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HEMICELLULOSIC POLYMERS FROM THE LEAVES OF *COFFEA ARABICA*

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Key Word Index—*Coffea arabica* var. *Mundo Novo*; Rubiaceae; leaves, hemicelluloses; arabinoxylan; xyloglucan; peptidoglycan; hydroxyproline, chemical composition.

Abstract—Polysaccharide fractions from leaves of *Coffea arabica* var. *Mundo Novo* were obtained by extraction with 24% potassium hydroxide solution and were found to contain rhamnose, arabinose, xylose, mannose, galactose, glucose, glucuronic acid and 4-*O*-methylglucuronic acid in different proportions. 2-Acetamido-2-deoxygalactose was detected in all fractions. The structures of the carbohydrate portions were analysed by methylation and Smith degradation. A high amount of 2,3,5-tri-*O*-methylarabinose and 2,3,4-tri-*O*-methylxylose units, which are related through end groups, suggested a large degree of branching in the polysaccharide fractions. Glucose was present mainly as (1 → 4)-linked residues, as indicated by the presence of 2,3,6-tri-*O*-methylglucitol in the hydrolysates of the methylated fractions. A greater proportion of monomethylxylitol in acidic fraction B-IV indicated that it was more branched than the others. The glucose and galactose residues are 4,6- and 3,4-di-*O*-substituted, respectively. Three successive Smith degradations gave mainly glycerol with some erythritol and threitol. In the linkage of carbohydrate–protein, the presence of *O*-glycosyl linkages between arabinose and hydroxyproline was indicated. A phenolic compound was detected in all polysaccharide fractions from leaves of the coffee tree and is probably derived from chlorogenic acid.

INTRODUCTION

In a previous paper, Wenzel and Correa [1] identified hemicellulose A from leaves of *Coffea arabica* var. *Mundo Novo* as a 4-*O*-methylglucuronoxylan. Furthermore, a comparison was made of the polysaccharides from two cultivars of *Coffea arabica* var. *Mundo Novo* which were resistant and susceptible to infection by *Hemileia vastatrix* Berk & Br. [2]. We now report a study on the hemicellulosic polymers obtained from leaves of the coffee tree.

RESULTS AND DISCUSSION

The powdered material from leaves of *Coffea arabica* var. *Mundo Novo* was extracted with hot MeOH–$CHCl_3$ (1:1) and successively extracted with hot water, 0.25% acid ammonium oxalate, and 4% and 24% potassium hydroxide solutions, and then was fractionated successively by the Gaillard method [3–6] and Cetavlon precipitation [7], yielding the fractions indicated in Table 1. The interaction of linear and branched polysaccharides in aqueous $CaCl_2$ solution with aqueous I_2–KI to give dark blue complexes has been described by Gaillard *et al* [3–6]. Many linear and slightly branched polysaccharides have been shown to give a dark blue colour with iodine, and highly branched polysaccharides do not react with iodine under these circumstances. Acidic polysaccharides are easily separated from neutral polysaccharides by precipitating them directly with reagents such as cetyltrimethylammonium bromide (Cetavlon) in mildly alkaline solution. Purification of polysaccharide fractions was effected by centrifugation of an alkaline solution, dialysis and precipitation with ethanol, which gave polysaccharides that were homogeneous by electrophoresis [8]. Optical rotations were obtained for the neutral fractions, and are shown in Table 1. The five polysaccharide fractions were subjected to gel filtration on Sepharose 6B and the protein remained associated with the carbohydrate-containing material in three fractions, B-I, B-II and B-IV, suggesting the possibility that they were peptidoglycans. Fraction B-I gave a good elution pattern and was studied further (Fig. 1). Table 2 shows the chemical composition of the five fractions analysed by gas chromatography in columns A and B. Fractions B-V and B-III are xyloglucans [9–12] and fraction B-IV consists of arabinoxylans.

The acidic fractions contained a higher proportion of uronic acid and proteins than the neutral polysaccharide fractions. The uronic acids were identified by paper chromatography with solvents b and c, and after carboxyl reduction [13] by gas chromatography as 4-*O*-methylglucuronic acid and glucuronic acid, as shown in Table 1. All the fractions when hydrolysed with 6 M hydrochloric acid showed the presence of amino acids as indicated in Table 2. The presence of 2-acetamido-2-deoxygalactose was demonstrated by paper chromatography using solvent d, after hydrolysis with 4 M hydrochloric acid at 100° for 6 hr and elution on a column (2 × 6 cm) of Dowex 50 X-8f H^+ resin with 2 M hydrochloric acid (40 ml). The 2-acetamido-2-deoxygalactose content was determined by the Boas reaction [14] and by the Elson–Morgan reaction, as modified by Blix [15], and is indicated in Table 1.

The polysaccharide fractions were permethylated first by the Haworth method (3 times) and then by the Hakomori method, until the products showed no hydroxyl absorption band in their IR spectra. The permethy-

Table 1 Properties, chemical composition, and alditol acetates (GC) of the polysaccharide fractions from leaves of *Coffea arabica* var. *Mundo Novo*

	B-I	B-II	B-III	B-IV	B-V
Weight					
Yield (g)	2.42	0.33	0 53	1.05	0.42
Total carbohydrates (%)*	48.0	40.0	56.5	30.5	51 0
Uronic acid (%)†	8.5	9.0	4 0	15.0	6.0
Hexosamine (%)‡	4 0	4.5	5.0	9.0	6.0
Protein (%)§	5.5	6.0	—	0.5	1.0
Total N_2 (%).	0.5	0.4	0 4	0.3	n.d.
$[\alpha]_D^{25}$	—	—	+46.0°	—	+41 7°
Carbohydrates (%)¶					
Rhamnose	6 3	4.6	0.3	5.8	1.5
Arabinose	29.5	25.7	7 8	32.5	16.0
Xylose	38 5	44.8	32.8	28.5	36.0
Mannose	tr	1.2	1.5	0.5	4 0
Galactose	11.3	10.0	4.3	21.0	9.5
Glucose	13.0	25 5	53.5	11.5	38 5
4-*O*-Methyl glucuronic acid	6.5	6.5	2.0	10.5	3.7
Glucuronic acid	2.0	2.5	2.0	4.2	2.4
Phenolic compound	2.0	3.0	1.5	2.8	2.6

*Phenol–H_2SO_4 reagent.

†Carbazole method. Values were calculated as % of glucuronic acid over total sugar.

‡Blix method and Boas reaction. Values were calculated as % of galactosamine over total sugar.

¶Area of the peak given by GC on columns A and B at 190°.

tr = trace, n d. = not determined.

lated polysaccharides were hydrolysed and analysed by gas chromatography using columns A and B, and GC/MS as shown in Table 3.

2,3,5-Tri-*O*-methylarabinitol, 2,3,4-tri-*O*-methylxylitol, 2,3,4,6-tetra-*O*-methylgalactitol and 2,3,4,6-tetra-*O*-methylglucitol or mannitol were derived from non-reducing end-units.

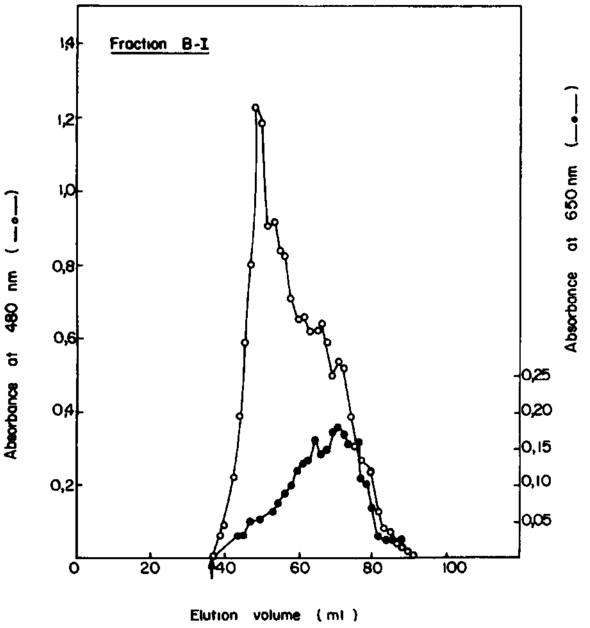

Fig 1 Gel filtration of the fraction on Sepharose 6B (see Experimental). The arrow represents the void volume determined with blue dextran. Each eluate was analysed for carbohydrate (○) and protein (●).

2,3,5-Tri-*O*-methylarabinitol was detected in greater proportions in the acidic fractions than in the neutral fractions. 2,3,4-Tri-*O*-methylxylitol was identified in greater quantities in polysaccharide fractions B-II, B-III and B-V than in B-I and B-IV, indicating that the former are more branched than the latter. The presence of 2,3-di-

Table 2. Amino acids of polysaccharide fractions from leaves of *Coffea arabica* var. *Mundo Novo*

Amino acid	Mol %				
	B-I	B-II	B-III	B-IV	B-V
Lys	2 2	1 8	1.0	2.0	1.3
His	1 1	1 0	*	1.0	*
Arg	1 3	1 0	—	*	—
Asp	7 0	4 5	2.3	4.8	5.3
Thr	2 2	1 3	*	*	*
Ser	3.0	1.9	1 3	2.2	2.0
Glu	7.4	5.0	2.3	5.4	4.0
Pro	4 2	3 1	*	*	*
Gly	4.9	2.9	1.8	3.4	3.0
Ala	5.2	3.2	1.5	3.4	2.0
Val	3.7	3.7	*	3.6	*
Ile	2.0	1.7	1 0	1.8	*
Leu	2.9	3.0	1.5	3 0	1 0
Tyr	1.9	1.2	*	*	*
Phe	2.3	1.3	*	*	*
Met	1.0	—	—	—	—
Hyp	13 0	2.1	*	5.0	11.5

* < 1%

Table 3. Identification of the methylated sugars obtained from the polysaccharides of leaves of *Coffea arabica* var. *Mundo Novo*

R_t*				Approximate amount†				
Column A	Column B	Alditol acetates of	Primary fragments (m/z)	B-I	B-II	B-III	B-IV	B-V
	0.35	?			0.48	0.99		0.4
0.48	0.41	2,3,5,-Me$_3$-Ara	45, 117, 161	5.84	9.22	2.98	15.54	3.24
	0.62	?		4.06				
	1.03	?		1.87				
1.40	1.10	2,4-Me$_2$-Ara	117, 233	8.61	9.00	2.80	9.96	3.86
	1.07	2,3-Me$_2$-Ara	117, 189	3.10	6.00	2.00	7.00	4.01
0.68	0.54	2,3,4-Me$_3$-Xyl	117, 161	5.49	10.68	17.43	7.82	11.52
1.54	1.10	2,3-Me$_2$-Xyl	117, 189	33.65	21.38	21.67	16.43	20.27
	1.35	?		0.77	2.29	2.35	1.40	2.22
	1.43	?		2.50	3.69	1.58		0.99
	1.92	?		2.26				
	2.09	?		1.12				
	2.12	?		2.18				
	2.14	?			1.60	1.49	2.49	0.52
2.92	2.15	3-Me-Xyl	189, 189	2.96	3.98	0.60	12.02	1.69
1.00	1.00	2,3,4,6-Me$_4$-Glu/or Man	45, 117, 161, 205	2.56	3.00	1.00	5.00	2.43
	2.21			0.58				
2.50	2.32	2,3,6-Me$_3$-Glu	45, 117, 233	10.23	13.02	22.77	9.26	21.80
	2.41	?			0.8	1.89		0.32
	2.87	?		0.36				
	3.45	?		0.90				
4.40	3.73	3,6-Me$_2$-Glu	45, 189, 233		1.17	0.64	1.41	
5.39	4.50	2,3-Me$_2$-Glu	117, 261	2.93	9.79	15.83	5.90	11.02
5.27	4.26	3,4-Me$_2$-Glu	189, 189	0.13				0.03
5.10	4.21	2,4-Me$_2$-Glu	117, 189					0.71
1.25	1.19	2,3,4,6-Me$_4$-Gal	45, 117, 161, 205	1.48	2.21	1.47	2.5	7.88
3.41	2.89	2,3,4-Me$_3$-Gal	117, 161, 189, 233	0.18	0.51	0.33	0.63	0.91
3.65	3.14	2,6-Me$_2$-Gal	45, 117	1.24			1.8	
4.35	2.60	3,6-Me$_2$-Gal	45, 189, 233	0.11	1.14		0.77	5.05
	5.66	?		0.74				
	6.07	?		1.91	1.7			

*Relative retention times with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as standard.

†Area of the peak by GC relative to the same standard. Column A = ECNSS-M. Column B = OV-225.

O-methylarabinitol in all fractions reveals 4-*O*-substituted linked arabinopyranose and/or 5-*O*-substituted arabinofuranose residues. This is consistent with glycerol being formed by Smith degradation. A high proportion of 2,3-di-*O*-methylxylitol was detected in all polysaccharide fractions containing (1 → 4)-linked xylopyranose. The proportion of monomethylxylitol indicates that the polymer is highly branched in all polysaccharide fractions, especially in fraction B-IV.

The formation of a large amount of 2,3,6-tri-*O*-methylglucitol indicates a large proportion of (1 → 4)-linked hexoses. This is in agreement with the presence of higher quantities of erythritol and threitol by Smith degradation. It was detected more in the neutral fractions B-III and B-V than in the acidic fractions B-I, B-II and B-IV. 2,3-Di-*O*-methylglucose was obtained in a greater proportion in the polysaccharide fractions B-III and B-V 2,6-Di-*O*-methylgalactose was detected in fractions B-I, B-IV and B-V. The occurrence of these two methylated derivatives indicates branch points which are linked O-4 and O-6 glucose residues, through O-3 and O-4 of the galactose residues, respectively. The presence of 2,6- and 3,6-di-*O*-methylgalactose residues (in arabinoxylans) is in accord with the resistance to periodate of galactose by Smith degradation.

Other methylated sugars were detected in small proportions which were derived from very minor polysaccharide structures. The methylation analysis of each fraction showed a high degree of branching. The overall structures are very complex, but part of polymer sugars seems to have a main chain of (1 → 4)-linked glucosyl residues from the presence of a high proportion of 2,3,6-tri-*O*-methylglucitol in the hydrolysed methylated sugars.

The results of methylation are in agreement with the observed release of arabinose on mild acid hydrolysis [16], and also with the formation of glycerol as the major alcohol in the first Smith degradation (arabinoxylan) and erythritol (xyloglucans). The glycerol content was greater in the arabinoxylans after three successive Smith degradations. This is in agreement with the fact that glycerol arises mainly from arabinofuranose and also glucopyranose end-groups. The erythritol and threitol contents were more in the polysaccharide fractions B-II, B-III, B-IV and B-V than in fraction B-I, which would result from

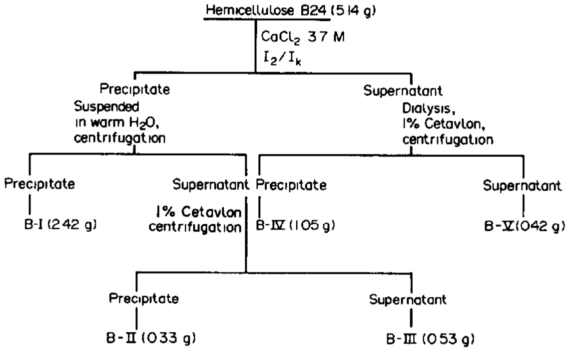

Fig 2 Fractionation of crude hemicellulose B24 from the coffee-tree leaves by precipitation and complex formation methods

4-linked glucose and galactose, respectively. Rhamnose was more susceptible to Smith degradation in fractions B-I, B-II, B-III and B-IV. Arabinose was more oxidized in fractions B-I, B-II and B-IV than in fractions B-III and B-V. Xylose was more vulnerable to oxidation in fractions B-IV and B-V than in B-I, B-II and B-III. More galactose was released in fraction B-I, and more glucose was released in fractions B-III and B-IV. The phenolic compounds were more oxidized in fractions B-III, B-IV and B-V than in B-I and B-II.

A close association between carbohydrate and protein in three polysaccharide fractions from leaves of *Coffea arabica* var. *Mundo Novo* suggests the presence of peptidoglycans. Lamport [17, 18] demonstrated the presence of an *O*-glycosyl linkage between arabinose and hydroxyproline in the polypeptide chain. Hydroxyproline-rich glycoproteins (or peptidoglycans) seem to play a fundamental role in plant cell-wall structure by stabilizing the extension polypeptide backbone, conferring on it a rigid, rod-like structure. The occurrence of hydroxyproline arabinosides in the cell walls of many plants [17–23] has been shown. Two methods were employed to characterize the glycosyl-peptide bond present in the peptidoglycan of fraction B-I. The sample (500 mg), partially hydrolysed, liberated the oligosaccharides which were eluted from a column of charcoal–celite, first with distilled water and then with aqueous 5, 10, 15, 30 and 50 % ethanol solutions, and the eluates (oligosaccharides) were detected by paper chromatography. Part of the eluate was hydrolysed with 6 M hydrochloric acid and analysed for amino acids by thin-layer chromatography (solvent e). The only amino acid detected was hydroxyproline. Another part of the same eluate was hydrolysed with 1 M trifluoroacetic acid at 100° for 5 hr, and the products were analysed by gas chromatography (alditol acetates). Rhamnose, arabinose, xylose, galactose and glucose were obtained in each oligosaccharide fraction. To confirm the possibility that the sugar attached to the amino acids was arabinose, the following method was used.

The sample (B-I:50 mg) was submitted to alkaline degradation with barium hydroxide, and the product analysed on Bio Gel P-2. The eluates were analysed for carbohydrate by phenol–H_2SO_4, and amino acids were analysed using thin-layer chromatography (solvent e). Different amino acids were detected, but no hydroxyproline. After subsequent alkaline treatment with simultaneous reduction of the carbohydrate component, the eluates were chromatographed by paper chromatography (solvent a) and gas chromatography as alditol acetates, showing the presence of arabinitol (eluates 4, 7 and 8); and by TLC, showing the presence of hydroxyproline (eluates 4 and 8).

Methylation analysis (see Table 3) of fraction B-I indicated the presence of two arabinosyl residues, one at the non-reducing end (2,3,5-tri-*O*-methylarabinose) and the other as a (1 → 3)-linked residues. This group is probably involved in the glycosyl-amino acid bond in the peptidoglycan.

A phenolic compound detected in every polysaccharide fraction is probably derived by hydrolysis of chlorogenic acid and is widespread in the plant kingdom [24]. The phenolic compound derivative was characterized as follows: The hydrolysates from the phenolic compounds (from leaves of the coffee tree) and the chlorogenic acid standard showed the same R_f value, 0.57 (in thin-layer chromatography, solvent g), and the same R_t, 10.4 (in gas

Table 4. Products obtained on three successive Smith degradations of the polysaccharide fractions from leaves of *Coffea arabica* var *Mundo Novo*

	Relative amount* (%)									
	B-I		B-II		B-III		B-IV		B-V	
Components	ND	D	ND	D	ND	D	ND	D	ND	D
Glycerol	8 1	16.0	10 6		18 5		2.0		7 0	4 2
Erythritol	21.5	7.0	8 0	38 0	13.5	41 0	1 5	14 5	6 2	38 5
Threitol	2.0	3 1	10 0	7.0	tr	6 5	2 5	7 5	tr	8 0
Rhamnitol	12 9	11 0	7 0	10.5	30.5	12 0	15 0	16 5	30 5	4.0
Arabinitol	7 1	32 0	12.5	31.5	12 7	10 3	14.0	36 5	19.5	18 5
Xylitol	2 2	4.5	17 5	4 6	tr	4.5	3.0	8 5	4 5	11 7
Mannitol	15.5	3 5	2 5			1 5				3 5
Galactitol	16 5	11 7	20.5		11 0	3 1	33.0		18.7	5 0
Glucitol	14 0	11.5	11.2	9 0	13 5	21 1	29.0	19 5	14.0	6 5
Phenolic compound	18 5	3.0	92 2	4 5	98.0	33.2	82 5	21 5	95.0	35.0

*Area of the peak of the derived acetates by GC relative to the mixture of known acetylated standards ND = Not dialysable, D = dialysable; tr = trace.

chromatography, column B). Afterwards, a mixture of the two compounds (analysed by gas chromatography) showed that both had migrated together as a single peak (R_t = 10.4). The IR spectra of the two compounds were identical. The UV spectra analysis in ethanol showed a shift near 274–275 nm in both compounds.

EXPERIMENTAL

General methods. Optical rotations were measured using aq solns at 25° with a Perkin–Elmer Model 141 automatic polarimeter. Evapns were carried out under red. pres at 40° or less Electrophoresis of the dyed polysaccharides was carried out on cellulose acetate according to the procedure of ref [8]. Carbohydrate was determined using PhOH–H_2SO_4 reagent [25] Reducing sugars were assayed by the Somogyi–Nelson method [26, 27] Hexosamine was estimated by the Elson–Morgan reaction, as modified in ref [15], and by the Boas reaction [14]. 2-Amino-2-deoxy-D-galactose was used as the standard. Uronic acid was determined by the carbazole method [28], with D-glucuronic acid as the standard. Protein was determined by the method of ref [29], with bovine serum albumin as the standard. Total nitrogen was assayed by the micro-Kjeldahl method [30, 31]. Amino acids were analysed using an automatic amino acid analyser. IR spectra were prepared using KBr discs with a Beckman Acculab ^{R}10 spectrophotometer. PC was performed on Whatman No 1 (3 MM filter paper) with the following solvent systems: (a) C_6H_6–*n*-BuOH–C_6H_5N–H_2O, 1:5:3:3; (b) EtOAc–HOAc–H_2O, 9:2.2; (c) EtOAc–HOAc–HCO_2H–H_2O, 18:3:1:4; (d) EtOAc–C_6H_5N–HOAc–H_2O, 5.5:1·3. TLC was performed on silica gel 60 with solvent systems (e) *n*-BuOH–HOAc–H_2O, 3:1.1, (f) PhOH–H_2O, 3·1; (g) EtOAc–petrol–$CHCl_3$, 3.3:1 Sugars were detected by spraying with alkaline $AgNO_3$ [32, 33], amino acids with ninhydrin [34], and phenolic substances with MeOH–H_2SO_4. GC was performed using a Varian Chromatography Model 2440 (flame ionization detector) with N_2 as carrier gas. Analyses were carried out using columns (200 × 0 15 cm) containing· (A) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° for alditol acetates of sugars and 170° for alditol acetates of partially methylated sugars, (B) 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 170° for alditol acetates of partially methylated sugars, and 190° for alditol acetates of sugars GC/MS was performed in an OV-225 capillary column (25 m) The programme was 50–182° (hold), 40°/min and scanned for mass 40–420 every 2 sec at 70 eV. UV spectra were obtained using a UV–vis Varian Model 635 spectrophotometer. Alditol acetates were prepared as in ref. [13].

Extraction of the hemicelluloses [11, 35]. Dried leaves from cultivars of *Coffea arabica* var *Mundo Novo* were ground in a Wiley mill (40–60 mesh). The powdered material (374.0 g) was extracted (Soxhlet) first with a hot 1·1 MeOH–$CHCl_3$ mixture for 72 hr, and then with Me_2CO. The air-dried material was exhaustively extracted with hot H_2O (1 l. each, 3 ×), vacuum-filtered through cloth and then the filtrate (1.82 l.) was treated with EtOH (3 vols), yielding an insoluble P-I fraction (12.7 g %). The residue from extraction with hot H_2O was then extracted with 0 25% $(NH_4)_2C_2O_4$–$H_2C_2O_4$ (3 ×), which was vacuum-filtered through cloth. The filtrate (3 8 l.) was dialysed and treated with EtOH (3 vols) to yield an insoluble P-II fraction (1.89 g %). The residue from the previous extraction treatment was extracted with 4% aq. KOH (1 l. each, 2 ×) for 72 hr in N_2 atmosphere. The alkaline slurry was vacuum-filtered through cloth, and the residue was washed with H_2O The combined filtrates were immediately cooled in an ice-bath and adjusted to pH 5 with 50% HOAc Hemicellulose A was collected by centrifugation, washed successively with H_2O (at pH 5), EtOH and Me_2CO, and then dried to yield crude hemicellulose fraction A4 (2.43 g %). The supernatants and acid washings from the hemicellulose A extracts were poured into EtOH (3 vols), and the ppt. (hemicellulose B) was collected by centrifugation. The ppt. was dissolved in H_2O, (100 ml), dialysed and the clear soln poured into EtOH (3 vols) The ppt. was collected by centrifugation, washed with EtOH and Me_2CO, and then dried to yield crude hemicellulose fraction B4 (3.04 g %). The residue from the 4% KOH extraction procedure was suspended in 24% aq. KOH and extracted for 72 hr in an atmosphere of N_2. The combined extracts and washings were acidified with HOAc, and hemicelluloses A24 (3.82 g %) and B24 (4 34 g %) were recovered as described for hemicelluloses A4 and B4

Fractionation of hemicellulose B24. Hemicellulose B24 (4 g %) was dissolved in H_2O and deproteinized by the method of ref. [36] (12 ×). After precipitation with EtOH, the material was dissolved in 3.7 M $CaCl_2$ and fractionated in linear (ppt.) and branched polysaccharides (supernatant) by precipitation with iodine, according to the Gaillard method [3–6] The ppt., after suspending in warm H_2O and centrifugation, gave a ppt (B-I) and a supernatant, which precipitated with 1% aq. soln of cetyltrimethyl ammonium bromide (Cetavlon). After centrifugation, polysaccharide fractions B-II and B-III were obtained. The supernatant after dialysis against tap water (overnight) was precipitated with 1% aq soln of Cetavlon affording, after centrifugation, the polysaccharide fractions B-IV and B-V, indicated in Fig 2. Samples of the different polysaccharide fractions (10 mg) were submitted to total hydrolysis and analysed by GC as alditol acetates. The composition and properties of the polysaccharide fractions are summarized in Table 1

Acid hydrolysis For complete hydrolysis the hemicellulose fractions were hydrolysed with 0 5 M H_2SO_4 for 5 hr at 100°. For isolation of the aldobiuronic acid and oligosaccharides, the hemicelluloses were hydrolysed with 1 M TFA for 5 hr at 100° and 0 5 M TFA for 2 hr at 100°, respectively. Methylated polysaccharides were hydrolysed initially by heating with 85% HCO_2H for 2 hr at 100°, the HCO_2H then being removed by co-evapns with H_2O, and then with 0.5 M H_2SO_4 for 16 hr at 100°.

Isolation of aldobiuronic acids. The polysaccharide fractions (50 mg) were treated with 1 M TFA (5 ml) for 5 hr at 100° After acid removal by evapn, the hydrolysate was concd and applied to a column of Ag 1-X 10 (AcO^-) resin (200–400 mesh). The column was washed with H_2O to remove neutral sugar. Acidic components were obtained by elution with 30% HOAc and were concd to a syrup

PC of the acidic components revealed components having R_{xyl} of 0.64 and 0.30 (solvent c) and R_{xyl} of 0 42 and 0.08 (solvent b) The syrup was converted into the methyl ester methyl glycoside by treatment with boiling 3% methanolic HCl (2 ml) for 8 hr This product was reduced with $NaBH_4$ and after deionization it was hydrolysed with 0.5 M TFA for 5 hr at 100° GC (alditol acetates) of the hydrolysate revealed rhamnose, xylose, 4-*O*-methylglucose and glucose.

Smith degradation Polysaccharide fractions (50 mg) were treated with 0.05 M $NaIO_4$ (15 ml) in the dark for 120 hr at 4° [37, 38]. Excess periodate was decomposed with ethylene glycol (2 ml), and the reagents were then removed by dialysis The products were reduced with $NaBH_4$ overnight at room temp., the soln was acidified with HOAc and then dialysed. The dialysates were concd to a small vol., and then hydrolysed with 1 M TFA for 5 hr on a boiling H_2O bath The hydrolysates, after the usual treatment, were converted into alditol acetates and analysed by GC using column B.

Methylation analysis. The samples (20 mg) were methylated by the Haworth procedure (3 ×) [39, 40] and then by the method of ref. [41]. After dialysis (to remove reagents), the methylated products were extracted with $CHCl_3$ and evapd to dryness. The IR spectrum showed no absorption bands at 3600–3300 cm^{-1}, indicating the absence of hydroxyl groups. The fully methylated polysaccharides were hydrolysed by heating with 85% HCO_2H for 2 hr at 100° and then with 0.5 M H_2SO_4. The hydrolysates were converted into their alditol acetates and analysed by GC using columns A and B, and GC/MS [42, 43] (see Table 3).

Gel filtration. The polysaccharide fractions (25 mg) in 25 mM Tris–HCl buffer (pH 7.2) were applied to a column (38 5 × 2.0 cm) of Sepharose 6B, which was eluted with the same buffer, 2 ml portions being collected. The carbohydrate and the protein contents were determined by the PhOH–H_2SO_4 method and the method of refs [25, 29] The column V_0 (void vol) was determined with blue dextran (MW 2 000 000)

Mild acid treatment of fraction B-I The polysaccharide (500 mg) was hydrolysed with 0.5 M TFA for 2 hr at 100° and the ppt. which formed was removed by centrifugation. The supernatant was concd to dryness to remove residual acid, redissolved in H_2O (2 ml) and applied to a column (3.5 × 22 cm) of charcoal–celite (1.1). The material was eluted first with H_2O (1 l.) and then with aq. 5, 10, 15, 30 and 50% (v/v) EtOH solns (1 l. of each). Fractions (50 ml) were collected and total carbohydrate, reducing sugars and protein were assayed. Portions of these eluates were hydrolysed with 6 M HCl and assayed for amino acids in TLC, using solvent e. Another portion was hydrolysed with 1 M TFA at 100° for 5 hr, and the products were analysed as alditol acetates by GC (column B)

Alkaline degradation [18] *of fraction B*-I The polysaccharide (50 mg) was treated with refluxing saturated aq. $Ba(OH)_2$ (5 ml) for 15 hr After neutralization with 0.5 M H_2SO_4 and filtration, the soln was evapd to dryness. The residue was dissolved in 0.1 M HOAc (2 ml) and applied to a column (32 × 1 0 cm) of Bio-Gel P-2 (200–400 mesh), which was eluted with 0 1 M HOAc The fractions (2 ml) were assayed for total carbohydrate and analysed by TLC (solvent e) for amino acids The eluates were dissolved in $NaBH_4$ (20 mg)–5 M NaOH and kept at 100° for 5 hr To dilute the alkali, H_2O (9 ml) was added and the mixture desalted by MeOH and C_6H_5N. Part of each eluate was analysed for amino acids using TLC (solvents e and f). Another part was subjected to PC using solvent a, and GC, as alditol acetates.

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