12)	26:74	8.8	19
Tylliria	HWS (13)	18:82	12.0	27
	CWS (6)	25:75	9.8	22
Arnele	HWS (6)	19:81	12.4	25
	CWS (13)	25:75	10.4	20
Clifford	HWS (8)	20:80	11.7	27
	CWS (10)	25:75	9.8	18
SFAX	HWS (20)	17:83	11.0	27
	CWS (4)	25:75	9 3	21
Santa Fe	HWS (19)	18:82	12.9	27
	CWS (10)	25 75	10.2	21
Commercial carob flours				
Indal	Total (69)	22:78	12.0	23
	HWS (41)	17:83	13.1	25
	CWS (28)	26·74	10.8	18
Margel MG	Total (56)	22:77	11.6	22
	HWS (27)	18:82	13.1	25
	CWS (29)	25:75	9.2	18
Vihep Merinos	Total (66)	22:78	11.3	22
	HWS (34)	18:82	13.4	26
	CWS (32)	25:75	9.7	18
Cerasol	Total (55)	23:77	9.9	23
	HWS (21)	19:81	10 4	25
	CWS (34)	25:75	7 4	18

[a]Samples from Dr. P. J. Orphanos. [b]Samples from Dr. H Esbenshade. [c]Determined by g.l.c. of the alditol acetates. [d]Limiting viscosity number, determined using an Ubbelohde suspended level viscometer.

edge of a pyranoid ring with an equatorial hydroxymethyl group, while α, λ, and ε bind to the HO-2,3 edge (Scheme 1). Rapid hydrolysis of the (1→4)-linked β-D-mannan chain by guar-seed β-D-mannanase requires binding across five D-mannosyl residues (A-E) and hydrolysis at X is prohibited by D-galactose substitution on sugar residues B, C, or D. In contrast, rapid hydrolysis by *A. niger* β-D-mannanase requires binding across only four D-mannosyl residues (B–E), and D-galactose substitution on sugar residue C apparently has no effect on hydrolysis at point X, although substitution on sugar residues B or D prevents hydrolysis.

The patterns of amounts of oligosaccharides produced on hydrolysis of HWS carob galactomannan by *A. niger* and guar-seed β-D-mannanases have been reported[20]. In Fig. 1, the patterns obtained on hydrolysis of the HWS, CWS, and total carob galactomannan fractions from the commercial flour, Vihep Merinos, by *A. niger* β-D-mannanase are shown. The experimental conditions were designed to give essentially complete hydrolysis of susceptible bonds but to minimise transglycosylation reactions. There was essentially no change in these oligosaccharide patterns, even if a 20-fold higher enzyme–substrate ratio was employed (see Fig. 3 in ref. 21). Transglycosylation is minimal at the substrate concentrations employed in these incubations; however, if very high levels of β-D-mannanase are incubated with high concentrations (10 mg/mL) of particular oligosaccharides, *i.e.*, Gal^1Man_3, further degradation slowly occurs. It should be noted that, under the conditions of galactomannan hydrolysis by β-D-mannanase employed in the current experiments, the concentration of Gal^1Man_3 in the hydrolysate was only 0.25 mg/mL. The small amount of D-mannose produced is thought to result from the limited ability of the enzyme to cleave the glycosidic linkage of the penultimate D-mannosyl residue (from the reducing end) of mannotetraose and higher manno-oligomers. From earlier studies[20] of the relative rates of cleavage of Gal^4Man_4, Gal^3Man_4, and mannotetraose, it appears that this linkage is hydrolysed at about one tenth of the rate for the central linkage.

The patterns of amounts of oligosaccharides obtained on hydrolysis of the HWS fractions from all the carob samples currently studied (see Experimental) were indistinguishable within experimental error, as were the patterns of amounts for the various CWS fractions. There were slight differences in the amounts of the

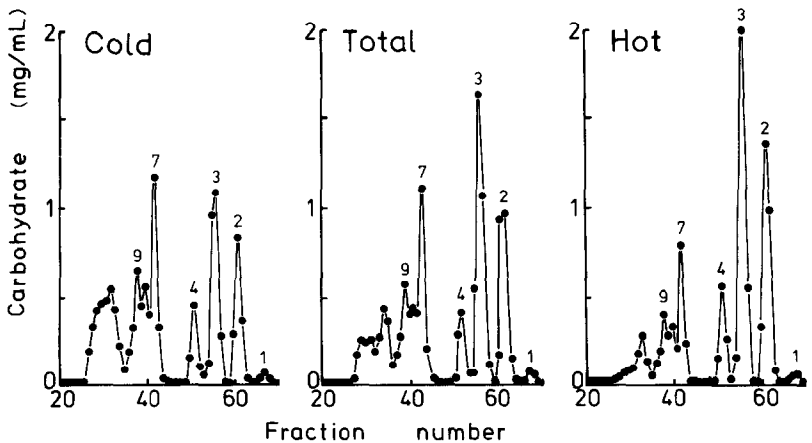


Fig. 1. Chromatography [column (2.5 × 80 cm) of Bio-Gel P-2 (<400 mesh); distilled water at 60°; 5.4-mL fractions] of the oligosaccharides produced on hydrolysis of cold-water-soluble, total, and hot-water-soluble, carob galactomannan by *A. niger* β-D-mannanase. Numbers represent the d.p. of eluted oligomers: disaccharide, Man_2; trisaccharides, Man_3 + Gal^1Man_2; tetrasaccharide, Gal^1Man_3; and heptasaccharide, $Gal^{3,4}Man_5$.

oligosaccharides produced on β-D-mannanase hydrolysis of the total carob-galactomannan fraction obtained from different seed varieties, but this can be explained simply in terms of the different proportions of HWS and CWS fractions in these samples.

Computer simulation of galactomannan synthesis and cleavage. — To obtain information on the distribution of D-galactosyl groups along the mannan chain of galactomannan, computer programmes were developed to simulate galactomannan synthesis either by a mechanism in which D-galactosyl groups are randomly distributed along the D-mannan backbone, or by a mechanism in which the probability of a given D-mannosyl residue being substituted is dependent on the nature of substitution of the previous two residues (a nearest-neighbour/second-nearest-neighbour model). These simulated polymers were then subjected to cleavage by computer-simulated *A. niger* and guar-seed β-D-mannanases, in which the specific sub-site binding requirements of these two enzymes were accounted for. A random, multi-chain mechanism of enzymic cleavage was assumed rather than a multiple-attack mechanism. The latter mechanism requires that, after cleavage, the enzyme "slides" along the polysaccharide chain towards the non-reducing end, making multiple hydrolytic attacks before releasing the polymer. This mechanism operates in the hydrolysis of amylose by porcine pancreatic alpha-amylase[32]; however, the alpha-amylases from human saliva, *Bacillus subtilis*, and malted rye function by multi-chain mechanisms over a wide range of conditions[33]. A multiple-attack mechanism may operate in the cleavage of the essentially unsubstituted regions of mannan chain but will not operate in regions containing even a low degree of substitution by D-galactose, as the D-galactose branch-units will preclude "sliding" of the substrate across the active site of the enzyme. Consequently, only the ratios of the unbranched manno-oligomers are likely to be altered.

The theoretical extents of enzymic cleavage of simulated galactomannans with a main-chain d.p. of 1000, a random D-galactose distribution, and proportions

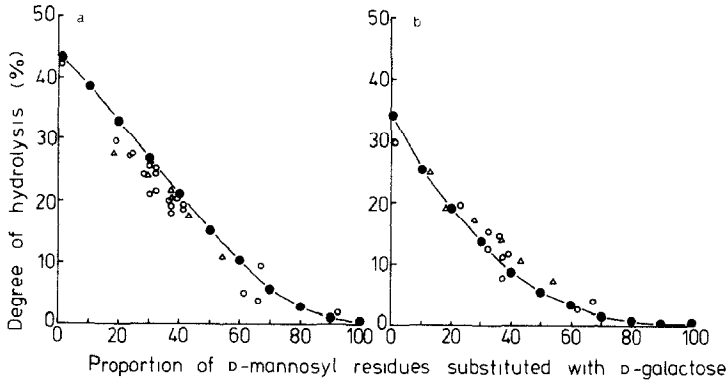


Fig. 2 Theoretical (●) and experimental [native galactomannans (○) and α-D-galactosidase-modified fenugreek-galactomannan samples (△)] values for the degree of cleavage of galactomannans by β-D-mannanase from (a) *A niger* and (b) guar-seed.

of substituted D-mannosyl residues ranging from 0 to 100% are shown in Fig. 2. As expected, the predicted extent of cleavage by *A. niger* β-D-mannanase is greater than that for the guar-seed enzyme, consistent with experimental data for a range of galactomannans. With both enzymes, the fit of theoretical to experimental data is quite good, but, in general, the experimentally measured extents of hydrolysis by the *A. niger* enzyme are slightly lower than the model predicts. With guar-seed enzyme, the experimental data are slightly higher than the theoretical data. The predicted patterns of amounts of oligosaccharides of d.p. 2–9, produced on cleavage of simulated galactomannans of increasing content of D-galactose and with a random distribution of D-galactose by simulated *A. niger* β-D-mannanase, are shown in Fig. 3. As the content of D-galactose increases, the amounts of mannobiose and mannotriose decrease, the amount of enzyme-resistant material of high d.p. increases, and the proportion of D-galactose-containing manno-oligomers of d.p. 2–9 increases and then decreases. Of particular note is the fact that the only D-galactose-containing oligomers of d.p. 2–9 predicted by the model are those previously characterised experimentally by the authors and colleagues in the *A. niger* β-D-mannanase hydrolysate of HWS-carob galactomannan. The oligomers include $Gal^{1}Man_2$, $Gal^{1}Man_3$, $Gal^{3,4}Man_5$, the three octasaccharides $Gal^{1,3,4}Man_5$, $Gal^{3,4}Man_6$, and $Gal^{4,5}Man_6$, and the four nonasaccharides $Gal^{3,4,5}Man_6$, $Gal^{1,4,5}Man_6$, $Gal^{1,3,4}Man_6$, and $Gal^{4,5}Man_7$[23,24]. These results clearly demonstrate that the model developed to simulate the action pattern of *A. niger* β-D-mannanase is valid.

The patterns of amounts of oligosaccharides present in the hydrolysates of several galactomannans, including those of the carob galactomannan fractions (Tables II and III, Figs. 4 and 5), are significantly different from those predicted on cleavage of galactomannans having a random D-galactose distribution (Fig. 3).

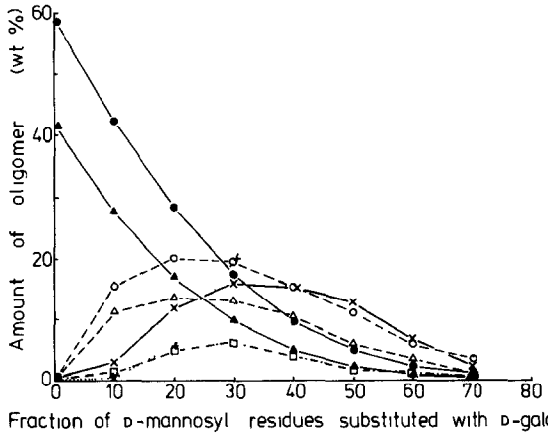


Fig. 3. Theoretical amounts of oligosaccharides produced on cleavage of galactomannans, with a random distribution of D-galactose and various extents of substitution, by a computer-simulated *A. niger* β-D-mannanase: Man_2 (●), Man_3 (▲), $Gal^{1}Man_2$ (○), $Gal^{1}Man_3$ (△), $Gal^{3,4}Man_5$ (□), octa- plus nonasaccharides (×), oligosaccharides of higher d.p. (+).

TABLE II

OLIGOSACCHARIDES RELEASED ON HYDROLYSIS OF HOT-WATER-SOLUBLE CAROB GALACTOMANNAN BY *A. niger* AND GUAR-SEED β-D-MANNANASES

Measured parameter	*Experimental data*	*Theoretical data*	
		Random model	*Nearest-neighbour/second-nearest-neighbour model*[a]
A. niger *β-D-mannanase*			
$Man_2 + Man_3$ (wt. %)	45	45	43
Gal^1Man_2	16	19	14
Gal^1Man_3	7	14	11
$Gal^{3,4}Man_5$	11	4	7
Octa- + nona-saccharides	11	11	16
>Nonasaccharides	11	7	9
D-Galactose content (%)	18 ±1	17	17
Degree of hydrolysis (%)	26 ±1	28	26
Guar-seed β-D-mannanase			
$Man_2 + Man_3$ (wt. %)	26	20	21
Man_4	8	7	8
Gal^3Man_4	10	9	8
$Gal^3Man_5 + Gal^4Man_5$	11	10	9
$Gal^3Man_6 + Gal^4Man_6 + Gal^{3,4}Man_5$	10	8	10
Octa- + nona-saccharides	13	13	14
>Nonasaccharides	22	33	30
D-Galactose content (%)	18 ±1	17	17
Degree of hydrolysis (%)	20 ±1	18	18

[a]For $P_{00} = 0.19$; $P_{01} = 0.14$; $P_{10} = 0.34$; $P_{11} = 0.06$.

Hot-water-soluble carob galactomannan. — The patterns of amounts of oligosaccharides present in the *A. niger* or guar-seed β-D-mannanase hydrolysates of HWS-carob galactomannan are shown in Table II. In the *A. niger* β-D-mannanase hydrolysate, all of the oligosaccharides up to, and including, the four nonasaccharides have been characterised and quantified. This represents 89% of the total hydrolysate. The remaining 11% consists of fragments of average d.p. of 10–15 (based on the chromatographic pattern on Bio-Gel P-2) with a preponderance of fragments of d.p. 12. Treatment of this material of high d.p. with α-D-galactosidase, followed by concentration, results in the formation of an insoluble precipitate of manno-oligomers. However, if an aliquot (5 mL) of this fraction (at 1 mg/mL) is treated with α-D-galactosidase (20 nkat, 30 min) and applied directly to Bio-Gel P-2 at 60°, the oligomers do not precipitate and can be recovered quantitatively through the column. The major mannosaccharide eluted (80%) was manno-octaose. Based on the substrate sub-site binding requirements of the enzyme, at least eight different oligosaccharides having a manno-octaose backbone could theoretically be present. However, since the bulk of this material has a d.p. of 12

TABLE III

OLIGOSACCHARIDES RELEASED ON HYDROLYSIS OF COLD-WATER-SOLUBLE CAROB GALACTOMANNAN BY *A. niger* AND GUAR-SEED β-D-MANNANASES

Measured parameter	*Experimental data*	*Theoretical data*	
		Random model	*Nearest-neighbour/second-nearest-neighbour model*[a]
A. niger *β-D-mannanase*			
Man_2 + Man_3 (wt. %)	21	28	23
Gal^1Man_2	11	19	7
Gal^1Man_3	6	13	7
$Gal^{3,4}Man_5$	18	5	14
Octa- + nona-saccharides	18	16	26
>Nonasaccharides	26	19	23
D-Galactose content (%)	24 ±2	22	22
Degree of hydrolysis (%)	18 ±1	23	21
Guar-seed β-D-mannanase			
Man_2 + Man_3 (wt. %)	13	10	10
Man_4	2	3	3
Gal^3Man_4	8	6	5
Gal^3Man_5 + Gal^4Man_5	8	6	5
Gal^3Man_6 + Gal^4Man_6 + $Gal^{3,4}Man_5$	15	6	15
Octa- + nona-saccharides	17	12	18
>Nonasaccharides	37	57	44
D-Galactose content (%)	24 ±2	22	22
Degree of hydrolysis (%)	15 ±1	13	14

[a]For $P_{00} = 0.31$; $P_{01} = 0$; $P_{10} = 0.62$; $P_{11} = 0$.

and a D-galactose content of ~35%, the most probable structures are $Gal^{3,4,6,7}Man_8$, $Gal^{1,4,5,6}Man_8$, and $Gal^{3,4,5,6}Man_8$. Methods currently available do not allow further refinement of the structures in this mixed fraction.

This detailed experimental analysis of HWS-carob galactomannan clearly demonstrates that the major structural features are the presence of a very high proportion of substituted couplets and of isolated D-mannosyl residues substituted by D-galactose. There is a virtual absence of blocks of more than four contiguous substituted D-mannosyl residues, and the potential amount of substituted tetramers is also low.

Hydrolysis of HWS-carob galactomannan by guar-seed β-D-mannanase, due to the different sub-site binding requirements, results in a quite different pattern of amount of oligosaccharide products to that produced by *A. niger* β-D-mannanase. The oligomers of d.p. 2–7 (65% of the total hydrolysate) have been characterised and quantified. The combined octa- plus nona-saccharide fractions and the fraction of higher d.p. have been quantified, but the exact structures of the individual oligosaccharides have not been determined.

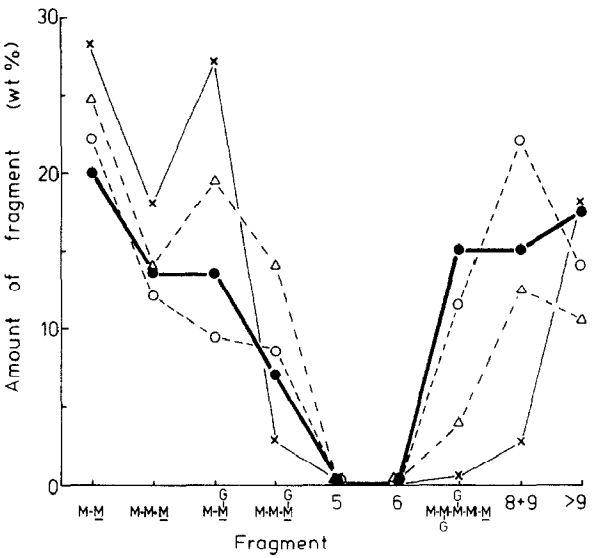


Fig. 4. Oligosaccharides released on cleavage of carob galactomannan (total) by *A. niger* β-D-mannanase: a comparison of experimental data (●) and theoretical data based on (*1*) a statistically random distribution of D-galactose (△, $P_{00} = P_{10} = P_{01} = P_{11} = 0.23$), (*2*) the current non-regular model (○, $P_{00} = 0.23$, $P_{10} = 0.50$, $P_{01} = 0.07$, and $P_{11} = 0.03$), and (*3*) the Gonzalez–Painter non-regular model (×, $P_{221} = 0.05$, $P_{211} = 0.17$, $P_{121} = 0.82$, and $P_{111} = 0.60$).

The experimental data for HWS-carob, particularly the high level of $Gal^{3,4}Man_5$ in the *A. niger* β-D-mannanase hydrolysate (Table II), is clearly not consistent with a statistically random distribution of D-galactosyl groups for the polymer. Rather, the pattern appears to be non-regular. In the end-wise, step-by-step synthesis of a mannan chain having a random distribution of D-galactosyl groups, the probability that D-galactose is transferred to the growing chain is independent of the nature of substitution of the rest of the chain. There is a single probability and this is equivalent to the proportion of D-mannosyl residues substituted by D-galactose. In contrast, in a galactomannan with a non-regular distribution of D-galactosyl groups, the probability of transfer of a D-galactosyl group to a D-mannosyl residue in the growing D-mannan chain is likely to be affected by the nature of substitution of adjacent D-mannosyl residues. We have developed a computer model in which the probability of a given D-mannosyl residue being substituted is dependent on the nature of substitution of the previous two residues, *i.e.*, a nearest-neighbour/second-nearest-neighbour model (Scheme 2; also see Experimental). Using this model, a better fit of the experimentally determined and the theoretically calculated values for degree of hydrolysis by β-D-mannanase, the D-galactose substitution of the polymer, and the amounts of the various oligosaccharides produced on cleavage by the two enzymes (Table II) can be obtained.

The fine-structure of the galactomannan is defined in terms of the four probability factors, P_{00}, P_{01}, P_{10}, and P_{11} (see Experimental). The high value for P_{10} indicates that the polymer contains a high proportion of substituted couplets and is

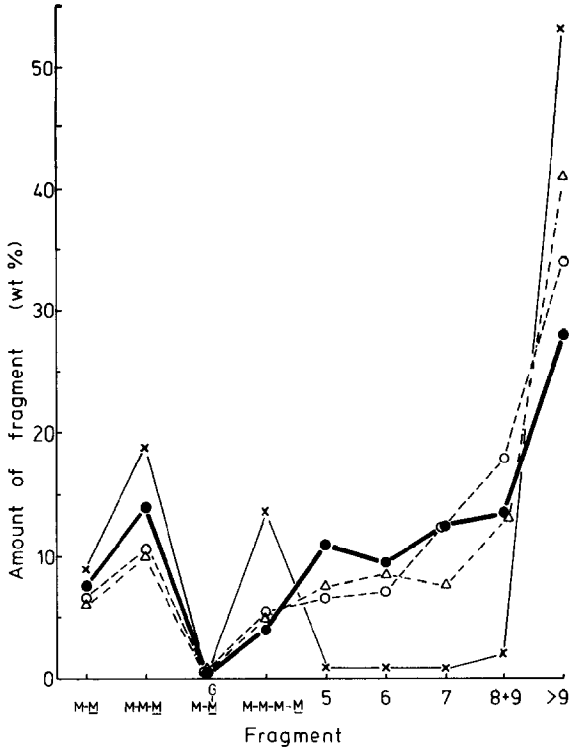
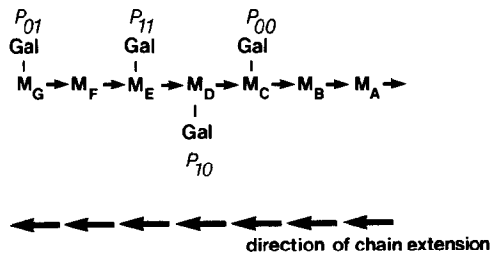


Fig. 5. Oligosaccharides released on cleavage of carob galactomannan (total) by guar-seed β-D-mannanase: a comparison of experimental data (●) and theoretical data based on (*1*) a statistically random model (△), (*2*) the current non-regular model (○), and (*3*) the Gonzalez–Painter non-regular model (×).



Scheme 2. Representation of galactomannan synthesis.

consistent with the very high proportion of the heptasaccharide $Gal^{3,4}Man_5$ and of octa- and nona-saccharides containing substituted couplets. The comparatively low value for P_{01} indicates that the proportion of alternating sequences of substituted and unsubstituted D-mannosyl residues is low. Again, this is consistent with experimental observations. The amount of Gal^1Man_2 in the *A. niger* β-D-mannanase hydrolysate of HWS-carob galactomannan is even less than that predicted to be produced on hydrolysis of a galactomannan with a random distribution of D-galactosyl

groups. Further, on hydrolysis of this galactomannan by guar-seed β-D-mannanase, significant proportions of the oligosaccharides Gal^3Man_4, Gal^3Man_5, Gal^4Man_5, Gal^3Man_6, and Gal^4Man_6 are produced. Each of these oligosaccharides will yield Gal^1Man_2 or Gal^1Man_3 on hydrolysis by *A. niger* β-D-mannanase, but none of them could have been derived from regions in the polymer molecule consisting of alternating sequences of substituted and unsubstituted D-mannosyl residues. The very low value for P_{11} suggests that the frequency of occurrence of substituted triplets would be quite low and that the probability of occurrence of larger blocks of substituted D-mannosyl residues would be even lower. This conclusion is consistent with the experimental observation that most of the D-galactosyl groups occur either on isolated D-mannosyl residues or as substituted couplets, and that even though substituted triplets are present, their frequency is low. The presence of substituted tetramers has yet to be experimentally demonstrated.

Cold-water-soluble carob galactomannan. — This fraction had a D-galactose content of 25% (Table III) and consequently was hydrolysed to a lesser extent (18%) by *A. niger* β-D-mannanase than was HWS-carob galactomannan (26%). Only the oligosaccharides up to d.p. 7, representing 58% of the hydrolysate, were characterised in detail, and the structures were identical to those of the products of hydrolysis of HWS-carob by this enzyme. The octasaccharide and nonasaccharide fractions were shown to be present in the ratio ~1:1. Since the structures of the reaction products are a consequence of the action pattern of the enzyme, it can be confidently concluded that the octa- and nona-saccharides will have the same structures as those present in the HWS-carob galactomannan hydrolysate. The exact amounts of the individual octa- and nona-saccharides were not determined. Of particular note is the high proportion of the heptasaccharide $Gal^{3,4}Man_5$ which, as in the hydrolysate of HWS-carob galactomannan, was equal to the combined amounts of octa- plus nona-saccharides. This feature is relatively peculiar to carob galactomannan, as the *A. niger* β-D-mannanase hydrolysates of a range of other galactomannans of similar D-galactose content, including those from *Caesalpinia pulcherima, Cassia fistula,* and *Parkinsonia aculatea*, and α-D-galactosidase pre-treated fenugreek (see Table IV) and guar-seed galactomannans of similar D-galactose content, contain a much lower proportion of this heptamer.

The oligomers produced on hydrolysis of CWS-carob galactomannan by guar-seed β-D-mannanase were the same as those produced on hydrolysis of HWS-carob, but there were significantly less unsubstituted manno-oligomers and significantly more branched oligomers of d.p. >9. The amounts of branched oligomers of d.p. 4–9 were only slightly altered.

The patterns of amounts of oligosaccharides produced on hydrolysis of this fraction by either *A. niger* or guar-seed β-D-mannanases are not consistent with what would be expected from a galactomannan having a random distribution of D-galactosyl groups. The high values for the probability factors P_{10} and P_{00} and the nil values for P_{01} and P_{11} are again consistent with experimental observations which suggest that the polymer contains a high proportion of substituted couplets and of isolated D-mannosyl residues substituted by D-galactose.

Total carob galactomannan. — Total carob galactomannan from the commercial flour Vihep Merinos contains approximately equal amounts of HWS- and CWS-fractions, and it would be expected that the properties of this material would be intermediate between those of the two fractions. This in fact is the case, as shown by the D-galactose content, the intrinsic viscosity values, the degree of hydrolysis by *A. niger* and guar-seed β-D-mannanases, and the patterns of amounts of oligosaccharides produced on hydrolysis by either of these enzymes. The most striking feature of the *A. niger* β-D-mannanase hydrolysate, as already noted for the fractions, is the high proportion of $Gal^{3,4}Man_5$. Oligosaccharides up to and including the heptasaccharide have been quantified and structurally characterised. These represent almost 70% of the total hydrolysate. The octa- and nona-saccharides in the *A. niger* digest of the HWS-fraction have been structurally characterised and, since these structures are a consequence of the sub-site binding requirements of the enzyme, it is concluded that the same structures are present in the digest of total carob galactomannan. In this latter digest, the combined octasaccharides represent ~8% of the total digest, as also do the combined nonasaccharides. The amounts of the individual octa- and nona-saccharides are not known, but since the D-galactose contents of the combined octasaccharide fractions from HWS-, CWS-, and total-carob galactomannan are the same (29%), as also is the D-galactose content of the combined nonasaccharide fractions (33%), and the ratio of combined octasaccharides to combined nonasaccharides in each of the three fractions is 1:1, it may be expected that the ratio of individual component oligosaccharides in these fractions might be similar. Only limited information is available on the structure of material of d.p. >9. The data for chromatography on Bio-Gel P-2 indicate that most of the material is in the d.p. range 12–15, with a preponderance of oligomers of d.p. 12. The fraction has a D-galactose content of 35 ±1% and, on treatment with excess of α-D-galactosidase, an insoluble, white precipitate was formed. Attempts to fractionate the manno-oligomers produced on such treatment were not successful. For the material of high d.p. to contain only 35% of D-galactose and still be resistant to hydrolysis by *A. niger* β-D-mannanase, the D-mannan chain must contain a high proportion of substituted couplets, although a lesser, but significant proportion of triplets may also be present. The presence of larger blocks of substitution would also require the presence of unsubstituted sections of D-mannan chain (to balance the Gal:Man ratio), but this is not consistent with the resistance of these fragments to further hydrolysis by *A. niger* β-D-mannanase.

The pattern of amounts of oligosaccharides produced on hydrolysis of total carob galactomannan by guar-seed β-D-mannanase is intermediate between those produced from the HWS- and the CWS-fractions. The structures are a consequence of the sub-site binding requirements of the enzyme and thus manno-oligomers substituted by D-galactose on either the reducing or non-reducing terminal D-mannosyl residue, or on the penultimate D-mannosyl residue from the reducing end, are not present. Guar-seed β-D-mannanase is unable to cleave the glycosidic linkage of a D-mannosyl residue substituted by D-galactose; thus, regions of the chain contain-

ing the repeating unit –Man–Man(Gal)– are resistant to hydrolysis by this enzyme, even though they are readily hydrolysed by *A. niger* β-D-mannanase. It is thus apparent that, by judicious use of these two enzymes, it should be possible to obtain information on the amount of such sequences in the native galactomannan. Total carob galactomannan was treated with guar-seed β-D-mannanase under standard conditions to the hydrolysis limit, the products were fractionated on Bio-Gel P-2, and the material of d.p. >7, representing 38% of the total hydrolysate, was treated with *A. niger* β-D-mannanase. The reaction products were again fractionated on Bio-Gel P-2 and the trisaccharide fraction was collected and shown to represent only 0.9% of the total hydrolysate; this fraction consisted of Gal^1Man_2 and Man_3 in the ratio 88:12. This result clearly demonstrates that the polymer does not contain significant proportions of the repeating unit –Man–Man(Gal)–. This conclusion is supported by the fact that high proportions of the oligosaccharides Gal^3Man_4, Gal^3Man_5, Gal^4Man_5, Gal^3Man_6, and Gal^4Man_6 are present in the guar-seed β-D-mannanase hydrolysate of this galactomannan. None of these oligosaccharides could be derived from sections of the polymer containing repeating sequences of –Man–Man(Gal)–.

As noted for the HWS- and CWS-carob galactomannan fractions, the pattern of amounts of oligosaccharides produced on hydrolysis of total carob by *A. niger* or guar-seed β-D-mannanases is not consistent with a random distribution of D-galactose for the galactomannan. Rather, the data are more consistent with what would be expected from a galactomannan having a non-regular distribution of these branch units and having a high proportion of substituted couplets. This experimental observation has been confirmed by the development of computer programmes that simulate β-D-mannanase action and galactomannan synthesis based on nearest-neighbour and second-nearest-neighbour interactions (see Experimental). By systematically altering the probability factors P_{00}, P_{01}, P_{10}, and P_{11}, the best fit of theoretical to experimental data for total carob galactomannan was obtained and is shown in Figs. 4 and 5 (for *A. niger* and guar-seed enzymes, respectively). It is apparent that, with the non-random model, a much better fit of data can be obtained than for a polymer in which the D-galactosyl groups are randomly distributed. The best fit of data, again, was obtained with high values for P_{10} and P_{00} and low values for P_{01} and P_{11} (Scheme 2), consistent with the polymer's containing high proportions of substituted couplets and of isolated D-mannosyl residues substituted by D-galactose. However, even with this non-regular model, the predicted amount of $Gal^{3,4}Man_5$ released by *A. niger* β-D-mannanase is significantly less than that obtained experimentally. This finding indicates that, in the transfer of D-galactosyl groups to the growing D-mannan chain, there are longer range interactions than allowed for in the current model. These interactions may extend over the previous three or four D-mannosyl residues. A model which better fits the experimental data could be developed by considering these longer range interactions and introducing more probability factors, *i.e.*, for the various trimer combinations. However, with the amount of experimental observables available, it was considered

that the introduction of more probability parameters could not be justified. The need for a more elaborate model may also be indicated by the tendency of the present calculations to underestimate the amount of D-galactose present in the systems studied. For example, a good overall fit to the complete data set for total carob (see Figs. 4 and 5), using both the non-random and completely random computer models, implied a D-galactose content of 19% and a degree of substitution of 0.23. The corresponding experimental values were 22% and 0.28 (Table I). The discrepancy is less serious for the separate fits to cold- and hot-water-soluble carob, as may be seen from Tables II and III, though the trend to underestimation of D-galactose content is maintained.

Deviation from a strictly random distribution of D-galactosyl groups could, of course, either be towards a more regular distribution or a more block-like distribution. A consequence of the significantly higher proportion of substituted couplets in the carob galactomannan fractions is that there will be also a higher proportion of relatively unsubstituted regions along the chain. This is visualised in Fig. 6, where the theoretical distribution functions for substituted and unsubstituted blocks of D-mannosyl residues for total carob galactomannan are compared for a strictly random distribution of D-galactose and the optimised non-random model. From Fig. 6, it can be seen that there is a higher proportion of unsubstituted blocks of intermediate size for the non-random model (30% increase in unsubstituted blocks of d.p. 5–10). Furthermore, in reality, total carob galactomannan has an

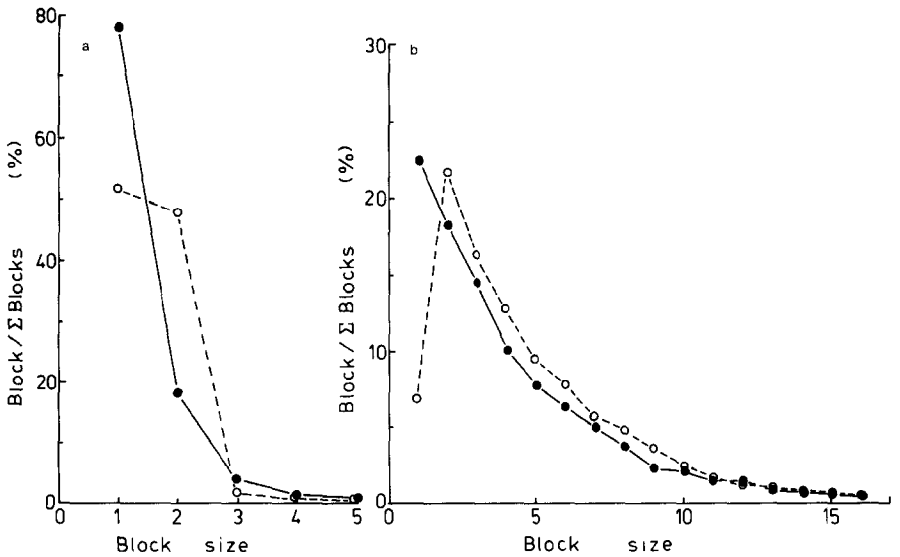


Fig. 6. The theoretical substituted (a) and unsubstituted (b) distribution functions of D-mannan block-size (*i.e.*, the numbers of blocks of a particular d.p. as a percentage of all substituted, or unsubstituted, blocks) for galactomannans having a D-galactose content of 19% and either a random (●, $P_{00} = P_{01} = P_{10} = P_{11} = 0.23$) or a non-random (○, $P_{00} = 0.23$, $P_{10} = 0.50$, $P_{01} = 0.07$, $P_{11} = 0.03$) pattern of distribution of D-galactose.

even higher proportion of substituted couplets than that predicted by the non-random model described in this study, and so the relative proportion of unsubstituted blocks will be even greater than that predicted.

As detailed in the Experimental section, the statistical treatment of data in the current model is essentially identical to that employed in an earlier study where experimental data were obtained from controlled periodate oxidation. However, for carob galactomannan, the probability values for trimer combinations were vastly different and consequently conclusions about structure are also very different. The values obtained in the previous study and in the current study (in brackets) were: P_{111} = 0.60 (0.03), P_{122} = 0.18 (0.93), P_{211} = 0.17 (0.50), P_{222} = 0.95 (0.77), P_{121} = 0.82 (0.07), P_{112} = 0.40 (0.97), P_{221} = 0.05 (0.23), and P_{212} = 0.83 (0.50). The former probability values lead to a non-random chain structure dominated by the repeating unit –Man–Man(Gal)–, as described previously[11]. In contrast, the current probability values give a different structure, dominated by the presence of substituted couplets, –Man–Man(Gal)–Man(Gal)–Man–, and of isolated, substituted D-mannosyl residues which, in general, appear not to occur as the repeating unit –Man–Man(Gal)–. On computer-simulated cleavage by *A. niger*

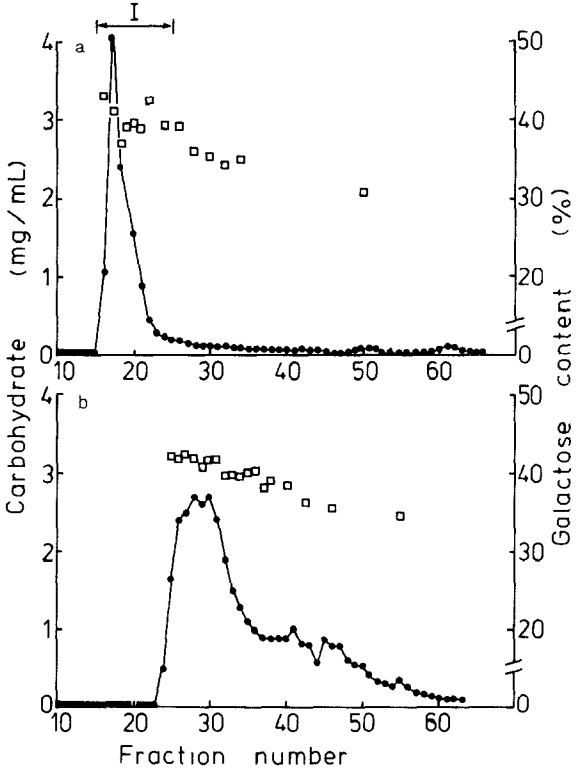


Fig. 7. Chromatography (5.4-mL fractions) on Bio-Gel P-6 (a) and Sephadex G-100 (b) of the oligosaccharides released on hydrolysis of guar-seed galactomannan by *A. niger* β-D-mannanase. Fraction "I" from Bio-Gel P-6 was applied to Sephadex G-100.

or guar-seed β-D-mannanase of a galactomannan synthesised using the probability factors of Gonzalez and co-workers[11], there was a poor fit of the patterns of amounts of oligosaccharides obtained to those observed experimentally (see Figs. 4 and 5).

Guar galactomannan. — The chromatographic pattern (Bio-Gel P-6) of the oligosaccharides produced on hydrolysis of guar galactomannan by *A. niger* β-D-mannanase is shown in Fig. 7. The galactomannans from all the guar varieties studied gave the same pattern of amount of oligosaccharide fragments. The bulk of the galactomannan hydrolysate was eluted near the void volume, and the fractions had D-galactose contents of 36–43%. This material could be partially fractionated on Sephadex G-100 and it is significant that none of the fractions had a D-galactose content approaching 50%. The degrees of hydrolysis of guar galactomannan by *A. niger* and guar-seed β-D-mannanases were 5 and 3%, respectively.

Analysis of the fine-structure of guar galactomannan by enzymic procedures is complicated by the fact that the polysaccharide is so highly substituted by D-galactose that it is only partially hydrolysed by β-D-mannanase. However, some useful and pertinent observations can be made. If guar galactomannan had a uniform distribution of D-galactose with a D-galactosyl group attached to every second D-mannosyl residue[6], then it would be resistant to hydrolysis by guar-seed β-D-mannanase[20] and would be quantitatively converted into Gal^1Man_2 by *A. niger* β-D-mannanase[16,20]. The fact that this does not occur is not surprising since the polymer contains 38% of D-galactose and not 33% as the model would require. *Leucaena leucocephala* galactomannan, which apparently has a high proportion of main chain composed of the repeating unit –Man–Man(Gal)–, yields significant quantities of Gal^1Man_2 on hydrolysis by *I. lacteus*[16] and *A. niger* β-D-mannanases. Also, the hexasaccharide $Gal^{1,3}Man_4$ is rapidly hydrolysed[20] by these enzymes to two mol of Gal^1Man_2. The degree of β-D-mannanase hydrolysis of guar galactomannan and the amount of Gal^1Man_2 released are actually less than the values theoretically predicted for a galactomannan having a D-galactose content of 38% and a random distribution of D-galactose. The results are more consistent with what would be expected from a galactomannan having a high proportion of substituted couplets, and thus support the model for guar galactomannan proposed by Hoffman and Svensson[8] in which the D-galactosyl groups are arranged mainly in pairs and triplets. A recent suggestion[9] that the D-galactosyl groups in guar galactomannan are arranged in blocks is totally inconsistent with the current results. Such a galactomannan would be hydrolysed by *A. niger* β-D-mannanase to fragments of high d.p. containing nearly 50% of D-galactose, plus unsubstituted mannosaccharides from the unsubstituted regions of the polymer chain. Such results were not obtained (Fig. 7).

Galactose-depleted fenugreek-seed galactomannan. — In an attempt to prepare a range of galactomannans having a random distribution of D-galactose for use in these studies, a highly substituted galactomannan from fenugreek seed (Gal:Man = 48:52) was modified to a range of D-galactose contents by treatment with α-D-

TABLE IV

OLIGOSACCHARIDES PRODUCED ON HYDROLYSIS OF α-D-GALACTOSIDASE-PRE-TREATED FENUGREEK GALACTOMANNAN BY *A. niger* β-D-MANNANASE

Oligosaccharide		*Amounts of oligosaccharides (wt. %) released from pre-treated galactomannans having different extents of* D-*galactose substitution*		
D.p.	*Structure*			
		43[a]	*37*[a]	*27*[a]
2	Man_2	8.5	12.0	17.6
3	Man_3	4.5	9.2	15.1
3	Gal^1Man_2	23.0	28.0	30.0
4	Gal^1Man_3	6.0	8.4	8.6
7	$Gal^{3,4}Man_5$	2.9	3.6	3.3
8 + 9	[b]	10.2	9 4	7.7
>9	[c]	44 9	29.4	16.0
Degree of hydrolysis by β-D-mannanase (%)		17.3	21 7	24.2

[a]The proportion of D-mannosyl residues substituted by D-galactose. [b]Structures determined[20], but not shown. [c]Structures not determined.

galactosidase. The patterns of amounts of oligosaccharides produced on hydrolysis of these polysaccharides by *A. niger* β-D-mannanase are shown in Table IV. The amount of $Gal^{3,4}Man_5$ is consistent with what would be expected from galactomannans having a random distribution of D-galactose, but the proportion of the trisaccharide Gal^1Man_2 is significantly greater than expected (see Fig. 3). It is thus apparent that this α-D-galactosidase does not remove D-galactosyl groups in a random manner, but rather has a tendency to remove them sequentially from one face of the mannan chain (which, in solution, has a fluctuating conformation that most probably averages at linkage to torsion angles close to those in the ordered two-fold form that has been characterised in the solid state), resulting in the release of D-galactose from alternate D-mannosyl residues and the production of a polymer with a high proportion of the repeating unit –Man–Man(Gal)–.

CONCLUSIONS

The current studies indicate that, in carob galactomannan (and in the HWS and CWS fractions), there is a non-regular distribution of D-galactosyl groups with a higher frequency of substituted couplets, of isolated, substituted D-mannosyl residues, and of unsubstituted blocks of intermediate length, than would be expected in a galactomannan with a statistically random distribution of D-galactosyl groups. The probability of the occurrence of substituted triplets or blocks, or of regions in which alternate D-mannosyl residues are substituted, is low. This defined pattern of non-regularity of the distribution of D-galactose in carob galactomannan appears to be unique to carob, and the galactomannan from all the carob varieties

currently studied showed the same pattern. This finding indicates that the synthetic system of the maturing seed is finely controlled and that the reported difference in the fine structure of carob galactomannan from different sources may be due to differences in the analytical procedures employed rather than to inherent differences in the material studied. Galactomannans (with the same D-galactose content as HWS-, CWS-, and total-carob galactomannans) from other seed sources, or produced on α-D-galactosidase modification of fenugreek, lucerne[19], or guar-seed galactomannan, have fine-structures different from those of the carob galactomannan fractions. The low values for the probability factors P_{11} and P_{01}, plus the fact that, in solution, the mannan chain of galactomannan is fluctuating in the region of the two-fold conformation, suggest that, in the synthesis of carob galactomannan, the transfer of D-galactose to the growing mannan chain by D-galactosyl transferase is sterically hindered by substitution of D-galactose on the D-mannosyl residue two units removed. Thus, in Scheme 2, transfer of D-galactose to M_G is sterically affected by substitution of D-galactose on M_E, but substitution on M_F has only a minor effect. This aspect of biosynthesis is discussed in greater depth elsewhere[34].

It has been proposed[8] that, in guar galactomannan, the D-galactosyl groups are arranged mainly in pairs and triplets. Results of the current enzymic studies are consistent with such a structure.

ACKNOWLEDGMENTS

The authors thank Mrs. E. Nurthen and Mrs. W. Sheppherson for expert technical assistance, and Dr. E. Morris for valuable discussions. Part of this work was performed by B. V. McCleary as visiting scientist at the Unilever Research Laboratory, Sharnbrook, and he thanks Unilever Research for financial support and the Nuffield Foundation for a Travelling Fellowship.

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