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Note

Isolation and characterization of feruloylated arabinoxylan oligosaccharides from bamboo shoot cell-walls

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Hydrolysis of bamboo shoot cell-walls with Driselase (a fungal enzyme preparation) gave arabinoxylan oligosaccharides containing ferulic acid. The structures of two feruloylated oligosaccharides are here determined to be *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose and *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-(*trans*-feruloyl-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose, on the basis of ^{1}H- and ^{13}C-n.m.r. spectroscopy, methylation analysis, and f.a.b.–m.s.

Ferulic acid is one of the phenolic acids esterified to polysaccharides in plant cell-walls. The linkage positions between ferulic acid and carbohydrate are determined by analysis of low-molecular-weight carbohydrate esters of ferulic acid obtained from enzymic hydrolyzates of cell walls[1–6]. In 1982, Fry[1] isolated two feruloylated disaccharides, 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and 3-*O*-(3-*O*-feruloyl-α-L-arabinofuranosyl)-L-arabinose, from cell walls of a suspension-cultured dicot (spinach), and suggested that these disaccharides were derived from pectin. Later, feruloylated oligosaccharides, probably derived from hemicellulosic arabinoxylan, were isolated from several numbers of the *Gramineae*[2–6]; these feruloylated arabinoxylan oligosaccharides include a trisaccharide, α-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose[3–5], and a tetrasaccharide, β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose[6].

It has been suggested[7–14] and recently summarized[15] that feruloyl residues undergo peroxidase-catalyzed oxidative coupling to yield cross-linked polysaccharides. Such coupling may contribute not only to the control of cell-wall extensibility and cell growth, but also to decreased digestibility by ruminant-

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secreted enzymes[16–18]. However, direct evidence for this hypothesis has not yet been obtained.

In the present study, carbohydrate–phenolic acid complexes in cell walls of growing bamboo shoots have been studied. Two feruloylated arabinoxylan oligosaccharides were isolated and their complete structures determined.

EXPERIMENTAL

General methods. — Evaporations were conducted under diminished pressure at <40°. Total carbohydrate was determined by the phenol–H_2SO_4 method[19]. Alditol acetates were prepared[20] and analyzed[21] as described, except that hydrolysis in 2M CF_3CO_2H was carried out for 20 min; g.l.c. was performed using a Shimadzu GC 14A instrument operated isothermally at 235° with a 15-m × 0.25-mm SP-2330 fused-silica column (Supelco). Absolute configurations were determined as described[22]. Per-*O*-methylation was performed using a modification[23] of the method of Hakomori[24], and per-*O*-methylated oligosaccharides and oligosaccharide alditols were purified as described[25]. Glycosyl linkage-compositions were determined by g.l.c.–m.s. of per-*O*-methylated alditol acetates[21]. Samples were per-*O*-acetylated for fast-atom-bombardment mass spectrometry (f.a.b.–m.s.) by the method of Bourne *et al.*[26].

Mass spectrometry. — G.l.c. was performed with a Hewlett–Packard 5890J g.l.c. coupled to a JEOL JMS-DX303 HF mass spectrometer, and JEOL JMA-DA5000 data system; ionization energy was 70 eV, emission current 300 μA, and source temperature 180°. For analysis of per-*O*-methylated alditol acetates, a 30-m × 0.25-mm SP-2330 column was used with a temperature program starting at 170° for 2 min, then increasing to 235° at 4° min^{-1}. Per-*O*-methylated oligosaccharide alditols were separated by using splitless injection and a 30-m × 0.25-mm fused-silica DB-1 column (J and W Scientific), with a temperature program starting at 50° for 3 min, then increasing to 150° at 30° min^{-1}, and finally to 340° at 6° min^{-1}.

F.a.b.–m.s. was performed in the positive-ion mode using a JEOL JMS-DX303 HF mass spectrometer. Native samples were dissolved in water, and loaded into glycerol on a stainless-steel target. Per-*O*-acetylated samples were dissolved in MeOH and loaded into 3-nitrobenzyl alcohol. Argon was used as the bombarding gas, and the gun was operated at 3 kV. Spectra were obtained at a scan rate that covered the mass range from 1 to 1800 mass units in 15 sec, using JEOL JMA-DA5000 data-system.

N.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were recorded at room temperature in D_2O with a Bruker AM-500 FT spectrometer operated at 125 MHz, using the noise-decoupled mode and deuterium lock. Chemical shifts were referenced to an external standard of MeOH (49.3 p.p.m.). ^1H-N.m.r. spectra were recorded using a JEOL GSX 400 spectrometer operated at 400 MHz and a temperature of 27°. Samples were dissolved in D_2O (99.996 atom%), and chemical shifts reported relative to H_2O (4.70 p.p.m.). 2D-^1H–^{13}C-COSY spectroscopy was performed with a JEOL GSX 400 spectrometer using the standard JEOL pulse program VCOSYN.

Phenolic acid determination. — Samples were saponified in 0.5M NaOH under N_2 for 24 h in the dark at room temperature, and then acidified with 6M HCl. The resultant solution was extracted with ether, and the ether phase was washed with water and evaporated. The total amount of phenolic compounds was estimated from the absorbance at 320 nm; values are expressed as ferulic acid equivalents. A portion of the ether extracts was fractionated by reversed-phase h.p.l.c. with an ODS column [(Shim-Pack Prep-ODS(H)-kit, 0.46 × 25 cm, from Shimadzu)]. Samples were chromatographed in 40% aq. MeOH containing 0.1% AcOH at a flow rate of 0.6 mL.min^{-1}. Phenolic acids were monitored at 320 nm. Ferulic, diferulic, and *p*-coumaric acids in u.v.-positive h.p.l.c. fractions were identified by using direct-insertion, electron-impact m.s. with an electron energy of 70 eV.

Plant materials. — Shoots (~30 cm long) of Mouso-chiku bamboo *(Phyllostachys edulis)* were collected in Tsukuba, Ibaraki Prefecture, in May 1984. The youngest growing parts (3 kg) were cut into slices and homogenized in 50mM Tris-HCl (pH 7.6) at 4° with a Waring blender. The homogenate was filtered through three layers of cheese cloth. The residue was washed successively with 50mM Tris-HCl buffer (pH 7.6), water, EtOH, 2:1 (v/v) benzene–ethanol for 5 h at 50° to remove lipids, and finally acetone, then air dried. The dried cell-wall material was extracted twice with 50mM ammonium oxalate for 1 h at 80° to remove pectic polysaccharides. The ammonium oxalate-extracted cell walls contained 4.6 mg of phenolic acids per g of dry weight. The ratio of *p*-coumaric acid, ferulic acid, and diferulic acid, determined by h.p.l.c., was 1:3:0.03 (w/w/w). Phloroglucinol reaction of the cell walls indicated lignin to be absent.

Isolation of compounds A and B. — Oxalate-extracted cell walls (20 g) were suspended in 1.0 L of 10mM NaOAc buffer (pH 5.0) and incubated for 16 h at 30° after addition of 3 mL of a 60 mg.mL^{-1} solution of Driselase [purchased from Kyowa Hakko, Tokyo, and purified as described[1]]. The suspension was heated for 20 min in a boiling-water bath to stop the reaction, and then centrifuged. The supernatant solution was passed through Dowex-50 (H^+ form) to remove Na^+ ions, and concentrated. Polysaccharides were removed from the digestion products by addition of 5 volumes of EtOH, the precipitate was removed by centrifugation, and the supernatant was evaporated to dryness. The EtOH-soluble, Driselase digestion-products were chromatographed on a column (2.5 × 90 cm) of Sephadex LH-20 eluted with water. Fractions of 5.0 mL were collected and assayed for total carbohydrate and phenolic acids. Two fractions, A and B, having K_{av} values of 1.94 and 3.14, respectively, contained predominantly feruloylated oligosaccharides. These two fractions were further purified using preparative reversed-phase h.p.l.c. with a 25 × 2.0 cm (i.d.) column [Shim-Pak Prep ODS(H)-kit from Shimadzu] at 40° eluted with 30% (v/v) aq. MeOH at 4 mL.min^{-1}. The eluates were monitored at 320 nm, and single peaks of u.v.-positive material were collected and lyophilized to give compounds A and B. (Using and analytical h.p.l.c. column at 40° eluted with 30% MeOH at 0.6 mL.min^{-1}, compound A was resolved into one major and one minor peak, probably corresponding to the α and β anomers of compound A.

Compound B, when chromatographed under the same conditions, yielded only one peak).

Alkaline hydrolysis and reduction of compounds A and B. — Compounds A and B (~300 μg of each) were separately dissolved in 250 μL of a 10 mg.mL^{-1} solution of $NaBD_4$ in 1.5M NH_4OH. After 3 h at room temperature, the solutions were acidified with AcOH, and extracted with ether. The ether phases were washed with water and evaporated. The aqueous phases were passed through Dowex 50W (H^+ form), to remove sodium ions, and dried. Evaporations with 1:9 (v/v) AcOH–MeOH (250 μL) and MeOH (250 μL) were performed 3 and 4 times, respectively, to remove borate. The ether extracts were analyzed for phenolic acids, and the oligosaccharide alditols A and B in the aqueous phases were per-*O*-methylated and analyzed, as already described.

RESULTS

Characterization of compound A. — Compositional analysis by g.l.c. of alditol acetates showed that compound A consisted of arabinose and xylose in a molar ratio of 1:2. The absolute configurations of arabinose and xylose were L- and D-, respectively. Methylation analysis (Table I) gave three derivatives, namely, 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol (derived from terminal arabinofuranosyl residues), 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylxylitol (derived from 3-linked xylopyranosyl residues), and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol (presumably derived from 4-linked xylopyranosyl residues). *trans*-Ferulic acid was the only phenolic acid released from compound A by alkali treatment and detected by h.p.l.c.

The positive-ion f.a.b. mass spectrum of the native material contained an intense ion at *m/z* 613, corresponding to the $(M + Na)^+$ quasimolecular ion of a molecule composed of one ferulic acid and three pentose residues. Weaker ions at *m/z* 591 and 629 corresponding to $(M + H)^+$ and $(M + K)^+$ quasimolecular ions, respectively, also were observed. In addition, fragment ions at *m/z* 441, 309, and

TABLE I

METHYLATION ANALYSIS OF COMPOUNDS A AND B, AND OLIGOSACCHARIDE ALDITOLS A AND B

Glycosyl residue	*Methylated derivative*	*Linkage positions*	*Compound*		*Oligosaccharide alditol*	
			A mol%	*B mol%*	*A mol%*	*B mol%*
Arabinofuranosyl	2,3,5	Terminal	25.8	18.0	53.8	23.5
Xylopyranosyl	2,3,4	Terminal	[a]	21.1		28.6
	2,3	4	28.6	24.3		
	2,4	3	45.6		22.3	
	2	3,4		35.6		43.5
Xylitol	1,2,3,5	4			24.0	4.5[b]

[a]Not determined. [b]In this case, the highly-volatile derivative was probably lost.

177, generated by loss of one, two, and three pentose residues, respectively, were observed; the same fragmentation pattern was observed by Meuller-Harvey *et al.*[5] when a feruloylated arabinoxylan trisaccharide was analyzed by the same method. The positive-ion f.a.b. mass spectrum of the per-*O*-acetylated material contained ions at *m/z* 949 and 907 corresponding to $(M + Na)^+$ quasimolecular ions of a fully acetylated and underacetylated feruloylated trisaccharide, respectively. In addition, fragment ions were observed at *m/z* 867, 651, 435, and 219, generated by loss of acetate and up to three pentose residues from the fully acetylated compound. A less-intense series of fragment ions derived from the underacetylated compound also was observed at *m/z* 609, 393, and 177; the expected fragment ion at *m/z* 825 was not observed, probably because of its low abundance. It is likely that the phenolic hydroxyl group of the feruloyl residue was the site of partial *O*-acetylation, as intenser fragment ions corresponding to the fully and under acetylated residues were observed at *m/z* 219 and 177, respectively.

Compound A was reduced with $NaBD_4$ to give ferulic acid and oligosaccharide alditol A. Methylation analysis (Table I) revealed terminal arabinofuranosyl and 3-linked xylopyranosyl residues, and 4-linked xylitol, but no 4-linked xylosyl residues, indicating that 4-linked xylose is present at the reducing terminus. E.i.-m.s. of per-*O*-methylated oligosaccharide alditol A gave ions at *m/z* 143 (cA_2, 42%), 175 (cA_1, 30), 192 (aJ_2, 100.0), 238 (aJ_0, 4.0), 303 (cbA_2, 2.4), 335 (cbA_1, 3.0), 412 (abJ_1, 4.8), 453 (ald, 1.4), and 497 (ald, 0.9). The presence in the e.i. mass spectrum of an aJ_0 fragment ion, and absence of an aJ_1 fragment ion at *m/z* 252, indicated the penultimate glycosyl residue was 3-linked[27]. On the basis of these results, oligosaccharide alditol A was assigned the structure L-arabinofuranosyl-(1→3)-D-xylopyranosyl-(1→4)-D-xylitol.

TABLE II

ASSIGNMENTS OF SIGNALS IN ^{13}C-N.M.R.[a] SPECTRA OF COMPOUNDS A AND B

Compound A	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*	*C-9*	*OCH_3*
α-L-Arabinose	110.0	83.2	78.3	83.7	66.9					
β-D-Xylose	103.6	74.6	83.0	69.6	65.7					
α-D-Xylose	93.8	73.3	72.8	78.6[c]	60.7					
β-D-Xylose	98.3	75.8[b]	75.9[b]	78.7[b]	64.8					
Ferulic acid	127.2	113.0	150.2	152.4	114.7	125.7	148.0	118.0	171.1	57.7
Compound B										
α-L-Arabinose	108.1	81.9	78.1	82.2	64.5					
β-D-Xylose″	101.6	73.4[d]	75.8	69.4	65.3					
β-D-Xylose′	101.6	73.3[d]	81.0	74.0	63.2					
α-D-Xylose	92.2	71.6	71.1	76.7	59.1					
β-D-Xylose	96.7	74.1[e]	74.2[e]	78.0	63.1					
Ferulic acid	127.1	111.6	147.9	148.1	115.4	123.7	146.6	115.7	169.3	56.2

[a]In D_2O at 27°. Values are chemical shifts relative to methanol. [b–e]These assignments may be interchanged.

TABLE III

ASSIGNMENTS OF SIGNALS IN ^{1}H-N.M.R. SPECTRA OF COMPOUNDS A AND B[a]

Compound	*X*		*X'*	*X''*	*A*	*F*					
	H-1α	*H-1β*	*H-1*	*H-1*	*H-1*	*H-2*	*H-5*	*H-6*	*H-7*	*H-8*	OCH_3
A	5.01	4.42	4.41		5.18	6.91	6.64	6.88	7.38	6.08	3.56
	(3.6)	(7.8)	(7.9)		(–)	(–)	(8.4)	(8.4)	(16.0)	(16.0)	(–)
B	5.07	4.46	4.37	4.30	5.29	7.09	6.78	7.01	7.52	6.27	3.75
	(3.6)	(7.9)	(7.9)	(7.8)	(–)	(–)	(8.3)	(8.3)	(16.1)	(16.1)	(–)

[a]Values in parentheses are coupling constants (*J*, Hz).

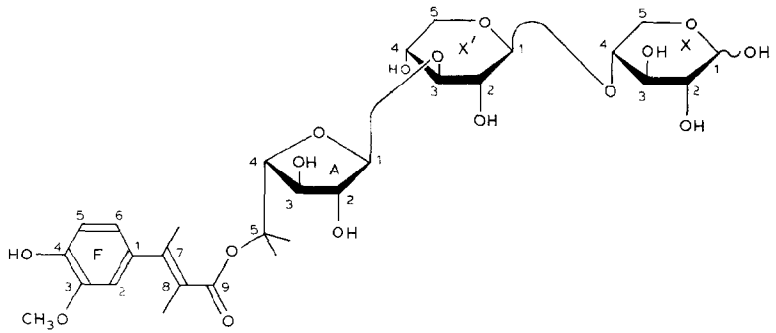
Analysis of compound A by ^{1}H- and ^{13}C-n.m.r. spectroscopy was used to elucidate the position to which ferulic acid is linked to the oligosaccharide, and the anomeric configurations of the glycosyl residues. Assignments of signals (Table II) were based on published values[28–31]. The resonance of the arabinofuranosyl C-5 at 67.26 p.p.m. indicated that ferulic acid is linked to the oligosaccharide at that position[3,4]. The presence of a signal at 107.9 p.p.m. suggested that the arabinofuranosyl residue is α-linked[30]. The xylopyranosyl residue is β-linked, as indicated by the coupling constant of the corresponding anomeric proton. The proposed structure of compound A is *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose, based on these results.

Characterization of compound B. — Compositional analysis by g.l.c. of the alditol acetates revealed arabinose and xylose in a molar ratio of 1:3. The absolute configurations of the arabinose and xylose were L- and D-, respectively. Treatment of compound B with alkali released *trans*-ferulic acid as the only phenolic acid. methylation analysis (Table I) gave four derivatives, namely, 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol (derived from terminal arabinofuranosyl residues), 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol (derived from terminal xylopyranosyl residues), 1,3,4,5-tetra-*O*-acetyl-2-mono-*O*-methylxylitol (presumably derived from 3,4-linked xylopyranosyl residues), and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol (presumably derived from 4-linked xylopyranosyl residues).

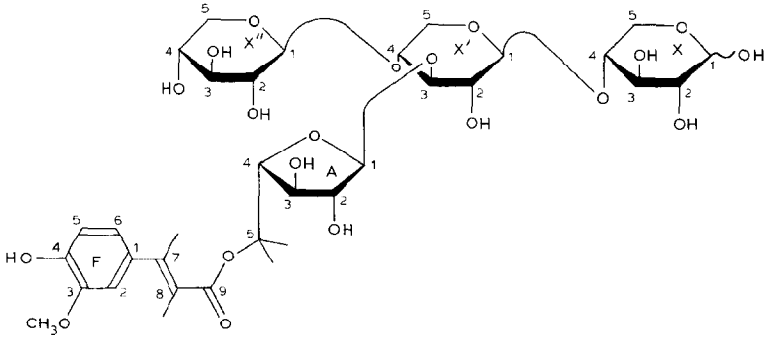
The positive-ion f.a.b. mass spectrum of native compound B contained an intense ion at *m/z* 745 and a weaker ion at *m/z* 723, correspond to the $(M + Na)^+$ and $(M + H)^+$ ions, respectively, of a compound containing one ferulic acid and four pentose, residues. Fragment ions also were evident at *m/z* 573, 441, 309, and 177, resulting from losses of one, two, three, and four pentose residues, respectively. The fragment ion at *m/z* 441 could have been derived either from a linear tetrasaccharide by a single cleavage, or from a branched tetrasaccharide by double cleavage (see later). The f.a.b. mass spectrum of the per-*O*-acetylated material contained ions at *m/z* 1165 and 1123 corresponding to the $(M + Na)^+$ quasi molecular ions of a fully acetylated and underacetylated feruloylated tetrasaccharide, respectively. Fragment ions were observed at *m/z* 435 and 219, generated by

loss three or four reducing-terminal pentose residues, respectively, from the fully acetylated compound. Less-intense fragment ions derived from the underacetylated compound also were observed at *m/z* 393 and 177. A fragment ion at *m/z* 651 was observed only in very low abundance, and was probably derived from a small amount of contaminating feruloylated trisaccharide (evidenced by quasi molecular ions at *m/z* 907 and 949). The absence of a fragment ion at *m/z* 651 suggests that the tetrasaccharide is branched with two terminal residues linked to the same residue. Larger fragment ions were not observed, probably due to their low abundances (see results already given of analysis of compound A).

Compound B was reduced with $NaBD_4$ to give ferulic acid and oligosaccharide alditol B. Methylation analysis (Table I) revealed 4-linked xylitol as the reducing-terminal residue (and no 4-linked xylosyl residues); the presence of branched (3,4-linked) xylosyl residues after reduction indicated that they were penultimate, not reducing terminal. The e.i. mass spectrum of per-*O*-methylated oligosaccharyl alditol B contained ions at *m/z* 143 (cA_2 and dA_2, 25%), 175 (cA_1 and dA_1, 24), 192 (aJ_2, 100), 238 (aJ_0, 1.8), and 572 ($acbJ_1$ and $adbJ_1$, 4.3). The presence in the e.i. mass spectrum of an aJ_0 fragment ion, and absence of an aJ_1 fragment ion at *m/z* 252, confirmed that the penultimate glycosyl residue was 3-linked[27].



Compound A



Compound B

Compound B was analyzed by ^{1}H- and ^{13}C-n.m.r. spectroscopy. Assignments (Table II) were made by comparison to published data for feruloylated arabinoxylan[6,28–30] and other[31] oligosaccharides. Results obtained by 2-dimensional C-H COSY n.m.r. spectroscopy (data not shown) indicated that the published values for the chemical shifts of C-2 and C-3 of the reducing terminal xylose have been assigned incorrectly; the positions of cross peaks indicated that in fact C-2 resonates downfield from C-3, not upfield as previously suggested[6]. The resonance of C-5 of the arabinofuranosyl residues at 64.5 p.p.m. indicated the linkage of ferulic acid at that position[3,4]. A value of $J_{7,8}$ of 16 Hz observed in the ^{1}H-n.m.r. spectrum indicated the ferulic acid to have the *trans*-configuration. The ratio of signal intensities of the anomeric protons of arabinofuranosyl and xylopyranosyl residues was 1:2, in accordance with the compositional analysis. The presence of a signal at 108.1 p.p.m. suggested that the arabinofuranosyl residues were α-linked. The xylopyranosyl residues were in the β-configuration, as indicated by the $J_{1,2}$ values. The proposed structure of compound B is *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose.

DISCUSSION

We have isolated two feruloylated arabinoxylan oligosaccharides from bamboo shoot cell-walls. This is the first report of *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (compound B) in young growing *Gramineae;* Kato *et al.*[6] have isolated the same feruloylated tetrasaccharide from maturated sugar-cane bagasse. The feruloylated trisaccharide *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (compound A) has been isolated from several sources, including, sugar-cane bagasse[3], maize cell-walls[4], barley straw[5], and barley aleurone layers after treatment with gibberelic acid[32]. Thus, bamboo shoot cell walls contain the same two feryloylated arabinoxylan oligosaccharides that have been found in other graminaceous monocots. Further studies are in progress to characterize the remaining feruloylated oligosaccharides released by enzymic hydrolysis from bamboo shoot cell-walls.

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