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Note

Isolation of diferulic bridges ester-linked to arabinan in sugar beet cell walls

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Abstract—After degradation of sugar beet cell walls with Driselase® and fractionation of the solubilised products by hydrophobic interaction chromatography, a dehydrodiferuloylated oligoarabinan was isolated. Its structure was assigned to two dimers of (1 → 5)-linked arabinose units esterified by a central 8-O-4′ ferulic dimer. These results provide the first direct evidence that pectic arabinans in sugar beet cell walls may be covalently cross-linked through dehydrodiferulates.

Keywords: Sugar beet; *Beta vulgaris*; *Chenopodiaceae*; Pectin; Ferulic acid; Dehydrodiferulate; Cell wall

Polysaccharides with ester-linked feruloyl groups occur in the primary cell walls of several plants, especially in the orders *Caryophyllales* and *Poales*. In the order *Poales*, most feruloyl groups are attached to arabinoxylans through *O*-5 of L-Ara residues.[1] In *Caryophyllales*, feruloyl groups are ester-linked to pectic polysaccharides. The cell walls of several members of the family *Chenopodiaceae*, namely beet (*Beta vulgaris*) roots,[2] spinach (*Spinacia oleracea*) leaves,[3] glasswort (*Salicornia ramosissima*) immature stems,[4] and quinoa (*Chenopodium quinoa*) different tissues,[5] all contain ferulic acid bound to pectic polysaccharides. Pectin main structural features include homogalacturonic and rhamnogalacturonic regions. In the last, some rhamnosyl residues are substituted by arabinose- and galactose-containing side chains. Feruloyl groups are mainly ester-linked to *O*-2 of Ara residues of the main core of α-(1 → 5)-linked arabinan chains and to *O*-6 of Gal residues of the main core of β-(1 → 4)-linked type I galactan chains.[6,7] Recently, minor amounts of feruloyl groups were assumed to be also ester-linked to *O*-5 of Ara residues of the main core of α-(1 → 5)-linked arabinan chains, indicating a peripheral location of some ferulic acids on pectic 'hairy' regions[8]

Biological interest in ferulic acids arises from the fact that they can undergo in vivo oxidative coupling reactions to form dehydrodimers,[9] thereby covalently cross-linking the polysaccharides they esterify.[10,11] Such coupling may contribute to wall assembly, promote tissue cohesion and restrict cell expansion.[9,12,13] Dehydrodiferulates have been identified and quantified in sugar beet cell walls where 15 to over 20% of the quantified alkali-soluble wall-bound phenolics comprised dehydrodiferulates, indicating a potentially high degree of polymer cross-linking.[13,14] However, the isolation of dehydrodiferulates linked to neutral sugars—hence providing direct evidence for these cross-links in the cell wall—has only been reported for monocotyledons, namely bamboo shoot and maize bran.[10,11] We describe here the isolation and partial structure determination of a dehydrodiferulate oligoarabinan from sugar beet, which provides direct evidence for covalent (intra- or inter-molecular) cross-linking of pectic arabinans through diferulic bridges in the cell wall.

Sugar beet cell wall material (CWM) was degraded with Driselase®. The solubilised material was loaded onto a column of Sephadex LH-20 eluted successively by water, EtOH 25%, EtOH 50% and EtOH 100%.

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Feruloylated galactobiose, arabinotriose and arabinobiose were eluted with water at 2.0, 2.7 and 3.7 column volumes as previously reported.[8] The elution was then carried out with EtOH 25% and three well-defined peaks eluting at 9.0 (fraction EO_1), 9.7 (fraction EO_2) and 12 (fraction EO_3) column volumes were recovered. Fractions EO_2 and EO_3 were identified as oligoarabians esterified by two ferulic acids.[8] Small badly defined peaks were eluted in EtOH 25% at 8.5, 11 and 13.3 column volumes. To tentatively achieve a better separation, these fractions were pooled together, concentrated and re-injected on a smaller Sephadex LH-20 column eluted by EtOH 25% at a lower flow rate. Four peaks were recovered at 1.1 (EO_4), 1.4 (EO_5), 1.7 (EO_6) and 2.3 (EO_7) column volumes (not shown). Arabinose was the sole neutral sugar detected in those fractions together with ferulic dimers (Table 1). The repartition of the dimers (5-5′, 8-O-4′ and 8-5′) differed within these fractions. Fractions EO_4 and EO_5 were rich in 8-O-4′ dimers and contained also large amounts of 5-5′ dimers but 8-5′ dimers were only detected as minor components. Fraction EO_6 contained the three dimers in similar amounts. Fraction EO_7 contained almost exclusively 8-O-4′ dimers (88% w/w). Fractions EO_4 and EO_5 exhibited a very high arabinose over dehydrodiferulate (Ara/diFA) ratio and fraction EO_6 was recovered in too small amounts to be fully characterised. These three fractions were not further analysed. Fraction EO_7 was structurally characterised by HPAEC at pH 13 and by ESI-IT-MS.

The negative ESI-MS analysis of fraction EO_7 showed four peaks at *m/z* 193, 913, 949 and 1011 (Fig. 1). The peak at *m/z* 193 corresponds probably to traces of free ferulic acid. The peak at *m/z* 913 could correspond to a dimer of ferulic acid associated with four arabinose residues, the peak at *m/z* 949 to the same compound carrying two water molecules, and the peak at *m/z* 1011 to the same compound carrying a sulfate group. This is in agreement with the molar ratio Ara/diFa of 3.6 observed for fraction EO_7 (Table 1). The degree of polymerisation of the carbohydrate moiety was checked by HPAEC at pH 13, since these alkaline conditions allow instant deesterification of the feruloylated compound. By comparison with true arabinose, (1 → 5)-linked arabino-biose, -triose and -tetraose, fraction EO_7 appeared majorly constituted of arabinose dimers (Fig. 2), which suggests that fraction EO_7 is made up of a central ferulic dimer associated to (1 → 5)-linked arabinobiose on each side. In order to determine the structure of this compound, MS^n experiments were performed by negative electrospray ionisation ion trap mass spectrometry. The fragment ions generated were assigned according to the nomenclature established by Domon and Costello,[15] as previously reported for neutral disaccharides[16] and oligogalacturonates.[17] After isolation and collision-induced dissociation of the $[M-H]^- + 2\ H_2O$ ion at *m/z* 949, a series of fragment ions appeared (Fig. 3). The peak at *m/z* 913 results from the loss of the two extra water molecules. The ions at *m/z* 853 and 823, corresponding to losses of 60 Da ($C_2H_4O_2$) and 90 Da ($C_3H_6O_3$), respectively, resulted from consecutive cross-ring cleavages from *m/z* 913 ion.[18] By analogy with the fragmentation of other carbohydrates,[19] they were assigned to the $^{0.2}A_4$ and $^{0.3}A_4$ fragment ions. One of the two reducing arabinose residues (arbitrarily designed Ara 1 on the figure) is therefore, as indicated by HPAEC data, linked through *O*-5. A fragment ion at *m/z* 781 (loss 132 Da), corresponding to the C_3 fragment ion resulting from the loss of this arabinose unit, was produced. This C_3 fragment ion undergoes further fragmentations that were assigned to H_2O loss (*m/z* 763) and to $^{0.2}A_3$ and $^{0.3}A_3$ fragment ions (*m/z* 721 and 691, respectively). The ion at *m/z* 649 corresponds to the C_2 fragment ion due to the loss of the second arabinose

Table 1. Composition of fractions isolated on Sephadex LH-20

Fractions	Ara (nmol)	Dehydrodiferulic acids (diFA) (nmoles)			Ara/diFA (molar ratio)
		5–5′	8-O-4′	8–5′	
EO_4	600	7.3	17.2	1.6	23.1
EO_5	625	8.6	25.9	1.4	17.3
EO_6	325	12.1	12.9	14.0	8.4
EO_7	665	7.3	160.2	14.6	3.6

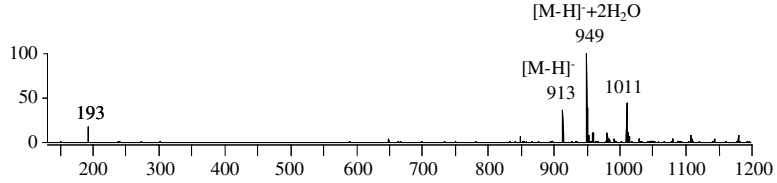

Figure 1. Negative full MS spectrum of EO_7.

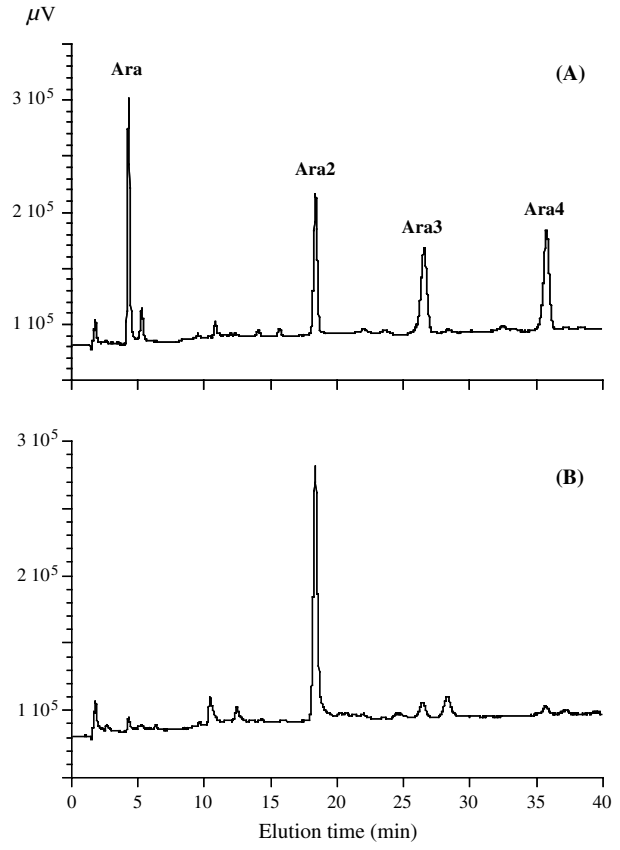

Figure 2. HPAEC-PAD profiles of : (A) oligoarabinan standards; (B) fraction EO_7.

reducing residue (arbitrarily quoted Ara 4 on the figure). The peak at *m/z* 631 could be assigned to the loss of a water molecule from the ion at *m/z* 649. The second reducing arabinose residue (Ara 4) is also, as indicated by HPAEC data, linked through *O*-5. MS^3 analysis of the C_3 ion (*m/z* 949 > 781 > products) provided the four fragment ions at *m/z* 763, 721, 691 and 649 (not shown) that were already observed in MS^2 analysis. The C_2 fragment ion at *m/z* 649 was not produced in sufficient quantity (either from MS^2 or MS^3 analyses) to allow further fragmentation. The exact position on which dehydrodiferulate is linked to arabinose units (arbitrarily quoted Ara 2 and Ara 3 on the figure) could therefore not be clarified.

In conclusion, we have isolated and partially characterised an oligoarabinan diester containing mainly 8-O-4′ diFA from sugar beet cell walls. Further work is however needed to elucidate the exact location of diFA on arabinan chains. The oligoarabinan diester isolated here accounted for only ~1% of the ferulic dimers released by NaOH treatment. The quantities of feruloylated arabinobiose, galactobiose and arabinotriose generated from sugar beet pulp and from the cell walls of spinach leaves with Driselase® accounted for 29.0% and 9.5%, respectively, of the ferulic acid released by NaOH treatment.[6,20] As previously shown for maize bran cell walls,[11] the presence of diferulic bridges as well as the high level of ferulic acid esterified to pectin sidechains probably restrict enzyme accessibility. Most of the ferulic dimers might therefore be still attached to polymeric glycosidic fragments and not readily separated by hydrophobic interaction chromatography. The use of other degradative means leading to more complete depolymerisation of pectic substances and allowing the release of higher quantities of oligosaccharide diesters of low degree of polymerisation is presently under investigation. Our results cannot discriminate

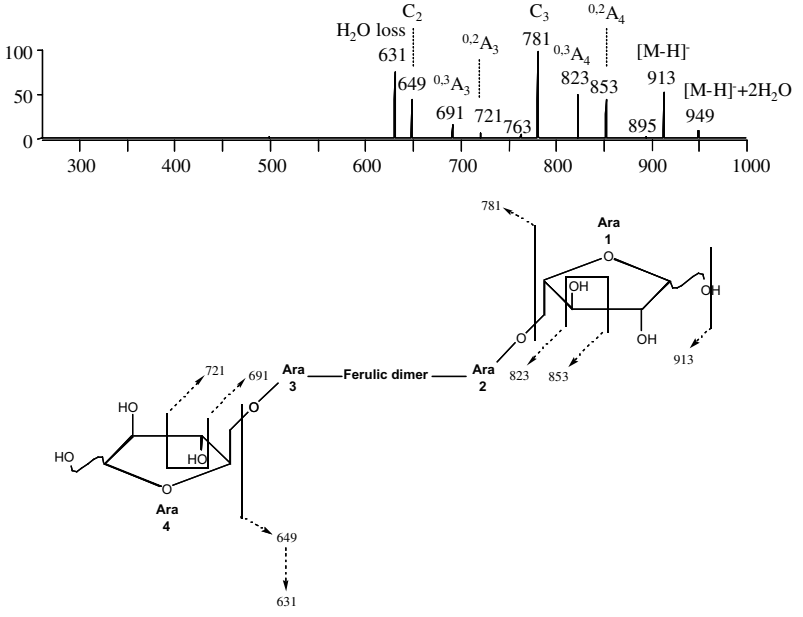

Figure 3. MS^2 spectrum (*m/z* 949 > products) of EO_7 and observed cleavages.

between inter- and intra-polymeric crosslinking even-though inter-polymeric diferuloyl cross-links are widely speculated to exist.[21]

1. Experimental

1.1. Materials

The cell wall material (CMW) was prepared from fresh sugar beet roots as previously described.[22] Sugar beet cell wall material (CWM) was rich in galacturonic acid (213 mg/g), glucose (221 mg/g), arabinose (212 mg/g) and galactose (56 mg/g). Rhamnose (20 mg/g), xylose (14 mg/g), mannose (14 mg/g) and fucose (2 mg/g) were detected as minor carbohydrate components. The total amount of ferulic acid (8.2 mg/g) and ferulic dehydrodimers (0.65 mg/g), as well as the proportion of dehydrodimers (5-5′/8-O-4′/8-5′: 4/48/48 by weight) were in agreement with previously reported values.[13,14] Driselase® was obtained from Sigma.

1.2. Isolation of feruloylated and diferuloylated oligosaccharides

Hydrolysis of CWM of sugar beet (10 g in 1 L) was performed at 37 °C during 48 h after Driselase® (1 g) addition. The hydrolysate was filtered on G4 sintered glass and the supernatant (91% of the CWM, w/w) concentrated to 50 mL by vacuum evaporation at 40 °C and loaded onto a column of Sephadex LH-20 (80 × 2.6 cm) eluted at 34 mL/h successively by water (8 column volumes), EtOH 25% (7 column volumes), EtOH 50% (5 column volumes) and EtOH 100% (5 column volumes). Fractions (11 mL) were collected and the absorbance was measured at 325 nm. As the EtOH 50% and EtOH 100% pools were devoid of ferulic compounds, these fractions were not studied further.

Water-eluted fractions were also analysed colorimetrically for galacturonic acid[23] and total neutral sugars.[24] Selected fractions eluting in EtOH 25% were pooled, concentrated and injected onto a column of Sephadex LH-20 (42 × 1.6 cm) eluted at 20 mL/h by EtOH 25%. The absorbance of the eluent was monitored continuously at 325 nm and fractions (6 mL) were collected. Pools corresponding to separate peaks of absorbance were concentrated and freeze-dried for further analysis.

1.3. Carbohydrate analysis

The individual sugars were analysed as their alditol acetate derivatives by gas chromatography after hydrolysis by 2 M TFA at 121 °C for 2 h.[25] HPAEC was performed on a Dionex system with pulsed amperometric detection. The Carbopac PA1 column was eluted with 500 mM NaOH, 200 mM sodium acetate and water at 1 mL/min as follows: initial conditions, 20/30/50; 30 min, 20/60/20; 30–34 min, 20/60/20; 35 min, 20/30/50. Oligoarabinans (dp 1–12) were obtained by acid hydrolysis (50 mM H_2SO_4, 90 min, 85 °C) of sugar beet linearised arabinan (Megazyme) followed by size-exclusion chromatography (Biogel P2).[26] Arabinose monomer and (1 → 5)-linked dimer, trimer and tetramer were used as standards. BORWIN software (JMBS Développements, Grenoble, France) was used for data acquisition and processing.

1.4. Identification and quantification of ferulic and dehydrodiferulic acids

Phenolic compounds were determined by HPLC after saponification and extraction. The pectins were saponified by 2 M NaOH at 35 °C under argon during 30 min in the dark. After an internal standard (*o*-coumaric acid) has been added, the solution was neutralised with 2 M HCl. Phenolic compounds were extracted with ether. The ether phase was evaporated at 40 °C, 1 mL of MeOH/H_2O (1:1 v/v) was added and samples (20 μL) were injected on an HPLC system equipped with a C18 column (Purospher, Merk). Gradient elution was performed using MeOH/acetic acid (1:0.01) (A) and H_2O/acetic acid (1:0.01) (B) at 0.7 mL/min at 25 °C: (0 min, A = 20%; 20 min, A = 60%; 21 min, A = 80%; 30 min, A = 80%; 31 min, A = 20%). Phenolic compounds were detected at 320 nm. Elution time and response factors relative to *o*-coumaric acid at 320 nm were established using commercial ferulic acid and dehydrodiferulic acid standards obtained as reported by Saulnier et al.[11]

1.5. Mass spectrometry

ESI-ITMS experiments were achieved on a LCQ Deca ion trap mass spectrometer (ThermoFinnigan, US) using negative (nano)electrospray as the ionisation processes. Samples were dissolved in MeOH/water 1/1 before their infusion into the electrospray source of LCQ Deca spectrometer. The MS analyses were carried out under automatic gain control conditions, using a typical needle voltage of 4.2 kV and a heated capillary temperature of 150 °C. For MS^n experiments, the various parameters were adjusted for each sample in order to optimise signal and get maximal structural information from the ion of interest.

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