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Phenolic components of the primary cell wall

Feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide

Stephen C. FRY
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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1. Cell walls from rapidly growing cell suspension cultures of *Spinacia oleracea* L. contained ferulic acid and *p*-coumaric acid esterified with a water-insoluble polymer. 2. Prolonged treatment with trypsin did not release any feruloyl esters from de-arabinofuranosylated cell walls, and the polymer was also insoluble in phenol/acetic acid/water (2:1:1, w/v/v). 3. Treatment of the cell walls with the fungal hydrolase preparation 'Driselase' did liberate low-M_r feruloyl esters. The major esters were 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and 3?-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose. These two esters accounted for about 60% of the cell-wall ferulate. 4. It is concluded that the feruloylation of cell-wall polymers is not a random process, but occurs at very specific sites, probably on the arabinogalactan component of pectin. 5. The possible role of such phenolic substituents in cell-wall architecture and growth is discussed.

Lignin is a well-known phenolic component of secondary (non-growing) cell walls, where it replaces the water and completely prevents further growth (Northcote, 1972). The occurrence of phenols in primary (growing) walls, in contrast, is less well documented, although potentially important in determining the physical properties of such walls. Lignin itself does not seem to occur in primary cell walls (Whitmore, 1974), but other phenols may be quite widespread, for example, in the primary walls of mosses (Czapek, 1899), monocotyledons (Harris & Hartley, 1980) and dicotyledons (Mort & Lamport, 1977; Fry, 1979). In addition, the cell-wall proteins contain phenolic amino acids (Lamport, 1977; Fry, 1982).

One major phenol identified in both primary and secondary cell walls of grasses is ferulic acid (Harris & Hartley, 1976). It also occurs in many other monocotyledons (Harris & Hartley, 1980) and in the walls of rapidly-growing cell suspension cultures of the dicotyledons spinach (Fry, 1979) and woad (S. C. Fry, unpublished work). The ferulic acid is probably ester-linked to wall polymers (Whitmore, 1974).

A hypothesis was advanced in which such phenolic units within the primary cell wall were subject to oxidation by extracellular peroxidases to yield hydrophobic and cross-linked derivatives that would lower the wall's extensibility (Fry, 1979). Gibberellic acid was found to favour cell expansion in the spinach cultures (Fry & Street, 1980), possibly through suppression of peroxidase secretion (Fry, 1980). Direct evidence for such cross-linking was recently obtained for the tyrosine residues of wall protein, by identification of an oxidatively coupled dimer, named isodityrosine (Fry, 1982).

To assess more fully the possible consequences of phenol-oxidation, it is important to discover where the phenolic units are linked in the primary cell wall. Secondary cell walls from mature wheat endosperm contain a feruloylated arabinoxylan (Neukom, 1976), and the ferulate from secondary walls of mature leaf blades of *Lolium* appeared to be associated with xylose, arabinose and glucose (Hartley, 1973). The precise linkages were not determined, but both these studies suggest a feruloylated hemicellulose. Ferulate has also been found linked through an amide bond to the *N*-terminus of a protein (van Sumere *et al.*, 1973). However, nothing was known about the sites of feruloylation within rapidly growing cell walls. Here I report the isolation

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and characterization of two disaccharide fragments from the primary cell wall of spinach with their ferulic acid units still attached.

Materials and methods

Sources of special materials

Ferulic acid and *p*-coumaric acid were from Sigma, cinnamic acid and caffeic acid were from Koch–Light, Colnbrook, Bucks., U.K., and sinapic acid was from Aldrich, Milwaukee, WI, U.S.A. Diferulic acid (Geissmann & Neukom, 1971) was a generous gift from Professor Dr. H. Neukom, E.T.H., Zürich, Switzerland. Cinnamoyl β-D-galactoside was from Travenol Laboratories, Los Angeles, CA, U.S.A. Radiochemicals were from Amersham International, Amersham, Bucks., U.K. Trypsin (specific activity 33 units/mg) was from Boehringer-Mannheim, 'Driselase' was from Kyowa Hakko Europe G.m.b.H., Düsseldorf, Germany, and galactosidases were from Sigma.

Plant material

An auxin-independent green suspension culture (line G10 of Dalton & Street, 1976) of spinach (*Spinacia oleracea* L., cv. Monstrous Viroflay) was maintained in Murashige & Skoog's (1962) salts solution (with $FeCl_3$ replaced by equimolar Fe–EDTA) plus 1% sucrose (final pH 4.4). No hormones or vitamins were added. The cultures were grown at 20–25°C under continuous fluorescent lighting (6×10^{-5} einstein·m^{-2}·s^{-1}) with orbital shaking (diameter of orbit = 3 cm; frequency = 100 min^{-1}), at 700 ml of suspension per 1-litre flask. The flasks were plugged with cotton wool and capped with aluminium foil. The cells were subcultured every 2 weeks by approx. 8-fold dilution.

Cell walls were prepared by sonication (with a Dawe 'Soniprobe') in 100 mM-sodium acetate buffer (pH 5.0) containing 3 mM-$CaCl_2$, followed by bench centrifugation. The treatment was repeated until the pellet was no longer green; nuclei were also shown to be absent at this stage by u.v.-microscopy after staining with ethidium bromide (Blaschek *et al.*, 1974). Where necessary, the cell walls were further treated as follows. Removal of non-covalently bound protein and glycoprotein was performed in phenol/acetic acid/water (2:1:1, w/v/v) at 70°C for 1 h. This treatment was shown to remove over 90% of the radioactivity from the walls of cells cultured for several days in the presence of L-[^{35}S]methionine. Arabinofuranose residues of wall polymers were hydrolysed with 30 mM-oxalic acid at 100°C for 3 h; this treatment was shown to remove essentially all the arabinose from spinach cell walls, but little of the other sugars.

Enzymic hydrolysis of cell walls

De-arabinofuranosylated cell walls (100 mg dry wt.) were incubated for 5 days with the trypsin (6 ml at 8 mg/ml in 20 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 8.0) at 30°C under a trace of toluene.

Driselase (a fungal enzyme preparation containing polysaccharide exo- and endo-hydrolases including cellulase, pectinase, β-xylanase and β-mannanase) contained phenolic contaminants. These did not include ferulate or *p*-coumarate, but they were removed as follows. The incompletely soluble powder (5 g) was gently stirred for 2 h at 4°C with 50 ml of 50 mM-sodium acetate buffer (pH 5.0), and centrifuged (25 000 *g* at 4°C for 15 min). The clear supernatant was brought to 75% saturation with $(NH_4)_2SO_4$ and re-centrifuged. The pellet was washed once with 75% saturated $(NH_4)_2SO_4$, and re-dissolved in a minimal volume of water. The enzyme was de-salted on Sephadex G-25, freeze-dried and stored at −20°C.

For digestion with Driselase, cell walls were suspended (20 mg dry wt.·ml^{-1}) in 25 mM-sodium acetate buffer (pH 5.0) containing 0.01% sodium azide, and mixed thoroughly to form a paste. The paste was dispensed on a wet-weight basis into the enzyme solution (final enzyme concentration usually 0.6%) in the same buffer. In non-radioactive experiments, control incubations were always performed simultaneously in the absence of cell walls. Digestion was stopped by boiling (5 min) or by drying on chromatography paper.

Enzymic hydrolysis of galactose-containing disaccharides

For determination of the anomeric configuration of galactosides, samples were incubated at 20°C for 16 h either with green-coffee-bean α-galactosidase (1 unit/ml of 50 mM-pyridine acetate buffer, pH 6.5) or with *Escherichia coli* β-galactosidase (100 units/ml of 50 mM-Tris/HCl buffer, pH 7.5). Control hydrolyses with melibiose and lactose showed that the enzymes were absolutely specific, within the limits of the chromatographic assay.

Alkaline hydrolysis

Feruloyl and related esters were completely hydrolysed by incubation in 0.1 M-NaOH at 20°C for 16 h under N_2 in the dark. (For the partial hydrolysis shown in Fig. 1, shorter treatments with 0.5 M-NaOH were used.) For analysis of total reaction products, the solution (10–100 μl) was acidified with 2 equiv. of acetic acid and spotted or streaked on chromatography paper. Alternatively, where only the phenolic acids were to be analysed, the pH was adjusted to 2.0 with H_3PO_4 and the solution was partitioned against ethyl acetate; the organic phase was chromatographed.

Analysis of sugars

Periodate oxidation was performed with tracer levels of radioactive sugars in 0.1 ml 50 mM-$NaIO_4$ in 0.25 M-sodium formate buffer, pH 3.5, at 4°C for 6 days. Unreacted periodate was destroyed with 20 μl of ethane-1,2-diol for a further 16 h.

Borohydride reduction was achieved with excess 0.25 M-KBH_4 in 0.5 M-NH_4OH at 20°C for 2 h. Remaining borohydride was then destroyed by addition of acetic acid to pH 4.7. Cations were removed on a short column of Zeo-Karb (H^+ form) and the eluate was dried *in vacuo* to remove acetic acid, and re-dried from methanol several times to remove H_3BO_3 as its volatile methyl ester.

Acid hydrolysis was performed in 40–100 μl of 0.01, 0.1 or 0.5 M-trifluoroacetic acid in a sealed glass tube at 120°C for 1–1.5 h. The cooled samples were loaded directly on paper (without heating) for chromatography.

Crude preparations of D-galactokinase and L-arabinokinase were obtained from mung-bean seedlings as described by Neufeld *et al.* (1960). The preparation was incubated with radioactive sugar in the presence of 20 mM-ATP, 40 mM-KF, 10 mM-$MgCl_2$ and 40 mM-Tris/HCl buffer (pH 7.5); total final volume was 20 μl. After 6 h at 20°C, the reaction mixture was electrophoresed at pH 3.5. The radioactive sugar 1-phosphates (co-electrophoresing approximately with a picric acid marker) were assayed.

Chromatography and electrophoresis

Unless otherwise stated, Whatman no. 1 paper and 'MN Polygram' silica gel G thin layers were used for chromatography. Solvents are listed in Table 1. High-voltage electrophoresis was performed on Whatman no. 1 paper (effective path length 50 cm) in tanks of white spirit as coolant (20–30°C). Buffers, voltages and times are listed in Table 1.

Hydroxycinnamic acids were located by fluorescence under long-wavelength u.v. light (with and without NH_3), or by their brown staining with I_2 vapour (this also stains cinnamic acid itself, although weakly), or by their blue staining with Folin and Ciocalteu's phenol reagent followed by NH_3. On paper, free ferulic acid fluoresced blue, turning bright blue with NH_3; authentic feruloyl esters fluoresced blue, turning intensely blue–green with NH_3. *p*-Coumaric acid was invisible before treatment with NH_3, but fluoresced violet in its presence; authentic *p*-coumaroyl esters were also invisible without NH_3, but fluoresced blue in its presence.

Sugars, sugar alcohols and saccharinic acids were stained with $AgNO_3$/NaOH, and amino acids and peptides with ninhydrin (Harborne, 1973).

Preparation of authentic hydroxycinnamoyl esters

Several feruloyl and *p*-coumaroyl esters were synthesized to assist in the recognition of natural esters. Ferulic acid or *p*-coumaric acid (10 mg) was dissolved in 2.5 ml of the appropriate alcohol (glycerol, ethane-1,2-diol, propane-1,2-diol, methanol, ethanol or butanol). Concentrated H_2SO_4 (0.1 ml) was added, and the mixture was incubated at 50°C for 2 h. The products were diluted into 100 ml of 0.4 M-sodium phosphate buffer, pH 8.3, and partitioned against 10 ml of diethyl ether to extract the ester. The organic phase was evaporated to dryness and the residual ester was re-dissolved in acetone and stored at 4°C in the dark. Since the alcohols were in excess, the di- and tri-hydric alcohols would yield mainly their mono-esters. Each preparation gave one major spot on t.l.c. in system 9 (R_F values: glyceryl ferulate, 0.015; 2-hydroxyethyl ferulate, 0.09; hydroxypropyl ferulate, 0.12; methyl ferulate, 0.46; ethyl ferulate, 0.50; butyl ferulate, 0.57; cf. ferulic acid, 0.42). The six feruloyl esters were indistinguishable from each other by u.v.-fluorescence and staining, but were quite different from the corresponding *p*-coumaroyl esters.

Isolation of natural hydroxycinnamoyl esters

The two major natural feruloyl esters investigated in this work were named compounds (29) and (59) from their R_F values (×100) in system 1. For detailed studies, they were isolated and purified as follows. After removal of non-covalently bound protein, cell walls (200 mg dry wt.) were incubated with 0.6% purified Driselase (10 ml) in 25 mM-sodium acetate buffer, pH 5.0, at 25°C with gentle shaking for 16 h under a trace of toluene. Methanol was then added to 35% (v/v), the reaction was stopped by boiling (5 min) and insoluble material was removed by centrifugation. The supernatant was chromatographed in the dark on Sephadex LH-20 (gel bed 2.5 cm × 40 cm) in 35% methanol, and 150 1.2-ml fractions were collected. Four peaks of feruloyl esters were detected (see Table 2). Each fraction was dried *in vacuo* (with a 'Speed-Vac'; Savant Instruments), re-dissolved in a little water and chromatographed in system 1. Fluorescent material of R_F 0.29 (compound 29) was eluted with water, and further purified by paper electrophoresis at pH 2.0 and 6.5, followed by chromatography of the neutral fraction in systems 3, 4 and 8. System 4 resolved compound (29) from the corresponding *p*-coumaroyl derivative, compound (29′).

Compound (59) was eluted from the above system-1 chromatogram ($R_F = 0.59$) and purified as described for compound (29). System 4 resolved compound (59) from the *p*-coumaroyl derivative compound (59′).

Quantitative assay of ferulic acid and its esters

Ferulic acid and the feruloyl esters were estimated spectrophotometrically in aqueous solution by use of the constants given in Table 2. Unidentified feruloyl esters were assayed at pH 10 from A_{370}, using the value $\log \varepsilon (M^{-1} \cdot cm^{-1}) = 4.45$, as determined for compound (29).

Radioisotopic methods

For uniform ^{14}C-labelling, a suspension culture (10ml) was grown in a 50-ml flask in the normal medium, except that the sucrose was replaced by 1.0% D-[U-^{14}C]glucose (2.0×10^7Bq) as sole carbon source. After 11 days, approx. 70% of the ^{14}C had been removed from the medium; cell walls were then isolated as normal.

Radioactivity was determined on strips of chromatography paper by liquid-scintillation counting in 0.5% (w/v) 2,5-diphenyloxazole in toluene with a Searle Mk III counter at an efficiency of approx. 20%. For re-chromatography after scintillation counting, the strips were washed with toluene, dried and eluted with water. This was not possible with free ferulic acid, which is soluble in toluene and therefore had to be re-chromatographed from a replicate profile that was not scintillation-counted.

Results and discussion

Hydroxycinnamic acid composition

Cell walls isolated from rapidly growing spinach cultures showed NH_3-enhanced u.v.-fluorescence. The fluorescent material was removed from the wall during mild alkaline hydrolysis. Essentially all the fluorescent material in the alkaline digest (after acidification) partitioned into ethyl acetate, showing that it was no longer associated with polymers. T.l.c. in system 9 showed that the major phenolic component thus released was ferulic acid (about 13nmol/mg dry walls), which would account for the cell-wall fluorescence. Smaller amounts of *p*-coumaric acid and several unidentified phenols were also detected.

Treatment with phenol, oxalic acid and trypsin

Removal of non-covalently bound proteins with phenol/acetic acid/water (2:1:1) did not affect cell-wall fluorescence. About half the ferulate also remained in the cell walls during hydrolysis of the arabinofuranose residues with oxalic acid. Subsequent prolonged treatment of the residue with a high concentration of trypsin [conditions devised by Lamport (1977) for proteolysis of wall protein] failed to diminish the cell-wall fluorescence and did not release any u.v.-fluorescent material that was mobile on chromatography (system 1) or electrophoresis (pH 2.0). Intensely ninhydrin-positive spots of lysine, arginine and numerous peptides were detected, demonstrating the activity of the enzyme. The results show that the oxalic acid-resistant feruloyl residues were not attached to proteins linked either covalently or non-covalently in the cell wall.

Of the ferulate that was removed from the cell walls with oxalic acid, only about 8% was free ferulic acid, about 25% was polysaccharide-bound (insoluble in 85% ethanol) and the rest was in the form of chromatographically mobile compounds with the characteristics of feruloyl esters. The major one of these showed an R_F of 0.59 in system 1.

Digestion with Driselase

Partially purified Driselase was applied to spinach cell walls for 24h, and the entire reaction mixture was then chromatographed in system 1. Four spots were found with the fluorescence characteristics of feruloyl esters at R_F 0.00, 0.20 (faint), 0.29 and 0.59. These are referred to as compounds (00), (20), (29) and (59), and they accounted for 33, 7, 31 and 29% respectively of the total cell-wall ferulate (values obtained by assay of sodium ferulate released from strips of the chromatogram by NaOH). No free ferulic acid (R_F 0.88) was present. Incubation of Driselase in the absence of cell walls did not result in the formation of the fluorescent spots. Time courses showed that compound (59) was exhaustively released by much shorter Driselase treatments than those required for compound (29). Prolonged treatment of isolated compounds (29) and (59) with more Driselase revealed no interconversion.

Compound (29) appeared to be a single molecular species. Impure compound (29), eluted from a system-1 chromatogram, gave single spots with the fluorescence characteristics of a feruloyl ester on re-chromatography in systems 2, 3, 4 and 8 (Table 1). Only systems 5 and 15 gave two spots: these were interconvertible by exposure to u.v.-light, suggesting *cis/trans* isomerism of the feruloyl moiety. Compound (29) was neutral on electrophoresis at pH 2.0 and 6.5, demonstrating the absence of free amino and carboxy groups. It moved towards the anode in borate buffer (pH 9.4) and gave a single spot in this system too. Borate electrophoresis would probably have resolved pairs of feruloyl sugars differing in the nature of the sugar moiety or in the position of feruloylation. Compound (59) was also a single neutral species by all the above criteria.

The absorbance maxima of purified compounds (29) and (59) and of authentic feruloyl esters are given in Table 2. In 0.2M-$NaOH/Na_2CO_3$ buffer (pH 12.83) at 22°C, the spectra of compounds (29) and (59) rapidly decayed (Table 2) and came to resemble that of sodium ferulate. The fluorescent products of such alkaline treatment were shown by t.l.c. in system 9 to be ferulate and a trace of *p*-coumarate. Treatment of compounds (29) and

Table 1. *Chromatographic and electrophoretic properties of feruloyl esters released by Driselase*

Abbreviations used: p.c., paper chromatography; p.e., paper electrophoresis; c.c., column chromatography; Amm, 2M-NH_4OH; BuOH, butan-1-ol; Bz, benzene; EtOAc, ethyl acetate; EtOH, ethanol; HOAc, acetic acid (glacial); MeOH, methanol; Py, pyridine; n.d., not determined.

	Mobility* of compound	
	(29)	(59)
1. p.c., BuOH/HOAc/H_2O (15:3:5)†	0.29	0.59
2. p.c., EtOAc/Py/H_2O (8:2:1)	0.10	Streak
3. p.c., BuOH/Py/H_2O (4:3:4)	0.60	0.75
4. p.c., BuOH/EtOH/H_2O (20:5:11)	0.42	0.65
5. p.c., HOAc/H_2O (1:9)	0.61, 0.78	0.63, 0.71
6. p.c., BuOH/Amm (1:1, upper)	0.04	0.19
7. p.c., Bz/HOAc/H_2O (2:2:1, upper)	0.00	0.00
8. t.l.c., chloroform/MeOH/H_2O (65:25:4)	0.25	0.45
9. t.l.c., Bz/HOAc (9:1), under u.v.‡	0.00	0.00
10. p.e., pH 2.0, formic acid/HOAc/H_2O (1:4:45), 5kV, 25min	0.00	0.00
11. p.e., pH 3.5, HOAc/Py/H_2O (10:1:189), 5kV, 25min	0.00	0.00
12. p.e., pH 6.5, HOAc/Py/H_2O (3:100:897), 3kV, 45min	0.00	0.00
13. p.e., pH 9.4, 1.9% (w/v) $Na_2B_4O_7,10H_2O$, 2kV, 60min	0.50	0.60
14. p.e., pH 5.0, 2.0% (w/v) $Na_2MoO_4,2H_2O$, adjusted to pH 5.0 with H_2SO_4, 2.5kV, 60min	n.d.	n.d.
15. c.c., Sephadex LH-20 in 35% (v/v) MeOH	2.5, 3.5	4.6, 6.5

* Mobilities given for p.c. and t.l.c. are R_F. Mobilities for p.e. at pH 2.0 and 5.0 (molybdate) are $m_{\text{Bromophenol Blue}}$ (corrected for electroendo-osmosis with 2,3,4,6-tetramethylglucose). Mobilities at pH 3.5 and 6.5 are m_{picrate} (corrected), and those at 9.4 (borate) are m_{glucose} (corrected). Mobilities on Sephadex are $K_{\text{av.}}$ relative to Blue Dextran ($K_{\text{av.}} = 0.0$) and glucose ($K_{\text{av.}} = 1.0$).

† Freshly prepared.

‡ A long-wavelength u.v. lamp was shone on the t.l.c. plate through the side of the tank during the run so as to maintain phenolic acids as *cis/trans* equilibrium mixtures.

Table 2. *Physical properties of hydroxycinnamoyl esters*

Samples were prepared under normal laboratory lighting, and were therefore equilibrium mixtures of the *cis* and *trans* isomers. The spectra were prepared in aqueous solution containing dilute acetic acid (pH approx. 3), dilute NH_4OH (pH approx. 10) or 1.0M-NaOH (pH approx. 14). Abbreviations used: d, decomposition; n.d., not determined. The ε values were obtained for the esters by spectrophotometric assay of the ferulate or *p*-coumarate liberated by 1.0M-NaOH. The apparent pK of the phenolic hydroxy group was obtained by measurement of the A_{370} in a series of 0.2M-Tris/HCl, glycine/HCl, NH_3/HCl and NaOH/Na_2CO_3 buffers (pH 7–13). The half-lives ($t_{\frac{1}{2}}$) were calculated from semi-logarithmic plots of [(absorbance at initial $\lambda_{\text{max.}}$)−(absorbance of equimolar ferulic acid at that wavelength)] versus time.

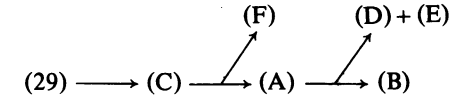
Parameter (and units)	Ferulic acid	Methyl ferulate	Ethyl ferulate	Hydroxyethyl ferulate	Compound (29)	Compound (59)	*p*-Coumaric acid	Compound (29′)	Compound (59′)
$\lambda_{\text{max.}}$ at pH 3 (nm)	321	320	320	320	322	323	308	310	311
log ε ($M^{-1}\cdot cm^{-1}$)	4.26	4.25	4.30	4.33	4.30	4.31	4.26	4.30	4.31
$\lambda_{\text{max.}}$ at pH 10 (nm)	343	366	366	369	371	373	331	358	361
log ε ($M^{-1}\cdot cm^{-1}$)	4.32	4.42	4.46	4.50	4.45	4.46	4.21	4.39	4.39
$\lambda_{\text{max.}}$ at pH 14 (nm)	343	d	d	d	d	d	331	d	d
log ε ($M^{-1}\cdot cm^{-1}$)	4.37	—	—	—	—	—	4.33	—	—
Apparent pK	9.2	8.6	8.6	8.6	8.6	nd	9.2	n.d.	n.d.
$t_{\frac{1}{2}}$ at pH 12.83 and 22°C (min)	$>10^3$	11	19	11	6	6	$>10^3$	n.d.	n.d.

(59) with 35% (w/v) NH_3 at 20°C for 5 days yielded ferulamide (R_F in system 6 = 0.36; in system 7 = 0.32) and a trace of *p*-coumaramide (R_F in system 6 = 0.49; in system 7 = 0.18). These observations together show that compounds (29) and (59) were low-M_r feruloyl esters. The rapidity with which they were hydrolysed at pH 12.83 and their rather high $\lambda_{\text{max.}}$ values at pH 10 suggest that they

were esters of polyhydric alcohols (cf. ethyl ferulate and hydroxyethyl ferulate in Table 2).

Identification of compound (29)

An attempt was made to discover what components besides ferulate were present in compound (29). Radioactive compound (29) was isolated from uniformly ^{14}C-labelled cell walls, and purified by chromatography and electrophoresis. The ^{14}C-labelled compound (29) was treated with 0.5 M-NaOH for various periods and then re-chromatographed in butan-1-ol/formic acid/water (15:3:5, by vol.). Brief treatments (3 min) with NaOH yielded three products (A, C and F) (Fig. 1*b*). During longer treatments (30 min), product (C) disappeared and only products A and F were present (Fig. 1*c*). Isolated product (C) treated with NaOH for 30 min also gave only products (A) and (F) (results not shown). Prolonged ($7\frac{1}{2}$h) treatment of either compound (29) or purified product (A) with NaOH caused decomposition to products (B), (D) and a trace of product (E) (Fig. 1*d*). These kinetic data indicate the reaction sequence:



Compound (F) was identified as ferulic acid by t.l.c. in system 9.

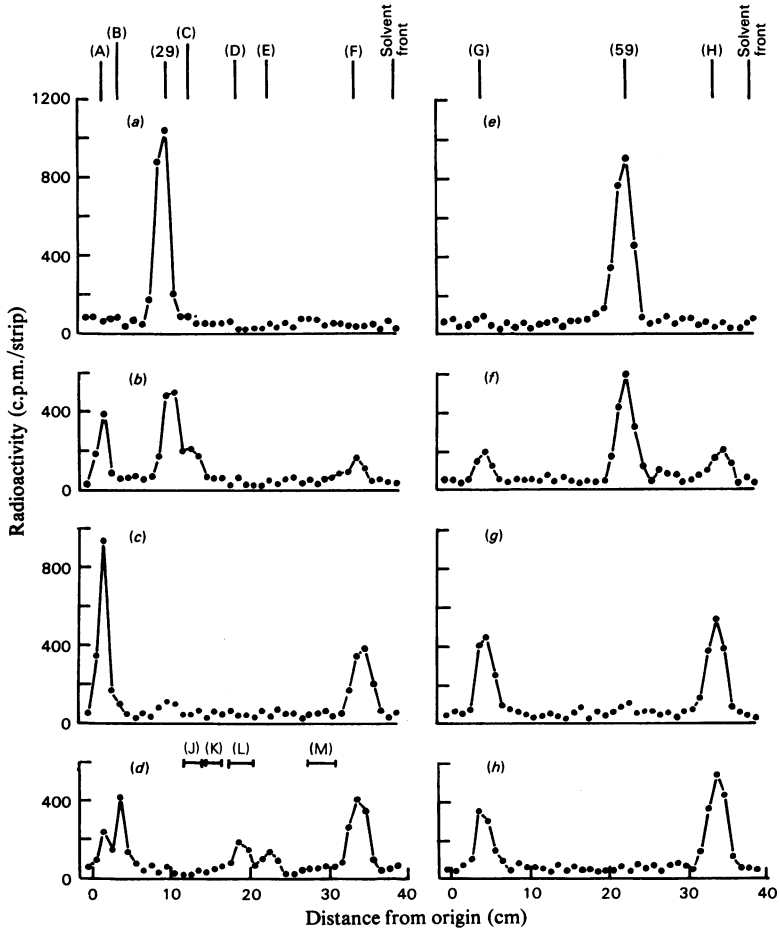


Fig. 1. *The action of alkali on the feruloyl esters (29) and (59)*

The uniformly ^{14}C-labelled esters (*a–d*, ^{14}C-labelled compound 29; *e–h*, ^{14}C-labelled compound 59) were treated with 0.5 M-NaOH at 20°C for (*a*, *e*) 0 min, (*b*, *f*) 3 min, (*c*, *g*) 30 min or (*d*, *h*) 440 min. The entire neutralized reaction products were chromatographed in butan-1-ol/formic acid/water (15:3:5, by vol.), and the profiles of radioactivity were obtained. Counts given are uncorrected for background, which was approx. 55 c.p.m. Radioactive compounds (A)–(H) are further characterized in the text. The markers (J), (K) and (L) are non-radioactive glucometasaccharinate, galactometasaccharinate and isosaccharinate respectively, synthesized by alkali treatment (440 min at 20°C in 0.5 M-NaOH) of laminarin, D-galactose and lactose respectively. Marker (M) is authentic DL-lactate.

Compound (B) was chromatographed in system 2 and electrophoresed in borate; in both systems it ran precisely with an internal marker of D-galactose (stained with aniline hydrogen phthalate after scintillation counting).

Compound (A) was converted completely and solely into compound (B) (galactose) on treatment with trifluoroacetic acid (0.5 M; 120°C for 1 h), Driselase or β-galactosidase, as monitored by chromatography in systems 1 and 2. α-Galactosidase was without effect. A sample of ^{14}C-labelled compound (B) obtained by acid hydrolysis of compound (A) was 96% converted into galactose 1-phosphate by D-galactokinase in the presence of ATP; this shows that compound (B) was D-galactose. Reduction of compound (29) or (A) with KBH_4, followed by acid hydrolysis, yielded two equal peaks with the mobilities of galactitol (dulcitol) and galactose (Fig. 2*a*). These results together show that compound (A) was a reducing disaccharide, β-D-galactosyl-D-galactose.

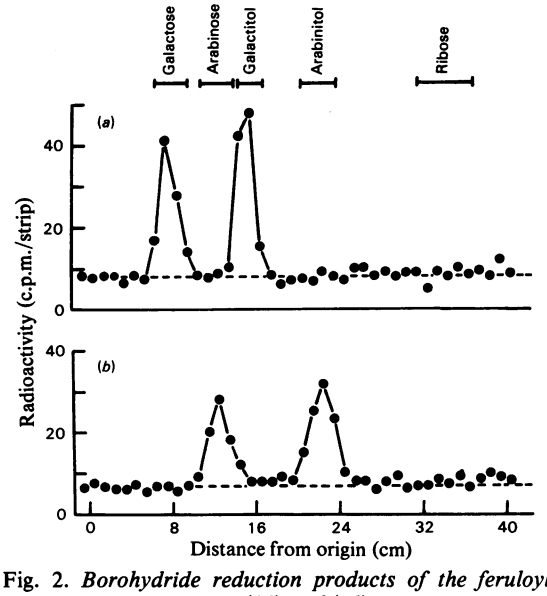


Fig. 2. *Borohydride reduction products of the feruloyl esters (29) and (59)*

Uniformly ^{14}C-labelled compounds (29) (*a*) and (59) (*b*) were treated with KBH_4, freed of cations on Zeo-Karb and hydrolysed in 0.5 M-trifluoroacetic acid (120°C for 1 h). The products were chromatographed for 16 h in butan-2-one/acetic acid/boric acid-saturated water (9:1:1, by vol.) and the profile of radioactivity was obtained. The positions are shown of non-radioactive sugars and alcohols, added as internal markers and stained after scintillation counting. Marker ferulic acid had a very high R_F and was lost off the end of the paper. The counts are uncorrected for background, which is shown as a broken line.

Mild acid hydrolysis (10 mM-trifluoroacetic acid at 120°C for 1½h) did not affect the R_F of compound (A) in solvent 1, but stronger hydrolysis (100 mM-trifluoroacetic acid at 120°C for 1½h) gave over 95% conversion into galactose. In the same experiment, lactose behaved like compound (A), whereas a mixture of hydroxyproline [^3H]arabinofuranosides (isolated from spinach cell-wall glycoprotein by alkaline hydrolysis; S. C. Fry, unpublished work) was 70% hydrolysed to [^3H]arabinose by the 10 mM acid. This suggests that the D-galactose residue of compound (A) was in the relatively acid-resistant pyranosyl form.

Electrophoresis of reduced disaccharides (glycosyl alditols) in the presence of molybdate has been proposed as a means of determining sugar linkages (Weigel, 1963). Mobility in the case of galactosyl galactitols is bestowed solely by the galactitol moiety and may be unaffected, halved or nullified by substitution (or removal) of hydroxy groups, depending on their position. Since galactitol is symmetrical, the results will be ambiguous. However, since reduced compound (A) had a mobility in molybdate approximately half those of 1-deoxy-L-galactitol ('D-fucitol'), 1-*O*-methyl-L-galactitol ('6-*O*-methyl-D-galactitol') and 2-deoxy-D-*lyxo*-hexitol ('2-deoxy-D-galactitol'), and equal to that of maltitol (Table 3), it follows that the galactitol moiety of reduced compound (A) was 3-*O*- (or '4-*O*-') substituted. Thus, compound (A) was 3- or 4-*O*-galactosyl galactose.

Electrophoresis of compound (A) (unmodified) in borate gave a single peak with $m_{Glc} = 0.45$ (Table 3). By comparison with the mobilities of marker sugars run simultaneously (Table 3) and with the mobilities of 3-*O*-β-D-Gal*p*-D-Gal ($m_{Glc} = 0.69$), 4-*O*-β-D-Gal*p*-D-Gal ($m_{Glc} = 0.48$), 6-*O*-β-D-Gal*p*-D-Gal ($m_{Glc} = 0.84$) and D-galactose ($m_{Glc} = 0.95$) reported by Bouveng & Meier (1959), this strongly suggests a 1→4 linked disaccharide.

Support for this conclusion came from the identification of compound (D). The alkali-breakdown products of disaccharides are characteristic of the linkage (Whistler & BeMiller, 1958). The non-reducing residue may be released as the monosaccharide, whereas the reducing moiety is degraded to a metasaccharinate, an isosaccharinate or lactate, from 1→3, 1→4 and 1→6 linked disaccharides respectively (1→2 linked disaccharides are stable to alkali). The chromatogram shown in Fig. 1(*d*) resolved isosaccharinate, glucometasaccharinate, galactometasaccharinate and lactate; and compound (D) ran exactly with authentic isosaccharinate.

From all these results together, it can be deduced that compound (A) is 4-*O*-β-D-galactopyranosyl-D-galactose.

The feruloylated disaccharide (29), unlike the

Table 3. *Electrophoretic mobility of sugars and sugar alcohols*

Compound*	m_{Glc} in borate	$m_{Bromophenol\ Blue}$ in molybdate
D-Galactose	0.93	0.18
L-Arabinose	0.95	0.20
Cellobiose	0.23	
Maltose	0.29	
Lactose	0.36	
Melibiose	0.75	
Compound (A)	0.45	
Compound (G)	0.58	
2,3,4,6-Tetramethyl-D-glucose	0.00	0.00
D-Glucitol		1.73
Galactitol		1.80
L-Arabinitol		1.90
6-Deoxy-D-galactitol (1-deoxy-L-galactitol)		1.52
6-*O*-Methyl-D-galactitol (1-*O*-methyl-L-galactitol)		1.68
2-Deoxy-D-galactitol		1.42
Cellobiitol		0.71
Maltitol		0.93
Lactitol		0.72
Melibiitol		1.43
Reduced compound (A)		0.95
Reduced compound (G)		0.60

* The marker sugars were obtained commercially. The alcohols were synthesized from the corresponding sugars by reduction with KBH_4.

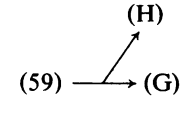
de-feruloylated derivative (A), was completely resistant to Driselase and β-galactosidase. Glycosidases are generally rather non-specific for the aglycone, and Driselase readily hydrolysed cinnamoyl β-D-galactopyranoside (S. C. Fry, unpublished work). This suggests that the feruloyl residue of compound (29) was on the (non-reducing) β-D-galactopyranosyl unit. In an investigation of the linkage of this ferulate, ^{14}C-labelled compound (29) was oxidized with $NaIO_4$ under conditions that would retain the ester linkage intact and also minimize overoxidation (Neumüller & Vasseur, 1953) and then reduced with KBH_4, acidified on Zeo-Karb, washed with ethyl acetate to remove some ferulate-oxidation products, hydrolysed (1 M-trifluoroacetic acid at 120°C for 1 h) and chromatographed in system 1. The principal non-volatile radioactive products co-chromatographed with authentic threitol and glycerol (Fig. 3*b*). These identities were confirmed by re-chromatography in system 2. Similar results (Fig. 3*a*) were obtained if the ferulate was first split off the disaccharide of compound (29) (*in situ* in 0.5 M-NaOH at 20°C for 30 min, before the addition of formic acid to pH 3.5); in this case, the [^{14}C]-glycerol would arise from C-4, C-5 and C-6 of the non-reducing galactose unit, and the [^{14}C]threitol from C-3 to C-6 of the reducing unit (Panayotatos & Villemez, 1973). If the non-reducing residue in compound (29) were *O*-feruloylated at C-2, C-3, C-4 or C-6, it would yield glyceraldehyde + glycerol, galactose, threitol or glycerol respectively. However, non-radioactive carrier glyceraldehyde was completely degraded to $AgNO_3$/NaOH-negative products during the trifluoroacetic acid treatment. The results (Fig. 3*b*) therefore suggest *O*-feruloylation at C-2 or C-6.

(The unidentified peak running 5 cm from the origin of Figs. 3*a*–3*d* only arose during prolonged periodate-oxidation, and, unlike the glycerol and threitol, was not present in 16-h oxidations. It was probably derived from ferulate.)

Since product (C) (see Fig. 1*b*) was composed of ferulate and disaccharide (A), it must have been an isomer of compound (29) with the feruloyl residue on a different hydroxy group. 6-*O*-Feruloyl sugars have markedly lower R_F values than the corresponding 1-, 2-, 3- or 4-*O*-feruloyl sugars, which are not well resolved from each other (Birkofer *et al.*, 1966). It is therefore likely that compound (29) was 6′-*O*-feruloyl-disaccharide (A), and that the faster migrating product (C) was a mixture of 2′-, 3′-, and 4′-*O*-feruloyl-disaccharide (A), formed from compound (29) by alkali-catalysed acyl-migration. Compound (29) is therefore proposed to be 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose.

Identification of compound (59)

^{14}C-Labelled compound (59) was isolated from uniformly ^{14}C-labelled cell walls and purified by chromatography and electrophoresis. The kinetics of alkaline hydrolysis (Figs. 1*e*–1*h*) were simpler than for compound (29), suggesting the reaction:



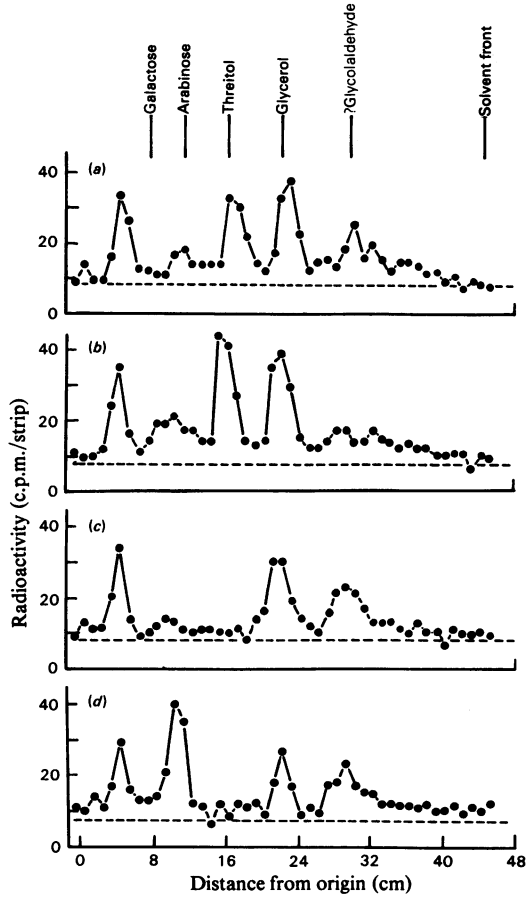


Fig. 3. *Periodate oxidation products of the feruloyl esters (29) and (59), and of their disaccharide moieties (A) and (G)* (*a*) Compound (29) oxidized after NaOH treatment; (*b*) compound (29) oxidized without NaOH treatment; (*c*) compound (59) oxidized after NaOH treatment; (*d*) compound (59) oxidized without NaOH treatment. For (*a*) and (*c*), the radioactive ester was first treated with 0.5 M-NaOH at 20°C for 30 min to split off the feruloyl groups (cf. Figs. 1*c* and 1*g*), and then sufficient dilute formic acid was added to convert the medium into 0.25 M-sodium formate buffer, pH 3.5 (final volume approx. 0.1 ml). For (*b*) and (*d*), the intact esters were dissolved directly in 0.1 ml 0.25 M-sodium formate buffer, pH 3.5. After cooling to 4°C, $NaIO_4$ was added to 0.05 M, and oxidation was conducted for 6 days at 4°C. After destruction of excess $NaIO_4$ with 0.02 ml of ethane-1,2-diol, the products were reduced with KBH_4, freed of cations on Zeo-Karb, washed with ethyl acetate, acid-hydrolysed and chromatographed in system 1. These radioactive profiles are uncorrected for background, which is shown as a broken line.

Compound (H) was identified as ferulic acid by t.l.c. in system 9.

Compound (G) ran in system 1 with authentic pentoses (arabinose and xylose; not resolved). In system 2 it ran slightly faster than arabinose but slower than xylose. Acid hydrolysis (100 mM-trifluoroacetic acid at 120°C for 1½h) or Driselase treatment converted it solely and completely into arabinose (identified by chromatography in system 2 and electrophoresis in borate). This arabinose was 90% phosphorylated by L-arabinokinase in the presence of ATP, showing it to be L-arabinose. Reduction of compound (59) or (G) with KBH_4 followed by acid hydrolysis yielded two equal peaks corresponding to arabinose and arabinitol (Fig. 2*b*). These data show that compound (G) was a reducing disaccharide of L-arabinose.

Mild acid hydrolysis (10 mM-trifluoroacetic acid at 120°C for 1½h) had no effect on compound (G), suggesting an arabinopyranosyl unit. However, mild oxalic acid-hydrolysis of cell walls liberated a feruloyl ester that co-chromatographed with com-

pound (59) (see above). Therefore the reducing arabinose unit of compound (59) was probably furanosidically linked to a wall polymer. Thus the glycosidic linkage in compound (59) could not have been 1→4.

Reduced compound (G) (L-arabinopyranosyl-L-arabinitol) had a moderate electrophoretic mobility in molybdate (Table 3). Methyl arabinopyranosides are immobile in molybdate, but arabinitol has a high mobility (Weigel, 1963). Therefore, as with reduced compound (A), the mobility of reduced compound (G) will be bestowed solely by the alditol moiety. Removal (and therefore presumably also substitution) of the C-2 hydroxy group of arabinitol completely prevents molybdate binding, as shown by the immobility of 2-deoxy-*erythro*-pentitol ('2-deoxyarabinitol') (Weigel, 1963). Removal of the C-5 hydroxy group of arabinitol has no effect on molybdate binding, as shown by the high mobility of 1-deoxylyxitol ('5-deoxyarabinitol') (Weigel, 1963). The effect of removal or substitution of the hydroxy group at C-3 of arabinitol has not been studied; however, the four hydroxy groups of 3-*O*-substituted L-arabinitol can adopt positions identical with those at C-1, C-2, C-4 and C-5 of 3-*O*-methyl-L-gulitol, which has a moderate mobility in molybdate (Weigel, 1963). The moderate mobility of reduced compound (G) is therefore likely to be due to *O*-substitution of the arabinitol moiety at C-3. Thus the glycosidic linkage in compound (G) is 1→3.

3-*O*-β-L-Arabinopyranosyl-L-arabinose has been isolated from gum, and has a relatively low R_F in several chromatographic systems (Andrews & Jones, 1954). Compound (G), in contrast, is remarkable in being a disaccharide with an R_F (in system 2) higher than that of its constituent monosaccharide. This characteristic is also exhibited by 3-*O*-β-D-xylopyranosyl-D-xylose (Howard, 1957), and indicates that compound (G) is 3-*O*-α-L-arabinopyranosyl-L-arabinose, since the β-D linkage is identical with the α-L.

The electrophoretic mobility of compound (G) (unmodified) in borate ($m_{Glc} = 0.58$; Table 3) is also compatible with a 1→3 linkage (Weigel, 1963).

Periodate oxidation of compound (G) gave negligible amounts of [^{14}C]erythritol (which co-chromatographs in system 1 with threitol), suggesting that extensive overoxidation was occurring despite the mild conditions (Fig. 3*c*). Disaccharides with 1→3 linkages are known to be extremely susceptible to overoxidation (Neumüller & Vasseur, 1953). The only recognizable product was [^{14}C]-glycerol, which would arise from C-2, C-3 and C-4 of the reducing arabinose unit by overoxidation. However, periodate-oxidation of compound (59) with the ferulate group still attached gave (in addition to [^{14}C]glycerol) a prominent peak of ^{14}C in the pentose zone (Fig. 3*d*). This was identified in system 2 as arabinose, showing that the non-reducing arabinose unit of compound (59) was *O*-feruloylated at C-3.

The structure of compound (59) is concluded to be 3-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose.

p-Coumaroyl derivatives

Chromatography of crude Driselase-digests in system 1 yielded fluorescent spots, the leading edge of which fluoresced blue in NH_3 vapour (cf. blue–green for feruloyl esters). Two-dimensional chromatography (systems 1 and 4) resulted in more complete resolution of the blue and blue–green spots. In the case of both compounds (29) and (59), alkaline hydrolysis of the blue–green spot released ferulate, whereas the blue spot gave *p*-coumarate (identified in system 9). The faster-running blue-fluorescing compounds (29′ and 59′; for spectral properties see Table 2) are concluded to be *p*-coumaroyl versions of the major (feruloyl) esters.

The possible nature of the feruloylated polymer

At least 60% of the ferulate of spinach cell walls occurs in two highly specific linkages, characterized here as 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and 3-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose. The stability of these disaccharides to Driselase was probably due to the feruloyl groups preventing glycosidases from recognizing the (non-reducing) glycosyl residue.

D-Galactose and L-arabinose are characteristic of three major cell wall polymers: (1) pectins, (2) covalently-bound glycoprotein ('extensin'), and (3) salt-soluble arabinogalactan-proteins. Of these, only the pectins contain the sequence Galpβ1→4Gal. In contrast, the covalently bound protein has single galactose residues, which are α-linked to serine; and the arabinogalactan-proteins contain only 1→3 and 1→6 sequences of galactose (O'Neill & Selvendran, 1980; Aspinall, 1980). These facts, together with the insolubility of the feruloylated polymer in phenol and in trypsin, suggest that the ferulate was attached to pectin rather than protein.

The present work does not establish the number of consecutive galactose residues in the feruloyl polymer. Firm evidence was obtained for two. However, the results are compatible with a feruloyl β-galactan, as previously conjectured in potato cell walls (Friend, 1976).

The arabinose residues of pectins (and other wall polymers) are usually in the furanose form, but a few arabinopyranose residues are also present (Northcote, 1972; Darvill *et al.*, 1978). My results show that a large proportion of the cell wall's ferulate is linked to such arabinopyranose residues. This implies that the addition of phenols to cell-wall polymers is not a random process. Feruloyltrans-

ferases must therefore exist with a very high specificity for the feruloyl acceptor, although possibly also functioning in *p*-coumaroyl transfer. Feruloyl-coenzyme A and *p*-coumaroyl-coenzyme A are likely to act as donors.

Functional aspects

Peroxidase has been shown *in vitro* to catalyse the covalent cross-linking of an artificially feruloylated polysaccharide (guaran) through diferuloyl (biphenyl) bridges, and of *p*-coumaroylated guaran through unidentified bridges (Geissmann & Neukom, 1971). Diferuloyl bridges also occur naturally in cell walls (Markwalder & Neukom, 1976; Hartley & Jones, 1976; Harris & Hartley, 1980). In addition, it has been suggested that ferulate could oxidatively couple with tyrosine to form a hybrid biphenyl, which would link polysaccharides to proteins (Neukom & Markwalder, 1978). A further type of oxidative coupling has been demonstrated between carbohydrates and phenols (probably as quinone methides) to form sugar-phenol ethers (Freudenberg & Grion, 1959; Harkin, 1967; see also Whitmore, 1976). Another plausible mode of cross-linking involves oxidation of the phenol (e.g. caffeate) to a hydroxyquinone, followed by chelation of Ca^{2+} by two adjacent hydroxyquinones (Painter & Neukom, 1968). Any of these coupling reactions would result in a binding together of the phenol-bearing polysaccharides within the cell wall, perhaps thereby restricting cell expansion.

A similar hypothesis was recently advanced for the cross-linking of wall glycoprotein through isodityrosine, an oxidatively coupled dimer of tyrosine (Fry, 1982).

Phenolic coupling reactions are feasible in the cell wall, since there are abundant peroxidase isoenzymes, and systems exist for the generation of cell-wall H_2O_2 (Gross *et al.*, 1977). Numerous examples have been reported of a negative correlation between rates of cell expansion and peroxidase levels (van Overbeek, 1935; Kamerbeek, 1956; McCune & Galston, 1959; Palmieri *et al.*, 1978; Fry, 1979, 1980). It is possible that this reflects a causal relationship, the peroxidase restricting growth by catalysing the oxidative coupling of pectin-bound phenols. The growth promoter gibberellic acid, for example, by inhibiting the secretion of peroxidase into the cell wall (Fry, 1979, 1980), may prevent the occurrence of phenolic coupling and thereby favour cell expansion. In this connection it is interesting that gibberellic acid-treated cells appear to shed pectins (Fry, 1980), presumably owing to reduced cross-linking within the cell wall.

Evidence that phenolic compounds do contribute to the binding of polymers in the cell wall comes from degradative studies. Acidified sodium chlorite, originally developed as a de-lignifying agent by virtue of its ability to cleave aromatic polymers, is able to solubilize some otherwise water-insoluble cell-wall protein and polysaccharides (O'Neill & Selvendran, 1980; Fry, 1982). Similarly, pretreatment of cell walls with methanolic sodium methoxide will render some hemicelluloses water-soluble, possibly by splitting phenolic esters (Morrison, 1977); and 0.1 M-Na_2CO_3 will solubilize some galacturonan, possibly by hydrolysis of phenolic esters (Jarvis *et al.*, 1981).

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