THE FINE STRUCTURE OF CAROB AND GUAR GALACTOMANNANS

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ABSTRACT

The fine structures of carob and guar galactomannans have been studied using highly purified, well characterised β-mannanases from culture filtrates of A. niger and from germinating guar seed. Oligosaccharide reaction products up to a degree of polymerisation of nine were quantitated and characterised. The patterns of amounts of oligosaccharides were not consistent with the galactomannan having a random D-galactose distribution. Computer programs were developed which simulated both β-mannanase action and galactomannan synthesis by a chain-extending process. Analysis of the experimental data using these programs indicated that the distribution of the D-galactosyl residues in both carob and guar galactomannans is in an irregular pattern. Carob galactomannan is typified by the presence of high proportions of substituted couplets but not triplets. Carob galactomannans from different seed varieties have essentially the same properties, including fine-structure. The same is true for galactomannans from different guar-seed varieties.

KEYWORDS

Galactomannan, carob, guar, fine-structures, β-mannanases, oligosaccharide products, computer analysis, galactose distribution.

INTRODUCTION

Galactomannan polysaccharides occur as reserve materials in a wide range of legume seeds in amounts varying from 0.1 to 38% of seed weight (Dea and Morrison, 1975). They are composed of a linear (1→4)-β-D-mannan backbone to which (1→6)-α-linked D-galactopyranosyl residues are attached as single unit side chains. The fine-structure of distribution of these D-galactosyl residues has received considerable attention in recent years (Painter, 1982) and will be discussed in detail in this article.

On seed germination, galactomannan is degraded and utilised. In guar seed the enzymes involved in galactomannan degradation are α-galactosidase (EC 3.2.1.22) for removal of the (1→6)-α-D-galactose side chains; β-mannanase (EC 3.2.1.78) for hydrolysis of the (1→4)-β-D-mannan backbone into oligosaccharides; and exo-β-mannanase (β-mannoside mannohydrolase, EC.3.2.1.25) for complete hydrolysis of the β-D-mannosaccharides to D-mannose (McCleary, 1983; Lee, 1965). Other enzymes involved in galactomannan degradation include β-mannosidase, from micro-organisms and intestinal tracts of certain animals, and a 1,4-β-D-mannan mannobiohydrolase from culture fluid of Aeromonas hydrophilia (Araki and Kitamikado, 1982). Several of these enzymes are useful in the characterisation of galactomannan fine-structure and in the characterisation of the structures of oligosaccharides released on partial degradation of galactomannans (McCleary, Taravel and Cheetham, 1982).

Galactomannans interact with several polysaccharides, resulting in a substantial viscosity increase and gel formation (Dea and Morrison, 1975). In general the degree of interaction is inversely proportional to the degree of D-galactose substitution on the D-mannan backbone. Guar galactomannan, in which 64% of the D-mannosyl residues are substituted by D-galactose, shows only a very limited interaction with xanthan, resulting in a viscosity increase rather than gelation. In contrast, carob galactomannan, in which only 30% of the D-mannosyl residues are substituted, interacts strongly with agarose, xanthan and κ-carrageenan resulting in the formation of a three-dimensional gel complex. This difference in interaction cannot be attributed to molecular weight differences as the polymers have similar intrinsic viscosities (McCleary, 1979) and for guar galactomannan, at least, this has been shown to be directly proportional to molecular weight (Robinson et al., 1982). The effect of D-galactose content on solution and interaction properties of guar galactomannan was clearly demonstrated in a recent study (McCleary et al., 1981) in which this polysaccharide was modified by α-galactosidase treatment to produce a series of galactomannans of decreasing D-galactose content. The enzyme employed was absolutely devoid of the chain-splitting enzyme β-mannanase. As the D-galactose content of the polysaccharide decreased, the degree of interaction with xanthan increased. Modified guar galactomannan with a D-galactose content of 19-25% had similar, but not identical, solution and interaction properties to carob galactomannan. Initial studies on the fine-structures of modified guar galactomannan with a D-galactose content of 19-25%, and of carob galactomannan, indicated that in both the D-galactosyl stubs are distributed in an irregular to random pattern.

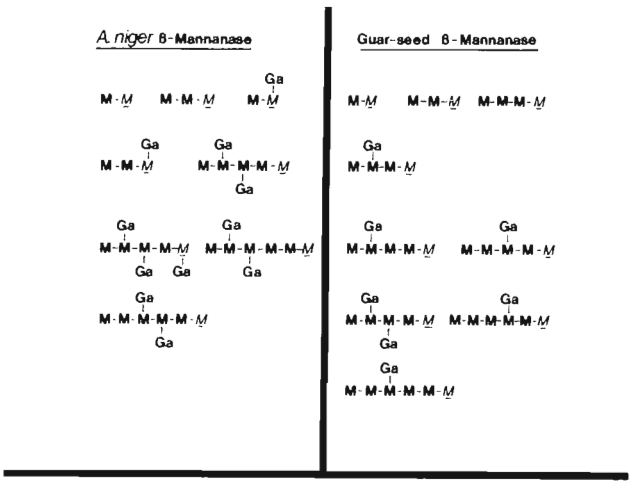
Enzymic Analysis of Galactomannan Fine-Structure

β-Mannanase degradation of galactomannans is dependent on both the proportion of D-mannosyl residues substituted by D-galactose and on the fine-structure of distribution of these D-galactosyl stubs. Evidence that fine-structure is important was clearly demonstrated in a study on the hydrolysis of galactomannans from guar and Leucaena leucocephala (McCleary, 1979). These galactomannans have the same D-galactose content, but the degree of hydrolysis and products released on hydrolysis by Irpex lacteus β-mannanase indicates that there are fine-structure differences. The significance of such studies was, however, limited by the lack of specific information on the subsite-binding and catalytic properties of the particular β-mannanase employed.

In the current study, two highly purified, well characterised β-mannanases with significantly different subsite-binding requirements have been used (McCleary and Matheson, 1983). The β-mannanases were purified from germinating guar seed and from Aspergillus niger culture filtrate preparations, by substrate affinity chromatography on glucomannan-AH-Sepharose 4B. Both enzymes appeared as single protein bands on SDS-gel electrophoresis, but the guar enzyme gave three major protein bands on gel isoelectric-focusing (McCleary, 1983). Each of these protein bands displayed β-mannanase activity and the action patterns were identical. The β-mannanases were absolutely devoid of α-galactosidase, β-mannosidase and exo-β-mannanase.

Information on the catalytic properties of the enzymes was obtained from detailed kinetic studies on the rates of hydrolysis of mannosaccharides, galactomannosaccharides, galactomannans and glucomannans (McCleary and Matheson, 1983). Characterisation of the products released on hydrolysis of galactomannans, glucomannans, galactomannosaccharides and glucomannosaccharides gave information on the subsite binding requirements. The only oligosaccharides of d.p.≤8 released on hydrolysis of galactomannan by A. niger β-mannanase and those of d.p.≤7 released by guar-seed β-mannanase are listed in Table 1. These were the only structures released, independent of the galactomannan studied.

TABLE 1 Structures of Oligosaccharides Produced on Hydrolysis of Carob Galactomannan by A. niger and Guar-seed β-Mannanases



Such studies lead to the conclusion (McCleary and Matheson, 1983) that for rapid hydrolysis of the β-1,4-mannan chain by guar-seed β-mannanase, binding across five D-mannosyl residues (A-E) is required (Fig. 1) and hydrolysis at X is prohibited by D-galactose substitution on sugar residues B, C or D. Rapid hydrolysis by A. niger β-mannanase, in contrast, 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.

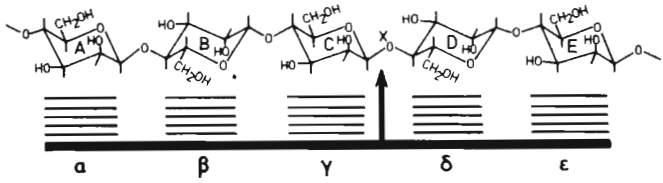


Fig. 1. Schematic representation of subsite binding between β-mannanase and the (1→4)β-mannan chain.

The Fine-Structure of Carob Galactomannan

Two galactomannan fractions can be readily and reproducibly extracted from finely milled carob-seed or commercial carob flour (Hui and Neukom, 1964). The fractions have been termed cold-water-soluble (CWS) and hot-water-soluble (HWS) and are extracted sequentially with water at room temperature (22°) then at 80°. The fractions differ significantly in their D-galactose content, intrinsic viscosities and susceptibility to hydrolysis by β-D-mannanase (Table 2). The patterns of amounts of oligosaccharides produced on hydrolysis of the carob galactomannan fractions by guar-seed β-mannanase (Fig. 2), and A. niger β-mannanase, are inconsistent with a block-type or a uniform pattern of distribution of D-galactosyl residues in the polysaccharides, but rather indicate that the residues are either irregularly or randomly distributed. In an attempt to define the degree of irregularity the experimental data was analysed using computer programs which simulated both β-mannanase degradation of galactomannan and galactomannan synthesis.

In these studies, the computer simulated galactomannan was 1,000 D-mannosyl residues long and the D-galactose distribution was either statistically random or was biased towards particular distribution patterns by nearest-neighbour and second-nearest-neighbour influences (non-random model) in a chain-extending program. A random pattern of D-galactose distribution was achieved if in the growing D-mannan chain the probability of a given D-mannosyl residue being substituted by D-galactose was totally independent of the nature of substitution of the rest of the chain. There is a single probability and this is equal to the proportion of D-mannosyl residues substituted by D-galactose. In the second chain building program the

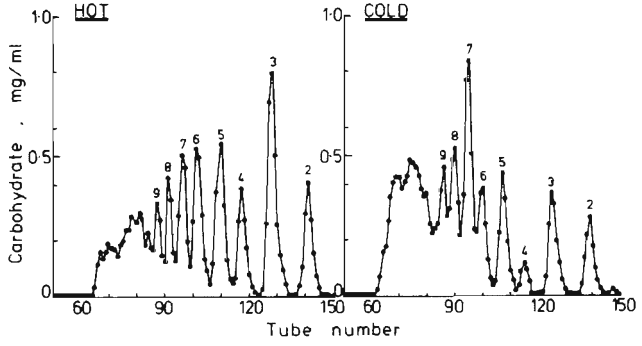


Fig. 2. Bio-Gel P-2 chromatography of the oligosaccharides produced on hydrolysis of hot-water-soluble and cold-water-soluble carob galactomannan with guar-seed β-mannanase. The numbers denote the d.p. of the eluted oligomers.

probability of a given D-mannosyl residue being substituted was dependent on the nature of substitution of the previous two residues, i.e. the nearest-neighbour and second-nearest-neighbour. Thus, four probabilities are involved, P_{00} (in which the previous two residues are unsubstituted), P_{01}, P_{10} and P_{11} (Fig. 3). These probabilities were optimised, in turn, through a minimisation of the sum of squared differences between the 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 both the A. niger and the guar-seed β-mannanases, and the amounts and structures of oligosaccharides of degree of polymerisation (d.p.) 2-9 produced on exhaustive hydrolysis by each of the enzymes.

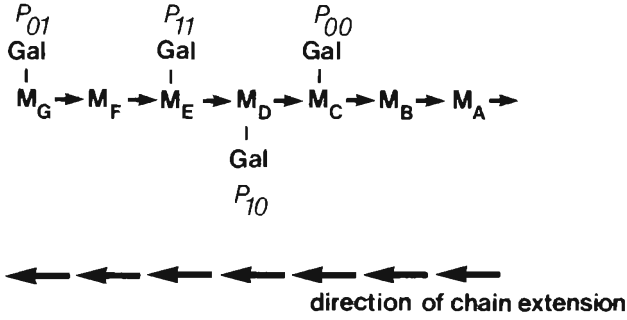


Fig. 3. Schematic representation of galactomannan synthesis.

The computer simulated β-mannanase program picked a point "X" at random on the simulated mannan chain and then examined the residues on the left-hand-side and right-hand-side of that point (Fig.1). With guar-seed β-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 may be unsubstituted (O) or substituted (I). With A. niger β-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 may 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 and after 100 the program recorded the total number of fragments as well as the structures and weight percentages of the individual fragments.

To reduce variability, the whole program of galactomannan synthesis (by either the random or non-random process) and degradation was repeated 100 times and averages were taken.

The theoretical degrees of cleavage by A. niger and guar-seed β-mannanases of galactomannans substituted to different extents and having a random D-galactose distribution, are shown in Fig. 4. Also shown is the experimental data obtained on hydrolysis of a range of native galactomannans and of several α-galactosidase pre-treated fenugreek galactomannan samples. The experimental and theoretical data are in reasonable agreement except that with A. niger β-mannanase the theoretical values are, in general, slightly higher than the experimentally derived values, and vice versa with the guar-seed enzyme.

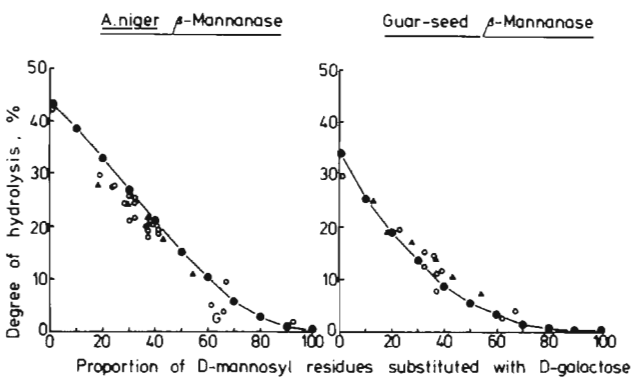


Fig. 4. Theoretical (●) and experimental [native galactomannans (○) and α-galactosidase modified fenugreek galactomannan samples (△)] values for the degree of hydrolysis of galactomannans by A.niger and guar-seed β-mannanases.

The structures of the oligomers produced by the computer simulated β-mannanases were in complete agreement with experimentally determined structures (McCleary et al, 1983), indicating that the defined rules for enzyme binding and hydrolysis are correct. However, the patterns of amounts of oligomers obtained experimentally on hydrolysis of carob galactomannan fractions are not consistent with the polymer having a random D-galactose distribution. There is a higher frequency of substituted couplets and a lower frequency of isolated D-galactosyl residues than predicted as shown by the larger than expected level of $Gal^{3,4}Man_5$ (see Table 1 for structure) and the lower than expected levels of $Gal^{1}Man_2$ and $Gal^{1}Man_3$ (Fig. 5). The D-galactosyl residues in carob galactomannan are thus irregularly distributed and we have attempted to define this degree of irregularity in terms of nearest-neighbour and second-nearest-neighbour interactions in a chain-extending computer program, as described. Using this program, the theoretical values obtained for A. niger β-mannanase cleavage of carob galactomannan are shown in Fig. 5. Also shown are the arrays of oligosaccharides obtained experimentally and those expected on hydrolysis of a galactomannan having a random D-galactose distribution. It is apparent that the best non-random model gives a better fit of theoretical to experimental data than does the random model. The discrepancies between this non-random model and experimental data are considered to be mainly due to longer range interactions, but allowance for these in the computer program would require the introduction of a large number of probability factors and this could not be justified with the number of experimental observables available.

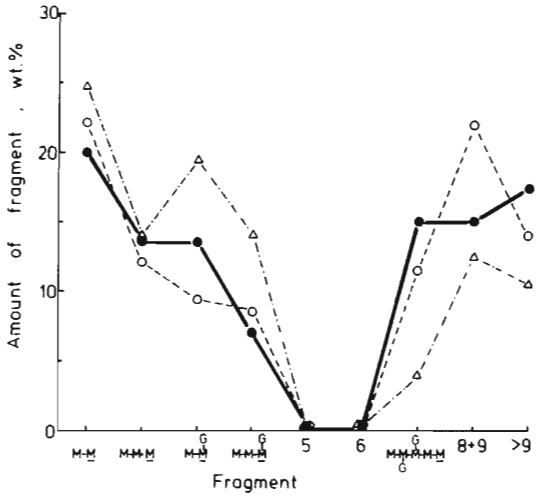


Fig. 5. Oligosaccharides released on hydrolysis of carob galactomannan by A.niger β-mannanase - a comparison of experimental data (●) and theoretical data for galactomannans having either a random (△) or a non-random (○) D-galactose distribution pattern.

The structure of the best non-random model for carob galactomannan is defined in terms of the four probability factors P_{00}, P_{01}, P_{10} and P_{11} (Fig. 3) where the values for P_{00} and P_{10} are large and those for P_{01} and P_{11} are small. Similar studies on the HWS and CWS carob galactomannan fractions showed that the values for P_{00} and P_{10} were greater than those for P_{01} and P_{11} (Table 3). Stated simply, these values show that in the synthesis of this galactomannan there is a high probability of transfer of a D-galactosyl residue to an isolated D-mannosyl residue and once such a residue has been substituted there is an even greater probability that the following D-mannosyl residue will be substituted, i.e. there is a high probability of substituted couplets. The low values for P_{11} and P_{01} show that the expected occurrence of substituted triplets or of small blocks of highly substituted regions or of regions in which every second D-mannosyl residue is substituted by D-galactose, is low.

It has been suggested (Painter, 1982) that the reported differences in the fine-structure of carob galactomannan may be due to differences in the material studied. It is well known (Hui and Neukom, 1964) that carob galactomannan can readily be fractionated into CWS and HWS material and that these fractions differ markedly in their D-galactose content. In the current study the HWS and CWS fractions and total carob galactomannan from a number of carob seed varieties and commercial seed and flour samples have been compared and the results are summarised in Table 2. It is evident that the properties of the three fractions are significantly different, but that within a polymer type the properties are very similar. There is slightly more variation in properties for the total galactomannans but this is to be expected since they are a mixture of HWS and CWS fractions and the ratio of these in a particular carob flour varies

TABLE 2 Properties of Carob* Galactomannan Fractions

PROPERTY	HOT WATER SOLUBLE CAROB	TOTAL CAROB	COLD WATER SOLUBLE CAROB
D-Galactose content, %	18 ± 1	22 ± 2	25 ± 1
Intrinsic viscosity, dL/g	13 ± 2	12 ± 2	10 ± 2
Hydrolysis by β-mannanase, %			
A. niger β-mannanase	26 ± 1	22 ± 1	18 ± 1
Guar-seed β-mannanase	20 ± 1	17 ± 1	15 ± 1

* Samples analysed included, seeds from ten varieties of carob, four commercial carob flour samples, and commercial seed samples from eleven different countries.

from 1:2 to 4:1 (unpublished data). Of particular note is the difference in intrinsic viscosities between fractions. If these are calculated as a function of the concentration of "mannan-backbone" (McCleary et al., 1981) rather than as a function of galactomannan concentration, an intrinsic viscosity of 14.5 ± 2 dL/g is obtained for all three fractions. If the direct proportionality between molecular weight and intrinsic viscosity, which has been found for guar galactomannan samples (Robinson et al, 1982), holds for carob galactomannan fractions, then it can be concluded that the molecular weight of carob galactomannan fractions are very similar.

The galactomannan fractions from each of the carob samples studied have been treated with A. niger β-mannanase and the oligosaccharide fragments separated on Bio-Gel P-2 chromatography. The patterns obtained for all the HWS fractions were, within the limits of experimental error, identical, indicating that the fine-structures were the same. The same results were obtained for the CWS fractions. Differences in the patterns between the HWS and CWS fractions could be attributed to their different degrees of D-galactose substitution. The ratio of the probability factors P_{00}, P_{01}, P_{10} and P_{11}, which in fact define the fine-structure, were similar.

TABLE 3 Nearest-Neighbour/Second-Nearest Neighbour Probability Factors for Carob Galactomannan Fractions

Probability Factor	Values of Probability Factors		
	HWS-Carob	Total-Carob	CWS-Carob
P_{00}	0.19	0.23	0.31
P_{10}	0.34	0.50	0.62
P_{01}	0.14	0.07	0.00
P_{11}	0.06	0.03	0.00

The Fine-Structure of Guar Galactomannan

Guar galactomannan is highly substituted with D-galactose and as a result is only slightly degraded by β-mannanase. Thus, β-mannanase treatment of this polymer yields less information on fine-structure than is obtained on similar treatment of carob galactomannan. However, from a knowledge of the subsite binding and catalytic properties of A. niger β-mannanase it is possible to conclusively demonstrate that guar galactomannan has neither a

block-type nor a uniform distribution of D-galactosyl residues. A galactomannan with a uniform D-galactose distribution with substitution on every second D-mannosyl residue, as proposed by Baker and Whistler (1975) for guar galactomannan, would be highly susceptible to degradation by A.niger β-mannanase yielding Gal^1Man_2 as the major or sole reaction product. The hexasaccharide $6^1,6^3$-di-α-D-galactosyl (1→4)β-D-mannotetraose is rapidly and completely hydrolysed by A.niger β-mannanase to Gal^1Man_2 (McCleary and Matheson, 1983) and Leucaena leucocephala galactomannan which appears to contain a high proportion of the repeating unit $-(Man-(Gal)Man)_n-$ yields Gal^1Man_2 as the major low d.p. product on treatment with this enzyme.

Recently, a block-type distribution for the D-galactosyl residues in guar galactomannan has been proposed (Hall and Yalpani, 1980). Guar galatomannan was oxidised using galactose oxidase and catalase and then subjected to further modification. Information on the distribution of nitroxide groups introduced by such a process, and by inference the distribution of D-galactosyl groups of guaran, was obtained from the dipole-coupling contribution to the electron-spin-resonance line width.

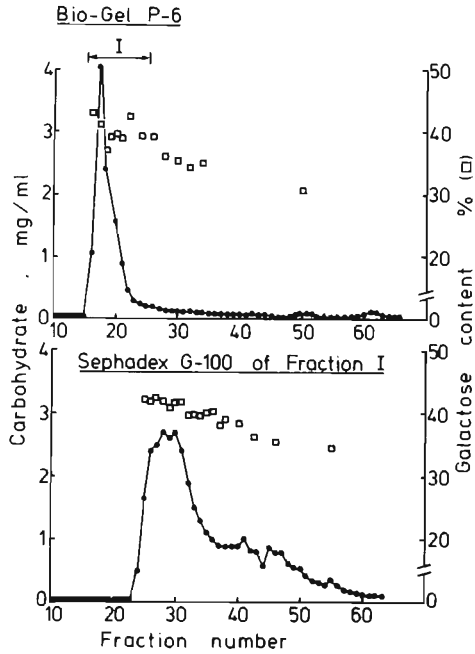


Fig. 6. Bio-Gel P-6 and Sephadex G-100 chromatography of the oligosaccharides released on hydrolysis of guar-seed galactomannan by A.niger β-mannanase.

The value of the nearest neighbour spins was considered to indicate that the D-galactosyl groups are distributed in blocks. These results contrast with those obtained in the current studies from β-mannanase degradation of guar galactomannan. The chromatographic pattern of the A.niger β-mannanase hydrolysate of guaran on Bio-Gel P-6 is shown in Fig. 6. Also shown is the pattern obtained on rechromatography of the high d.p. fraction on Sephadex G-100, together with the D-galactose content of the recovered fractions. It is evident that none of the fractions contain significantly more D-galactose than the native galactomannan, and the values are not consistent with a block-type distribution of D-galactosyl residues. The degree of hydrolysis of guar galactomannan (5%) by A.niger β-mannanase is significantly less than that predicted for a galactomannan with this D-galactose content and a random D-galactose distribution ("G" in Fig. 4). Rather the data tends to support the model for guar galactomannan proposed by Hoffman and Svensson (1978) in which the D-galactosyl residues are arranged mainly in pairs and triplets.

The chromatographic patterns on Bio-Gel P-2 of β-mannanase treated galactomannans from seeds of eleven guar varieties were indistinguishable and quite different to the pattern obtained on hydrolysis of Leucaena leucocephala galactomannan. This result together with the similar D-galactose contents of the polymers (McCleary, 1981) and the very similar intrinsic viscosities (15 $\pm$ 2 dL/g) indicates that galactomannans from seeds of different guar varieties are essentially identical.

CONCLUSIONS

The D-galactosyl residues in both carob and guar galactomannans are irregularly distributed along the D-mannan backbone. Carob galactomannan can be readily separated into hot- and cold-water-soluble fractions but the ratio of probability factors, which define the fine-structure of D-galactose distribution, are essentially the same. This polysaccharide is characterised by having a very high proportion of substituted couplets, but not triplets. The low value for P_{01} indicates that the proportion of blocks in which every second D-mannosyl residue is substituted by D-galactose is low. The proportion of blocks of unsubstituted D-mannosyl residues appears to be similar to that found in a galactomannan having a random D-galactose distribution. This proposed fine-structure is significantly different to that suggested by Painter et al. (1979).

Guar galactomannan also has an irregular distribution of D-galactosyl residues. The pattern appears to be similar to that proposed by Hoffman and Svensson (1978) in which there is a high proportion of substituted couplets and triplets. The results are totally inconsistent with a uniform or block type pattern of D-galactose distribution.

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