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O-FERULOYLATED, *O*-ACETYLATED OLIGOSACCHARIDES AS SIDE-CHAINS OF GRASS XYLANS

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Key Word Index—*Festuca arundinacea*; Gramineae; tall fescue grass; cell cultures; feruloyl esters; acetyl esters; oligosaccharides; arabinoxylans; plant cell walls.

Abstract—Partial acid hydrolysis of cell wall material from *Festuca arundinacea* cell cultures yielded a novel *O*-feruloylated trisaccharide (**3**). Treatment of **3** with Driselase, which contains β- but not α-D-xylosidase, released xylose plus the known compound, β-D-xylopyranosyl-(1 → 2)-(5-*O*-feruloyl)-L-arabinose. Since **3** contained one $NaIO_4$-resistant xylose residue, it was concluded to be β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-(5-*O*-feruloyl)-L-arabinose. Partial acid hydrolysis of *Festuca* cell walls also yielded several higher-M_r feruloylated oligosaccharides, including a feruloylated pentasaccharide, **4** (sugar composition: Ara + Xyl_2 + two non-pentose residues) and a feruloylated heptasaccharide, **5** (Ara + Xyl_3 + three non-pentose residues). Compounds **4** and **5** were endogenously *O*-acetylated but **3** was not. Similar or identical compounds were found in hydrolysates of 20 additional species of the Gramineae. These products represent a series of complex side-chains which, *in vivo*, are attached via Ara*f* residues to the parent xylan. Their possible biological roles are discussed. Copyright © 1997 Elsevier Science Ltd.

INTRODUCTION

The main hemicelluloses of grasses and cereals are glucuronoarabinoxylans, in which the Xyl*p* residues of a β-(1 → 4)-D-xylan backbone are substituted at *O*-3 with α-L-Ara*f* and at *O*-2 with α-D-Glc*p*A and/or its 4-*O*-methyl ether [1, 2]. In addition, *O*-acetyl [1, 2] and *O*-feruloyl esters [2, 3] are present.

The *O*-feruloyl groups are of interest because of their high reactivity *in vivo*. They can be enzymically dimerized (e.g. by peroxidase + H_2O_2) to form diferuloyl and other putative inter-polysaccharide cross-links, which may tighten the cell wall and, therefore, limit cell expansion [3]. They may also be subject to photo-dimerization [4] and photo-isomerism [5]. In addition, they may protect the xylan against enzymic hydrolysis of glycosidic linkages; the feruloyl groups are themselves the target of attack by microbial esterases [6]. The precise molecular environment of the *O*-feruloyl groups is likely to affect their ability to participate in these and other metabolic reactions.

The major site of *O*-feruloylation in xylans is position 5 of the L-Ara*f* residues, because mild acid hydrolysis releases 5-*O*-feruloyl-L-arabinose (**1**). Many of the residues of **1** themselves carry a β-D-Xyl*p* residue attached to *O*-2 [7–9]. Thus, mild acid hydrolysis of all graminaceous species tested released substantial amounts of 2-*O*-β-D-xylopyranosyl-(5-*O*-feruloyl)-L-arabinose (**2**) [9]. In the present paper, we have explored what additional sugar residues and/or ester groups may be attached to **2**.

RESULTS AND DISCUSSION

A suspension culture of *Festuca* readily incorporates radioactivity from L-[1-^{3}H]arabinose into the pentose residues of cell wall polysaccharides [9]. ^{3}H-Labelled alcohol-insoluble residue (AIR) was heated in mild acid, under conditions that hydrolyse ~10% of *O*-feruloyl ester bonds, ~60% of β-D-xylopyranosyl linkages and essentially all the α-L-arabinofuranosyl linkages [9]. On PC in BAW, the hydrolysate gave eight zones (1–8) [9] that exhibited the fluorescence characteristics of feruloyl esters (blue, turning intense blue–green in NH_3); in the present work, fractions 3–5 have been investigated.

Fractions 3–5 were eluted from a preparative PC (BAW), enriched in aromatic material by low-pressure reverse-phase chromatography (LP-RPC) and further

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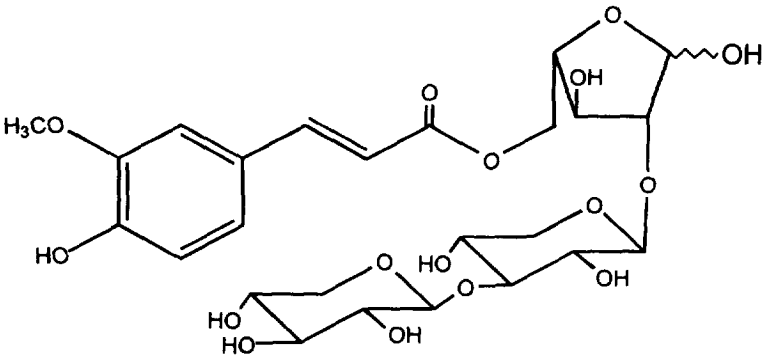
Table 1. Chromatographic data for the feruloyl esters present in fractions 1–5 and for the corresponding de-esterified sugars **1a–5a**

Compound	R_{Ara} (PC in BAW)	R_f (PC in BEW)	R_{Ara} (PC in EPW_1)	R_{Ara} (PC in BPW)
1	2.21	0.74	n.d.	n.d.
1a	1.00	n.d.	1.00	1.00
2	1.86	0.57	n.d.	n.d.
2a	0.64	n.d.	0.27	n.d.
3	1.36	0.38	n.d.	n.d.
3a*	0.50	n.d.	0.11	n.d.
3a′*	0.70	n.d.	0.34	n.d.
4	0.95	0.33	n.d.	n.d.
4a	0.38	0.15	n.d.	0.81
5	0.73	0.29	n.d.	n.d.
5a	0.25	0.09	n.d.	0.74

*3a and 3a′ = major and minor alkaline hydrolysis products, respectively, of fraction 3.
n.d., not determined.



3

purified by PC in BEW. R_f values (Table 1) indicated that they were of higher M_r than **1** and **2**. Since compounds 3–5 (the major fluorescent compounds present in fractions 3–5, respectively) were efficiently released from AIR by mild acid hydrolysis, it is likely that the reducing terminal sugar moiety of each compound had been present as Ara*f* within the parent polymer.

(pentosyl-^{3}H)-Labelled compounds **3–5** were subjected to alkaline hydrolysis to yield the corresponding de-esterified sugar moieties (**3a–5a**) (Fig. 1; Table 1).

Complete acid hydrolysis of the major (pentosyl-^{3}H)-labelled oligosaccharide (**3a**) obtained from **3** yielded [^{3}H]arabinose and [^{3}H]xylose in the ratio 1.31:1 on a ^{3}H-basis (data not shown). Assuming that the pentose residues in **3a** had the same specific activity as those in the bulk AIR, this corresponds to a molar ratio of Ara:Xyl = 1:1.93; GPC on Bio-Gel P-2 indicated that **3a** was a trisaccharide (Fig. 2a). For identification of the reducing terminus, ^{3}H-labelled **3a** was reduced then hydrolysed. The products, separated by PC in EPW_1, were [^{3}H]arabinitol and [^{3}H]xylose in the molar ratio 1:1.78 (data not shown), indicating a trisaccharide ($Xyl_2 \cdot Ara$) with Ara as reducing terminus.

The fungal glycanase preparation, 'Driselase', converted **3** to [^{3}H]xylose and a ^{3}H-labelled product with a higher R_f (Fig. 3(c)), which co-chromatographed with an internal marker of non-radioactive **2** [9]; **2** itself was resistant to Driselase (Fig. 3(a) and (b)). Therefore, the major compound in fraction 3 is **2** with one additional xylose residue. Since Driselase lacks α-D-xylosidase and **3** had resisted mild acid hydrolysis, the additional Xyl residue must be in the β-pyranose ring form.

For identification of the glycosidic linkage of the additional Xyl residue, the $NaBH_4$-reduction product of **3a** was subjected to Smith degradation, followed by an additional reduction with $NaBH_4$. The radioactive products (Fig. 4) were [^{3}H]glycerol and [^{3}H]xylitol. [^{3}H]Glycerol would arise from the [1-^{3}H]arabinitol moiety of the D-Xyl*p*-(1 → 2)-L-arabinitol 'core'. [^{3}H]Xylitol would arise from the [^{3}H]Xyl*p* residue of the core only if that residue were itself substituted at *O*-3. The additional non-reducing terminal [1-^{3}H]Xyl*p*

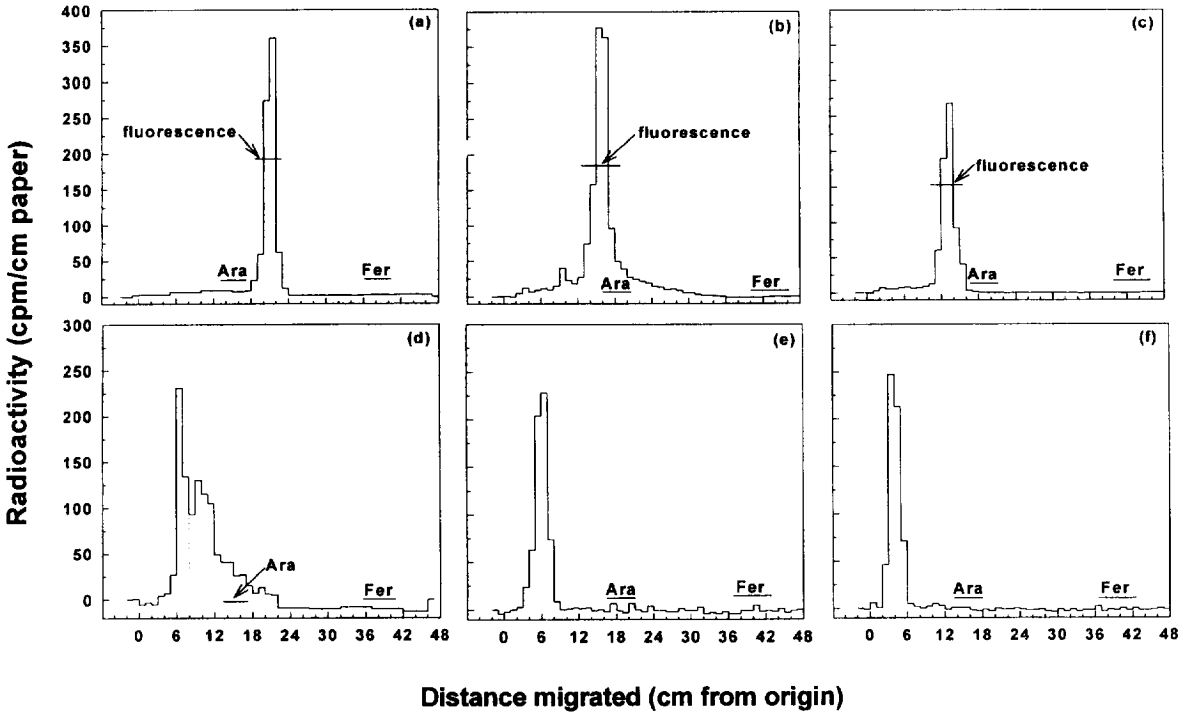


Fig. 1. PC of untreated and alkali-hydrolysed ^{3}H-labelled fractions 3–5. (a–c) Untreated fractions 3–5, respectively; (d–e) fractions 3–5, respectively, after incubation in 0.5 M NaOH at 25° for 1 hr. Ara used as external marker. The chromatography solvent was BAW for (c) and BEW for the others.

residue present in the $NaBH_4$-reduction product of **3a** would yield [^{3}H]glycollaldehyde, which is lost as volatile products during the work-up [10]. Thus, **3a** was β-D-Xyl*p*-(1 → 3)-β-D-Xyl*p*-(1 → 2)-L-Ara.

To test for the presence of multiple esters in **3**, we conducted partial alkaline hydrolysis of (feruloyl-^{14}C)-labelled **3**. The half-life of **3** in 0.5 M NaOH was ~8 min (from data in Fig. 5 and similar profiles, data not shown). An *O*-acetyl group, if present, would have been released by alkaline hydrolysis more rapidly than the *O*-feruloyl group and a transient product with an R_f lower than that of **3** would have been expected. No such intermediate product was formed (Fig. 5) (the peak of ^{14}C at $R_{Ara} = 2.0$ is likely to be an impurity as it was already present in the 0-min sample). No intermediate was found in similar experiments with ^{3}H-labelled **3** (data not shown). We conclude that a single *O*-feruloyl residue was the sole ester-linked group in **3**, which was thus β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-(5-*O*-feruloyl)-L-arabinose.

Complete acid hydrolysis of (pentosyl-^{3}H)-labelled **4a** yielded [^{3}H]arabinose and [^{3}H]xylose in the molar ratio Ara:Xyl = 1:2.11 (data not shown); GPC on Bio-Gel P-2 indicated a DP of ~5 (Fig. 2b). These data suggest the presence of two Xyl, one Ara and two non-pentose sugar residues.

^{3}H-Labelled **4** was hydrolysed by Driselase yielding a complex range of radiolabelled products including compounds that co-migrated with **2** and **3** (Fig. 3(d)). The material with $R_{Ara} \approx 1$ that remained after 96 hr in Driselase (Fig. 3(d)) was found, by LP-RPC followed by PC in BAW and EPW_1, to be a mixture of unchanged **4** and free [^{3}H]xylose with a trace of free [^{3}H]arabinose (data not shown). The ability of Driselase to release [^{3}H]xylose indicates that it was β-D-Xyl.

The half-life for the hydrolysis of (feruloyl-^{14}C)-labelled **4** in 0.5 M NaOH at 25° was ~4 min. However, the initial radioactive product was not [^{14}C]ferulic acid but a fraction that migrated between **4** and **4a** (Fig. 6). This indicates the presence in **4** of two ester groups, the more alkali-labile of which could be *O*-acetyl.

The ability of Driselase to convert a proportion of ^{3}H-labelled **4** to compounds with lower R_f values (Fig. 3d) could be due to partial deacetylation. To test whether the additional alkali-labile bond was indeed an *O*-acetyl group, we fed *Festuca* cells with [^{14}C]acetate for 32 hr (found in preliminary work to give maximal labelling of AIR), then subjected the radioactive AIR to mild acid hydrolysis and PC (BAW), which revealed peaks of ^{14}C co-migrating with **4** and **5** (Fig. 7). Together, these results suggest that the compounds in fraction 4 have a core of **3** with, on average, two additional non-pentose sugar (NPS) residues and an *O*-acetyl ester group. Thus, **4** is suggested to have the constitution [*O*-acetyl] · [NPS_2] · [β-D-Xyl*p*-(1 → 3)-β-D-Xyl*p*-(1 → 2)-(5-*O*-feruloyl)-L-Ara·], where Ara· is the reducing terminal arabinose group, formerly Ara*f* within the parent polysaccharide.

Complete acid hydrolysis of (pentosyl-^{3}H)-labelled **5a** yielded [^{3}H]arabinose and [^{3}H]xylose in the molar

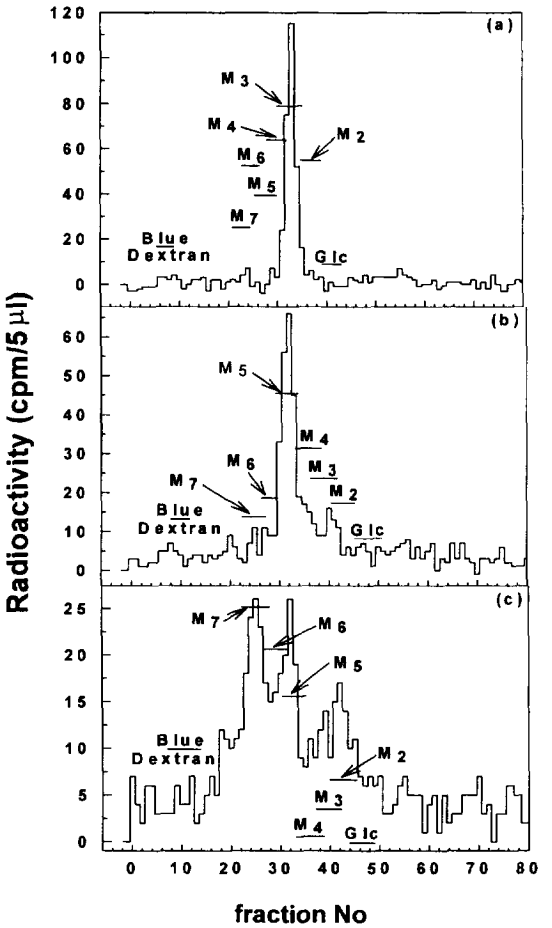


Fig. 2. Behaviour of ^{3}H-labelled **3a** (a), **4a** (b) and **5a** (c) on Bio-Gel P-2. Blue dextran, glucose and maltose to malto-heptaose (M_2–M_7) as internal markers.

ratio 1:3.04 (data not shown). GPC of **5a** on Bio-Gel P-2 indicated that it was a mixture, with peaks centred on internal marker malto-oligosaccharides of DP 5 and 7 (Fig. 2(c)). The data thus suggest the presence of three Xyl, one Ara and one to three NPS residues.

Driselase digestion of **5** yielded a complex mixture of hydrolysis products including substances that approximately co-migrated with **4**, **3** and **2** (Fig. 3(e)). The ^{3}H-labelled breakdown products that co-migrated with **4** were fractionated by LP-RPC and then re-chromatographed. The retained (aromatic) material co-migrated (on PC in BAW) with internal marker **4**; the non-retained material co-migrated (on PC in EPW_1) with Xyl and a trace with Ara. These results show that the compounds in fraction 5 are based on a core containing **4** and, therefore, also **3**. The half-life for the hydrolysis of (feruloyl-^{14}C)-labelled **5** in 0.5 M NaOH at 25° was ~3 min. However, the initial radioactive product was not [^{14}C]ferulic acid but a fraction that migrated between **5** and **5a** (Fig. 8). Complete alkaline hydrolysis of this intermediate released [^{14}C]ferulate, as judged by PC in BAW (data not shown). This indicates the presence in **5** of two alkali-labile groups. Incorporation of [^{14}C]acetate

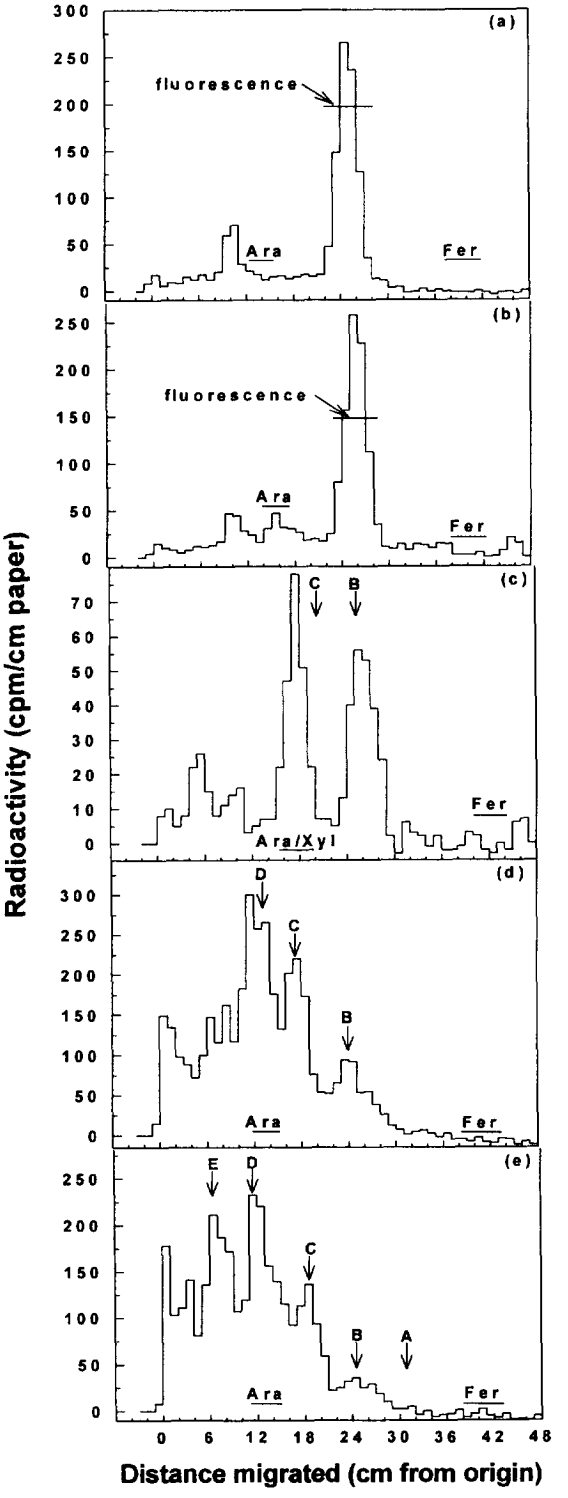


Fig. 3. PC in BAW of Driselase hydrolysis products of ^{3}H-labelled feruloyl oligosaccharides. (a) The feruloyl disaccharide **2**, untreated; (b) **2** after 48 hr treatment with Driselase; (c) **3** after 96 hr treatment with Driselase; (d) **4** after 96 hr treatment with Driselase; (e) **5** after 96 hr treatment with Driselase. (A–E = positions expected of compounds **1**–**5**, respectively; — = positions of external markers.)

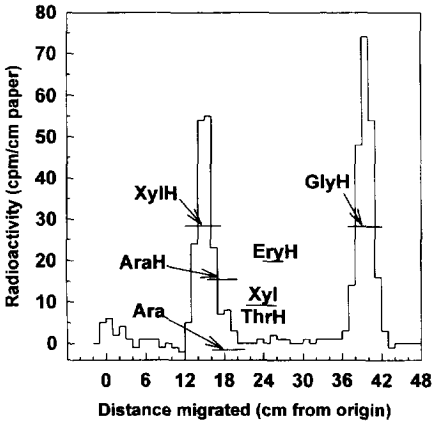


Fig. 4. PC in EPW_1 of products obtained from $NaBH_4$-reduced, ^3H-labelled, **3a** by Smith degradation followed by a second $NaBH_4$ reduction. AraH, arabinitol; XylH, xylitol; EryH, erythritol; ThrH, threitol; GlyH, glycerol.

again suggested that one of these was an acetyl ester (Fig. 7). Taken together, these results suggest that fraction 5 contains feruloylated, acetylated penta- to heptasaccharides with a backbone of **3**.

Mild acid hydrolysis of AIR from leaves of all grasses and cereals tested (*Festuca arundinacea*, *F. gigantea*, *F. pratensis*, *F. rubra*, *Lolium multiflorum*, *L. perenne*, *Dactylis glomerata*, *Bromus sterilis*, *Triticum aestivum*, *Avena sativa*, *Arrhenatherum elatius*, *Anthoxanthum odoratum*, *Agrostis tenuis*, *Phleum pratense*, *Hordeum vulgare*, *Secale sentinal*, *Sorghum verticilliflorum*, *S. vulgare*, *Dichanthium sericeum*, *Bothriochloa ambigua* and *Zea mays*) yielded a feruloyl oligosaccharide which, by PC in BAW, appeared to be **3**. In addition, most of the 21 species tested produced a series of feruloyl oligosaccharides resembling **4** and **5**, as well as the slower-migrating **6**–**8** (cf. [9]).

It is clearly an over-simplification to regard the glucuronoarabinoxylans of grasses and cereals as possessing a backbone of β-(1 → 4)-D-xylan with side-chains of α-D-Glc*p*A and α-L-Ara*f*, some of which are 5-*O*-feruloylated. We recently showed that many of the feruloyl arabinose groups are further substituted with β-D-Xyl*p* to form the feruloyl disaccharide **2**. In the present work, we have shown that the unit **2** is often further substituted with an additional β-D-Xyl*p* residue, giving rise to **3**. This appears to be a general feature, common to most, if not all, graminaceous plants. The additional β-D-Xyl*p* residue is attached at position *O*-3 of the existing D-Xyl*p* residue, indicating a highly specific biosynthetic system.

The data presented here also show that **3** itself acts as a 'core' to which further sugar residues are frequently attached. The larger structures thus formed have not been completely characterized; however, they include an additional β-D-Xyl*p* residue, NPS residues and *O*-acetyl esters. The NPS residues may include β-D-Gal, a well-known minor constituent of xylans [1, 2]. We did not investigate to which sugar residue the acetyl group was attached but since only

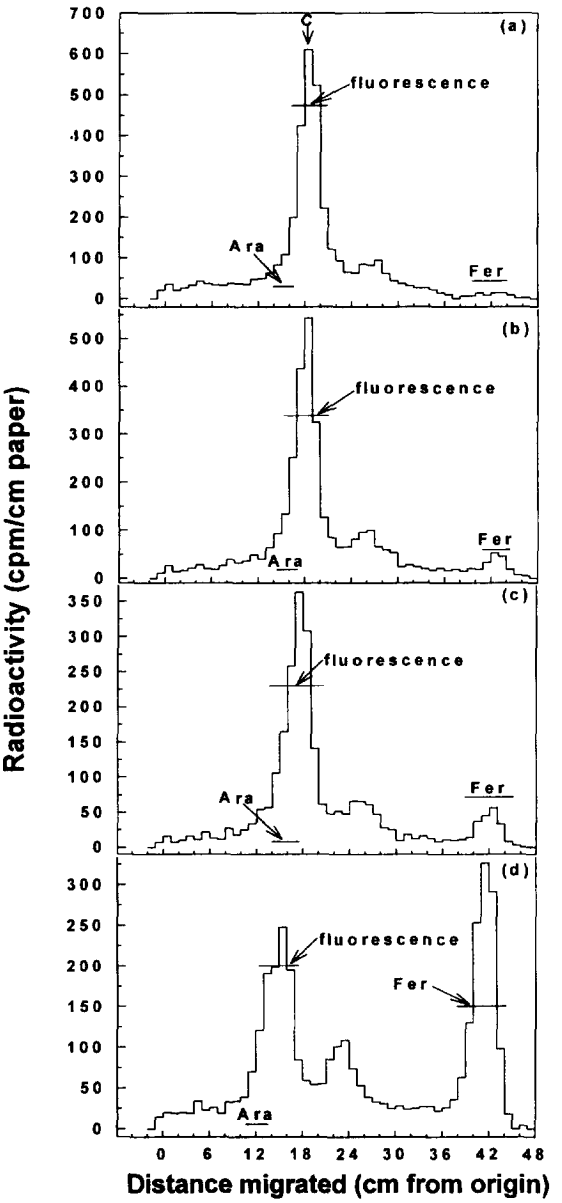


Fig. 5. PC in BAW of partial alkaline hydrolysis products of ^{14}C-labelled **3**. Hydrolysis was stopped after (a) 0 min, (b) 0.5 min, (c) 1 min and (d) 8 min in 0.5 M NaOH at 25°. (C = position expected of compound **3**.)

the larger structures (penta- to heptasaccharides) were *O*-acetylated, it seems probable that the acetyl group was associated with one of the more peripheral sugar residues, possibly the NPS.

It is of interest to consider the roles of the complex array of additional sugar and acetyl groups linked to (**1**). Possibilities include the following. Feruloylated complex carbohydrates may be secondary products with no universal role in cell biology. The additional sugar residues could decrease the accessibility of the feruloyl groups to enzymes, such as peroxidases, which oxidatively cross-link them, tightening the cell wall and rendering it more resistant to turgor-driven

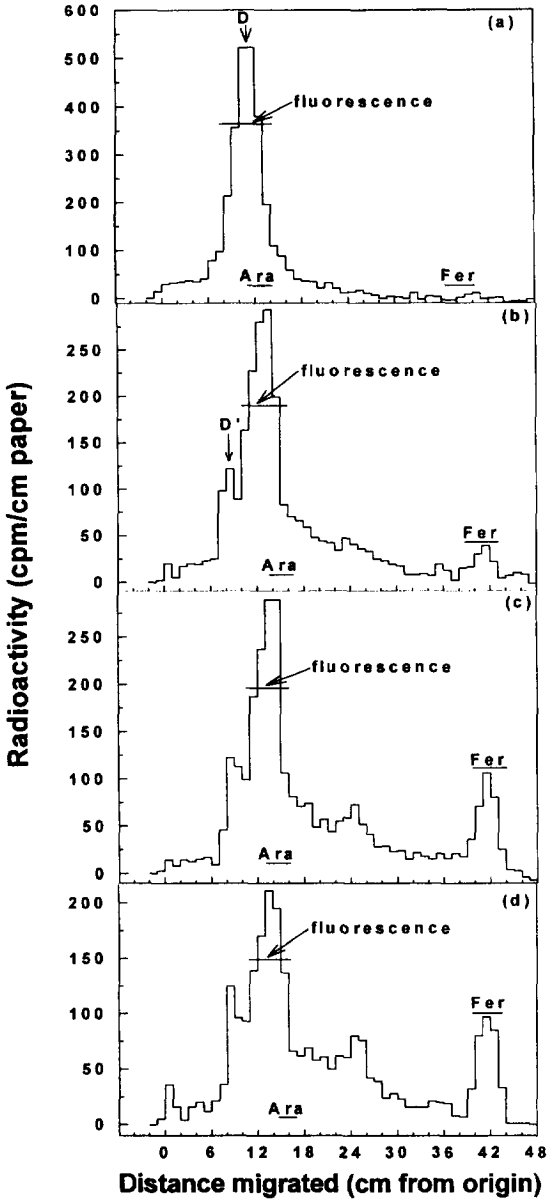


Fig. 6. PC in BAW of partial alkaline hydrolysis products of ^{14}C-labelled **4**. Hydrolysis was stopped after (a) 0 min, (b) 1 min, (c) 2 min and (d) 4 min in 0.5 M NaOH at 25°. (D = position expected of compound **4**; D′ = intermediate degradation product referred to in text.)

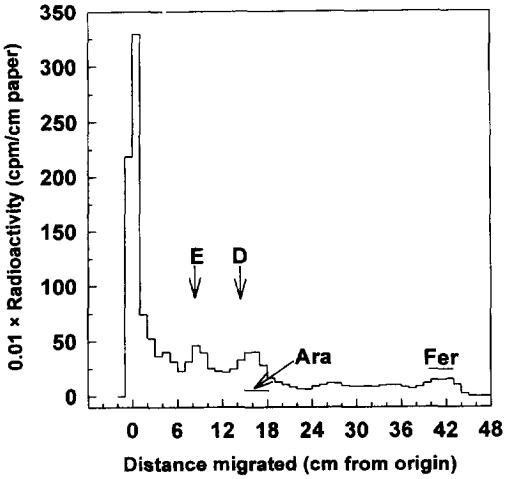


Fig. 7. PC in BAW of mild acid hydrolysate of alcohol-insoluble residue from [^{14}C]acetate-fed *Festuca* cells. E and D indicate the positions expected for compounds **5** and **4**, respectively, based on their R_{Ara} values and the position of the external marker arabinose.

extension or to enzymic hydrolysis [3]. They could protect the feruloyl group from enzymic removal by feruloyl-esterases. They could also affect the susceptibility of the feruloyl group to photo-dimerisation or to (*E*)↔(*Z*) photo-isomerism. Alternatively, they could affect the polysaccharide as a whole. For example, they could decrease the susceptibility of the xylan backbone to enzymic hydrolysis, increase the water-solubility of the polysaccharide [11–13] or decrease its ability to hydrogen-bond to the cellulosic microfibrils [14]. Finally, in view of their highly specific chemical structures, they might be the latent forms of oligosaccharins (signalling molecules releasable from the cell wall in response to given stimuli [15]); in this respect, it is interesting to note that exogenous xylanases [16] and feruloylated oligosaccharides [17] can influence plant growth and metabolism.

EXPERIMENTAL

Radiochemicals. L-[1-^{3}H]Arabinose (97 MBq μmol^{-1}) and (*E*)-[U-^{14}C]cinnamic acid (15 MBq μmol^{-1}) as described in ref. [9]. Na [1-^{14}C]acetate (1.4 MBq μmol^{-1}) was from Amersham.

Plant material. Cell suspension cultures of *F. arundinacea* Schreber were maintained as described in ref. [9]. For radiolabelling, a 4-day-old culture was incubated with 47 MBq of L-[1-^{3}H]arabinose for 5 hr or (aseptically) with 2 MBq of (*E*)-[U-^{14}C]cinnamate for 7 days or (aseptically) with 18.5 kBq [^{14}C]acetate for 32 hr. For prepn of AIR from cell cultures, cells were packed into a column and washed for at least 24 hr with flowing 80% EtOH. Seedlings of the following species were grown as described in ref. [9]: *Festuca arundinacea*, *F. gigantea*, *F. pratensis*, *F. rubra*, *Lolium multiflorum*, *L. perenne*, *Dactylis glomerata*, *Bromus sterilis*, *Triticum aestivum*, *Avena sativa*, *Arrhenatherum elatius*, *Anthoxanthum odoratum*, *Agrostis tenuis*, *Phleum pratense*, *Hordeum vulgare*, *Secale sentinal*, *Sorghum verticilliflorum*, *S. vulgare*, *Dichanthium sericeum*, *Bothriochloa ambigua* and *Zea mays*. AIR was prepd from seedling leaves [9].

Acid and alkaline hydrolysis. Mild acid hydrolysis (to hydrolyse preferentially furanosidic bonds) and 'complete' acid hydrolysis were performed with 0.1 and 2.0 M TFA at 100° and 120°, respectively, for 1 hr [9]. Feruloyl ester-linkages were cleaved by treatment with 0.5 M NaOH at 25° for 1 hr, and then the soln was adjusted to pH 4.7 with HOAc [9]; for unhydrolysed controls, HOAc was added before the NaOH so that

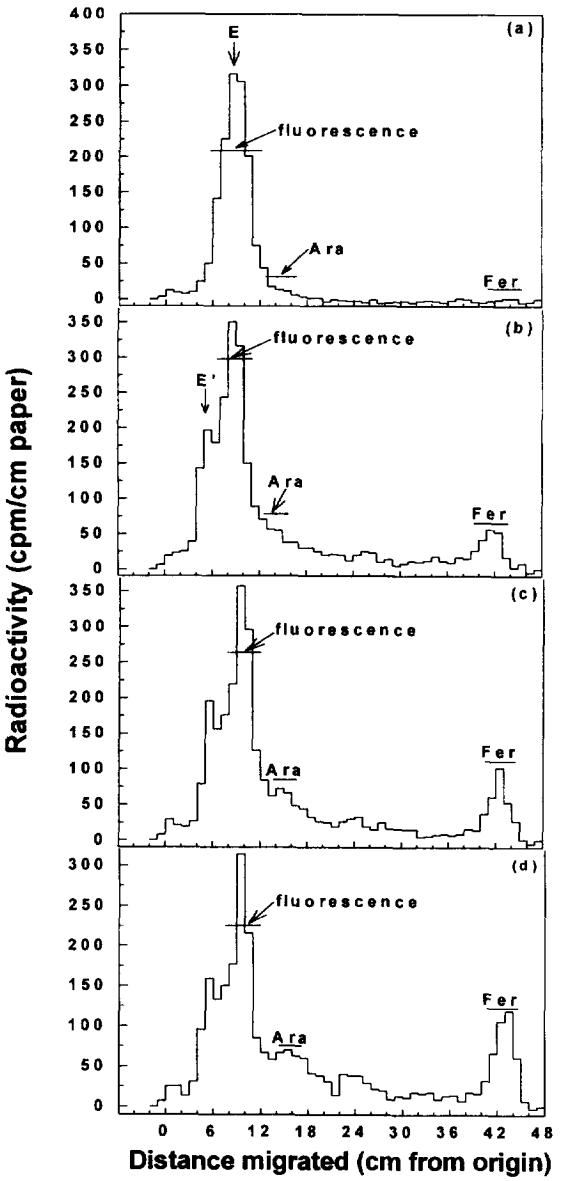


Fig. 8. PC in BAW of partial alkaline hydrolysis products of ^{14}C-labelled fraction **5**. Hydrolysis was stopped after (a) 0 min, (b) 1 min, (c) 2 min and (d) 4 min in 0.5 M NaOH at 25°. (E = position expected of compound **5**; E′ = intermediate degradation product referred to in text.)

control chromatograms would receive the same NaOAc loading.

Enzymic hydrolysis. Driselase was de-salted [18] and used as a 0.5% soln in pyridine–HOAc–1,1,1-trichloro-2-methylpropan-2-ol–H_2O (1:1:0.5:98, v/v/w/v) at 25° for 2, 4 or 6 days. Digestion was stopped by addition of 0.3 vols of HCO_2H.

Paper chromatography. PC was performed on Whatman 3MM paper by the descending method in the following solvents: BAW, *n*-BuOH–HOAc–H_2O (12:3:5); BEW, *n*-BuOH–EtOH–H_2O (20:5:11); BPW, *n*-BuOH–pyridine–H_2O (4:3:4); EPW_1, EtOAc–pyridine–H_2O (8:2:1) and EPW_2, EtOAc–pyridine–H_2O (10:4:3).

Internal and external markers. 'Internal' and 'external' markers [19] were used as described in ref. [9]. Reducing and non-reducing sugars were stained by the modified [19] aniline hydrogen-phthalate method [20] and silver ($AgNO_3$–NaOH–$Na_2S_2O_3$) [21], respectively.

Elution of compounds from PC. For recovery of radioactive samples, paper was freed of scintillant, if any, with PhMe and methylcyclohexane, then eluted ×5 with H_2O.

Low-pressure, reverse-phase chromatography. C_{18}-Substituted silica columns were used to separate *O*-feruloyl esters from simple mono- and oligosaccharides [9].

Gel-permeation chromatography (GPC). Samples were mixed with non-radioactive int. markers (blue dextran, glucose and maltose to maltoheptaose; 2 mg each) and passed through Bio-Gel P-2 [9].

Reduction and Smith degradation. Compound **3** was reduced to the corresponding alditol with $NaBH_4$ [9]. For Smith degradation [22], the alditol was oxidised with $NaIO_4$, reduced with $NaBH_4$ and then hydrolysed with TFA [9]. In the present work, the Smith degradation products were again reduced with $NaBH_4$.

Assay of radioactivity. ^{3}H and ^{14}C activity and sp. act. were assayed as described in ref. [9]. The sp. act. of [^{3}H]arabinose and [^{3}H]xylose residues in the AIR used in the present work were 3.78 MBq μmol^{-1} and 1.50 MBq μmol^{-1}, respectively. These values served as the basis for estimation of the molar Ara:Xyl ratios in **3**–**5**.

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