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Enzyme specificity in galactomannan biosynthesis

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Abstract. Membrane-bound enzymes from developing legume-seed endosperms catalyse galactomannan biosynthesis in vitro from GDP-mannose and UDP-galactose. A mannosyltransferase [mannan synthase] catalyses the extension of the linear (1→4)-β-linked D-mannan backbone towards the non-reducing end. A specific α-galactosyltransferase brings about the galactosyl-substitution of the backbone by catalysing the transfer of a (1→6)-α-D-galactosyl residue to an acceptor mannosyl residue at or close to the non-reducing terminus of the growing backbone. Labelled galactomannans with a range of mannose/galactose (Man/Gal) ratios were formed in vitro from GDP-[^{14}C]mannose and UDP-[^{14}C]galactose using membrane-bound enzyme preparations from fenugreek (*Trigonella foenum-graecum* L.), guar (*Cyamopsis tetragonoloba* (L.) Taub.) and senna (*Senna occidentalis* (L.) Link.), species which in vivo, form galactomannans with Man/Gal ratios of 1.1, 1.6 and 3.3 respectively. The labelled galactomannans were fragmented using a structure-sensitive *endo*-(1→4)-β-D-mannanase and the quantitative fragmentation data were processed using a computer algorithm which simulated the above model for galactomannan biosynthesis on the basis of a second-order Markov chain process, and also the subsequent action of the *endo*-mannanase. For each galactomannan data-set processed, the algorithm generated a set of four conditional probabilities required by the Markov model. The need for a second-order Markov chain description indicated that the galactomannan subsite recognition sequence of the galactosyltransferase must encompass at least three backbone mannose residues, i.e. the site of substitution and the two preceding ones towards the reducing end of the growing galactomannan chain. Data-sets from the three plant species generated three distinctly different sets of probabilities, and hence galactose-substitution rules. For each species, the maximum degree of galactose-substitution consistent with these rules was closely similar to that observed for the primary product of galactomannan biosynthesis in vivo. The data provide insight into the mechanism of action and the spatial organisation of membrane-bound polysaccharide synthases.

Key words: Biosynthesis (computer simulation) – Cell wall (plant) – *Cyamopsis* – Galactomannan – *Senna* – *Trigonella*

Abbreviations: Gal = galactose; Man = mannose

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Introduction

The galactomannans present in the endosperm cell walls of leguminous seeds all have a common structural pattern, namely a (1→4)-β-linked D-mannan chain substituted laterally by (1→6)-α-linked D-galactose residues (Reid 1985). The degree of galactose-substitution of the backbone is variable within the Leguminosae (Man/Gal ratio from ca. 1.1 to ca. 3.5), but is constant for a given species (Reid and Meier 1970a; Dea and Morrison 1975). Lower Man/Gal ratios (higher substitution) are typical of those taxonomic groups which are considered to be phylogenetically advanced (Leguminosae-faboideae), and higher Man/Gal ratios of the more primitive Leguminosae-caesalpinioideae (Reid and Meier 1970a). There is good evidence also that low-galactose species with identical Man/Gal ratios may differ in the statistical distribution of galactosyl residues along the mannan backbone (McCleary 1979a; Dea et al. 1986). Variations in galactose content and/or distribution within the legume-seed galactomannans clearly arise from differences either in the pathway of biosynthesis or in the post-synthetic processing of the galactomannan molecules.

The biosynthesis in vitro of galactomannans, catalysed by membrane preparations from the developing endosperms of fenugreek [*Trigonella foenum-graecum* L. (Leguminosae-faboideae, tribe Trifolieae; in vivo Man/Gal ratio = 1.1)] and guar [*Cyamopsis tetragonoloba* (L.) Taub. (Leguminosae-faboideae, tribe Indigoferae; Man/

Gal = 1.6)] has been studied in some detail (Campbell and Reid 1982; Edwards et al. 1989; 1992). Two membrane-bound enzymes, a GDP-mannose-dependent (1→4)-β-D-mannosyltransferase (or mannan synthase), and a UDP-galactose-dependent α-D-galactosyltransferase interact. The mannan synthase catalyses the elongation of the mannan backbone by successive transfer of mannosyl residues to the non-reducing terminus. The highly specific galactosyltransferase catalyses the transfer of an α-D-galactosyl residue *only* to a mannosyl residue at or close to the non-reducing terminus of the growing (galacto)mannan chain (Edwards et al. 1989).

Comparative studies on galactomannan deposition in developing fenugreek, guar and senna [*Senna occidentalis* (L.) Link (Leguminosae-caesalpinioideae; Man/Gal ratio in vivo = 3.3)] seeds have shown that in fenugreek and in guar there is no post-depositional modification of the Man/Gal ratio (Reid and Meier 1970b; Edwards et al. 1992). The different Man/Gal ratios characteristic of the two species are therefore determined in some way by the pathway of galactomannan biosynthesis itself. By contrast, in senna, the primary product of the biosynthetic pathway had a significantly higher galactose content (Man/Gal = ca. 2.3) than that observed in the galactomannan of the mature seed (Man/Gal = ca. 3.3). This was linked to a process of α-galactosidase-catalysed galactose loss which occurred during the final stages of galactomannan deposition, when the developing seed was already undergoing desiccation (Edwards et al. 1992).

We now present the results of experiments to determine the biochemical level at which the Man/Gal ratio is controlled within the pathway of galactomannan biosynthesis. Our earlier observation that the Man/Gal ratio can be manipulated in vitro via substrate levels (Edwards et al. 1989) indicated the possibility of regulation in vivo by the strict control of sugar-nucleotide levels. On the other hand, our observations that the degrees of galactose-substitution exhibited in vivo by the fenugreek and guar seeds could be approached but not surpassed in vitro (Edwards et al. 1989) suggested some measure of control via the transfer specificities of the glycosyltransferase enzymes themselves. Experimental data from the enzymatic fragmentation of galactomannans synthesised in vitro have been processed using a computer algorithm (McCleary et al. 1985) simulating our experimental model for galactomannan biosynthesis (Edwards et al. 1989) and the action of a structure-sensitive *endo*-(1→4)-β-mannanase (McCleary 1979a,; McCleary and Matheson 1983). We have in this way obtained quantitative statistical parameters to describe the transfer specificities of the galactomannan-biosynthesising enzymes from fenugreek, guar and senna. The quantitative statistical data provide insight into the specificities and molecular organisation of the membrane-bound galactomannan-synthesising enzyme complexes.

Materials and methods

Chemicals. Radiochemicals were purchased from DuPont NEN, Stevenage, UK. Purified carob (*Ceratonia siliqua* L.) seed galactomannan was prepared as before (Edwards et al. 1989). Specialised biochemicals were from Sigma, Poole, UK. General laboratory chemicals were at least of AR quality.

Membrane-bound enzyme preparations. Fenugreek *Trigonella foenum-graecum* L., guar *Cyamopsis tetragonoloba* (L.) Taub and senna *Senna occidentalis* L. plants were grown to flowering and fruiting under conditions which have been described elsewhere (Edwards et al. 1992). Membranes sedimenting at 40 000·*g* were prepared as described previously (Edwards et al. 1989) from fenugreek, guar and senna endosperms, hand-isolated at a stage of seed development during which intensive galactomannan synthesis was taking place [35–40 d, 35–40 d and 33–38 d after anthesis for fenugreek, guar and senna respectively (Edwards et al. 1992)].

Synthesis in vitro of labelled galactomannans. Membranes equivalent to 0.2–0.3 endosperms were incubated at 30°C for 1 h, in 25 mM Tris-HCl buffer containing 2.5 mM dithiothreitol (DTT), 2.5 mM $MgSO_4$, 5.0 mM $MnCl_2$, with UDP-galactose (800 μM) and GDP mannose (varied between 0.8 μM and 80 μM) – total volume 100 μl. The GDP-mannose and/or the UDP-galactose substrate was labelled with the appropriate nucleotide diphospho-[U-^{14}C]-sugars. Specific radioactivities were adjusted to 25–250 Bq·nmol^{-1}, and were checked by scintillation counting in each experiment. At the end of the incubation time, glacial acetic acid (50 μl) was added and the mixture was heated at 100°C for 2 min. Carrier galactomannan [100 μl of a 0.2% (w/v) solution of carob galactomannan] was then added and a pellet containing any labelled galactomannan product was isolated as described previously (Edwards et al. 1989).

Enzymatic fragmentation and analysis of galactomannans labelled in vitro. Labelled pellets prepared as above were digested with *Aspergillus niger endo*-(1→4)-β-D-mannanase (purified according to McCleary and Matheson 1983), under conditions which had been optimised to give complete fragmentation of the carrier galactomannan, and minimal transglycosylation. Digests were separated on silica-gel TLC plates (Kieselgel 60; E Merck, Darmstadt, FRG) using the solvent system *n*-propanol 5, nitromethane 2, water 3, by volume (three developments). In our first experiments, radioactive spots on the dried plates were detected by linear scanning of individual lanes (Dünnschichtscanner II; Berthold Instruments UK, St. Albans, UK). Zones corresponding to each labelled compound were scraped from the plate directly into a polypropylene microcentrifuge tube, followed by water (100 μl) to dissolve the radioactive saccharide. After 30 min. scintillation fluid (1 ml Optiphase X; LKB) was added and the radioactivity was measured by scintillation counting (Packard Tri-Carb 2000CA; Canberra Packard, Pangbourne, UK). In subsequent experiments the dried TLC plates were subjected to digital autoradiography (Berthold Digital Autoradiograph), and the relative amount of radioactivity asociated with each of the separated components of a hydrolysate was estimated by one-dimensional evaluation of each lane. This procedure was much more rapid, and gave results which were identical to those obtained by scanning, scraping of zones and scintillation counting of the separated compounds.

Data analysis by computer. Fragment data, obtained by enzymatic digestion of galactomannans synthesised in vitro, were analysed by computer methods described previously (McCleary et al. 1985). This involved computer generation of an ensemble (fifty) of galactomannan chains (one thousand mannose residues each) whose level and pattern of galactose-substitution were determined by four Markov chain conditional probabilities P_{00}, P_{10}, P_{01} and P_{11} (for more details see later discussion). An exhaustive enzyme [*A. niger endo*-(1→4)-β-D-mannanase] attack on each chain was simulated following rules of enzyme action established previously (McCleary and Matheson 1983). By averaging results over the full chain ensemble, predicted values were obtained for the weight fractions of the various fragment types produced, together with an estimate of the limiting degree of hydrolysis achieved. From the fragment data, five quantities were assembled: i.e. the weight fractions of species

M2+M3, M2G, M3G, M5G2 (for structures see *Results and Discussion*) and the sum of all octameric and nonameric fragments. These predictions were compared with corresponding experimental values obtained as described earlier, and the four probabilities adjusted by a numerical iterative, least-squares minimisation method to establish a best fit. Final values for the probabilities were output together with estimated standard deviations, and details of the final fit achieved. For the purpose of comparing probability values calculated for different synthetic galactomannans of the same type (fenugreek, guar or senna) the individual probability vectors were rescaled by dividing throughout by P_{00}. Error limits for these ratios were assigned using normal methods of error combination. In addition, for each set of probabilities (and hence sample analysed) an upper limit of possible galactose-substitution (Man/Gal ratio) was estimated by a further rescaling of the probabilities such that in each case the largest component was adjusted to unity. The limiting Man/Gal ratio was then calculated by simulating one thousand chains substituted according to these new probabilities and then averaging the degree of substitution predicted for a chain. Finally, for each galactomannan type, results were tabulated in order of increasing galactose content, and overall average values for the scaled probabilities and limiting Man/Gal ratios (together with estimated error limits) obtained by standard methods.

Results and discussion

Preparation and analysis of in-vitro biosynthetic products. Endosperms were excised manually from fenugreek, guar and senna plants at the stage of development when galactomannan was being formed (Edwards et al. 1992), and membrane fractions capable of catalysing galactomannan biosynthesis in vitro were isolated. Labelled galactomannans were formed by incubating the membranes in the presence of labelled sugar nucleotide precursors (GDP-[U-^{14}C]-D-mannose and UDP-[U-^{14}C]-D-galactose) of known specific radioactivity and molar concentration (Edwards et al. 1989). The labelled, in-vitro products were subjected to exhaustive digestion by a pure *endo*-(1→4)-β-D-mannanase from *A. niger* (McCleary and Matheson 1983). The optimum substrate subsite-binding requirement of this enzyme is a stretch of five (1→4)-β-linked D-mannosyl residues. Unsubstituted mannotetraose is nonetheless hydrolysed, but unsubstituted mannotriose is hydrolysed only very slowly. The influence of the lateral galactosyl substituents present in galactomannans on the action of the *A. niger* mannanase is well understood. Galactosyl-substitution at the second and/or the fourth mannosyl residue of the subsite recognition sequence prevents hydrolysis, whereas a galactosyl substituent elsewhere within the recognition site does not prevent the enzyme from acting (McCleary and Matheson 1983) (Fig. 1). Thus galactomannans with higher degrees of galactose-substitution are hydrolysed to a lesser extent than those with lower degrees of substitution. Also only certain well-defined oligosaccharide fragments are released from galactomannans by the action of this enzyme (McCleary 1979b; McCleary and Matheson 1983) (Table 1). Furthermore, the relative proportions of such structures released reflect not only the degree of galactose-substitution of the galactomannan substrate but also the statistical distribution of the substituents along the mannan backbone (McCleary et al. 1985).

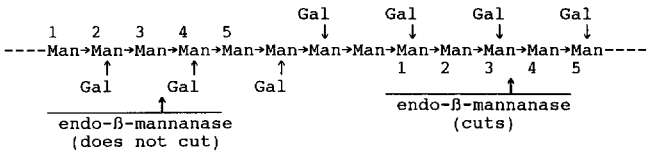

Fig. 1. Action of the structure-sensitive *endo*-(1→4)-β-D-mannanase from *Aspergillus niger* (McCleary and Matheson 1983)

Table 1. Structures of all oligosaccharides[a], up to the level of heptasaccharides, which can be released from galactomannans on exhaustive digestion with the *A. niger endo*-(1→4)-β-D-mannanase. Three octasaccharides, four nonasaccharides and several higher oligosaccharides are also allowed products (McCleary and Matheson 1983)

Structure	Name	Abbreviation
Man→Man	Mannobiose	M2
Man→Man→Man	Mannotriose	M3
Man→Man ↑ Gal	Galactosyl-mannobiose	M2G
Gal ↓ Man→Man→Man	Galactosyl-mannotriose	M3G
Gal ↓ Man→Man→Man→Man→Man ↑ Gal	Digalactosyl-mannopentaose	M5G2

[a] Slight traces of mannose (M) may also be generated, by slow hydrolysis of M3

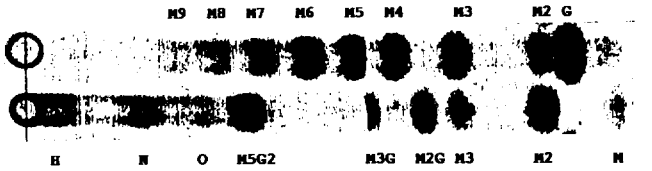

Fig. 2. Thin-layer chromatographic separation of oligosaccharide products formed on digestion of a low-galactose galactomannan [from carob (*Ceratonia siliqua* L.; Man/Gal = 3.5)] with the *A. niger endo*-β-mannanase. *M2* = mannobiose, *M3* = mannotriose, etc.; *M2G* = galactosylmannobiose, *M5G2* = digalactosylmannopentaose, etc. – structures as in Table 1; *O* = galactomannan octasaccharides; *N* = galactomannan nonasaccharides; *H* = higher galactomannan oligosaccharides; *G* = galactose. *Lower track* = galactomannan hydrolysate. *Upper track* = standards of manno-oligosaccharides + galactose

Clean separations of all fragment oligosaccharides up to the heptasaccharide level, and group separations of the octa- and nonasaccharides were achieved by TLC (Fig. 2, Table 1), and the *A. niger endo*-β-mannanase digests of the labelled in-vitro polysaccharides were separated in this way. The quantitative distribution of radioactivity on the TLC plates was analysed by digital autoradiography (Fig. 3). On comparing Figs. 2 and 3 it is clear that our labelled in-vitro products were galactomannans.

Labelled galactomannans with Man/Gal ratios ranging from 5.99 to 1.83 were synthesised in vitro using the biosynthetic membrane preparations from fenugreek,

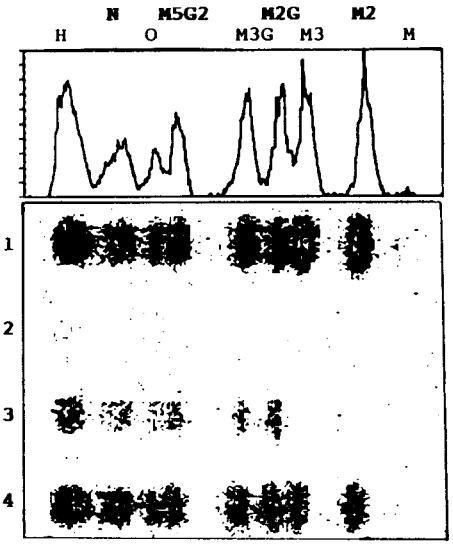

Fig. 3. Illustration of digital autoradiographic procedure. *Aspergillus niger endo*-(1→4)-β-D-mannanase digests of labelled polysaccharides synthesised in vitro using the senna enzyme preparation were separated by TLC and the dried plate was subjected to digital autoradiography. *Lower panel*, digital autoradiogram. *Lane 1*, digest of product formed from labelled GDP-mannose and unlabelled UDP-galactose. *Lane 2*, digest of product formed from labelled UDP-galactose alone. *Lane 3*, digest of product formed from unlabelled GDP-mannose and labelled UDP-galactose. *Lane 4*, digest of product from labelled GDP-mannose and labelled UDP-galactose. *Upper panel*, one-dimensional scan, for integration, of lane 1. *M* = Mannose; *M2* = mannobiose; *M2G* = galactosylmannobiose; *O* = octasaccharides etc. as in Fig. 2. Structures of (galacto)mannooligosaccharides as in Table 2

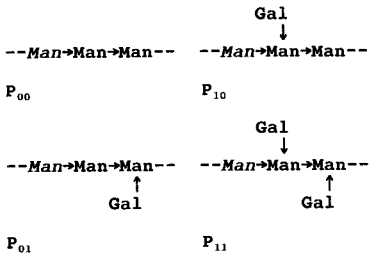

Fig. 4. The four possible patterns of galactosyl-substitution at the mannosyl residues nearest and second-nearest to the galactosyl-acceptor mannosyl residue (*italics*) at or near the non-reducing chain terminus. In our computer simulation of galactomannan biosynthesis it was assumed that the probability of obtaining galactosylsubstitution at the acceptor mannosyl residue is influenced by the pattern of galactose-substitution at the nearest and second-nearest mannosyl residues. Thus there are four independent probabilities P_{00}, P_{10}, P_{01} and P_{11} as illustrated

guar and senna endosperms. Experimentally, the Man/Gal ratios were varied by fixing the concentration of UDP-galactose at a saturating value (800 μM), and decreasing the concentration of GDP-mannose from a saturating level (80 μM) (Edwards et al. 1989). The relative amounts of the fragments M, M2, M3 M2G, M3G, M5G2, octasaccharides, nonasaccharides and higher saccharides released were calculated on a weight basis from the quantitative autoradiographic data (Fig. 3, lower panel). This information was processed using the computer algorithm.

The computer algorithm. Data-processing was carried out using a computer algorithm designed to investigate the statistical distribution of galactose substituents in natural galactomannans susceptible to hydrolysis with the *A. niger endo*-β-mannanase, i.e. those with a relatively high Man/Gal ratio (McCleary et al. 1985). It simulates a biosynthetic process in which the mannan chain is elongated towards the non-reducing end, galactose-substitution is permitted only at the extreme terminal (newly transferred) mannosyl residue, and the probability of obtaining galactose-substitution at the terminal mannose residue is influenced by the existing state of galactose-substitution at the nearest-neighbour and second-nearest-neighbour mannose residues only (a second-order Markov chain assumption). Thus there are four independent probabilities of obtaining galactose-substitution, namely P_{00}, P_{10}, P_{01} and P_{11}, defined as illustrated in Fig. 4. On inputting a set of four P-values (P_{00}, P_{10}, P_{01}, P_{11}) the algorithm will simulate first the biosynthesis of a galactomannan according to the statistical rules defined by the four probabilities, and then simulate the exhaustive digestion of that galactomannan using the *Aspergillus* mannanase, outputting a Man/Gal ratio and the molar ratios of the product galactomannan oligosaccharides which would be liberated by the action of the mannanase. Conversely, an input of sufficient quantitative experimental data from the hydrolysis of natural galactomannans in vitro by the *A. niger* mannanase will allow the generation of a set of four P-values. The P-values thus obtained for several natural, low-galactose galactomannans indicated inter-species differences in the distribution of the galactose residues on the mannan backbone (McCleary et al. 1985; Dea et al. 1986). Galactomannans with higher galactose contents, such as those from guar and fenugreek, were not analysed in this way because of the very low degrees of hydrolysis obtained using the mannanase. The biosynthetic model, assumed when the algorithm was written, is now known to be valid (Edwards et al. 1989). The second-order Markov chain assumption remained untested.

Processing of quantitative data from enzymatic fragmentation of galactomannans synthesised in vitro. The quantitative fragmentation data (M, M2, M3, M2G, M3G, M5G2, pooled octasaccharides, pooled nonasaccharides and pooled higher saccharides) obtained for each in-vitro galactomannan was sufficient to allow the calculation of a set of four probabilities P_{00}, P_{10}, P_{01}, P_{11} and a Man/Gal ratio. To allow direct comparison of probability sets derived from galactomannans with different Man/Gal ratios, each was scaled linearly, to give P_{00} the arbitrary value of unity. It was then clear that all the probability sets derived from products synthesised using enzymes from a given plant (fenugreek, guar or senna) were, within estimated error limits, indistinguishable (see Table 2 for fenugreek results). Yet the fenugreek, guar and senna consensus values were distinctly different (Table 3). The close similarity of all the probability values for a single biosyn-

Table 2. Computer-generated probability sets for all fenugreek in-vitro galactomannan products. Individual data-sets were scaled linearly for comparison by letting $P_{00} = 1.00$. Thus the values for P_{00}, P_{10}, P_{01}, P_{11} in this Table were obtained by dividing each numerical value within a set by the numerical value of P_{00} for that set. Each scaled value is accompanied, in parentheses, by the degree of uncertainty in the value. The average probability set is accompanied by the degrees of uncertainty in the average values

Man/Gal ratio of in vitro galactomannan	Scaled probabilities			
	P_{00}	P_{10}	P_{01}	P_{11}
5.64	1.00 (0.00)	1.15 (0.36)	2.85 (0.89)	3.31 (1.62)
4.74	1.00 (0.00)	1.75 (0.38)	3.92 (1.06)	4.58 (1.07)
4.46	1.00 (0.00)	1.25 (0.15)	2.38 (0.46)	3.19 (0.43)
4.37	1.00 (0.00)	1.11 (0.21)	1.94 (0.60)	2.50 (0.77)
4.29	1.00 (0.00)	1.19 (0.10)	2.69 (0.21)	3.25 (0.28)
3.88	1.00 (0.00)	1.15 (0.12)	1.95 (0.27)	2.20 (0.32)
3.62	1.00 (0.00)	1.04 (0.21)	1.33 (0.53)	1.92 (0.55)
3.42	1.00 (0.00)	1.14 (0.15)	2.33 (0.44)	2.00 (0.47)
3.33	1.00 (0.00)	1.20 (0.19)	2.60 (0.56)	2.35 (0.55)
3.28	1.00 (0.00)	1.08 (0.17)	1.62 (0.43)	1.27 (0.48)
2.79	1.00 (0.00)	1.41 (0.24)	2.73 (0.59)	2.09 (0.46)
2.78	1.00 (0.00)	1.21 (0.20)	2.33 (0.51)	2.13 (0.43)
2.60	1.00 (0.00)	0.95 (0.09)	0.95 (0.21)	0.93 (0.14)
2.53	1.00 (0.00)	1.35 (0.24)	2.19 (0.57)	2.00 (0.44)
2.38	1.00 (0.00)	1.19 (0.48)	2.10 (1.00)	1.03 (0.63)
2.30	1.00 (0.00)	1.15 (0.27)	2.06 (0.57)	0.82 (0.38)
2.16	1.00 (0.00)	1.26 (0.24)	2.00 (0.50)	1.87 (0.38)
2.01	1.00 (0.00)	1.64 (0.55)	1.44 (1.12)	1.53 (0.65)
1.97	1.00 (0.00)	1.80 (0.46)	3.12 (0.91)	2.08 (0.59)
1.93	1.00 (0.00)	1.44 (0.49)	2.25 (0.94)	1.72 (0.66)
1.89	1.00 (0.00)	1.25 (0.11)	1.55 (0.19)	1.45 (0.13)
1.83	1.00 (0.00)	1.39 (0.19)	2.03 (0.35)	2.03 (0.29)
Average[a]	1.0(0.0)	1.2(0.2)	2.1(0.6)	1.9(0.7)

[a] Weighted according to individual standard deviations

thetic system was a strong indicator that the second-order Markov chain assumption built into the computer algorithm was adequate. The differences between the P-values for fenugreek, guar and senna demonstrated that the fenugreek, guar and senna enzyme systems differed in their transfer specificities. In fenugreek, the value of P_{10} was not significantly different from P_{00}, indicating that galactosyl-substitution at the nearest-neighbour mannosyl residue did not alter the likelihood of obtaining galactose-substitution at the non-reducing terminal (or near-terminal) mannose residue which was the acceptor for galactosyltransfer. The values of P_{01} and P_{11} were, however, markedly higher than P_{00}, demonstrating clearly that a galactosyl substituent at the second-nearest-neighbour mannose enhanced strongly the likelihood of obtaining galactose-substitution at the acceptor mannose. The high value of P_{11} is particularly significant, implying that once a doublet of galactosyl substituents has been achieved by this enzyme system, there is an increased probability of obtaining a triplet and higher numbers of adjacent, substituted mannosyl residues. In guar, P_{10} is slightly, but significantly, greater than P_{00}. Consequently, substitution at the nearest-neighbour mannosyl residue increases the probability of galactose-substitution at the acceptor mannose: thus the formation of substituted doublets is slightly favoured. Neither P_{01} nor P_{11} is significantly different from P_{00}, indicating that neither alternations of substituted and unsubstituted mannose residues nor substituted triplets are favoured or discriminated against. In senna, P_{10} is slightly greater than P_{00}, but the difference is not so great as in guar. Furthermore, in senna, both P_{01} and P_{11} are very much smaller than P_{00}, P_{01} not differing significantly from zero. Thus, in sharp contrast to fenugreek, galactosylsubstitution at the mannosyl residue second-nearest to the acceptor mannose has a strong negative effect on the probability of attaining galactosyl-substitution at the acceptor mannose.

The values of the scaled probabilities P_{00} to P_{11} are quantitative expressions, defining the statistical transfer rules followed by each of the three galactomannan-synthesising enzyme complexes. These rules are independent of the Man/Gal ratio of the product galactomannans, at least over the range of Man/Gal ratios covered by our experiments. The values of the scaled probabilities incorporate a theoretical maximum galactose content for any galactomannan synthesised according to them. This is the Man/Gal ratio of the galactomannan defined by rescaling the probabilities linearly to let the highest value equal unity (100% probability). The consensus probability-sets for fenugreek, guar and senna were rescaled in this way and the predicted maximum galactose contents were calculated using the computer algorithm. In Table 3 they are presented as Man/Gal ratios alongside the in-vivo

Table 3. Consensus probability sets (P_{00}, P_{10}, P_{01} and P_{11}) for fenugreek, guar and senna, with predicted maximum Man/Gal ratios allowed by each set and Man/Gal ratios observed in vivo for each species. Uncertainties in each value are given in parentheses. The average values were obtained using 22 fenugreek data sets (Man/Gal ratio 5.65 to 1.83), 20 guar data sets (Man/Gal ratio 5.85 to 2.51), and 20 senna data sets (Man/Gal ratio 5.99 to 2.72)[a]

Plant Species	Average scaled probability values				Calculated maximum Man/Gal Ratio	Man/Gal ratio in vivo
	P_{00}	P_{10}	P_{01}	P_{11}		
Fenugreek	1.00 (0.0)	1.2 (0.2)	2.1 (0.6)	1.9 (0.7)	1.1 (0.2)	1.1
Guar	1.00 (0.0)	1.5 (0.2)	1.1 (0.2)	1.0 (0.4)	1.4 (0.2)	1.6
Senna	1.00 (0.0)	1.2 (0.1)	0.1 (0.1)	0.3 (0.2)	1.9 (0.2)	3.3[b] 2.3[c]

[a] The processing of some of the data sets used to compile this table has been reported in preliminary form (Reid et al. 1992)

[b] Man/Gal ratio of galactomannan in mature seed endosperm

[c] Man/Gal ratio of primary biosynthetic product before post-depositional modification (Edwards et al. 1992)

Table 4. Probability sets for native guar and senna galactomannans. Oligosaccharides released on enzyme-fragmentation of the polysaccharides were separated and estimated quantitatively. The data were used for the computer-generation of the probability sets

Species	Scaled probabilities			
	P_{00}	P_{10}	P_{01}	P_{11}
Guar[a]	1.0 (0.0)	1.5 (0.3)	1.1 (0.6)	1.2 (0.3)
Senna[b]	1.0 (0.0)	1.3 (0.2)	0.1 (0.3)	0.0 (0.3)

[a] Average of three determinations (one commercial guar gum; seeds from two laboratory-grown plants)
[b] Single determination on seeds from a laboratory-grown plant

Man/Gal ratios characteristic of the galactomannans present in the mature seeds of each species. In the case of fenugreek, the in-vivo Man/Gal ratio was identical to the predicted value. The in-vivo Man/Gal ratio for guar was slightly higher than the predicted value, but the difference may not be statistically significant. In senna, the predicted Man/Gal ratio (1.9), was very much lower than the Man/Gal ratio of the galactomannan in the mature seed (3.3). It was, however, very close to (although still significantly lower than) the Man/Gal ratio (2.3) of the primary product of biosynthesis, which, in senna, undergoes a process of post-depositional modification (Edwards et al. 1992). These observations provide strong evidence for the operation, in vivo in all three species, of the biosynthetic enzyme systems under conditions where the statistical transfer rules delineated in Table 3 apply, and the biosynthetic product has a degree of galactose-substitution which approaches the maximum allowed by those rules.

In principle further evidence for or against the operation of the deduced statistical rules in vivo would have been obtained by enzyme-fragmenting samples of the native galactomannan from seeds of each species and obtaining probability sets for comparison with those obtained using the in-vitro galactomannans. In practice this cannot be done for fenugreek, since native fenugreek galactomannan is almost fully substituted and is not appreciably attacked by the *A. niger* mannanase. Guar galactomannan is very highly substituted, and is hydrolysed only to the extent of 4–5% by the enzyme. Thus the estimation of the fragment oligosaccharides may involve relatively large errors. In the case of senna, enzyme fragmentation of the native polysaccharide does not present a practical problem, but the galactomannan in the senna seed results from biosynthesis followed by limited galactose-removal from the primary galactomannan product by an α-galactosidase (Edwards et al. 1992). Thus the deduced probabilities for senna will reflect the specificity of the biosynthetic process only if the action of the α-galactosidase is random. Nonetheless, probability values were obtained for the native galactomannans from mature guar and senna seeds (Table 4), and they were in very close agreement with those obtained for the corresponding in-vitro products (compare Tables 3 and 4).

A biochemical reading of the statistical rules for galactomannan biosynthesis. Our experimental observations generate the following three statistical statements. (i) The second-order Markov chain assumption built into our computer simulation of the biosynthetic process is adequate. (ii) The specificities of the biosynthetic enzyme systems from fenugreek, guar and senna are different, giving different statistical patterns of galactose-substitution along the mannan backbone. (iii) For each species the deduced statistical substitution rules define maximum permitted degrees of galactose-substitution which are approached by the degrees of galactose-substitution exhibited by the primary products of galactomannan biosynthesis in vivo.

The adequacy of the second-order Markov chain assumption implies that the (galacto)mannan substrate subsite recognition of the galactosyltransferases from fenugreek, guar and senna must encompass at least three backbone mannosyl residues: the one which is the site of reaction, and the two preceding ones, towards the reducing end of the chain. Other backbone mannosyl residues may be recognised by the galactosyltransferase, but their states of substitution do not influence greatly the probability of obtaining galactosyl-substitution at the reacting mannosyl residue. The existence of four different values for the statistical probability of obtaining galactosyl-substitution at the non-reducing terminal mannosyl residue indicates that the four structures depicted in Fig. 4 are not equally good substrates for the galactosyltransferases. In fenugreek, structures (01) and (11) are substituted most readily, and the others in the order $(10) \simeq (00)$. In guar the order is $(10) > (01) \simeq (00) \simeq (11)$, and in senna it is $(10) > (00) > (11) \simeq (01)$.

The observed maintenance, for a given species, of a constant ratio between the probabilities of obtaining galactosyl-substitution at the four possible galactomannan chain-ends depicted above, independently of Man/Gal ratio (i.e. of relative GDP-mannose and UDP-galactose substrate concentrations during galactomannan synthesis in vitro) is less easy to rationalise. It has been shown experimentally that the rate of mannosyltransfer by the mannan synthase is unaffected by the UDP-galactose concentration (i.e. by the rate of galactosyl-transfer), yet the rate of galactosyl-transfer, at saturating UDP-galactose concentration, is influenced by the rate of mannosyl-transfer (Edwards et al. 1989). Thus, the mannosyltransferase is insensitive to the state of galactosyl-substitution at or near the growing chain-end, and a constant rate of backbone chain elongation will be established for any given GDP-mannose concentration. Given a constant rate of backbone elongation, a constant ratio between the probabilities of obtaining galactose-substitution at the four different chain-end configurations could be maintained if each chain-end were exposed to the action of the galactosyltransferase only for a fixed time. This implies a precise, close spatial relationship between the mannosyl- and galactosyltransferases in the membrane. During this fixed time-window, given constant levels of UDP-galactose (saturating in our in-vitro experiments), the statistical probabilities of obtaining galactose-substitution would reflect the relative rates of galactosyl-substitution of the four different structures depicted in Fig. 4.

Our observation that the Man/Gal ratios of the primary galactomannan products formed in vivo in fenugreek, guar and senna approach the maxima predicted under the experimentally deduced statistical transfer rules implies that the relative rates of mannosyl- and galactosyl-transfer in vivo are such that the rate of galactosyltransfer is always close to that necessary to galactosyl-substitute the galactosyl-acceptor mannosyl residue of the growing mannan chain with 100% certainty when the pattern of galactosyl-substitution of the two neighbouring mannose residues is optimal. This is remarkable, and it is difficult at present to offer a full molecular rationalisation of this constraint. Nonetheless, it is clear that the different Man/Gal ratios characteristic of the primary products of galactomannan biosynthesis in fenugreek, guar and senna are a direct result of differences in the statistical patterns of galactosyl-substitution in the three species. These differences may reflect differences in the molecular structures and substrate-binding properties of the mannosyl- and galactosyltransferases in the three seed systems, their orientations in the membranes, or both. Current work is directed at the characterisation and purification of the biosynthetic transferases.

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