Intracellular feruloylation of pectic polysaccharides

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Abstract. The pectic polysaccharides of spinach cell walls carry feruloyl groups on arabinose and galactose residues. The following experiments were designed to discover whether the arabinose residues are feruloylated intra- or extracellularly. Cultured spinach cells started to incorporate exogenous [3H]arabinose into polymers at a linear rate after a lag period of approx. 3-4 min, although radioactive polysaccharides and extensin did not start to appear outside the plasmalemma until after an approx. 25-min lag. In the same cells, polysaccharide-bound feruloyl-[3H]arabinose units started to accumulate radioactivity at a linear rate after a lag period of approx. 4-5 min. Therefore, arabinose residues of polysaccharides began to be feruloylated while still intracellular. The rate of formation of polysaccharide-bound feruloyl-[3H]arabinose units did not appreciably increase after 25 min, showing that any additional extracellular feruloylation of the polysaccharide was relatively slow. This conclusion was supported by two different types of pulse-chase experiments, one of which was designed to detect feruloylation of polysaccharides up to 6 d after synthesis.

Key words: Cell wall – Ferulic acid – Pectic arabinogalactan – Polysaccharide – Secretion (polysaccharide) – Spinacia (cell wall).

Introduction

Polysaccharides of the primary cell wall often have phenolic groups linked to them. For instance, the arabinoxylans of Monocotyledons (Harris and Hartley 1980; Ahluwalia and Fry 1986) and the pectic arabinogalactans of certain Dicotyledons (Hartley and Harris 1981; Fry 1983, 1986a) have ferulate and p-coumarate esterified to specific sugars. Pectic polysaccharides of spinach carry feruloyl groups linked in such a way that they can be isolated by enzymic digestion as a feruloyl-arabinobiose (Fer-Ara₂) and a feruloyl-galactobiose (Fer-Gal₂) (Fry 1982).

Wall-bound ferulic and related hydroxycinnamic acids are important because they are sites at which covalent cross-links may form between wall polymers by oxidative coupling (Geissmann and Neukom 1971). Such cross-linking, catalysed in the cell wall by peroxidase and requiring an oxidant (e.g. H₂O₂), could restrict wall extensibility and digestibility (Fry 1979, 1983, 1986a). The control of cross-linking in vivo is not understood, but is of considerable interest in studies of growth, defence, digestion and decay. Control might be effected at several levels such as feruloylation of polysaccharides, secretion of peroxidase, or production of oxidant. It is difficult to test the first of these possibilities since the mechanism of feruloylation is unknown. The present work was therefore undertaken in order to investigate the biosynthesis of the feruloyl-arabinopyranose linkage, which is characteristic of the pectic polysaccharides of spinach (Fry 1982).

The reactants involved in the feruloylation of polysaccharides are unknown. Free ferulic acid is unlikely to be the immediate reactant as synthesis of an ester bond requires the acyl group to be activated. Hydroxycinnamoyl-CoA and hydroxycinnamoyl-β-glucoside occur widely in plants and both these types of compound have been shown to be capable of acting as acyl donors in enzymic transesterification reactions in vitro (Zenk 1979; Tkotz and Strack 1980), though only with acyl acceptors other than polysaccharides.

The immediate feruloyl acceptor is also unknown. It is conceivable that UDP-arabinose is
first feruloylated and then used to introduce a pre-formed feruloyl-arabinopyranosyl unit into the nascent polysaccharide; alternatively, feruloyl groups might be transferred directly on to a pre-formed polysaccharide or on to a completed domain of a nascent polysaccharide.

Nor is it known where in the cell the feruloylation occurs. Yamamoto and Towers (1985) suggested extracellular feruloylation of polysaccharides that had already been deposited in the cell wall. Supporting this idea was the fact that the accumulation of feruloyl-polysaccharides in maturing barley coleoptile cell walls continued well after net deposition of wall polysaccharides had ceased. However, an alternative explanation of their data is that, in old coleoptiles, the feruloyl-polysaccharides are synthesis intracellularly de novo, and secreted, at relatively high rates after the deposition of total (non-feruloylated) polysaccharide has slowed down and perhaps been exceeded by turnover.

Since neither the sub-cellular site(s) of feruloylation nor the nature of the feruloyl-donor or -acceptor are known, it is difficult to begin a search for the feruloyltransferase(s) involved. I report here experiments designed to discover at what stage in the career of a pectic arabinogalactan molecule its arabinose residues acquire feruloyl groups.

Material and methods

**General.** 1-[1-²H]Arabinose (92 GBq·mmol⁻¹) was custom synthesised by Amersham International, Amersham, Bucks, UK, by the ‘TL7’ catalytic exchange method. The material was purified by paper chromatography in EPW and stored in 60% ethanol at −40 °C. It was re-purified immediately before use by chromatography in BEW.

Chromatography was on Whatman (Maidstone, Kent, UK) 3 MM paper by the descending method. Solvents used were:

- **BAW** = butan-1-ol/acetic acid/water (12:3:5, by vol.);
- **BEW** = butan-1-ol/ethanol/water (20:5:11, by vol.);
- **EPW** = ethyl acetate/pyridine/water (8:2:1, by vol.);
- **Electrophoresis** was on Whatman no. 1 paper either with 1.9% (w/v) Na₂B₄O₇; 10H₂O, pH 9.4, at 300 V for 3 h in a simple air-cooled apparatus (Fry 1984a) or with formic acid/acetic acid/water (1:4:45, by vol.), pH 2.0, at 5 kV for 45 min in a white-spirit-cooled apparatus.

Sugars were located on paper with aniline hydrogen phthalein, feruloyl-esters with ultra-violet fluorescence (Fry 1982), and hydroxyproline-tetraarabinobioside with isatin/ninhydrin (Kolor and Roberts 1957). Scintillation-counting of paper strips was by immersion in 2 ml of 0.5% 2,5-diphenyloxazole (PPO) + 0.05% 1,4-bis(5-phenyloxazole)benzene (POPPO) in toluene (counting efficiency approx. 4% for ²H and 25% for ¹⁴C).

**Driselase**, a glycanase mixture from *Irpex lacteus*, was from Sigma Chemical Co., Poole, Dorset, UK, and was partially purified (Fry 1982). Driselase lacks esterase activity, but effectively hydrolyses the major glycosidic linkages, except α-xylosyl, of cell-wall polysaccharides (Fry 1986b). It therefore cleaves the major polysaccharides of the primary cell wall to yield the following diagnostic pentose-containing products:

- Xyloglucan* → Xylosyl-α-(1→6)-glucose
- Pectic arabinogalactan → Arabinose
- Arabinoxylan** → Arabinose + Xylose

(* Contains α-xylene residues. ** Contains β-xylene residues.) Driselase hydrolyses the feruloylated pectic arabinogalactan of spinach to give arabinose plus a small amount of Fer-Ara₂ (Fry 1982). Driselase can therefore be used to distinguish feruloylated from non-feruloylated arabinose residues. In some experiments, Driselase digests were assayed for total hexose by treatment of a 0.2-ml sample (with any undigested material resuspended) with 0.3 ml of water plus 1.0 ml of 0.2% (w/v) anthrone in concentrated H₂SO₄ at 100 °C for 5 min followed by measurement of the A₆₂₅.

Suspension cultures of spinach (*Spinacia oleracea* L.) were maintained as described before (Fry 1983) and subcultured every two weeks.

**Specific experiments.** Cultures of the required age were adjusted to packed cell volume of approx. 15% by addition or removal of spent medium and incubated under standard culture conditions for approx. 4 h. [²H]Arabinose (2.5 MBq·ml⁻¹) was then added, with a minimum of disturbance, and the incubation was continued.

Samples (0.5 ml) were removed from the radioactive cultures at intervals and quickly filtered. An aliquot (30 µl) of the filtrate was chromatographed in BAW. The material that remained at the origin was assayed for radioactivity and is reported as ‘soluble extracellular [²H]polymers’. The cells were rapidly treated with 80% ethanol, repeatedly washed in 80% ethanol until the washings were no longer radioactive, dried, and incubated with shaking for 18 h at 25 °C in 400 µl of 1% ‘Driselase’ in 20 mM acetate (Na⁺) buffer containing 0.05% NaN₃.

The Driselase-digestion products (50 µl) were chromatographed in EPW to separate arabinose, xylose (RARA = 1.26) and xylosyl-α-(1→6)-glucose (RARA = 0.30) (Fry 1986b), which were assayed for radioactivity. A further 150 µl of the digestion products was chromatographed in BAW to separate free pentoses (arabinose and xylose, not completely resolved; R₂ = 0.33-0.35), xylosyl-α-(1→6)-glucose (R₂ = 0.16) and Fer-Ara₂ (R₂ = 0.7) (Fry 1982, 1986b). The Fer-Ara₂ spot was eluted in 50% methanol and further purified by chromatography in BEW, which separates Fer-Ara₂ (R₂ = 0.65) from the corresponding p-coumaroyl ester (R₂ = 0.70) (Fry 1982).

The purified Fer-Ara₂ was eluted in 50% methanol and dried in two halves. One half was de-feruloylated by incubation in 50 µl of 0.5 M NaOH at 25 °C for 1 h and then adjusted to pH approx. 4.7 with acetic acid; the other half was treated for 1 h with a corresponding amount of sodium acetate+acetic acid, pH 4.7. Both halves were re-chromatographed in BAW. The control showed > 95% of the radioactivity still at the R₂ of Fer-Ara₂, whereas the NaOH-treated sample had been converted to a substance which ran with arabinobiose (ARA₂), which is unusual for a disaccharide in that it approximately co-chromatographs with arabinose (Fry 1982). ARA₂ was assayed for radioactivity, and the results are reported as Fer-[³H]ARA₂.

Further evidence for the identity of ARA₂ was obtained by paper electrophoresis in borate, in which ARA₂ (mARA₂ = 0.6) clearly separates from arabinose (Fry 1982). Hydrolysis of the ARA₂ in 2 M trifluoroacetic acid at 120 °C for 1 h yielded only radioactive material that co-electrophoresed with arabinose.