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CARBOHYDRATE POLYMERS FROM UNDERGROUND PARTS OF *CISTANCHE DESERTICOLA*

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Key Word Index *Cistanche deserticola*; Orobanchaceae; holoparasite; underground parts; arabinogalactan; pectic polysaccharides; α-1,4-D-glucan; 4-*O*-methylglucuronoxylan.

Abstract—The subterraneous parts of the holoparasite, *Cistanche deserticola*, was extracted with methanol and the easily extractable polysaccharides were isolated from the insoluble residue using in succession cold water, hot water, 0.5 M NaOH, and 0.01 M EDTA. Based on the data of sugar composition analysis, methylation analysis and NMR spectroscopy, it was shown that the bulk of the easily extractable polysaccharides was composed of starch-like α-1,4-D-glucan, α-L-arabino-3,6-β-D-galactan, pectic polysaccharides and 4-*O*-methyl-D-glucurono-D-xylan.

INTRODUCTION

Cistanche deserticola is a holoparasite which is widely distributed in the Gobi desert. Apart from the flowers, the parasite is attached underground directly to the roots of the dicotyledonous plant *Haloxylon ammodendron*. The whole dried plant (without flowers) is known as the drug 'Cistanchis Herba' used in oriental medicine for the treatment of kidney pain, gynaecological diseases and intestinal infections. From related species (*C. tubulosa*, and *C. salsa*), various phenylethanoid and phenylpropanoid glycosides have been isolated and claimed to be anti-stress drugs [1–3]. Pharmacological studies carried out by Mongolian scientists have shown that a decoction of *Cistanche* exhibits anabolic and adaptogenic activities. As in the case of a number of medicinal herbs [4, 5], these properties might be associated with some of the polysaccharide components of *Cistanche* which have not yet been investigated.

In a preceding paper [6], the general chemical characteristics of underground parts of *C. deserticola*, as well as the low M_r saccharidic components have been described. Now, we report on the isolation, composition and main structural features of the easily extractable polysaccharide components from this drug.

RESULTS AND DISCUSSION

The methanol-insoluble residue (MIR) of *Cistanche* which amounted to 39.3% of its dry wt was successively treated with cold water, hot water, 0.5 M NaOH and 0.01 M EDTA. Gradual precipitation of the cold water extract with ethanol yielded a main (CP1) and minor (CP2) polysaccharide fraction, respectively. The hot water extract gave material on cooling (HCP) and further material on adding ethanol (HP1, HP2). From the dilute alkali extract, fraction AP was separated and fraction EP was obtained in the last extraction step. The yield and analytical characteristics of the polymeric fractions extracted are summarized in Table 1. Most of the fractions were contaminated by protein and lignin' yielding acid-resistant portions by acid treatment.

Once isolated in the lyophilized form, the crude polysaccharide fractions became only partially water soluble. Table 2, showing the ratios of the various sugars in the hydrolysates, also shows that the water-soluble (ws) fractions were poorer in protein than were the water-insoluble (wis) ones. For further studies, the ws-fractions were treated with Pronase. Except from ws-CP1, all fractions were rich in glucose and gave a positive iodine test, indicating the presence of starch.

The M_r distribution (MWD) of the ws-fractions evaluated from the HP gel permeation chromatograms (Table 3) indicated a high degree of polydispersity. The structural features in the main polysaccharide fractions were established by methylation analysis (Table 4).

Cold water extraction

CP1 contained galactose, arabinose and glucose as the main neutral sugar components and had a low uronic acid content, comprising mostly galacturonic acid. The MWD of the fraction was broad, ranging from M_r 10^3 to 10^6. CP2 was richer in xylose and glucose and showed a lower M_r (3000) similar to the ethanol-nonprecipitable polymeric fraction of the cold water extract (CNS).

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Table 1. Characteristics of crude polysaccharide fractions isolated from *Cistanche deserticola*

Fraction	CP1	CP2	CNS	HCP	HP1	HP2	AP	EP
Yield(%)*	0.66	0.02	0.50	0.53	0.50	0.04	2.90	3.20
Total carbohydrate ($mg\,g^{-1}$)†	55.5	44.5	40.0	82.7	41.0	47.1	83.6	90.5
Lignin(%)‡	14.9	19.0	ND	11.0	47.1	24.2	8.2	1.5
Protein(%)§	23.7	25.0	ND	13.4	26.9	11.6	7.5	1.3
Neutral sugars (mol%)								
Rhamnose	5.0	9.9	5.4	2.8	7.8	8.4	1.9	9.5
Arabinose	24.6	7.0	6.1	31.4	10.9	15.5	4.0	59.7
Xylose	6.0	24.2	15.7	4.1	1.7	9.0	4.4	0
Mannose	4.0	4.5	11.9	4.1	2.7	0	0.6	0
Glucose	23.2	50.7	60.9	45.8	64.6	64.0	88.6	23.3
Galactose	37.2	3.7	tr	11.8	12.2	3.1	tr	7.5
Uronic acid (%)¶	3.1	5.5	3..9	12.7	7.9	11.3	1.7	40.8
$[\alpha]_D$, degrees	− 16.1	− 24.2	− 22.0	+ 91.9	ND	ND	+ 108.0	+ 1.7

*Calculated on basis of original dry wt.
†Determined by phenol–H_2SO_4 assay.
‡Acid-resistant portion after hydrolysis with 2 M TFA.
§% Nitrogen × 6.25.
¶Determined by 3-hydroxydiphenyl assay.
ND, not determined.
tr, traces.

Table 2. Characteristics of polysaccharide subfractions from *Cistanche desirticola*

Fraction	Yield (%)	Protein (%)	Neutral sugar components (mol%)							Uronic acid (%)
			Rha	Fuc	Ara	Xyl	Man	Glc	Gal	
ws-CP1	60.0	9.2	3.8	2.5	43.1	13.1	–	8.7	28.1	6.8
wis-CP1	37.8	49.1	tr	–	22.4	0.3	0.3	51.0	18.4	ND
ws-HP	63.0	6.5	2.4	1.6	12.5	5.7	–	76.3	1.5	2.0
wis-HCP	31.1	17.3	3.1	–	1.9	1.1	tr	93.8	–	2.4
ws-HP1	83.8	11.2	3.1	0.4	3.3	1.2	0.2	89.8	2.0	4.9
wis-HP1	10.9	30.1			12.3	5.1		82.6	–	3.8
ws-AP	80.5	4.3	2.6		6.1	6.4	–	83.3	1.5	12.5
wis-AP	15.5	22.3	6.2		14.7	8.5	1.7	65.1	4.3	15.2
ws-EPα*	88.5	1.4	7.1		69.0	4.5		7.0	12.5	41.5

*EP after amylases digestion.

Ws-CP1 exhibited the most complex ^{13}C NMR pattern of the various fractions (Fig. 1). The signals at δ110.38 (a) and 108.56/108.28 (b–d) can be assigned to C-1 of α-L-arabinofuranosyl residues located in different environments. Usually, arabinofuranosyl units linked to β-D-galactans resonate at lower field [7–9] than those linked to α-L-arabinans [10–12]. The corresponding anomeric proton signals in the ^1H NMR spectrum of ws-CP1 (Fig. 2) were at δ5.26 (J = 1.5 Hz) and 5.18–5.10 (J = 1.5 Hz) indicating the α-configuration of the arabinofuranosyl units [13, 14]. The complex of signals at δ104.29–105.50 was assigned to C-1 of β-galactopyranosyl residues [7, 15] involved in different linkages, indicating the presence of various types of galactan chains. The minor signals centred at δ102.90, 101.85 and 100.85 were attributed to C-1 of 4-linked β-D-xylopyranose [16], α-L-rhamnopyranose [17] and α-D-glucopyranose [18]. In accordance, there were H-1 signals at δ5.43 (J = 3.4 Hz) of α-glucopyranose [19], as well as at least four signals at δ4.76 (*b*), 4.65 (J = 7.8 Hz), 4.55 (J = 7.7 Hz) and 4.50 (J = 9.5 Hz) characteristic of the β-configuration of galactopyranose [20] and xylopyranose [21] units. The signals at δ 100.1 and 5.0 are attributable to C-1 and H-1, respectively, of 4-linked α-D-galacturonic acid [20–22] of pectin chains. They are partially methyl-esterified (^{13}C: δ54.06; ^1H: δ3.80). The

Table 3. Molecular weight distribution of ws-polysaccharide fractions from *Cistanche deserticola*

Fraction	Distribution		
	M_r 10^{-3}	M_w M_n	Area(%)
CP1	20	1.39	40
	90	2.29	50
	> 1000		10
HCP	2	1.22	8
	51	1.55	81
	800–1000		11
HP1	3	1.08	11
	48	1.60	86
	800–1000		3
AP	47	2.76	95
	> 1000		5
EP	85	2.36	85
	> 1000		15

*Measured on Separon HEMA BIO-100 and BIO-1000 columns, calibrated with pullulan standards.

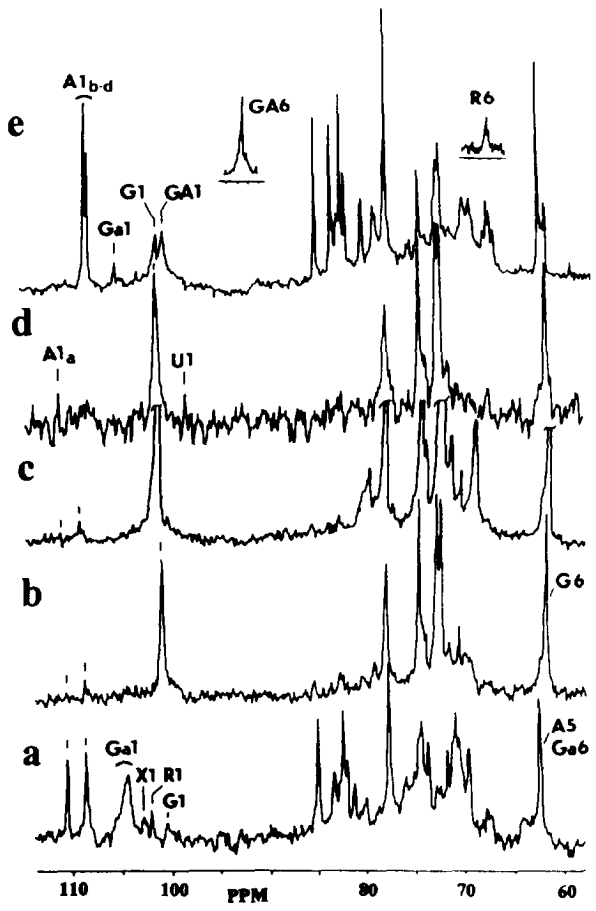

Fig. 1. ^{13}C NMR spectra in D_2O of *Cistanche* polysaccharide fractions (a) ws-CP1, (b) ws-HCP, (c) ws-HP1, (d) ws-AP and (e) EP. A, Arabinose linked to galactose (a), to arabinose (b–d); G, glucose; GA, galacturonic acid; R, rhamnose; X, xylose.

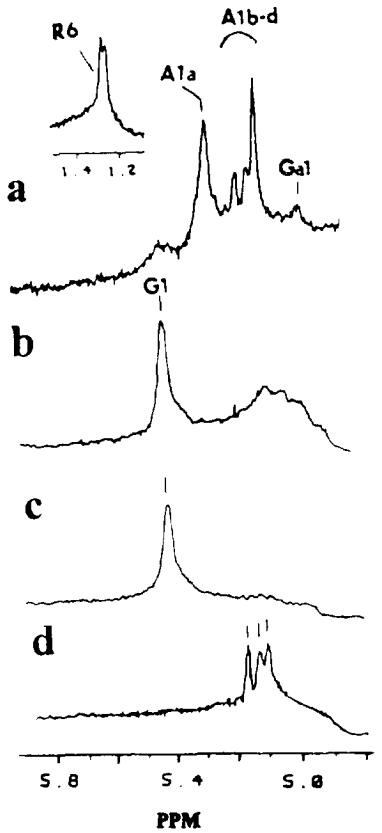

Fig. 2. Partial ^{1}H NMR spectra of *Cistanche* polysaccharide fractions (a) ws-CP1, (b) ws-HCP, (c) ws-AP and (d) EP after digestion with α- and γ-amylases. For symbols see Fig. 1.

low proportion of *O*-acetyl groups (^{13}C: δ21.45–21.68; ^{1}H: δ2.10) may substitute the pectin backbone. However, xylans are also known to occur *O*-acetylated in their native state [23]. The rhamnopyranosyl residues gave a broad, split signal of C-6 at δ17.8 and H-1 at δ1.26–1.31, respectively, indicating the presence of rhamnogalacturonan chains [20].

Methylation analysis data of ws- and wis-fractions of CP1 (Table 4) showed the same methyl ethers but in different proportions and confirmed the complex character of CP1. The arabinose of ws-CP1 were mainly terminal non-reducing (70%), while lower portions were 5- (19%) and 3,5-linked (7%); the rest was 2- and 3-linked. By simultaneous application of EI- and CI-mass spectrometry it was shown that a small proportion of terminal Ara was present in the pyranose form, as found in larch wood arabinogalactans [23]. From the galactosyl residues, 62% were 3,6-linked, 15% were non-reducing terminal, 16% 3-linked and the others were 4-, 3,4- and 3,4,6-linked. The ratio of terminal to branched glycosyl residues indicates that most of the Ara*f* units terminate galactose chains. These results suggest that fraction ws-CP1 consisted mainly of arabino-3,6-galactan with a lower proportion of the (1 → 4) analogue; they may be either independent or present as side-chains of pectic polymers.

Table 4. Methylation analysis of polysaccharide fractions isolated from *Cistanche deserticola*

Sugar unit	Linkage type	Glycosidic linkage composition (mol%)					
		ws-CP1	wis-CP1	wis-HCP	ws-HP1	ws-AP	EP*
Rha*p*	Terminal	1.5	5.5	1.0	0.3	0.7	0.4
	1 → 2	0.2		0.3		0.3	—
	1 → 3	0.1	1.6				
	1 → 4	0.1	0.3	0.1		—	—
	1 → 2,3	—			—	0.3	—
	1 → 2,4	1.6	0.2		—	0.9	4.8
Fuc*p*	Terminal	0.4	0.2				—
	1 → 2	0.5			—	—	—
Ara*f*	Terminal	27.2	8.3	2.1	0.5	2.1	31.3
	1 → 2	1.8	1.5			0.2	1.6
	1 → 3	0.5	1.7			0.3	2.0
	1 → 5	7.8	6.5	0.4	0.4	2.7	20.5
	1 → 3,5	2.6	1.7		0.2		23.7
	1 → 2,5			0.2		—	4.3
Xyl*p*	Terminal	0.5	0.2			tr	
	1 → 2	1.1	0.3	—	—	—	—
	1 → 4	8.4	1.4	0.1	0.4	1.6	0.3
	1 → 2,4	1.5	0.4		—	—	—
Man*p*	Terminal		0.2			—	—
Glc*p*	Terminal	2.5	7.7	4.6	5.0	7.5	1.2
	1 → 3	1.4	5.6	1.0	0.1	0.1	0.8
	1 → 4	5.6	29.5	83.4	86.8	69.0	—
	1 → 6	1.5			0.1	—	—
	1 → 3,4	1.1	1.2	1.0	1.4	0.5	0.9
	1 → 3,6		2.3			0.2	0.7
	1 → 4,6	2.6	1.9	3.3	3.8	8.1	—
	1 → 3,4,6		1.8	0.5		—	—
Gal*p*	Terminal	1.6	2.8	0.1	0.2	1.0	2.2
	1 → 3	0.8	3.5		—	0.3	2.2
	1 → 4	1.0	2.8	1.6	0.2	3.9	0.7
	1 → 6	0.5	1.8		0.1	—	—
	1 → 3,4	1.0	5.3		0.4	0.3	0.5
	1 → 4,6	1.6	0.3	1.0		—	—
	1 → 3,6	22.4	3.3	0.4	0.1	1.0	1.9
	1 → 3,4,6	0.6	0.1			—	—
Terminal Ara*f*/terminal Ara*p*		164:1	63:1	106:1	26:1	132:1	—

Methylation analyses carried out after Pronase digestion.
*After amylase digestion.

Mass spectrometry indicated minor proportions of the methyl ethers of rhamnose, xylose, fucose, mannose and glucose, indicating the presence of various 4-, 4,6-, 3-, 3,4- and 3,6-linkages containing glucan types, as well as of xylan. There are more frequent in wis-CP1. Terminal and 2-linked Xyl units may be present in xyloglucans [24].

Hot water extraction

In fractions HCP, HP1 and HP2, glucose was the main component with lower proportions of arabinose and uronic acids (galacturonic acid and 4-*O*-methyl-glucuronic acid were detected by paper chromatography in the ratio 9:1) (Table 1). The ws-fractions of HCP and HP1 showed a broad MWD with a preponderant peak at M_r 50000 (Table 3) and gave a positive iodine test, indicating the presence of starch-like glucans. This was supported by the high proportions of terminal, 4- and 4,6-linked glucopyranosyl residues, representing 94% of the methyl ethers (Table 4). The degree of branching derived from the methylation analysis was 4%.

The starch-like character of most of the glucan components of the ws- and wis-fractions indicated by methylation analysis was confirmed by the chemical shifts of C-1/C-6 of the (1 → 4)-α-D-glucan backbone at δ100.8, 72.6, 74.4, 79.4, 72.3 and 61.6 in the ^{13}C NMR spectra in D_2O (Fig. 1) in accordance with reference data for amylose and amylopectin [25–27]. The signals at δ100.5 (shoulder), 72.5, 70.6 and 61.0 were assigned to C-1, C-2, C-4, and C-6, respectively, of α-D-glucopyranosyl residues terminating the 6-linked side-chains [27, 28]. Only

the signals of C-4,5 of the 4,6-linked glucose, which appeared at δ70.6, are visible in the spectrum.

0.5 *M* NaOH *extraction*

Dilute alkali extracted further material (AP) from the hot-water residue. A positive iodine test, methylation analysis (Table 4) and the ^{13}C NMR spectrum of ws-AP (Figs 1 and 2) indicated the presence of amylose-rich starch. AP also contained a typical 4-*O*-methylglucuronoxylan, a cell wall component of monocot and dicot plants [23]. This was evident from the preponderance of 4-linked xylosyl units (Table 4) and the presence of signals at δ102.5/102.7 and 64.1, assigned to C-1 and C-5, respectively, of 4-linked xylopyranosyl units, and at δ98.7 and 60.1, corresponding to C-1 and OCH_3 groups of 4-*O*-methyl-α-D-glucuronic acid [16] in the ^{13}C NMR spectrum (Fig. 1).

0.01 *M EDTA extraction*

Pectic polymers held in the cell wall complex by divalent ions were extracted by dilute EDTA. The ethanol-precipitable fraction, EP, had a broad MWD with a main peak at M_r 85000 and a smaller peak at $M_r > 10^6$. Such high M_r fractions were found to various extents in all polysaccharide fractions isolated in the previous extraction steps. Based on the high content of uronic acid with a preponderance of galacturonic acid, neutral sugar composition (Table 1), methylation analysis (Table 4) and a positive iodine test, EP was considered to be a mixture mainly of pectic polysaccharides rich in 3,5-linked arabinan and of α-1,4-glucan.

The ^{13}C NMR spectrum of EP exhibited the complete set of signals of a (1 → 4)-α-D-galacturonan backbone at δ100.31, 69.21, 69.93, 79.15, 72.29, and 176.40 (C-1/C-6), in accordance with published data [22]. The C-1 and H-1 signals of arabinofuranosyl residues linked to galactan moieties (expected at δ 110.3 and 5.25, respectively) and C-1 of β-D-galactopyranosyl residues (δ 104.5–105.5) could not be distinguished from noise. The arabinose component, representing *ca* 70% of the neutral sugars in EP digested with amylases gave strong signals for terminal, 5- and 3,5-linked α-arabinofuranosyl residues (Table 5). The relative intensities of their C-1 signals agreed with methylation analysis data. Minor amounts of 2-, 3- and 2,5-linked arabinofuranosyl units were found, but no terminal arabinopyranosyl ones. These results, and the preponderance of 2,4-linked rhamnopyranose, suggest that the pectic polymers of EP are rich in 'hairy' regions containing highly branched (1 → 3, 5)-α-L-arabinan side-chains. The proportion of the arabino-3,6-galactan component present in the cold water extract was low.

Table 5. ^{13}C NMR chemical shifts (in D_2O) of arabinose component from EP fraction of *Cistanche deserticola*

Sugar linkages	Chemical shifts (δ)				
	C-1	C-2	C-3	C-4	C-5
α-L-Ara*f*	108.5	82.5	77.9	85.0	62.2
5-α-L-Ara*f*	108.5	82.0	77.9	83.4	67.7
3,5-α-L-Ara*f*	108.2	80.5	83.4	82.5	67.4

CONCLUSIONS

Although the results presented provide no exact chemical structures for any of the isolated polysaccharide fractions, it is obvious that the *Cistanche* drug contains, in comparison with the host hardwood plant, a very high amount of easily extractable non-hemicellulosic polysaccharides, particularly starch-like glucan, arabinogalactan and pectic polymers rich in arabinans. Such features are typical of primary cell walls of higher plants [23]. Most of the pectic polysaccharides isolated from medicinal plants, like *Panax ginseng* [29], *Angelica acutiloba* [30] and *Artemisia princeps* [31], were reported to be associated with arbino-3,6-galactans. They contain 5- and 3,5-linked arabinan moieties which were suggested to be important for expression of anticomplementary [32, 33] activity and to be potentiators of the reticuloendothelial system [34].

As both ws-CP1 and EP were found to exhibit significant mitogenic and comitogenic activities (unpublished results), further detailed studies on purified fractions are now in progress.

EXPERIMENTAL

Materials. Whole underground parts of *C. deserticola* Y.C.Ma were collected in 1990 in Baian-Chongor province, Mongolia. A voucher specimen of the plant is deposited in the Herbarium of the Institute of Botany of the Mongolian Academy of Sciences, Ulan-Bator. Air-dried material was frozen and then ground to yield a brownish powder with a moisture content of 22.2%. It contained 7.7% of Klason lignin and 14.8% of protein, based on dry wt [6]. Separon HEMA-BIO 100 and 1000 columns were supplied by Tessek Ltd (Prague, Czech Republic).

General methods. Moisture content was determined by drying at 105° to constant wt. All yields and composition calculations were made on a moisture-free basis. Protein was calculated from the N content (%N × 6.25) assayed using an elemental analyser. Total carbohydrate and uronic acid contents were determined by the phenol–H_2SO_4 [35] and 3-hydroxydiphenyl [36] methods, respectively, using glucose and galacturonic acid as respective standards. PC was performed by the descending method on Whatman No. 1 in the systems S1: EtOAc–pyridine–H_2O (8:2:1) and S2: EtOAc–HOAc–HCO_2H–H_2O (18:3:1:4); reducing sugars were detected with anilinium hydrogen phthalate. Polysaccharide frs were hydrolysed with 2 M TFA under reflux for 2 hr. The hydrolysate was sepd into neutral and acidic sugars by ion-exchange on Dowex 1 × 8 (acetate form) and the acidic sugars analysed by PC. Lignin content of polysaccharide frs were determined as the acid-resistant portion after TFA hydrolysis. Optical rotations were measured at 20 ± 1° in H_2O or 0.5% NaOH. HPGPC was performed

on Separon HEMA-BIO 100 and 1000 columns (calibrated with standard Pullulans P-5, P-10, P-20, P-50, P-100, P-200, P-600 and P-800, Macherey-Nagel) using aq. 0.1M $NaNO_3$ as solvent and eluent. The eluate was monitored by refractometry and UV-absorption at 254 nm. IR spectra were recorded in KBr pellets. For the quantitative determination of sugars in the form of their alditol trifluoroacetates [37], a GC capillary column (25 m × 0.32 mm i.d.) packed with PAS 1701 and a temp. range from 110° (1 min) to 125° (2° min^{-1}) were used. CI- and EI-MS were obtained in the positive-ion mode. Vapours of pyridine were directly introduced into the source of the mass spectrometer. The pressure of the pyridine (1.3 torr) was adjusted so that ratio of $PyrH^+$ (*m/z* 80) to Pyr^+ (*m/z* 79) was 10:1. CI spectral data were obtained by scanning in mass range 280–600 mu. In special cases, selected ion-monitoring was used. Samples were introduced via GC. A fused silica capillary column SP-2330 (30 m × 0.25 mm i.d.) and He as the carrier gas at a flow rate of 25 ml min^{-1} were used. Temp. programming was from 80 to 240° at 6° min^{-1}.

NMR. ^{13}C NMR spectra (75 MHz) of samples (2% in D_2O or DMSO-d_6) were recorded at 40° in the inverse-gated decoupling mode. Chemical shifts are reported relative to internal MeOH ($\delta_{TMS} = 50.15$). ^1H NMR spectra (300 MHz) were measured under the same conditions.

Fractional extraction. Air-dry ground plant (1286 g) was suspended in aq. MeOH (5 l) and extracted (× 5) under reflux. The residue after MeOH extraction (MIR) was washed with Me_2CO and dried at 40° (393 g). MIR (300 g) was macerated (× 4) with cold dist. H_2O (2 l) under stirring for 2 hr and filtered. The combined filtrates were concd under red. pres. and dialysed against dist. H_2O. The dialysable portion was concd to a syrup yielding fr. DS [6]. The non-dialysable part gave a ppt (CP1, 5 g) by adding 3 vols of EtOH. After addition of a further vol of EtOH to the supernatant, the ppted fr. CP2 (0.2 g) was sepd by centrifugation and the supernatant evapd to dryness yielding fr. CNS. The plant residue was washed with Me_2CO and air-dried (229 g). Then, it was extracted (× 4) with hot H_2O (3 l) at 80° for 2 hr. On cooling, the combined extracts yielded a ppt. which was sepd by centrifugation (fr. HCP, 5.3 g). From the supernatant, fr. HP1 (5 g) was ppted with three vols of EtOH and fr. HP2 (0.4 g) was sepd by addition of a further vol. of EtOH. The air-dry residue (257 g) was extracted with 0.5 M aq. NaOH (3 l) at room temp. for 2 hr. The alkaline extract was filtered and the residue washed with 1 l of 0.5 M NaOH. From the combined extracts, polysaccharides were ppted with three vols of EtOH, filtered, suspended in 3% HCl in EtOH and dialysed against dist. H_2O until free of Cl^- ions, The ppt was dried by solvent-exchange yielding fr. AP (29 g). The alkali-insoluble material was washed with dil. HOAc H_2O, EtOH and air-dried, yielding residue AIR (160.5 g; protein and lignin contents were 1.7 and 1.6%, respectively, based on original plant solids). AIR (20 g) was treated × 2 with 0.01 M EDTA (200 ml, pH 6.8) at room temp. for 2 hr. The extracts after concn and dialysis were ppted with 3 vols of EtOH. The formed ppt. was sepd by centrifugation and lyophilization yielding fr. EP (3.8 g). The EDTA-insoluble residue (15.7 g) represented 12.6% of the original solids.

Crude polysaccharide frs were dispersed in dist. H_2O in the ratio 1:100 (w/v) and left under stirring at room temp. for 2 hr. The ws portions were isolated by centrifugation, concd to a smaller vol., dialysed and finally lyophilized. The insoluble residues (wis) were dispersed in H_2O, dialysed and lyophilized.

Methylation analysis. Methylation of polysaccharides was performed with the DMSO–solid NaOH–MeI reagent [38]. The sample (50 mg) was dissolved in DMSO (2 ml), then finely powdered NaOH (200 mg) was added to the soln. After stirring under N_2 for 30 min, MeI (1–1.5 ml) was added slowly with external cooling and the reaction allowed to proceed at room temp. for 1 hr. Then, cold H_2O was added and the mixt. dialysed against dist. H_2O. The methylated product was extracted with $CHCl_3$ and dried over Na_2SO_4. The absence of free OH groups was checked by IR. Hydrolysis of permethylated samples was performed with 90% HCO_2H under reflux for 1 hr. After evapn of acid, hydrolysis was completed with 2 M TFA under reflux for 3 hr. The product was converted into alditols by reduction with $NaBD_4$ and acetylated for GC and GC-MS analysis.

Pronase digestion. Samples (200 mg) were dissolved in dist. H_2O (20 ml) and the pH was adjusted to 7.5 with NaOH. Pronase from *Streptomyces griseus* (3 mg 50 mg^{-1} sample) was added and the soln incubated at 37° for 48 hr. After digestion, the enzyme was inactivated by heating the mixt. at 100° for 10 min. The insoluble part was removed by centrifugation and the soluble part dialysed and lyophilized.

Amylase digestions. Samples were dissolved in 0.2 M NaOAC buffer (pH 6.1) to make a 1% soln. α-Amylase (1 mg 10 mg^{-1} sample) and γ-amylase (1 mg 20 mg^{-1} sample) solns in the same buffer were added and the mixt. incubated at 37° for 72 hr. Digestion was checked using the I_2–KI test for amylose. After inactivation of enzymes (100° for 10 min), the soln was centrifuged and the supernatant dialysed and lyophilized to give the amylose-free sample.

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