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On the galactosyl distribution of commercial galactomannans

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Abstract

A simple method was developed that enabled the enzymatic determination of the galactose distribution in galactomannans. *endo*-Mannanase of *Aspergillus niger* was used to degrade the galactomannan polymers and the degradation products were determined with high-performance anion-exchange chromatography. A whole range of commercial high-to-low substituted galactomannans was analyzed in this way. It was found that differences in the anion-exchange chromatograms reflected dissimilarities in the distribution of galactose and could be used directly to discern these dissimilarities. The differences among the various elution profiles were used to construct a similarity distance tree. In addition to this approach, the absolute amount of non-substituted mannose released by the enzyme was found to be a good discriminating factor. In this way, galactomannans with regular, blockwise, and randomly distributed galactose could be discerned. All guars and the highly substituted gum of *Prosopis juliflora* were found to have a blockwise distribution of galactose. For different batches of tara gum both random and blockwise distributions were found. Among batches of locust bean gum the greatest variation was observed: both random, blockwise, and ordered galactose distributions were present. Cassia gum was found to have a highly regular distribution of galactose. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Galactomannan; Mannose; Galactose; Distribution; Mannanase

1. Introduction

Galactomannans are plant reserve carbohydrates that occur in large amounts in the endosperm of the seeds of many Leguminoseae [1,2]. During sprouting they are degraded enzymatically and used as nutrient. Of the many galactomannans known, locust bean- (or carob), guar-, and tara gum are used predominantly on an industrial scale in food products [1]. Because of their high-water binding capacity, galactomannans are able to form highly viscous solutions even at low concentrations [1–3]. In combination with κ-carrageenan, agar, or xanthan gum a synergistic effect occurs which, depending on the concentration, can result in highly viscous solutions.

Galactomannans are composed of a β-(1 → 4)-linked D-mannose backbone to which single unit α-D-galactosyl residues are attached at

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Abbreviations: DB, degree of blockiness; DS, degree of substitution; HPAEC, high-performance anion-exchange chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAD, pulsed amperometric detection.

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O-6 [2]. The mannose–galactose ratio varies from about 10 to 1, depending on the source used. For locust bean-, guar-, and tara gum mannose–galactose ratios of 3.5, 1.5, and 3 are found commonly [1]. The distribution of the galactose units over the mannan backbone is important for the functional properties of the polysaccharide, especially for the synergistic effect of galactomannans on the gel forming properties of κ-carrageenan, agar, and xanthan [1,3]. The distribution of the galactosyl residues has been investigated by X-ray diffraction, NMR, and chemical and enzymatical degradation studies (reviewed in Refs. [2] and [4]). In particular, McCleary et al. have studied extensively the fine structure of galactomannans with enzymes [4–7]. Information on the galactosyl distribution was obtained by characterizing and quantifying the oligosaccharides released upon hydrolysis of galactomannans with highly purified *endo*-β-D-mannanases of *Aspergillus niger* and germinated guar seed [6,7]. These enzymes degrade specifically the non-substituted regions of galactomannans. The predominant degradation products were purified and identified using NMR, chemical, and enzymatic techniques [6]. From this, the action pattern and subsite binding requirements of the mannanases were obtained [7]. This information was put into a computer program that compared the amounts and structures of the oligosaccharides released with those that would be released after degradation of galactomannans with various simulated galactosyl residue distributions [4]. It was concluded that a more random distribution occurred in tara gum [8] while non-regular distributions were found in locust bean gum [4]. Guar was found to have few, if any, non-substituted regions [1,4].

Recent studies on the distribution of the functional groups over the polymeric backbone of the important food hydrocolloid pectin have resulted in the development of a new approach towards the use of enzymes in the determination of the substituent distribution of polysaccharides [9]. By determining the individual and total amounts of non-substituted (i.e., non-methyl esterified) residues released after enzymatic degradation of the homogalacturonan backbone of pectin, information on the occurrence and average size of the non-substituted regions could be obtained easily [10,11]. High-performance anion-exchange chromatography (HPAEC) and pulsed amperometric detection (PAD) were used for the separation and quantification of the oligomers formed. From this and the overall amount of methyl esterified residues determined, information on the occurrence and distribution of the galactosyl residues was derived [11] which could be coupled with (some of) the applications of the various pectin types discerned [12].

In this report, a method is described that enables the rapid determination of the fine structure of galactomannans with enzymes. The method is based on the approach developed for the determination of the methyl ester distribution of pectin. First, the galactomannan polymers are degraded extensively by *endo*-β-mannanase. The enzyme digests are analyzed subsequently by HPAEC and PAD detection. The galactosyl distributions of a whole range of commercial galactomannan preparations within a given series (e.g., locust bean-, guar-, tara- and cassia gums) were derived from their degradation profiles. The significance of this approach for the evaluation of a given commercial galactomannan and, even more importantly, the positioning of this sample *within* a certain group of galactomannans is discussed.

2. Results

Sugar composition of galactomannans.—The mannose and galactose content of the galactomannans investigated are listed in Table 1. Because the mannose backbone of galactomannans is only substituted by single-unit galactose residues, the amount of substituted mannose is expressed traditionally by the mannose–galactose ratio [1]. Alternatively, the extent of substitution was expressed as the percentage of the total amount of mannose present: the degree of substitution (DS in Table 1; standard deviation 0.2%). Upon high-performance anion-exchange chromatography (HPAEC) of the galactomannans, small amounts of mannobiose and mannotetraose were almost always observed. On average, the di- and tetramer amounts corresponded to 0.6 and 0.2% of the total amount of mannose present, respectively. Other monomeric–oligomers sugars were not found in any of the galactomannans with the exception of guar G64a. In this enzymatically modified sample [13], large amounts of monomeric galactose were detected which disturbed the DS determination (Table 1). To determine the DS of this galactomannan reliably, G64a was first dialyzed against demiwater and subsequently freeze dried. Determination of the sugar composition revealed a DS of 26%. From here on, dialyzed G64a will be denoted as G26-D.

Enzymatic degradation and chromatographic analysis of galactomannans.—All galactomannans were degraded extensively with *endo*-β-mannanase purified from the commercial *A. niger* preparation Gammanase. This was necessary because the commercial preparation contained some α-galactosidase activity that would remove galactose from the mannose backbone [14]. After 24 h of incubation the digests were analyzed with high-performance size-exclusion chromatography (HPSEC) and HPAEC. With the latter technique no galactose monomer was observed in any of the digests. The degradation profiles obtained with both chromatographic techniques were highly reproducible. The size-exclusion chromatograms of the enzymatically degraded samples almost depended entirely on the type of gum studied (Fig. 1). Hardly any differences were observed between the degradation profiles of samples of the same gum type (data not shown). HPAEC analysis, in contrast, revealed a much greater variation (Fig. 2). Differences between the elution profiles of digests of the same gum type are illustrated in Fig. 3. In this figure, the chromatograms of two locust bean gum digests are shown (L29a and L30). To enable an accurate comparison of the amount of mono- and oligomers produced, arabinose was included in all samples as an internal standard. Despite there almost identical DS (Table 1), the area of the peaks

Table 1
Mannose and galactose content and ratio, degree of mannose substitution (DS), degree of blockiness (DB), and the ratio of the galactose substituted- and non-substituted peak areas

Sample codes [a]	Mannose (w/w%)	Galactose (w/w%)	Man–Gal ratio	DS (%)	DB (%) [b]	Substituted- vs. non-substituted area ratio
C12	69.8	8.3	8.4	11.9	42.2	0.56
G26-D	66.0	17.1	3.9	25.9	36.8	1.06
G62	48.8	30.0	1.6	61.6	18.3	2.50
G64a	52.6	33.8	1.6	64.3		
G64b	51.9	33.5	1.6	64.5	15.3	1.93
G64c	52.5	33.5	1.6	63.7	14.9	2.01
G65	48.4	31.6	1.5	65.3	14.8	2.40
G66	47.1	31.2	1.5	66.2	17.3	2.11
G67a	40.3	27.2	1.5	67.4	11.5	3.02
G67b	50.3	33.7	1.5	66.9	10.8	2.60
G67c	47.7	31.8	1.5	66.6	11.6	2.58
G70	36.3	25.3	1.4	69.6	15.8	3.17
H80	48.3	38.8	1.2	80.5	6.4	3.37
L29a	73.8	21.3	3.5	28.9	48.0	1.40
L29b	71.8	20.8	3.5	28.9	32.9	1.23
L29c	66.4	19.0	3.5	28.7	46.0	1.25
L30	71.1	21.1	3.4	29.7	25.4	1.45
L32	45.0	17.3	3.2	31.6	36.3	1.38
M2	66.3	1.4	48.5	2.1	67.4	0.03
T34	64.9	22.3	2.9	34.3	41.1	2.19
T35	61.3	21.4	2.9	34.9	29.6	2.98

[a] C, cassia; G, guar; H, highly substituted galactomannan of *Prosopis juliflora*; L, locust bean; M, mannan; T, tara.

[b] Percentage of non-galactosylated mannose calculated from the formula given in the text (DB).

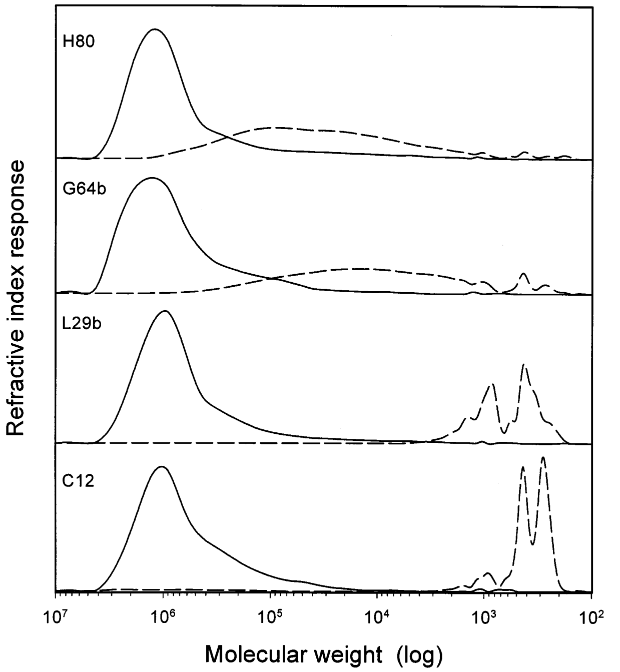

Fig. 1. Size-exclusion chromatograms of differently substituted galactomannans before (—) and after *endo*-mannanase (---) degradation. The capital letter in the sample code denotes the type of galactomannan, as listed in the legend of Table 1. The number in the sample code indicates the degree of substitution.

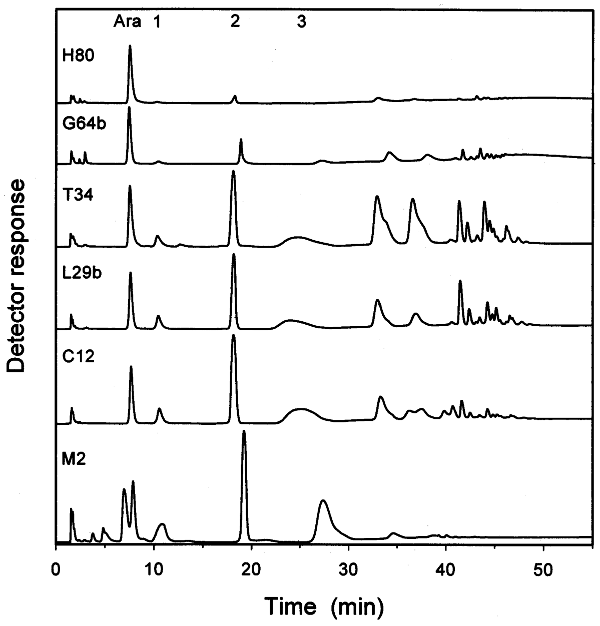

Fig. 2. High-performance anion-exchange chromatograms of *endo*-mannanase digests of the various types of galactomannanans. The numbers 1–3 indicate the peaks corresponding to the mono-, di-, and trimer of mannose, respectively. Ara indicates the arabinose internal standard.

observed for degraded L30 are much lower than those observed for degraded L29a, reflecting the fact that the enzyme is more restricted in its action on L30 than on L29a galactomannan.

In the high-performance anion-exchange chromatograms of the galactomannan digests, peaks with retention times identical to those of mannose and β-(1 → 4)-linked mannobiose and mannotriose (10.5, 19, and 26.5 min, respectively) were observed (Fig. 3). The retention times of the mono-, di-, and trimer varied somewhat within a series of digests (Fig. 2): maximum differences of ± 0.5 min were observed. Mannotetraose eluted at 34 min. Prolonged incubation of the galactomannan digests (up to 72 h tested) resulted in a gradual decrease of the mannotriose peak and an increase in the mannose and mannobiose peaks. Incubation of mannotriose with *endo*-mannanase revealed that the enzyme degraded mannotriose very slowly to a mono- and dimer. Because of this and the absence of changes in the other peak areas, digestion was routinely stopped after 24 h of incubation. To obtain information about the mass composition of the other peaks in the chromatograms, 80 μL of a tara gum digest was separated with the HPAEC-PAD system (Fig. 4). To the detector outlet of this system, two combined anion and cation desalting units and a microwell fraction collector were coupled [15]. A total number of 260 desalted fractions (of 250 μL) were collected during the first 65 min of

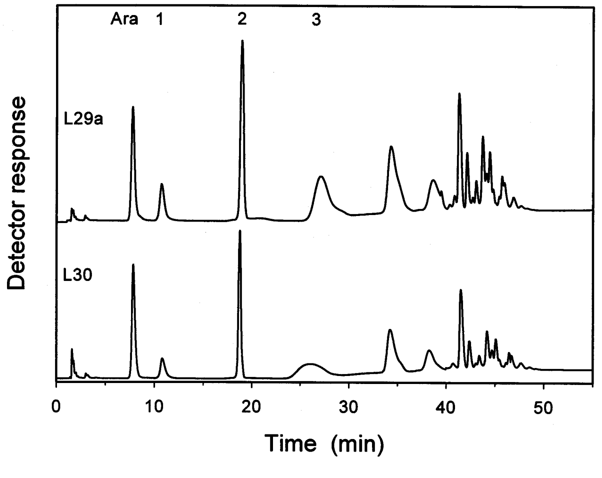

Fig. 3. High-performance anion-exchange chromatograms of *endo*-mannanase digests of locust bean gums L29a and L30. The differences in the elution patterns reflect that the enzyme is more restricted in its action on L30 than on L29a, indicating a non-identical distribution of galactose.

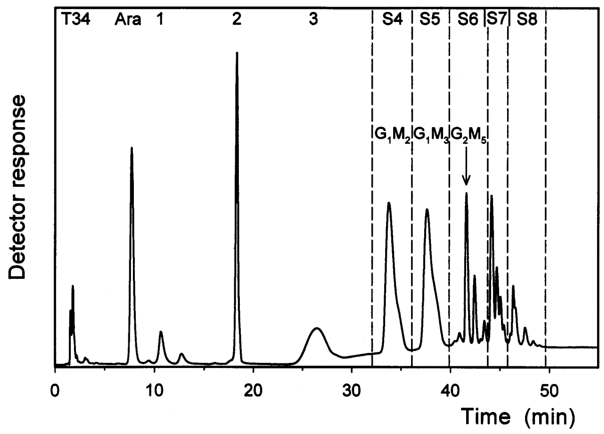

Fig. 4. High-performance anion-exchange chromatogram of the tara gum T34 digest. The peaks and peak sections used to calculate the degradation profile similarity are indicated. S4–S8 corresponds to the sections 4 through 8 as indicated by the dotted lines. Oligomers that could be assigned positively to a single peak after mass spectrometric analysis (see text) are indicated by G_xM_x, where G is the number of galactose and M is the number of mannose residues.

separation. After that time no peaks eluted and fractionation was stopped (Fig. 4). A Symbiot-I pipette–robot was used to automatically mix 1 μL of each fraction with matrix on a MALDI sample plate. Subsequently, the samples were analyzed by MALDI-TOF MS. Around the elution position of mannose monomer, which could not be detected by this MS technique because of matrix ion interference in that mass region, no components of higher mass were observed. Around the retention times of mannobiose and mannotriose, only components consisting of two and three hexose units were observed. The two peaks eluting at 34 and 37.5 min (Fig. 4) resulted from components with three and four hexose units, respectively. No components with four hexose units were detected around the elution position of the mannotetraose standard (34 min). Oligomers with seven and eight hexose units eluted at 41.6 and 42.4 min, respectively. After that, no clear distinction could be made between the elution position of the peaks and the occurrence of the various components as observed by MALDI-TOF MS. Oligomers of up to 19 hexose units were found. In the chromatogram of the mannan digest (Fig. 2) a peak is observed that eluted just in front of the arabinose internal standard. This product was only present after enzymatic incubation and did not co-elute with galactose, rhamnose, xylose, fucose, or glucose. Upon MALDI-TOF MS analysis of the corresponding fraction no components were observed.

Determination of the amount of non-substituted mannose residues liberated.—To enable quantification of the mannose mono-, di-, and trimer amounts released, the PAD-response factors were determined. The peak areas of various amounts of mono-, di-, and trimannose (0.1–1 mM) were obtained and compared to those of the arabinose internal standard (0.5 mM). For the mono-, di-, and trimer, the relative response factors were 0.85, 0.73, and 0.46, respectively. Mannotetraose had a relative response factor of 0.40. The peak area of arabinose was used to calculate the mono- to trimannose concentration of each sample accurately. Routinely, an area of about 4×10^8 was observed per μmol arabinose injected. After correction for the small amounts of mannobiose and mannotetraose present in the non-degraded sample, the total number of (non-galactosylated) mannose residues was calculated from the mono-, di-, and trimer amounts. The number of non-substituted mannose residues liberated was expressed as the percentage of the total number of non-substituted residues present per gram of galactomannan. This percentage is also known as the ‘degree of blockiness’ [10]. The calculation was carried out using the formula:

$$\begin{aligned}\mathrm{DB}\,(\%) = {} & \{[(\text{area } 1^0 \cdot \mathrm{RF}1^0) + (\text{area } 2^0 \cdot 2 \cdot \mathrm{RF}2^0) \\ & + (\text{area } 3^0 \cdot 3 \cdot \mathrm{RF}3^0)] \cdot \mu\text{mol/area Ara}\} \\ & \times [(\mu\text{mol Man/gram galactomannan}) \\ & \times (1 - (\mathrm{DS}/100))]^{-1} \times 100\end{aligned}$$

where Ara is arabinose, DB is the degree of blockiness, DS is the degree of galactose substitution, Man is mannose, RF is the relative response factor, and x^0 is a mannose fragment of 1–3 mannose residues.

The DBs as determined for all galactomannans studied are listed in Table 1. The results were obtained from *endo*-mannanase digestions performed in duplicate. A standard deviation of 1.5% was found for these values. In Fig. 5 the DBs are plotted versus the DS of the galactomannans. Interestingly, different

DBs can be observed for gums of similar DSs. For the mannan digest (M2) the highest DB value (67.4%) was found. By using the subsite model of *A. niger endo*-β-mannanase of McCleary and Matheson [7] (illustrated in Fig. 6) the DB of statistically randomly substituted galactomannans was calculated. A spreadsheet program was used to calculate the occurrence of non-galactosylated sequences of 1–1000 mannose units for randomly substituted galactomannans of DS 0–100% (in steps of 5%). The occurrence of sequences with a substituted mannose on both sides and those with a non- and substituted mannose on one and a substituted mannose on the other side were calculated, because these two types of 'blocks' are non-identically degraded by the enzyme [4]. For these two series of blocks the average amount of mannose residues liberated by the enzyme was calculated and multiplied by the chance of the occurrence of that type of sequence. By summation of these amounts for both types of blocks for all sequences, the total number of non-substituted mannose residues that could (theoretically) be released by the enzyme was obtained. This amount was expressed as the percentage of all non-substituted mannose present and is indicated by the dotted line in Fig. 5. Inclusion of mannose sequences greater than 1000 had no effect on the calculated DB values.

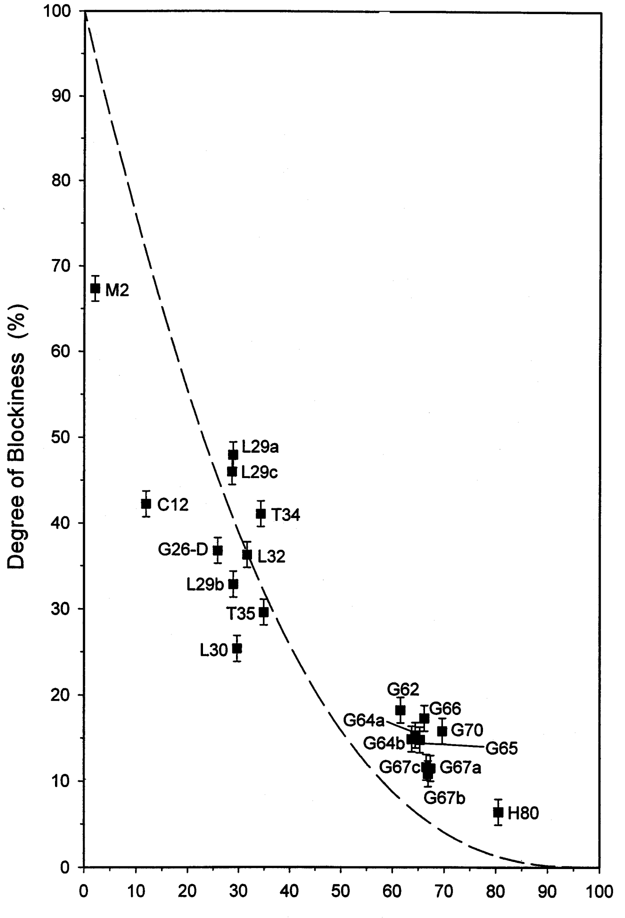

Fig. 5. Percentage of non-substituted mannose residues liberated after *endo*-mannanase degradation of galactomannans (DB, degree of blockiness) vs. the DS. The dotted line indicates the calculated results of *endo*-mannanase degradation of randomly substituted galactomannans of various DSs. The standard deviation is indicated by error bars.

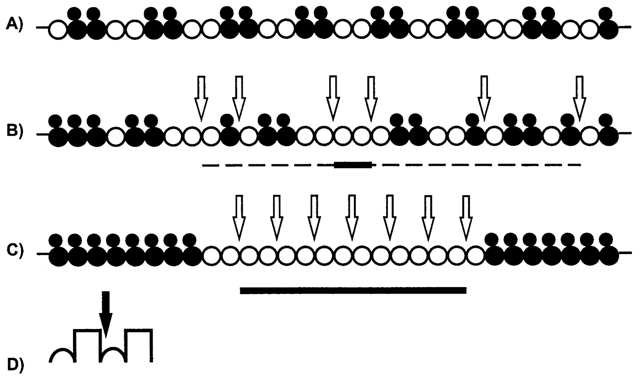

Fig. 6. Schematic representation of *endo*-mannanase action on three DS 50 galactomannans with a different galactose distributions. Galactose- and non-substituted mannose are represented by black and open circles, respectively. Galactose is drawn as a smaller black circle. Three typical distributions are shown: A, ordered; B, random; C, blockwise. White arrows indicate the sites of enzyme attack. The non- and galactose substituted mannose molecules released are drawn with solid and dashed underscores, respectively. In D the subsite model of *A. niger endo*-mannanase of McCleary et al. [4] is illustrated. Semicircles represent subsites that can only be occupied by a non-substituted mannose. The rectangular subsites can be occupied by both non- and galactose-substituted mannose residues. The black arrow indicates the catalytic group.

Comparison of the degradation profiles of galactomannans.—Because the PAD response factors of the galactose substituted mannose oligomers were not determined, the exact amount of these type of oligomers present in the digests could not be calculated. However, by comparison of the ratio of the total area of the peaks eluting after 32 min (all resulting from substituted oligomers) and the combined mono-, di-, and trimannose peak area, additional information regarding the distribution of substituents can be revealed [9,11]. From Table 1 it is clear that this ratio differs quite substantially between the various digests. Especially tara and locust bean gum, which both have quite a similar DS, had very different ratios. Among the guar digests the ratios also differed.

Since variations in the distribution of galactose results in the formation of dissimilar amounts and different types of degradation products (Fig. 6), the HPAEC elution profiles of the digests can also be used as an indicator for the overall (dis)similarity in the distribution of galactose. To enable this, the chromatograms were divided into sections indicative of specific chromatographic features. The eight features discerned are indicated as 1–3, and S4–S8 in Fig. 4. Splitting of the chromatograms into even more sections had no additional discriminating effect. The features 1–3 correspond to the mono-, di-, and trimannose peaks. The sections S4–S8 are comprised of the individual and/or assembled areas of the peaks eluting in specific regions as indicated by the dotted lines in Fig. 4. To assure a similar contribution of the eight chromatographic features, the total peak area of each section was normalized to numbers varying between 0 and 1; the latter corresponded to the maximum area found in each region for all the chromatograms. Next, a matrix was constructed for each of these sections in which the pairwise differences (in absolute value) of the galactomannan digests were plotted. The mean degradation profile similarity of all samples was calculated from the pairwise differences of the 1–3 and S4–S8 results. The mean distances obtained are listed in Table 2. The FITCH program [16] of the PHYLIP package [17] was used to construct a non-rooted similarity tree from the data of Table 2. Since previous studies had already revealed that the FITCH program was best suited for the construction of trees from data derived from HPAEC profiles [9,12] only the tree obtained with the FITCH algorithm is shown. With FITCH the global search option was used. The average tree obtained is shown in Fig. 7. Here, the largest variation in the distribution of galactose can be observed for locust bean gums. However, among the tara and guar samples non-identical distributions were also found.

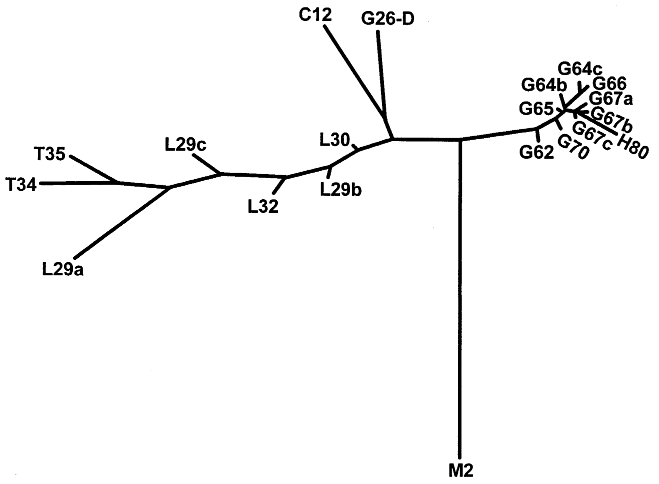

Fig. 7. Fitch–Margoliash tree showing the similarity of the high-performance anion-exchange chromatograms of the *endo*-mannnase digests of galactomannans. The tree was constructed from the data of Table 2.

3. Discussion

From the preceding section it is evident that with HPAEC analysis all non-substituted mannose molecules formed after extensive *endo*-mannanase degradation of galactomannans can be identified and quantified. Since no components of four hexose units were observed around the retention time of mannotetraose (34 min), this indicated that all components eluting after the retention time of the mannose trimer (26.5 min) contained one or more galactose units. Because the degradation mechanism of the enzyme employed [4,7] and the composition of the major degradation products [6,7] were described extensively before, the galactose containing degradation products could be identified by comparing their relative HPAEC elution position and mass composition. From this it was obvious that all identified peaks eluting after 32 min resulted from galactose substituted oligomers with two or more mannose units (Fig. 4). The component that eluted just in front of the arabinose internal standard of the mannan digest (Fig. 2) could, unfortunately, not be identified. Since this product was only formed after enzymatic incubation of the mannan sample and — most likely — resulted from some unidentified component present in the substrate, it was ignored.

Table 2
Sequence similarity distances as calculated from the HPAEC degradation profiles of galactomannans

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	C12	0	0.200	0.308	0.314	0.312	0.322	0.312	0.348	0.348	0.336	0.322	0.383	0.373	0.225	0.279	0.232	0.279	0.346	0.430	0.455
2	G26-D	0.200	0	0.261	0.298	0.289	0.292	0.292	0.284	0.297	0.304	0.299	0.336	0.366	0.220	0.255	0.235	0.292	0.478	0.353	0.338
3	G62	0.308	0.261	0	0.065	0.067	0.049	0.068	0.056	0.066	0.067	0.045	0.108	0.537	0.221	0.375	0.173	0.271	0.462	0.529	0.449
4	G64a	0.314	0.298	0.065	0	0.038	0.025	0.037	0.046	0.034	0.023	0.042	0.069	0.576	0.260	0.414	0.213	0.310	0.421	0.568	0.488
5	G64b	0.312	0.289	0.067	0.038	0	0.038	0.013	0.062	0.060	0.048	0.056	0.071	0.574	0.258	0.412	0.211	0.308	0.424	0.567	0.486
6	G65	0.322	0.292	0.049	0.025	0.038	0	0.038	0.026	0.034	0.022	0.025	0.076	0.568	0.252	0.406	0.205	0.302	0.442	0.561	0.480
7	G66	0.312	0.292	0.068	0.037	0.013	0.038	0	0.060	0.059	0.047	0.050	0.071	0.574	0.258	0.412	0.211	0.308	0.424	0.566	0.486
8	G67a	0.348	0.284	0.056	0.046	0.062	0.026	0.060	0	0.022	0.029	0.042	0.060	0.593	0.277	0.431	0.230	0.327	0.428	0.586	0.505
9	G67b	0.348	0.297	0.066	0.034	0.060	0.034	0.059	0.022	0	0.012	0.051	0.042	0.603	0.286	0.440	0.239	0.336	0.421	0.595	0.514
10	G67c	0.336	0.304	0.067	0.023	0.048	0.022	0.047	0.029	0.012	0	0.039	0.054	0.591	0.274	0.428	0.227	0.324	0.431	0.583	0.502
11	G70	0.322	0.299	0.045	0.042	0.056	0.025	0.050	0.042	0.051	0.039	0	0.093	0.552	0.235	0.390	0.188	0.286	0.463	0.544	0.464
12	H80	0.383	0.336	0.108	0.069	0.071	0.076	0.071	0.060	0.042	0.054	0.093	0	0.645	0.328	0.483	0.281	0.379	0.396	0.637	0.557
13	L29a	0.373	0.366	0.537	0.576	0.574	0.568	0.574	0.593	0.603	0.591	0.552	0.645	0	0.316	0.162	0.364	0.266	0.703	0.206	0.271
14	L29b	0.225	0.220	0.221	0.260	0.258	0.252	0.258	0.277	0.286	0.274	0.235	0.328	0.316	0	0.154	0.047	0.075	0.536	0.312	0.266
15	L29c	0.279	0.255	0.375	0.414	0.412	0.406	0.412	0.431	0.440	0.428	0.390	0.483	0.162	0.154	0	0.201	0.111	0.613	0.215	0.257
16	L30	0.232	0.235	0.173	0.213	0.211	0.205	0.211	0.230	0.239	0.227	0.188	0.281	0.364	0.047	0.201	0	0.102	0.529	0.356	0.277
17	L32	0.279	0.292	0.271	0.310	0.308	0.302	0.308	0.327	0.336	0.324	0.286	0.379	0.266	0.075	0.111	0.102	0	0.580	0.258	0.248
18	M2	0.346	0.478	0.462	0.421	0.424	0.442	0.424	0.428	0.421	0.431	0.463	0.396	0.703	0.536	0.613	0.529	0.580	0	0.776	0.801
19	T34	0.430	0.353	0.529	0.568	0.567	0.561	0.566	0.586	0.595	0.583	0.544	0.637	0.206	0.312	0.215	0.356	0.258	0.776	0	0.127
20	T35	0.455	0.338	0.449	0.488	0.486	0.480	0.486	0.505	0.514	0.502	0.464	0.557	0.271	0.266	0.257	0.277	0.248	0.801	0.127	0

Enzymatic degradation of galactomannans resulted in a variety of HPAEC elution profiles (Fig. 2). These differences reflect variations in the degradability of the samples that were caused by variations in the DS and the distribution of the galactose substituents over the polymeric chains. By direct comparison of the HPAEC elution profiles of the digests and constructing a similarity tree, these variations could be discerned (Fig. 7). The overall Y shape of the tree obtained can be largely attributed to two typical features present in the HPAEC profiles: (i) the total area of the non-substituted, and (ii) the total area of the galactose substituted mannose peaks. The occurrence of hardly any or few substituted and non-substituted mannose peaks results in a top right position (guars and the highly substituted gum H80). When a large amount of substituted and quite some non-substituted peaks are observed, a top left position is expected (tara, locust bean gum, modified guar, and cassia). When only non-substituted peaks are found, a position close to the bottom of the tree is found (linear mannan).

A more detailed study of the HPAEC elution profiles enabled the coupling of these differences to specific distributions of galactose. The information indicated by the total amount of non-substituted mannose residues liberated by the enzyme (DB) can be used to reveal the differences in the amounts and/or sizes of non-substituted mannose sequences (Fig. 6) [10]. Because of the lower DB data of randomly distributed galactomannans of similar DS, it is evident that all guar and the highly substituted H80 sample have a more blockwise galactose distribution. In between the guar samples, variations occur (Fig. 5) which, because of their almost identical DSs, must result from variations in the number and size of the 'enzyme degradable' mannose regions. Locust bean gums revealed the greatest variation in distribution of galactose. Comparison of their DBs demonstrated that in addition to more blockwise (L29a,c) also randomly (L32) and more orderly (L29b and L30) distributions occur (Fig. 5). Of the tara gums, one had a more random distribution (T34) while the other had a more blockwise distribution (T35) of galactose. The variations in the DBs of the locust bean gums are, up to a certain extent, reflected in their relative positions in the similarity tree of Fig. 7. The same holds for the guar and tara samples.

For tara gums the ratio of the substituted to non-substituted peak areas was approximately twice that for locust bean gums (Table 1). Because of their rather similar DS, this indicated that enzymatic degradation of tara galactomannan liberates much more substituted oligomers than a locust bean gum. Because of this, a tara gum must contain more enzyme degradable sequences than a locust bean gum which suggests a more regular distribution of the galactose over the substituted part of the mannose backbone of the former gum. In view of the composition of the active site of the enzyme employed (Fig. 6) and the relative high amount of single galactose-substituted di- and trimannose observed in tara gum (compare the chromatograms of tara and locust bean gum in Fig. 2), the tara gum has to contain many mixed sequences in which every other and/or every third mannose is substituted. Based on Fig. 7, the degradation pattern of the cassia gum most resembled that of the modified guar G26-D which both most resemble that of a locust bean gum digest. Since the DB of the modified guar is quite low in comparison to the DBs of randomly distributed galactomannans of similar DS (Fig. 5), the galactose substituents are somewhat distributed more regularly. The DB of the cassia gum is extremely low for a galactomannan of DS 12% (Fig. 5) revealing a highly regular distribution of galactose. Such a distribution will also result in the formation of (relative) large amounts of low (e.g., single) substituted oligomers. This is indeed observed for the single substituted mannobiose and mannotriose oligomers (Fig. 2). Both are present in amounts quite similar to those found in a locust bean gum digest.

4. Experimental

Materials.—All guars were extracted from the endosperm of *Cyamopsis tetragonoloba* seeds and all locust bean gums were derived from the endosperm of *Ceratonia siliqua* seeds.

Diaguar GH 200\5000 (G64b), Diagum CS (C12; from the endosperm of *Cassia tora/obtusifolia* seeds), Diagum 1.1 (H80; from the endosperm of *Prosopis juliflora*), and tara gum (T35; from the endosperm of *Caesalpina spinosa*), were obtained from BFGoodrich Diamalt GmbH (Munich, Germany). The locust bean gums SC1500-80μ (L29a) and SC1500-250m (L30) and the guar OP 3309 (G64c) were provided by SKW Biosystems (Baupte, France). Supercol U Guar (G62) was obtained from Aqualon (Wilmington, USA). The locust bean gum Genu Gum RL-200Z (L29b) was provided by Copenhagen Pectin A/S (Lilli Skensved, Denmark). The locust bean gum Meypro-LBG Fleur M-175 (L29c) and the processed guars Meyprogat 7&30 (G70 and G67a), Meyprodor 50&100 (G67b and G67c), and Meyprofin 500 (G66) were obtained from Meyhall Chemical AG (Kreuzlingen, Switzerland). The tara gum Ferwotar E417 (T34) was provided by Ferdiwo BV (Oudewater, The Netherlands). The locust bean gum Grindsted LBG 147 (L32) and Grindsted Guar 1400 (G65) were obtained from Danisco Ingredients (Brabrand, Denmark). The enzymatically processed guar [13] Sherex QSG81 (G64a) was provided by Quest International (Carrigaline, Ireland). Linear mannnan (β-(1 → 4)-linked; M2) was isolated from ivory nut by Megazyme International (Wicklow, Ireland). As indicated by the various manufacturers, the samples were not blended with any other saccharides. D-Mannose and L-arabinose were obtained from Merck (Darmstadt, Germany). Mannobiose, mannotriose, and mannotetraose (β-(1 → 4)-linked) were purchased from Megazyme International. *endo*-β-Mannanase (EC 3.2.1.78) was purified from Gammanase, a commercially available *A. niger* enzyme mixture (Novo Nordisk, Bagsvaerd, Denmark), as described by Düsterhoft et al. [14]. All chemicals used were of analytical grade.

Sample preparation and enzymatic degradation.—Galactomannans were weighed into glass vials and wetted with a few drops of EtOH (96%). Subsequently, 50 mM AcONa buffer (pH 5.0) containing 0.5 mM arabinose (as an internal standard) was added to obtain a final galactomannan concentration of 2 mg/mL. The samples were shaken vigorously and heated at 100 °C for 10 min. Part of these solns were degraded by addition of 0.1 units of purified *endo*-β-mannanase per mg galactomannan. To assure an optimal degradation of the substrate, incubations were mixed continuously (head-over-tail) for 24 h at 30 °C. The enzymes were inactivated by heating the samples at 100 °C for 5 min. Prior to chromatographic analysis, the samples were centrifuged for 5 min at 16,000*g* (eppendorf) to remove any insoluble materials. Partially digested mannan was prepared by incubating mannan (2 mg/mL) with 1 μL/mL *endo*-β-mannanase at rt for 4 h.

Chromatography.—High-performance size-exclusion chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three Bio-Gel TSK columns (each 300 × 7.5 mm) in series (40XL, 30XL, 20XL; Bio-Rad Labs) in combination with a TSK XL guard column (40 × 6 mm). Elution was at 30 °C with 0.4 M AcONa buffer (pH 3.0) at 0.8 mL/min. The eluate was monitored using a Shodex SE-61 refractive index detector. Amounts of 20 μL were injected.

Mannose, mannan- and galactomannan-oligomers were analyzed on a Spectra Physics P4000 HPLC system equipped with a Spectra Physics AS3000 autosampler. Separation was achieved on a CarboPac PA-1 column (250 × 4 mm), equipped with a CarboPac PA guard column (25 × 3 mm). The system was equilibrated with 0.04 M NaOH. After injection, 0.04 M NaOH was run through the column for 20 min after which a 7 min linear gradient from 0.04 to 0.1 M NaOH was applied. Subsequently, a second linear gradient of 0–0.15 M AcONa in 0.1 M NaOH was run through the column for 33 min after which the column was washed with 0.9 M AcONa in 0.1 M NaOH for 5 min. Next, the column was regenerated with 0.04 M NaOH for 20 min. Elution was at rt at 1 mL/min. Routinely, amounts of 19 μL were injected. Detection was with a Dionex PAD II pulsed electrochemical detector, working in the amperometric detection mode and equipped with a gold working electrode and an Ag/AgCl reference electrode. Integration was performed with PC1000 software (Thermo Separation Products). Retention times were compared with those of mannose, mannobiose, mannotriose, mannotetraose, and the oligomers observed in partially digested mannan. For the separation of galactose and mannose by HPAEC the gradient described by Tenkanen et al. was used [18]. To identify the molecular masses of the various peaks detected, fractionation of 80 μL of tara gum digest (T34) was performed using similar conditions. An anion and a cation ultra self-regenerating suppressor unit (Dionex) were connected in series to remove the sodium and acetate ions of the eluate on-line [15]. During the first 65 min of the separation, 260 fractions of 250 μL were collected using a Gilson FC-203B multiwell fraction collector. A Symbiot-I pipette–robot (PerSeptive Biosystems) was used to automatically transfer 1 μL of each fraction to a MALDIsample plate and add 1 μL of matrix. The matrix soln was prepared as described before [10]. Before analysis with MALDI-TOF MS, the samples were allowed to dry in a gentle stream of air at rt. In an identical way, 50 μL of mannan (M2) digest was fractionated.

Elution profile comparison and tree construction.—The areas of the mannose mono-, di-, and trimer peaks and the areas of the peaks eluting in sections 4 through 8 (see Section 2) were used to calculate the degradation profile similarity. Calculations were performed as described in Section 2. The phylogeny inference package PHYLIP [17] was used to construct a consensus tree using the FITCH (Fitch–Margoliash algorithm and least-squares method) program [16] according to the procedure described previously [12].

General methods.—Neutral sugars were determined by gas–liquid chromatography [19] after pretreatment (1 h, 30 °C) with aq 72% H_2SO_4 followed by hydrolysis with 1 M H_2SO_4 (3 h, 100 °C) and conversion of the products into alditol acetates [20]. A standard deviation of 0.1% was found for the sugar compositions. MALDI-TOF MS was performed as described before [10].

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