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O-ACETYLATED XYLOGLUCAN IN EXTRACELLULAR POLYSACCHARIDES FROM CELL-SUSPENSION CULTURES OF *MENTHA*

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Key Word Index—*Mentha arvenis* × *M. spicata*; Labiatae; cell-suspension cultures; extracelluar polysaccharide; hemicellulose; xyloglucan; *O*-acetylated xyloglucan.

Abstract—Extracellular polysaccharide produced by suspension-cultured *Mentha* cells consisted of 50% neutral sugars, 32% uronic acid and 10% of protein. The ammonium oxalate-soluble fraction of this polysaccharide contained 30% hemicellulose and 70% pectic substances. The purified hemicellulose contained xylose, glucose, arabinose, galactose, mannose and fucose residues in a molar ratio of 41.6:31.3:13.1:11.1:1.3:1.6. It was identified as a xyloglucan from its neutral sugar composition and by methylation analysis and cellulase treatment: the principal neutral sugar was arabinose. The presence of *O*-acetyl residues was confirmed with ^{1}H NMR, ^{13}C NMR and GC-mass spectrometry. The total acetyl content in the polysaccharide was 4%. The point of attachment of the *O*-acetyl residue was shown to be at position 6 of the galactosyl residue.

INTRODUCTION

Plant cell walls are composed of two types, primary and secondary. The primary cell wall plays an important role in controlling the growth rate of cells and morphogenetic processes, and acts as a barrier to plant pathogens. A cell wall is a complex network of several macromolecules, mainly polysaccharide in nature, comprising pectic substances, hemicelluloses and fibrillar cellulosic material.

Xyloglucans, commonly regarded as a hemicelluloses, account for 20–25% of the dry weight of the primary cell walls of dicotyledons; they appear to be tightly bound, throughout the primary cell wall, to the cellulosic microfibrils by means of hydrogen bonds [1]. Xyloglucans were first characterized as an amyloid in *Tamarindus indica* seeds [2, 3], and were isolated from the cell walls of dicotyledons, the medium of suspension-cultured cells [4] and then from its primary cell walls [5]. The basic structure of the polysaccharide was determined by Kooiman [2] in his study on the amyloid of *T. indica* seeds. They consist of a backbone of β-(1 → 4)-linked D-glucose residues, which have an α-D-xylopyranose residue attached at O-6. Xyloglucan, because of its possible role in wall-loosening in response to auxin action, has been the most thoroughly studied noncellulosic polysaccharide of primary cell walls [6–9]. In dicotyledenous walls, these polysaccharides appear to undergo bond-breakage in response to auxin [9].

Knowledge of the nature of polysaccharides in primary cell walls is important for clarifying the cause–effect relationship between auxin-induced elongation and chemical changes in the polysaccharides. The constituents of primary cell walls can be easily obtained from whole-plant tissues, but there is a major problem in the methods which require drastic chemical conditions for extracting hemicelluloses. Thus, conventional chemical-extraction procedures almost always involve the cleavage of covalent bonds and often alter the structures [10].

It was reported in a previous study that a suspension culture of *Mentha*, established from a callus formed on the tips of young shoots of a *Mentha* hybrid (*M. arvenis* × *M. spicata*), produced extracellular polysaccharides (ECP) in the medium [11]. The ECP of *Mentha* suspension-cultured cells is composed of pectic polysaccharides and hemicellulose. Xyloglucan separated from the hemicellulose fraction in ECP is high in *O*-acetyl residue content. *O*-acetylated xyloglucans, until recently, were rarely observed because of the strong alkaline conditions needed to solubilize xyloglucans from cell walls. However, ECPs are a source of soluble xyloglucans that can be isolated without using alkaline extraction. York *et al.* [12] found the isolated xyloglucan from sycamore ECP to have *O*-acetyl groups. Spellman *et al.* [13], based on the ^{1}H NMR spectrum of the heptasaccharide obtained

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from rhamnogalacturonan II of suspension-cultured sycamore cells by acid hydrolysis, also confirmed the presence of *O*-acetyl groups. This evidence supports the view that suspension-cultured cells provide a source of primary cell walls for studying their structures and roles.

In the present study, the xyloglucan from *Mentha* was isolated from ECP and the structure of the *O*-acetylated xyloglucan was determined. The roles of *O*-acetyl residues in the xyloglucan are discussed.

RESULTS

Extracellular polysaccharide composition

The ECP from *Mentha* was found to consist of 50% neutral sugars, 31.6% uronic acid and 11.1% protein. Glycosyl components of the ECP were 40.5% arabinosyl, 24.3% galactosyl, 17.4% xylosyl, 11.6% glucosyl, 3.5% rhamonosyl, 2.3% mannosyl and 0.5% fucosyl residues as mol%. The uronic acid in the ECP was galacturonic acid, determined as galactose by carboxyl reduction of the uronic acid [14].

Fractionation of extracellular polysaccharide

The ECP (1 g) was dissolved in 300 ml of 20 mM ammonium oxalate solution (pH 4) and stirred for 2 hr at room temperature. The solution was centrifuged at 10 000 *g* for 10 min and insoluble material separated. The dry wt of the insoluble material was 127.4 mg (12.7%); the material comprised 4.5% of neutral sugars, 3.9% uronic acid, 10.6% proteins and 48.8% inorganic materials. The most abundant inorganic substance was calcium (32.4%).

The supernatant solution was dialysed at 5° against deionized water, followed by dialysis against 50 mM phosphate buffer (pH 7) solution and treatment with Actinase E (5 mg added to 300 ml of the solution along with sodium azide (0.02%) as preservative) for 72 hr, at 45°. The solution was then dialysed against deionized water and freeze-dried: 784 mg (78.4%) of white powder (purified ECP) were obtained.

Purified ECP (200 mg) was dissolved in 10 mM phosphate buffer solution (pH 7), centrifuged at 10 000 *g* for 10 min and insoluble material removed. The supernatant solution was loaded onto a DEAE–Toyopal column (2.8 × 20 cm) pre-equilibrated with 10 mM phosphate buffer (pH 7) and eluted at 100 ml hr^{-1} with the same buffer (three volumes of column bed) and then a linear gradient of 0 to 500 mM NaCl containing the same buffer solution: 6.2 ml fractions were collected and analysed for neutral sugar (orcinol–H_2SO_4 method) and uronic acid content (*m*-hydroxydiphenyl method). Appropriate fractions were combined, dialysed and freeze-dried. Recovery of nonadsorbed hemicellulose and absorbed- uronic acid fraction was 58.1 mg (29%) and 133.1 mg (67%), respectively.

The uncharged hemicellulose fraction was purified by gel filtration chromatography. Gel filtration on a Sephacryl S-400 column (2.5 × 91 cm) pre-equilibrated with 50 mM phosphate buffer (pH 7) solution containing 0.1 M NaCl was carried out on the sample (50 mg) followed by elution with the same buffer solution. Fractions (6.8 ml) were assayed for the presence of neutral sugars by the phenol–H_2SO_4 method. Fractions 40 to *ca* 58 of peak II (Fig. 1) were pooled and dialyzed against deionized water and lyophilized. The yield of peak II (hemicellulose fraction) was 44 mg (88%). Hemicellulose (Peak II) was eluted as a single peak by repeated gel chromatography conducted under the same conditions. To confirm the homogeneity of the material, paper electrophoresis was carried out: the hemicellulose gave a single peak (Fig. 2).

Sugar composition of hemicellulose

The neutral sugar composition of the hemicellulose was determined by complete acid hydrolysis. Fucose, arabinose, xylose, mannose, galactose and glucose were found to be present with molar ratios of 1.6, 13.1, 41.6, 1.3, 11.1 and 31.3, respectively. Hemicellulose is characteristically rich in glucose, xylose and arabinose. The xylose content was much higher than that of glucose and the molar ratio of xylose to glucose was 1.4. No uronic acids could be detected in the polysaccharide.

Methylation studies

Hemicellulose was subjected to linkage analysis and the results are shown in Table 1. Overall recoveries of

Table 1. Methylation analysis of purified hemicellulose separated from ECP

Peak no.	Partially methylated alditol acetate	Deduced linkage	RR_t*	Mol%
1	2,3,5-Me_3-arabinitol	T-Araf (1 →	0.49	12.9
2	2,3,4-Me_3-xylitol	T-Xylp (1 →	0.66	24.5
3	3,4-Me_3-xylitol	→ 2) – Xylp (→	1.46	21.9
4	2,3,4,6-Me_4-mannitol	T-Manp (1 →	0.99	0.7
5	2,3,4,6-Me_4-galactitol	T-Galp (1 →	1.25	5.8
6	3,4,6-Me_3-galactitol	→ 2)-Galp (1 →	2.46	3.0
7	2,3,6-Me_3-glucitol	→ 4)-Glcp (1 →	2.53	10.0
8	2,3-Me_2-glucitol	→ 4, 6)-Glcp (1 →	5.50	16.9
9	3-Me-glucitol	→ 2,4,6) – Glcp (1 →	10.00	4.3

*Retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on DB-225. Mol% were determined based on the relative molar-response factors [27].

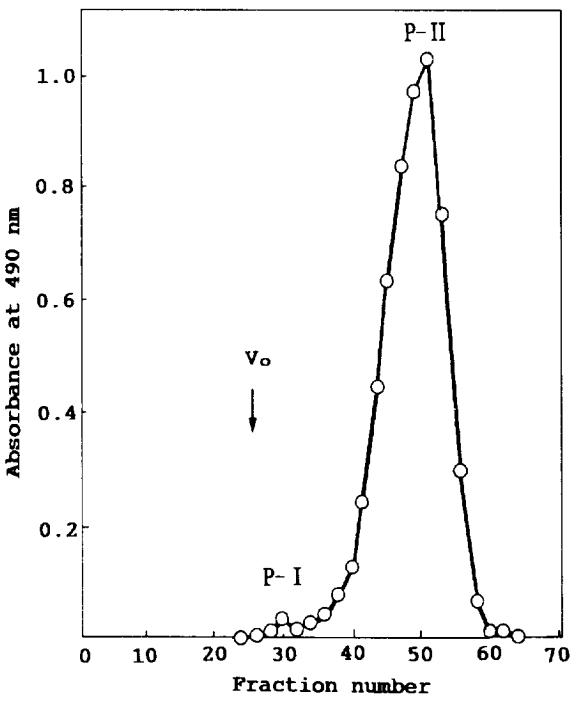

Fig. 1. Chromatogram of hemicellulose on Sephacryl S-400. Sample (50 mg) was introduced into the column (2.5 × 91 cm) pre-equilibrated with 50 mM phosphate buffer (pH 7) containing 0.1 M NaCl and eluted with the same buffer solution. The presence of neutral sugar was determined by the phenol–sulphuric acid method.

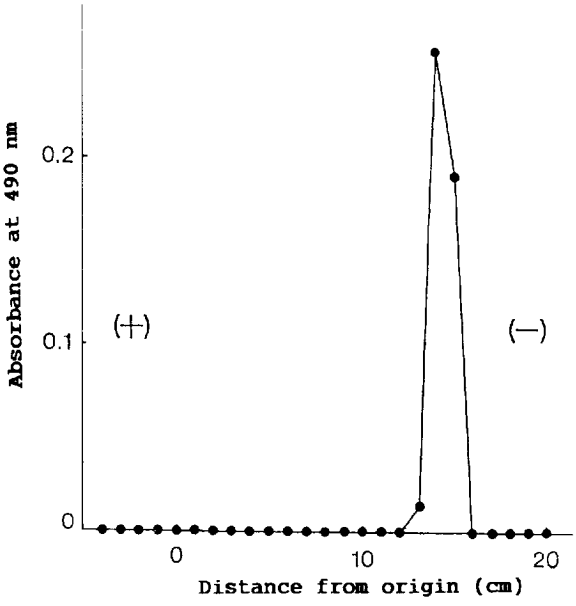

Fig. 2. Paper electrophoresis of purified hemicellulose. Electrophoresis was performed on Whatman GF/A glass fiber paper at 50 mA for 100 min with 0.05 M sodium borate buffer (pH 9.3). The paper was cut into 1-cm strips and sugars eluted from the strips with deionized water. Sugar content of each eluate was measured by the phenol–sulphuric acid method.

methylated alditol acetates (no methylated fucose could be detected), agreed well with sugar values obtained by direct analysis.

Methylation analysis indicated that the hemicellulose structure was based on a (1 → 4)-linked glucan backbone carrying substituents groups on C-6 and C-6, C-2 with *ca* 68% of glucose residues. The high proportion of terminal sugars (44%) indicated that side-chain oligosaccharides had a low degree of polymerization. Arabinose was furanoid and other sugars were pyranoid. Hemicellulose was highly substituted at C-2 and C-6 [15].

Enzymic hydrolysis

For further elucidation of its glucan backbone, the hemicellulose was treated with partially purified cellulase and purified β-1,3-glucanase. The polysaccharide was hydrolysed in the same manner as sodium carboxymethylcellulose (CMC) by cellulase, but not by β-1,3-glucanase (Fig. 3). The result of hemicellulose degradation with cellulase indicated the structure of the xyloglucan to be based on a (1 → 4)-linked D-glucan backbone.

Mild acid hydrolysis

Analysis following partial acid hydrolysis (in 10 mM oxalic acid for 2 hr at 100°) of the purified xyloglucan indicated that only the arabinose and fucose residues had been hydrolysed. Arabinose and fucose were the only sugars detected in the acid hydrolysate, indicating each monosaccharide to be a terminal component.

^1H NMR of xyloglucan

In the ^1H NMR spectrum of the xyloglucan, the signal at δ2.14 (*S*) was assigned to the methyl proton of *O*-acetyl residues and to methyl protons of the *O*-acetyl group on the 6-mono-*O*-acetyl-D-galactopyranosyl residues, based on published data [12, 16]. Signals at δ1.317, 1.303 (*d*) and 1.241 (*S*) indicated the presence of two fucosyl residues.

Signals in the ^{13}C NMR spectra at δ23.0 (methyl carbon of *O*-acetyl residue) and 176.6 (carboxyl carbon of *O*-acetyl residue) confirmed the presence of the *O*-acetyl residue in the xyloglucan.

The proportion of *O*-acetyl groups was estimated to be *ca* 2.66 mol% by comparison of the integrals for the methyl protons of fucosyl and *O*-acetyl residues.

Identification and estimation of O-acetyl residues

^1H and ^{13}C NMR signals indicated that acyl groups were possibly present in the polysaccharide. The acidic compound arising from the acyl groups was investigated and its retention time and mass spectrum were identical to those of acetic acid. The total acetyl content (as acetic acid) in the polysaccharide was 11% by HPLC and

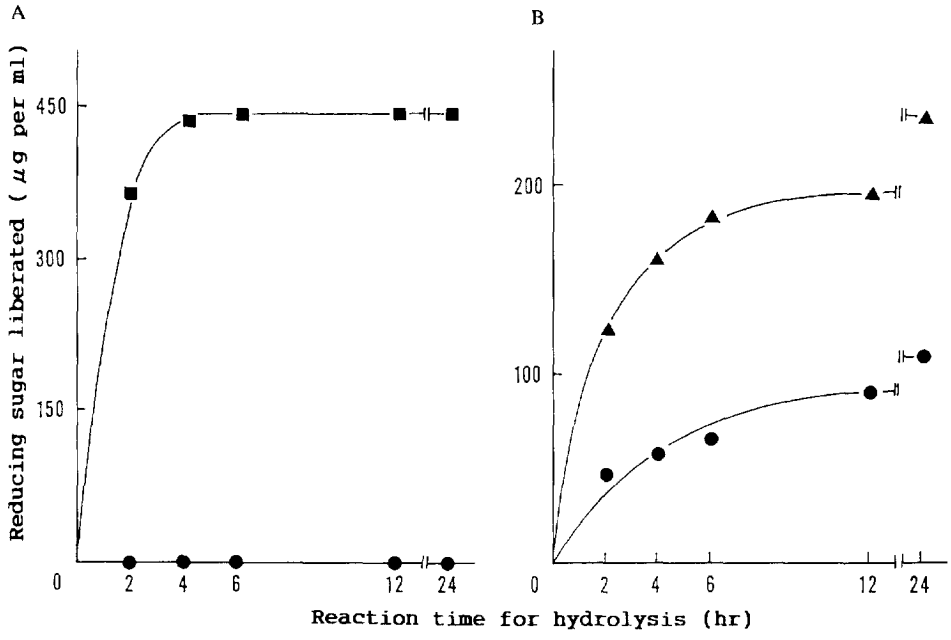

Fig. 3. Time-course of enzymic hydrolysis of purified hemicellulose with (A) *endo*-β-1,3-glucanase and (B) cellulase. Increase in reducing sugar content was measured by the Somogyi–Nelson method. Symbols: ●, xyloglucan; ■, laminarin; ▲, carboxymethylcellulose.

4.36% by the method of selected ion-monitoring using LC-mass spectrometry.

DISCUSSION

Suspension-cultured *Mentha* cells produced large quantities of ECP in the growth medium. This was found to consist of 50% neutral sugars, 32% uronic acid and 10% protein. No other oligo- and monosaccharides could be detected in the residual culture medium from which ECP had been separated by ethanol precipitation. The ammonium oxalate-soluble fraction of the ECP contained 30% hemicellulose and 70% pectic substances. This indicated that the ECP consisted mostly of matrix polysaccharides from the primary cell walls. By ion-exchange and gel chromatography, the neutral hamicellulose, homogeneous by gel chromatography and paper electrophoresis, was obtained.

The neutral polysaccharide was identified as a xyloglucan from its neutral sugar composition, methylation analysis and degradation studies with cellulase. It had a (1 → 4)-linked D-glucan backbone and was rich in glucosyl residues at positions 6 and 2,6 [15]. Arabinose was the most abundant neutral sugar in the polysaccharide.

^{1}H and ^{13}C NMR confirmed the presence of *O*-acetyl residues in the xyloglucan. The degree of *O*-acetylation of the β-D-galactosyl residue was estimated by ^{1}H NMR to be 55 to around 60% at *O*-6, 15 to around 20% at *O*-4 and 20 to around 25% at *O*-3. The only point of attachment of the xyloglucan extracted from *Mentha* ECP was position 6 of the galactopyranosyl residue (6-mono-*O*-acetyl-D-galactopyranosyl) [12,15].

There was a wide difference between the two measurements of the acetyl content of the polysaccharide. However, comparison of the integration of the methyl protons of the fucosyl and *O*-acetyl residues indicated the *O*-acetyl group content to be 2.7 mol%. This value shows a relatively good approximation to the value obtained using mass spectrometry.

Hara *et al.* [17] isolated a linear (1 → 3)-α-D-mannan from fruit bodies of *Dictyophora indusiata*. *O*-Acetyl groups in the polysaccharide comprised 11.4% and were located only at position 6 of the α-(1 → 3)-linked D-mannopyranosyl residue. This polysaccharide dissolved slowly in water to give a slightly viscous solution; the *O*-deacetylated polysaccharide was insoluble in water. O'Neil *et al.* [18] found that the partially *O*-acetylated polysaccharide produced by *Klebsilla* K54 gave a viscous aqueous solution and was capable of forming cation-dependent gels. In addition, they noted that the presence of *O*-acetyl groups in a polysaccharide causes significant changes to its secondary and tertiary structures. The solubility of *O*-acetylated xyloglucan may be dependent on the above features and be the cause of the liberation of matrix polysaccharides from primary cell walls and their dissolution in culture media.

Suspension-cultured *Mentha* cells consist mostly of sausage-like elongated cells which connect with each other longitudinally when grown in B-5 liquid medium containing 1 $mg\,l^{-1}$ of 2,4-D [11]. When the cells are transferred to a B-5 medium free of 2,4-D, remarkable morphological differences changes occur: cell elongation stops and elongated cells divide into smaller cells, similar to fungal mycelia. Conversely, partial dissolution of wall-bound polysaccharides (ECP) in the medium decreases

markedly in the absence of 2,4-D (unpublished data). These results indicate that *Mentha* cells show typical responses to auxin-induced wall-loosening and an increment of cell expansion in the presence of 2,4-D. Acetylation of the xyloglucan in *Mentha* cells appears to be requisite for increasing the elasticity of primary cell walls.

Auxin-induced wall-loosening mechanisms remain unknown, but much attention has been focused on the xyloglucans of the major hemicellulose in the wall polymer as the site of action. Enzymic cleavage [9] and transglycosylation [19] of xyloglucans are thought to promote wall-loosening and to promote cell expansion. Although there is little information related to the roles of *O*-acetylated xyloglucans in wall-extensibility, it is clear that *O*-acetyl residues cause considerable rheological changes [8] to the xyloglucans in primary cell walls.

EXPERIMENTAL

Cell suspension cultures. Suspension cultures of *Mentha* were prepd as previously described [11]. Cells were grown in Erlenmeyer flasks (300 ml) containing 100 ml B-5 liquid medium supplemented with 2,4-D (1 mg l^{-1}) and sucrose (30 g l^{-1}). All flasks were shaken on a rotary shaker at 100 rpm in the dark at 25°.

Preparation of extracellular polysaccharide. The liquid medium of 8- to 10-day-old cultures was sepd from the cells by filtration through a Kimwipe S-200 (Jujou Kimberly Co.) using red. pres. The filtrate was concd to an appropriate vol. by rotary evapn and the small ppt was removed by centrifugation (10000 *g*, 10 min) and discarded. Crude ECP was recovered by pptn with EtOH (3–4 vols) and allowed to stand overnight at 4°. The ppt. was collected by centrifugation at 2500 *g* for 15 min, redissolved in deionized H_2O and dialysed against deionized H_2O for 2 days at 4°. The dialysate was then freeze-dried and stored in a dessicator. The yield of ECP was 925 mg dry 1000 ml^{-1} medium.

Estimation of sugar contents. Uronic acid content was determined by the *m*-hydroxydiphenyl method [20, 21] and expressed as galacturonic acid equivalents. Total sugar was determined by the phenol–H_2SO_4 method [22] neutral sugars by the orcinol–H_2SO_4 acid [23] method. Sugar contents using both methods was expressed as arabinose equivalents.

Acid hydrolysis of polysaccharide and glycosyl composition. To determine neutral sugar composition, polysaccharides were hydrolysed with 1 N TFA in a N_2-purged test tube with a Teflon-lined screw cap for 1,2 and 3 hr and with 2 N TFA for 4,5 and 6 hr at 100°, respectively. After removal of TFA under red. pres., the sugars liberated were converted to alditol acetates [24] and estimated by GC using a glass column (200 × 0.3 cm) containing supelcoport (60/80 mesh) coated with 3% OV-225. Neutral sugar contents of the polysaccharides was determined from maximum peak areas of monosaccharides released under the test conditions. Methyl-β-D-glucoside served as int. standard [25].

Paper electrophoresis. This was conducted on Whatman GF/A glass micro-fibre paper with 0.05 M Na borate buffer (pH 9.3) at 50 mA for 100 min. Following electrophoresis, the paper was cut into 1-cm strips and the sugar on each strip eluted with deionized H_2O. Total sugar content of each eluate was measured by the phenol–H_2SO_4 method [22].

Methylation analysis. Methylation of carbohydrate samples was carried out by the method of Hakomori as described in ref. [26]. Sugar components of methylated polysaccharides were analysed as partially methylated alditol acetates (PMAA) by GC and GC-MS [27]. GC was carried out using (A) a DB-225 glass capillary column (30 m × 0.55 mm) and (B) a 3% ECNSS-M glass column (2 m × 3 mm). Sepns on column A were performed from 150–190° at 1° min^{-1} and, column B, at 170° isothermal. Peak identification was made by GC-MS analysis of PMAA and by comparision of GC R_ts with 2,3,4,6-Me_4 Glc. GC-MS was performed in the EI mode, using a wide-bore glass column (DB-225, 30 m × 0.55 mm, J & M Scientific) and 3% ECNSS-M-coated glass columns.

Enzymic degradation. Partially purified cellulase (Cellulase Onozuka R-10) from *Trichoderma viride*, (Seikagaku Kogyo Co., Ltd) was used. A soln of polysaccharide (10 mg) in 0.2 M Na acetate buffer (10 ml, pH 5) was incubated with 0.1 mg of cellulase at 45° for 24 hr. Carboxymethyl cellulose (CMC) (10 mg) was incubated under the same conditions as a control. *endo*-β-1,3-Glucanase from *Escherichia coli*: HB101 pNTOO3R [28] was kindly provided by Dr Watanabe in our university: enzyme activity toward laminarin was 4000 unit mg^{-1} protein. A soln of polysaccharide (10 mg) in 0.1 M succinate buffer (10 ml, pH 6) was incubated with 150 units of endo-β-1,3-glucanase at 37° for 24 hr. Laminarin (10 mg) was incubated under the same condition as a control. The time-course of reducing sugars liberated by enzymic hydrolysis was monitored by the Somogyi–Nelson method [22].

Estimation of inorganic salts. Insol. material (100 mg) was mineralized by dry ashing [29] (10 hr at 200° and 10 hr 500°) in an electric furnace. Mineralized material was dissolved in 5 ml of 2.4 N HCl soln and heated at 180° for 5 min. After cooling, the soln was adjusted to constant vol. with H_2O. Mg, Ca, Zn and Mn were determined by atomic absorption spectrometry, K and Na by flame photometry. P was estimated using the molybdenum-blue reaction [30].

^1H and ^{13}C NMR. ^1H NMR were recorded at 50° in D_2O (38 mg ml^{-1}). A spectral width of 5998.80 Hz was used with 32768 data points: 64 scans were made. Proton chemical shifts were measured relative to the int. standard Na 4,4-dimethyl-4-sila[2,2,3,3-$^2H_4$]pentanoate (TSP). ^{13}C NMR spectra were recorded at 55° in D_2O (30 mg ml^{-1}) with complete proton-decoupling. A spectral width of 24038.5 Hz was used with 32000 data points; 30000 scans were made. ^{13}C chemical shifts were measured relative to the int. standard TSP.

Identification and estimation of O-acetyl residues. To identify *O*-acetyl groups by GC-MS, xyloglucan (15 mg)

was dissolved in 3 ml of 1M NaOH soln and stirred at room temp. for 1 hr. The pH of the soln was then adjusted to 2 with HCl and the acidic compound liberated in the soln was extracted with Et_2O. The extract was dried (Na_2SO_4), the Et_2O removed in a stream of N_2 and the residue dissolved in a small amount of MeOH and analysed by GC-EI-MS using a wide-bore column (DB-Wax, 0.55 mm × 30 m, J&M Scientific) at 120°.

For estimation of *O*-acetyl residues, the xyloglucan was suspended in a 0.5 N soln of KOH in 95% EtOH and the mixt. stirred at room temp. for 1 hr. The reaction mixt. was then centrifuged at 5000 *g* for 10 min and the supernatant evapd to dryness. The residue was dissolved in a small amount of H_2O and treated with Amberlite GC-120 (H^+ form). After thorough mixing and filtering (Toyo Roshi No. 2), the supernatant was made up to 10 ml with H_2O and analysed by HPLC and LC-MS equipped with an atmospheric pressure chemical ionization (APCI) interface. HPLC of HOAc was performed on TSK gel OApak-P (6 mm i.d. × 4 cm) and TSK gel OApak-A (7.8 mm i.d. × 30 cm) columns. A UV detector was used to detect HOAc at 210 nm. The column was maintained at 40° and eluted at 0.8 ml min^{-1} with 0.75 mM H_2SO_4. Peak area determinations were made with a data module LC-MS for estimation of HOAc was performed using SIM at *m/z* 59 in the negative-ion mode. The sample soln containing HOAc and standard HOAc solns were fed into the LC-MS by a constant vol. flow injection method (1 ml min^{-1}).

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