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Note

Process for the isolation of preparative quantities of [2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose from sugarbeet

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Abstract

Specific enzymes were used to hydrolyse sugarbeet pulp to facilitate the isolation of [2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose in preparative amounts. The feruloylated arabinose disaccharide was purified by chromatography on Sephadex LH-20 and Bio-Gel P-2 and the structure confirmed by NMR and UV spectroscopy and high-performance liquid chromatography. © 1997 Elsevier Science Ltd.

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Ferulic and *p*-coumaric acid are the major phenolic acids found covalently linked to the cell walls of several plants. Cell wall-esterified ferulic acid comprises up to 3% (w/w) of monocot cell walls [1], and comprises up to 1% (w/w) of some dicot cell walls such as spinach [2] and sugarbeet [3]. In monocots, ferulic acid is found ester-linked at O-5 of arabinofuranose substituents of the arabinoxylans [4–6], whereas in sugarbeet, ferulic acid is linked either at O-2 of arabinofuranose or at O-6 of galactopyranose residues in the pectic side chains [7,8]. Esterified phenolics may contribute to cell wall extensibility and growth, and are known to decrease digestibility by ruminants [9]. Ferulic acid is able to form dimers and cross-link cell wall polysaccharides [10] and lignin [11], hence increasing cell wall strength and stability.

Small, soluble feruloylated oligosaccharides have been released from plant cell wall polysaccharides using enzyme or chemical treatments. [5-*O*-(*trans*-Feruloyl)-α-L-arabinofuranosyl]-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose can be isolated from wheat bran [6], while various feruloylated arabino- and galacto-oligosaccharides were obtained from sugarbeet [7]. In a previous publication, we reported that incubation of sugarbeet with a mixture of *endo*-arabinanase and α-L-arabinofuranosidase solubilised significant amounts of feruloylated material [3]. Here we describe the isolation and structure identification of the major feruloylated arabino-oligosaccharide re-

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leased from sugarbeet pulp by a mixture of *endo*-arabinanase and α-L-arabinofuranosidase.

Incubation of the sugarbeet pulp (20 g; containing 184.6 mg esterified ferulic acid) with a mixture of *endo*-arabinanase and α-L-arabinofuranosidase for 65 h at pH 4.0 solubilised 47.4% (87.5 mg) of the alkali-labile ferulic acid. HPLC analysis of the crude supernatant indicated that the major peak eluted with the same retention time as [2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose (Ara_2F), and accounted for > 90% of the soluble feruloyl groups in the sample. An absorption spectrum obtained at pH 6.0 indicated the presence of esterified feruloyl groups, with $\lambda_{max} = 325$ nm [12]. The feruloylated oligosaccharide was converted to ferulic acid by NaOH treatment. The crude supernatant contained large amounts of arabinose (18.0 weight% of starting material; 91% as monomer, 9% in oligomeric forms) with some glucose (3.32 weight%) and galactose (0.46 weight%; all oligomeric). After concentration and centrifugation (10000 × *g*, 30 min), the supernatant was separated by chromatography on Sephadex LH-20 (Fig. 1A). Ferulic acid (62.7%) eluted between the void and total volume of the column (peak A1), which revealed that this fraction of the solubilised feruloyl groups was associated with oligosaccharides of high dp. The remainder of the ferulic acid applied to the column (37.3%; 0.168 mol) eluted as a single, broad peak at 2.0–2.5 column volumes (peak A2). Peak A2 was collected and concentrated before purification on Bio-Gel P-2 (Fig. 1B). The major peak of absorbance at 325 nm eluted at 1.9 column volumes (peak B2). HPLC analysis of peak B2 gave a single peak of absorbance with a retention time similar to that for Ara_2F which contained 0.144 mmol ferulic acid (> 85% of that applied to the column), while sugar analysis indicated 0.276 mmol arabinose (all oligomeric), indicating a molar ratio (arabinose:ferulic acid) of 1.9. The absorption spectrum (pH 6.0) indicated the presence of esterified feruloyl groups, with $\lambda_{max} = 325$ nm. The feruloylated oligosaccharide was converted to ferulic acid by NaOH treatment. ^1H NMR spectroscopy confirmed that peak B2 was a pure feruloylated arabinobiose with the same NMR spectrum as that described previously [8] for [2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose (Ara_2F; Fig. 2). Pure Ara_2F (66.4 mg) was obtained, accounting for 15.2% of the feruloyl groups initially present in the sugarbeet pulp. The compound purified here is the same as that isolated and characterised by Ishii and Tobita [13]. The isolation of pure Ara_2F described here is a

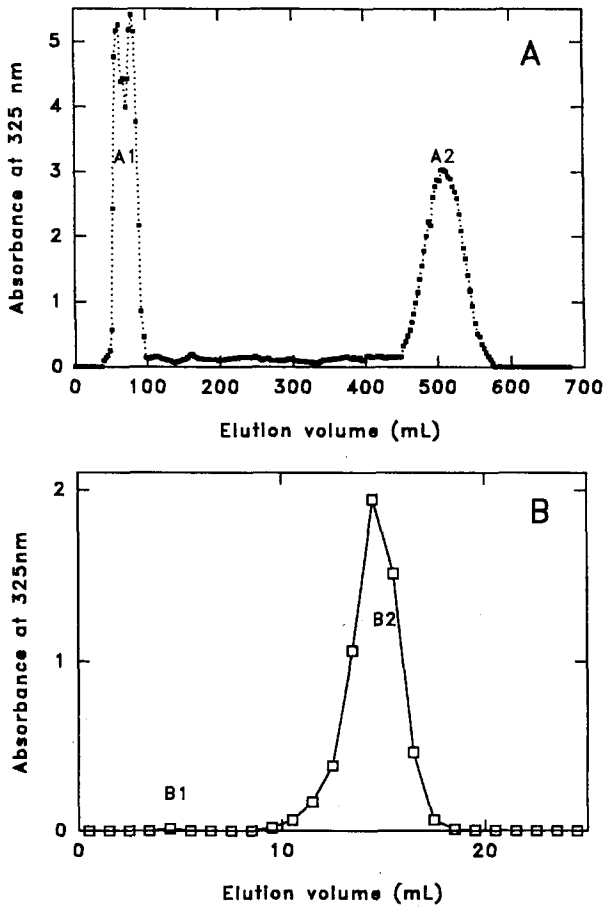

Fig. 1. Purification of the feruloylated arabinobiose from the soluble fraction after treatment of sugarbeet pulp with a mixture of *endo*-arabinanase and α-L-arabinofuranosidase by chromatography using, successively, columns of Sephadex LH-20 (A) and Bio-Gel P-2 (B). Fractions were analysed for absorbance at 325 nm which indicates esterified feruloyl groups.

relatively quick and simple procedure since this compound is the major (> 95%) feruloylated oligosaccharide released by the specific enzymic treatment.

The isolated feruloylated arabinobiose has potential applications as a substrate in enzyme screening and characterisation, and in addition, may possess biological activities. Ferulic acid esterases (FAE) release ferulic acid from plant cell walls (see [3] and references therein) and may provide useful tools for determining the physical and chemical role of phenolics in the wall. Assays for FAE activity have utilised (as substrate) methyl ferulate [14] or small, feruloylated oligosaccharides derived from plant cell walls such as [5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose from cereal brans [6,12]. Large, insoluble cell wall

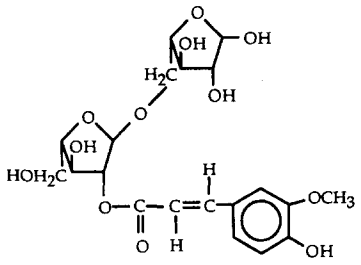

Fig. 2. Structure of [2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose (Ara_2F) from sugarbeet pulp.

materials (e.g. cereal brans, sugarbeet pulp) are poor substrates for FAEs, since these enzymes generally require the action of at least one carbohydrase to solubilise the polymers and release soluble feruloylated oligosaccharides; the latter are hydrolysed efficiently by FAEs [3,15–18]. Hence, cell wall materials are generally not useful as substrates in, for example, routine analysis of fractions during enzyme isolation, since the FAE activity will 'disappear' during separation from cooperating activities [19]. Methyl ferulate can be chemically synthesized [14]. However, although all FAE's are active on methyl ferulate, they possess different specificities for ferulic acid-arabinose ester linkages; specific feruloylated oligosaccharides have been used to show that some esterases are active only on 5-substituted Ara [12], while others are specific on 2-substituted Ara [3,12]. Hence, cell wall-derived substrates such as Ara_2F are essential tools for the detection, isolation and characterisation of specific FAE's capable of releasing free ferulic acid from esterified plant cell walls.

In addition, feruloylated oligosaccharides can be used to study the antioxidant potential of food components, and the hypersensitive response of plants to microbial invasion. Feruloylated oligosaccharides are potent antioxidants; it has been demonstrated that feruloylated oligosaccharides are as effective as free ferulic acid in (in vitro) antioxidant assays [20], and these compounds are likely released from fibre in the human gastrointestinal tract [21]. It has been shown that the heat-stable killing activities produced by the action of *Magnaporthe grisea* secreted enzymes on plant cell walls are esterified, and that the feruloyl substituents are essential for their activity [22].

1. Experimental

Materials.—The source and composition of the sugarbeet has been described previously [3]. *endo*-Arabinanase and α-L-arabinofuranosidase (both *Aspergillus niger*) were obtained from Megazyme (Australia) Pty. Ltd. These enzymes were shown to be > 95% pure by SDS-PAGE analysis. Ferulic acid was purchased from Sigma–Aldrich.

Enzymic hydrolysis of sugarbeet pulp.—Sugarbeet pulp (20 g) was incubated with 60 U *endo*-arabinanase and 200 U α-L-arabinofuranosidase in 20 mM NaOAc buffer (pH 4.0) containing 0.02% NaN_3 (final volume 500 mL) with constant agitation (flat-bed shaker, 150 rpm) at 37 °C. After 65 h incubation, the products were filtered through miracloth (Calbiochem Corporation), the retained material washed with distilled water, and the filtrate and washings concentrated (rotary evaporator).

Chromatography.—Chromatography on a column (32 × 3 cm) of Sephadex LH-20 was performed at 30 mL/h with distilled water; 3-mL fractions were collected. Chromatography on a column (9.5 × 1 cm) of Bio-Gel P-2 was performed at 30 mL/h with distilled water; 1.5-mL fractions were collected. For both chromatographic separations, fractions were analysed for their absorbance at 325 nm.

Analytical methods.—Levels of alkali-extractable ferulic acid were determined using an HPLC method [3] after treating samples with 1 M NaOH for 24 h. Untreated samples were analysed for esterified feruloyl groups using the same HPLC method. Absorption spectra (range 220–400 nm) were recorded in 100 mM MOPS buffer (pH 6) using a Beckman DU-70 spectrophotometer. Neutral sugars in crude supernatant and purified fractions were released by hydrolysis with 1 M H_2SO_4 for 2.5 h at 100 °C. After conversion to their corresponding alditol acetates [23], sugars were analysed by gas–liquid chromatography (RTX-225, 30 m × 0.32 mm). 2-Deoxyglucose was used as an internal standard.

NMR spectroscopy.—Samples (5 mg) were dissolved in D_2O and ^1H (400 MHz) NMR spectra recorded at 27 °C with a Jeol GX-400 spectrometer as described previously [8].

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