Carbohydrate Research, 219 (1991) 15–22
Elsevier Science Publishers B.V., Amsterdam

Isolation and characterization of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell-walls

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(Received October 2nd, 1990; accepted for publication, in revised form, March 15th, 1991)

ABSTRACT

Hydrolysis of bamboo shoot cell-walls with Driselase (a fungal enzyme preparation) released a diferuloyl arabinoxylan hexasaccharide. The structure of the diferuloyl oligosaccharide was determined to be 5,5′-di-*O*-(diferul-9,9′-dioyl)-[α-L-arabinofuranosyl-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose] on the basis of n.m.r. spectroscopy, methylation analysis, and fast-atom-bombardment mass spectrometry (f.a.b.-m.s.). This is the first reported evidence that arabinoxylans are covalently cross-linked via diferulic acid.

INTRODUCTION

Growing plant cell-walls contain polysaccharides which bear a small proportion of phenolic side-chains. Interest in these compounds arises from the fact that these side-chains appear to undergo *in vivo* oxidative coupling to yield cross-linked polysaccharides. It has been proposed that such coupling may contribute to the control of cell-wall extensibility and cell growth[1–9], and also to a decreased digestibility by ruminant-secreted enzymes[10–12]. However, direct evidence for these hypotheses has not yet been obtained.

In previous papers, we reported the isolation of two feruloylated arabinoxylan oligosaccharides[13], an acetylated feruloyl arabinoxylan trisaccharide[14], a novel feruloyl xyloglucan disaccharide[15,16], and a *p*-coumaroyl arabinoxylan trisaccharide[15,16] from cell-walls of growing bamboo shoot. In the present paper, we report the isolation and characterization of a novel diferuloyl arabinoxylan hexasaccharide (compound **1**) from cell-walls of the same plant. This is the first experimental evidence that diferulic acid cross-linkage of arabinoxylan actually exists in growing cell-walls.

RESULTS

Compositional analysis by g.l.c. of alditol acetates show that **1** contained arabinose and xylose in a molar ratio of ~1:2. The absolute configuration of arabinose and xylose were determined to be 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

0008-6215/91/$ 03.50

terminal arabinofuranosyl residues), 1,3,5-tri-*O*-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). Diferulic acid and a small amount of ferulic acid (2.5%) were released from **1** by alkali treatment. The small amount of ferulic acid may have originated from a contaminating monoferuloylated oligosaccharide. The positive-ion f.a.b.–m.s. of the native material showed an intense ion at *m/z* 1201 corresponding to the $(M + Na)^+$ quasimolecular ion of **1**. Weaker ions at *m/z* 1217 and 1309 corresponding to $(M + K)^+$ and (M + thioglycerol + $Na)^+$ quasimolecular ions were also observed. The negative-ion spectrum of the native material showed an intense ion at *m/z* 1177 $[(M - H)^-]$, indicating the molecular weight of **1** to be 1178, being thus consistent with a molecule containing one diferulic acid and six pentose residues. The positive-ion f.a.b.–m.s. of the per-*O*-acetylated material showed a less-intense ion at *m/z* 1873 and an intense ion at *m/z* 1831, corresponding to $(M + Na)^+$ quasimolecular ions of a fully acetylated and a mono-underacetylated diferuloyl hexasaccharide, respectively. Complete acetylation of the phenolic hydroxyl group of the feruloyl residue was difficult when acetic acid and trifluoroacetic anhydride were used as acetylating reagents[13]. Fragment ions derived from the mono-underacetylated compound were observed at *m/z* 1533, 1317, and 1101, generated by the loss of up to three pentose residues.

TABLE I

Methylation analysis of compound **1** and diglycosylalditol **2**

Glycosyl residue	*Methylated derivative*	*Linkage position*	*Compound* **1**	*Diglycosylalditol* **2**
			mol % recovered	
Arabinofuranosyl	2, 3, 5	Terminal	33.7	33.0
Xylopyranosyl	2, 3	4	27.4	[a]
	2, 4	3	38.9	51.5
Xylitol	1, 2, 3, 5	4		15.6[b]

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

Compound **1** was analyzed by ^{13}C-n.m.r. spectroscopy to define the positions at which diferulic acid is linked to the oligosaccharide and the anomeric configurations of glycosyl residues (Fig. 1). Assignments of chemical shifts (Table II) were made by comparison with published data for feruloylated arabinoxylan oligosaccharides[13] and diferulic acid. The resonance of C-5 of the arabinofuranosyl residues at 66.22 p.p.m. and the absence of a signal at 62.50 p.p.m. corresponding to C-5 of the unsubstituted arabinofuranose residues, indicated that diferulic acid is linked to O-5 of both arabinofuranosyl residues. The presence of signals at 109.52 and 102.96 p.p.m. suggested that the arabinofuranosyl and xylopyranose residues are α- and β-linked, respectively. For reference data for assignments of the diferulic acid residue in **1**, chemical shifts of *trans, trans*-diferulic acid (denoted DF) in $(CD_3)SO$ were assigned as follows: ^{13}C; δ 167.86

TABLE II

Assignments of signals in ^{13}C-n.m.r.[a] spectrum of compound 1

Component	*chemical shifts, p.p.m.*[b]									
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-7*	*C-8*	*C-9*	*OCH_3*
α-L-Arabinose	109.52	82.58	78.13	82.29	66.22					
β-D-Xylose'	102.96	73.84	83.24	69.06	64.21					
α-D-Xylose	93.22	72.18[c]	72.60[c]	77.87	60.08					
β-D-Xylose	97.73	75.20[d]	75.16[d]	77.71	65.37					
Diferulic acid	127.26	109.52	148.36	148.89	125.36	126.26	146.60	116.65	169.78	56.95

[a] In D_2O at 27°. [b] Value are chemical shifts relative to CD_3OD (49.30 p.p.m.). [c,d] These assignments may be interchanged.

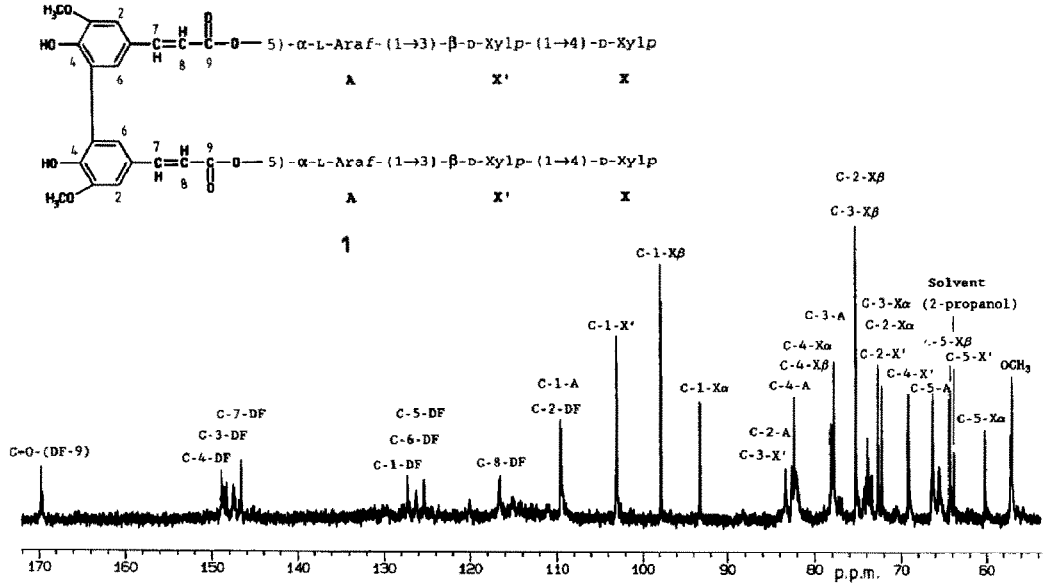

Fig. 1. Structure and 100-MHz ^{13}C-n.m.r. spectrum of compound **1**.

(DF-9), 147.90 (DF-4), 146.49 (DF-3), 144.51 (DF-7), 125.19 (DF-1), 124.97 (DF-6), 124.72 (DF-5), 115.68 (DF-8), and 109.39 (DF-2). The anomeric protons in the ^{1}H-n.m.r. spectrum were assigned as follows: δ 5.43 (broad signal, H-1 of arabinose), δ 5.25 (t, $J_{1,2}$ 3.7 Hz, H-1 of α-D-xylose), δ 4.64 (d, $J_{1,2}$ 7.7 Hz, H-1 of β-D-xylose), δ 4.52 (d, $J_{1,2}$ 7.0 Hz, H-1 of β-D-xylose). The xylopyranosyl residue is β-linked, as indicated by the coupling constant of the corresponding anomeric proton.

Compound **1** was reduced with $NaBD_4$ to give diferulic acid, a small amount of ferulic acid, and diglycosylalditol **2**. Methylation analysis (Table I) confirmed that 4-linked xylose was present at the reducing terminus of **2**. By comparison of the g.l.c. retention time and e.i. mass spectrum of per-*O*-methylated diglycosylalditol **2** with those of the authentic compound[13], diglycosylalditol **2** was determined to be L-arabino-

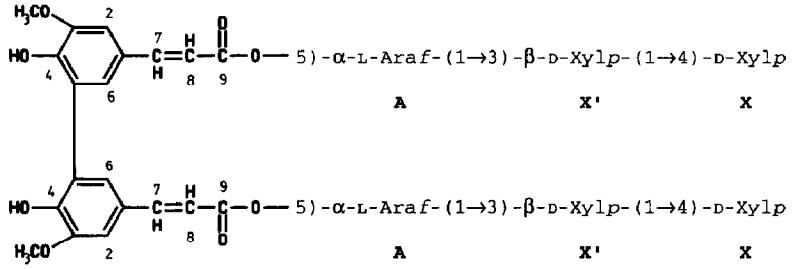

Fig. 2. Structure of compound **1**.

furanosyl-(1→3)-D-xylopyranosyl-(1→4)-D-xylitol. The presence in the e.i. mass spectrum of an aJ_0 fragment-ion at *m/z* 238 and absence of an aJ_1 fragment-ion at *m/z* 252, indicated that penultimate glycosyl residue of diglycosylalditol **2** was 3-linked[17].

From these results, the proposed structure of **1** is 5,5′-di-*O*-(diferul-9,9′-dioyl)-[α-L-arabinofuranosyl-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose] (Fig. 2).

Isomerization of compound **1**. — Compound **1** was found to be accompanied by isomers. Diferulic acid liberated from **1** by alkali treatment was analyzed by g.l.c.–m.s. The resulting mass spectra and retention times were compared to those obtained upon analysis of synthetic *trans,trans*-diferulic acid that contained a small amount of the *trans,cis*-isomer resulting from photoconversion over time[5]. These data indicated that the diferulic acid released from compound **1** consisted of the *trans,trans*-, *trans,cis*-, and *cis,cis*-isomers in the molar ratio of 1:0.16:0.71. Because of the presence of these isomers of the diferulic acid component, the ^{1}H- and ^{13}C-n.m.r. spectra were not clear enough to permit complete assignments of chemical shifts. These isomers might be artifacts formed from the *trans,trans*-isomer of diferulic acid residues during isolation. We previously reported the presence of *trans*- and *cis*-isomers of feruloylated xyloglucan disaccharide and *p*-coumaroyl arabinoxylan trisaccharide, and that the *cis*-isomers were artifacts[16].

DISCUSSION

The cross-linking of cell-wall polysaccharides has long been thought to control plant growth and effect ruminant digestion of plant material[1–12]. Diferulic acid can be released from cell-walls by treatment with alkali[4,5,18], but its association with cell-wall polysaccharides through esterification at both carboxyl groups has not previously been demonstrated.

The results presented here show that cell walls isolated from young growing bamboo cell-walls yield a diferuloyl arabinoxylan hexasaccharide after treatment with Driselase. This is the first reported evidence that growing plant cell-wall polysaccharides are covalently cross-linked through phenolic acid residues.

The calculated yield of **1** from cell-walls of bamboo shoot was 23 μg/g of cell-walls. The quantity of **1** released from cell-walls of bamboo shoot by treatment with Driselase accounted for 8.7% of the total diferulic acid released by treatment with

sodium hydroxide. The inability of Driselase to release all of the diferulic acid residues present in the cell-walls of bamboo shoot indicated that phenolic cross-links restrict the ability of endo-xylanase to completely depolymerize cross-linked arabinoxylan. In addition, it is likely that diferuloyl crosslinks, other than the one demonstrated here, exist in these cell-walls.

Our results demonstrate that cross-linkages of the arabinoxylan molecule through a diferuloyl bridge occur in cell walls of a young, growing monocotyledonous plant. It has been proposed[19,20] that arabinoxylan bonds strongly to cellulose fibrils through hydrogen bonds. It seems strongly probable that arabinoxylan molecules bind not only to cellulose but also to other arabinoxylan molecules through diferuloyl bridges, that is that intermolecular cross-linkage of arabinoxylan may occur through diferuloyl bridges. However, it is impossible to distinguish these diferuloyl arabinoxylan oligosaccharides derived from intermolecular cross-linkages from those associated with a single polysaccharide chain. In previous papers[15,16], we have isolated feruloyl xyloglucan disaccharides. We obtained another oligosaccharide fraction containing diferulic acid residues. Methylation analysis of this fraction showed it consisted mainly of an arabinoxylan trisaccharide and a small amount of xyloglucan disaccharide, and f.a.b.–m.s. analysis showed that it contained a small amount of diferuloyl xyloglucan–arabinoxylan oligosaccharides (unpublished data). Further studies are in progress to characterize these diferuloyl xyloglucan–arabinoxylan oligosaccharide fractions with the hope of demonstrating intermolecular cross-linking of two different types of polysaccharides (namely, xyloglucan and arabinoxylan). This result would clearly show intermolecular rather than intramolecular cross-linking by a diferuloyl bridge.

EXPERIMENTAL

Plant material. — Shoots (~20 cm in height) of Mouso-chiku bamboo (*Phyllostachys edulis*) were harvested in Tsukuba, Ibaraki Prefecture in early May 1989. Bamboo shoot cell-walls (~400 g) were prepared from the youngest growing parts (100 kg) as described previously[16]. The cell-walls contained 1.5 mg of phenolic acid per g of dry weight. The ratio of *p*-coumaric acid, ferulic acid, and diferulic acid obtaining by h.p.l.c. was 1:2.8:0.06 (mol/mol/mol). No other phenolic acids (e.g., dehydrodiferulic and isodiferulic acids) were detected when the products of saponification of bamboo shoot cell-walls were analyzed, as *O*-trimethylsilyl derivatives, by g.l.c.–m.s. The phloroglucinol test on the cell-walls indicated lignin to be absent.

Isolation of compound **1**. — The cell-walls were hydrolyzed with Driselase (purchased from Kyowa Hakko, Tokyo, and purified as described previously[21]). Driselase showed no peroxidase or laccase activity. Thus, enzymic hydrolysis of the cell-walls provided no oxidative coupling of phenolic acids. The Driselase digestion-products were chromatographed on a column (4.4 × 85 cm) of Sephadex LH-20 eluted with water and 50% (v/v) aq. 1,4-dioxane. A fraction eluted with 50% aq. 1,4-dioxane contained predominantly diferuloyl oligosaccharides. These oligosaccharides were fractionated using a slightly modified method described by Nishitani and Nevins[22] with

a Sep-Pack C_{18} cartridge previously washed with 15 mL of EtOH, and then 20 mL of water, followed by 2 mL of MeCN. One mL of the 50% aq. 1,4-dioxane fractions (containing < 2 mg sugar) was applied to a Sep-Pak C_{18} cartridge. Material retained by the cartridge was eluted successively with 10 mL of water (fraction A), 5 mL of 10% aq. EtOH solution (fraction B), 5 mL of 60% aq. EtOH solution (fraction C), and 2 mL of 100% MeCN (fraction D). This procedure was repeated with new cartridges until adequate quantities of the various fractions were obtained. Fractions A, B, C, and D were separately combined. Portions of each fraction were assayed for sugar and phenolic acid composition. Fraction C, which only contained phenolic acids, was applied to a column (1.7 × 85 cm) of Bio-Gel P-10 (100–200 mesh) which had been equilibrated with 25mM sodium acetate buffer (pH 5.2) and eluted with the same buffer solution. Fractions (1.5 mL) were collected and assayed for the total carbohydrate and phenolic acid. A fraction, having a K_{av} value of 1.4 contained predominantly diferuloylated oligosaccharides. These oligosaccharides were further fractionated by analytical normal-phase h.p.l.c. with a 0.46 (i.d.) × 25 cm column (Shim-Pack CLC-SIL from Shimadzu) at 40° and eluted with 150:150:1 (v/v/v) 2-propanol–$CHCl_3$–AcOH at 0.5 mL min^{-1}. The eluate was monitored at 320 nm, and a peak of u.v.-positive material eluting at 9.7 min was collected and freeze-dried to give **1**. (Compound **1**, when chromatographed under the same conditions as described, gave only one peak).

General methods. — Evaporations were conducted under diminished pressure at < 40°. Total carbohydrate was determined by the orcinol method[23]. Alditol acetates were prepared[24] and analyzed as described[25], except that hydrolysis in 2M CF_3CO_2H was performed for 20 min; g.l.c. was performed using a Shimadzu GC 14A instrument operated isothermally at 230° with a 30 m × 0.25 mm SP-2330 fused-silica column (Supelco). Absolute configurations were determined as described[26]. Per-*O*-methylation was performed by a modification[27] of the method of Hakomori[28], and per-*O*-methylated oligosaccharides and diglycosylalditol were purified as described[29]. A sample was per-*O*-acetylated for f.a.b.–m.s. by the method of Bourne *et al.*[30].

Alkaline hydrolysis and reduction of compound **1**. — Compound **1** (~ 300 μg) was dissolved in 250 μL of a 10 mg mL^{-1} solution of $NaBD_4$ in 1.5M NH_4OH. After 3 h under N_2 in the dark at room temperature, the solution was acidified with 6M HCl, and extracted with ether. The ether phase was washed with water and evaporated.

The diglycosyl-alditol **2** in the aq. phase was per-*O*-methylated and analyzed as described[13]. The ether extract was fractionated by reversed-phase h.p.l.c. with an ODS column [Inertsil ODS-2 from Gasukuro Kogyo]. A sample was chromatographed in 25% aq. MeCN acidified at pH 2.5 with CF_3CO_2H at a flow rate of 1.0 mL·min^{-1}. Phenolic acids were monitored at 320 nm. A portion of the ether extract was converted into *O*-trimethylsilyl derivatives by treatment with *N,O*-bis(trimethylsilyl)acetamide, and analyzed by g.l.c.–m.s.

Mass spectrometry. — G.l.c.–m.s. was performed with a Shimadzu GC-MS QP 2000A mass spectrometer. Per-*O*-methylated alditol acetates and per-*O*-methylated diglycosylalditols were separated as described[16]. Silylated phenolic acids were separated by split injection and a 30 m × 0.25 mm fused-silica DB-1 column, with a temperature program starting at 180° for 2 min, and then increased to 300° at 5° min^{-1}.

F.a.b.–m.s. was performed in the positive ion and negative-ion modes by using a Jeol JMS-SX 102 mass spectrometer with an accelerating voltage of 10 kV. Native sample was dissolved in water and loaded onto a stainless-steel target with thioglycerol as a matrix. Per-*O*-acetylated samples were dissolved in MeOH and *m*-nitrobenzyl alcohol was used as a matrix. Xenon was used as a bombarding gas. Spectra were obtained at a scan rate that covered the mass range from 100 to 2000 units in 20 s, using the Jeol JMA data system.

N.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were recorded at 27° in D_2O with a Jeol GSX 400 spectrometer operated at 100 MHz, using the noise-decoupled mode and a deuterium lock. Chemical shifts were referenced to CD_3OD (49.30 p.p.m.) as the internal standard. ^{1}H-N.m.r. spectra were recorded using a Bruker AM-500 spectrometer operated at 500 MHz with a probe temperature of 30°. Samples were dissolved in D_2O (99.996 atom %), and chemical shifts are reported relative to internal acetone (δ 2.234 p.p.m.).

ACKNOWLEDGMENTS

The author thanks Dr. T. Tobita (Japan Tobacco, Inc.) for the 500-MHz Bruker AM-500 spectroscopy measurement, Dr. M. Samejima (The University of Tokyo) for obtaining the f.a.b.–m.s., and Dr. N. Shibuya (National Institue of Agrobiological Resources) for kindly providing diferulic acid. The author also thanks Drs. T. Hiroi and S. Hosoya in this Institute, and J. Thomas (The University of Dundee, Scotland), William York, and Malcolm O'Neill (Complex Carbohydrate Research Center, The University of Georgia) for critical reading of this manuscript; Mrs. K. Ishizuka for providing fresh bamboo shoots; and Mrs. N. Nishida and Karen Howard for typing the manuscript. This work was supported by research grant (Biomedia Projects BMP-91-I-1-1) from the Ministry of Agriculture, Forestry and Fisheries.

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