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Novel *O*-D-Galacturonoyl Esters in the Pectic Polysaccharides of Suspension-Cultured Plant Cells[1]

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Driselase digestion of *uronate*-6-^{14}C-labeled primary walls of cultured spinach (*Spinacia oleracea* L.) cells yielded about 18 novel uronate-containing compounds, most of which could be hydrolyzed by cold dilute alkali to yield oligo-[^{14}C]galacturonides. One typical Driselase digestion product (compound 17) yielded α-(1→4)-D-[^{14}C]galacturonotriose ($GalA_3$) upon very mild treatment with alkali (50% yield of $GalA_3$ in 7.2 min at pH 11 and 25°C). One of the three galacturonate residues in compound 17 was reducible to a galactose residue with sodium borohydride, indicating that that GalA residue was esterified, via its —COOH group, to a putative alcohol. Compound 17 had a higher mobility than $GalA_3$ on paper chromatography, indicating that the putative alcohol was relatively nonpolar. The putative alcohol could not have been methanol because Driselase readily hydrolyzed mono-, di-, and trimethyl esters of $GalA_3$ to yield free galacturonic acid. Another Driselase digestion product (compound 12) was a derivative of $GalA_3$ that apparently possessed two nonpolar esterified substituents: one about as labile as in compound 17, and the other approximately 10 times more stable. Compounds 12 and 17 could not be labeled by in vivo feeding of [U-^{14}C]cinnamate, suggesting that they were not phenolic conjugates. Similar but chromatographically distinguishable *uronate*-^{14}C-labeled esters were obtained by Driselase digestion of walls of cultured carrot (*Daucus carota* L.), Paul's Scarlet rose (*Rosa* sp.), and tall fescue (*Festuca arundinacea* Schreber) cells. In spinach, the novel compounds constituted about 5% of the total galacturonate residues of the cell wall. The observations suggest that pectic polysaccharides are linked, via *O*-D-galacturonoyl ester bonds, to relatively hydrophobic constituents of the primary cell wall. Their possible role in wall architecture is discussed.

Pectic polysaccharides (pectins) usually make up about one-third of the dry weight of the primary cell walls of dicots; smaller amounts are present in the Gramineae. Pectins are rich in (1→4)-linked α-D-galacturonate residues and also contain α-L-arabinose, β-D-galactose, α-L-rhamnose, and numerous minor sugar residues (O'Neill et al., 1990). Several noncarbohydrate substituents are linked to pectins via ester bonds involving both carboxy and hydroxy groups of the polysaccharide. For example, many of the galacturonate residues are carboxy-esterified to methanol (Deuel and Stutz, 1958), a variable proportion of the galacturonate residues are *O*-acetylated (Komalavilas and Mort, 1989), and (especially in the Chenopodiaceae) a few of the Ara and Gal residues are *O*-feruloylated and *O*-*p*-coumaroylated (Fry, 1982a).

Several observations are consistent with the hypothesis that some pectins are cross-linked within the wall matrix via ester bonds (Fry, 1986). For example, a proportion of the CDTA- and endopolygalacturonase-inextractable pectin can be extracted from the cell wall in cold aqueous Na_2CO_3 (e.g. 0.5 M; Selvendran and O'Neill, 1987; Ishii et al., 1989; Renard et al., 1990), a reagent that would break many types of ester bond. Also, suspension-cultured tomato cells that had been adapted to growth in the presence of 2,6-dichlorobenzonitrile possessed abnormal cell walls almost lacking cellulose and xyloglucan but composed largely of pectins, which became water soluble after treatment with alkali (Shedletzky et al., 1990). However, this type of evidence for ester cross-links is indirect and cannot show whether the putative ester bonds involve carboxy groups or hydroxy groups of the pectins.

We are investigating the possible natural occurrence of *O*-D-galacturonoyl ester cross-links, i.e. esters that involve the carboxy group of a galacturonate residue and the hydroxy group of some other wall component. Evidence in favor of the existence of *O*-D-galacturonoyl esters, other than the methyl ester, is the discovery that alkaline hydrolysis of maize coleoptile cell walls releases less methanol (mol/mol) than the total apparent content of galacturonoyl esters (Kim and Carpita, 1992). The latter were estimated as galacturonate residues that were reducible to Gal residues by neutral $NaBH_4$, a reagent that has been shown to reduce carboxy-esterified but not nonesterified galacturonate residues in pectins (Maness et al., 1990).

To enable this study, we had previously synthesized and characterized several model *O*-D-galacturonoyl and *O*-D-polygalacturonoyl esters. One such model compound was 6-*O*-polygalacturonoyl-D-[1-^{3}H]Glc—that is, an α-(1→4)-D-galacturonan in which a small proportion of the galacturonate residues are esterified to O-6 of [^{3}H]Glc (Brown and Fry, 1993). At 25°C, the ester bonds in this model compound were relatively stable to 1 M TFA and to PAW (an excellent protein extractant [Selvendran and O'Neill, 1987]), but were very alkali labile, exhibiting a half-life of about 4 min at

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Abbreviations: BPW, butan-1-ol:pyridine:water (4:3:4, v/v); CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate; EAW, ethyl acetate:acetic acid:water (10:5:6, v/v); EPW, ethyl acetate:pyridine:water (8:2:1, v/v); EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; $GalA_2$, α-D-galacturonopyranosyl-(1→4)-D-galacturonic acid; $GalA_3$, α-D-galacturonopyranosyl-(1→4)-α-D-galacturonopyranosyl-(1→4)-D-galacturonic acid; PAW, phenol:acetic acid:water (2:1:1, w/v/v); PCV, packed cell volume.

pH 11. Driselase (a commercial mixture of fungal endo- and exo-polysaccharidases) did not hydrolyze the ester bond in 6-*O*-polygalacturonoyl-D-[^{3}H]Glc, but did hydrolyze the glycosidic bonds of the galacturonan chain except for two glycosidic bonds in the immediate vicinity of the [^{3}H]Glc group; thus, a major product of Driselase digestion was the esterified trisaccharide

D-[^{3}H]Glc
6
|
6
α-D-GalA-(1→4)-α-D-GalA-(1→4)-D-GalA

(Brown and Fry, 1993). These observations suggested that Driselase digestion of plant cell walls could generate small pectic oligosaccharides still bearing the hypothesized (non-methyl) ester-linked substituent(s). Such an approach could thus allow us to characterize novel, naturally occurring *O*-D-galacturonoyl esters in cell walls.

To facilitate the search for quantitatively minor *O*-D-galacturonoyl derivatives in vivo, we preincubated cells with D-[6-^{14}C]glucuronic acid, from which the radioactivity is incorporated into uronate residues and not neutral hexose or pentose residues (Fry, 1988).

MATERIALS AND METHODS

Cell Cultures

Suspension-cultured cells of spinach (*Spinacia oleracea* L.) were maintained as described (Fry, 1982a). Other suspension-cultured cells were of Paul's Scarlet rose (*Rosa* sp.), carrot (*Daucus carota*), and tall fescue grass (*Festuca arundinacia*). Each culture was grown with D-Glc as sole carbon source; where necessary, the culture was preadapted to growth in the absence of exogenous inositol.

Radiochemicals

D-[6-^{14}C]Glucuronic acid (55 Ci/mol) was synthesized by the method of Sowden (1952), which involves a cyanohydrin condensation between Na^{14}CN and the 5-carbon aldehyde formed by the periodate oxidation of commercially available 1,2-*O*-isopropylidene-α-D-glucofuranoside. The material appeared pure by HPLC on Bio-Rad Aminex HPX-87H in 5 mM H_2SO_4, and by TLC on silica gel in butan-1-ol:acetic acid:water (3:1:1, v/v); however, paper chromatography in BAW and paper electrophoresis at pH 3.5 revealed the presence of 15.1% [^{14}C]iduronic acid. The product was therefore repurified by preparative paper chromatography in BAW to remove the [^{14}C]iduronic acid. The purified D-[6-^{14}C]glucuronic acid was eluted from the paper with water and stored as an aqueous solution at −40°C.

Trans-[U-^{14}C]Cinnamic acid (500 Ci/mol; generous gift of Dr. G. Wallace, University of Edinburgh) was synthesized from L-[U-^{14}C]Phe (Amersham International) by incubation with Phe ammonia-lyase and purified by TLC.

In Vivo Labeling

At 4 to 5 d after subculture, i.e. during rapid growth, a 2-mL portion of each culture (PCV approximately 10%) was transferred aseptically into a 60-mL polycarbonate beaker (Sterilin) containing 100 μCi of D-[6-^{14}C]glucuronic acid or 10 μCi of *trans*-[U-^{14}C]cinnamic acid and incubated aseptically for a further 48 h under the standard conditions used for each culture. The cells were then filtered and washed briefly with water, and the filtrate was assayed for radioactivity.

Driselase Digestion

The radioactive cells were stirred in 2 × 10 mL of PAW at 25°C for a total of 30 h, and the insoluble (cell wall-rich) material was collected by centrifugation, washed repeatedly in buffer A (pyridine:acetic acid:H_2O, 1:1:98, pH approximately 4.5) to remove all detectable phenol, and then shaken gently in 200 μL of 5% Driselase (partially purified as described by Fry [1982a]) in buffer A containing 0.5% 1,1,1-trichloro-2-methylpropan-2-ol at 25°C for 24 h. The digestion products were stored at −20°C, without removal of the enzymes, and then analyzed by paper chromatography in EAW and/or BPW and by paper electrophoresis at pH 3.5.

Paper Chromatography

Chromatography was performed by the descending method on 57-cm lengths of Whatman 3MM paper in the solvents EAW for 24 h, BPW for 24 to 48 h, or EPW for 24 h. After autoradiography, the chromatogram was sometimes stained for reducing sugars by aniline hydrogen-phthalate (Partridge, 1949; modified by Fry, 1988).

High-Voltage Electrophoresis

Electrophoresis (flat bed method) was usually carried out on Whatman 3MM paper in acetic acid:pyridine:water (10:1:189, pH 3.5) at 2 kV for 3 h. To separate aldoses from alditols, paper electrophoresis in molybdate buffer (2.0% $Na_2MoO_4 \cdot 10H_2O$, adjusted to pH 5.0 with dilute H_2SO_4) was used at 2 kV for 3 h (Weigel, 1963). In both cases, picric acid was used as the mobile marker; the immobile marker (used to correct for electro-endo-osmosis) was Glc. Electrophoretic mobilities of compounds are reported as $m_{picrate}$ values, defined as (distance from Glc to compound)/(distance from Glc to picrate).

Partial Characterization of Driselase Digestion Products

Selected radioactive zones were eluted from unstained chromatograms by the method of Eshdat and Mirelman (1972), with buffer A as eluent, and the eluted solutes were analyzed. Acid hydrolysis was performed in 2 M TFA at 120°C in a sealed tube for 1 h, and the products were analyzed by paper electrophoresis at pH 3.5.

One portion of each eluate was dried, incubated in 100 μL of 50 mM NaOH for 3 min, and then slightly acidified with 10 μL of 1 M acetic acid; an identical dried portion was incubated in premixed 50 mM NaOH (100 μL):1 M acetic acid (10 μL) to check for interference of the Na^+ ion in the

subsequent analysis. Both samples were then reanalyzed by paper chromatography in EAW or paper electrophoresis at pH 3.5.

The kinetics of alkaline hydrolysis were investigated by incubation of compounds in 100 mM carbonate buffer (Na^+, pH 11) at 25°C, followed, at timed intervals, by slight acidification with acetic acid and analysis of the products by paper chromatography in EAW.

Reduction of compound 17 was based on the method of Kim and Carpita (1992), but with imidazole acetate instead of imidazole chloride. Dried compound 17 was dissolved in 250 μL of 1 M imidazole (acetate) buffer, pH 7.0, at 0°C, to which was added three 5-mg portions of $NaBH_4$ at 5-min intervals, followed by a further 60 min of incubation; the tubes were kept on ice throughout the reaction. (As a control, a sample of compound 17 was alkali hydrolyzed, neutralized, and then treated with $NaBH_4$ as above.) The solution was then treated with excess acetic acid to destroy the remaining borohydride, and the solution was decationized on a 2.5-mL bed volume column of Dowex 50 (H^+ form). The eluate was dried to remove acetic acid and then redried six times from methanol:acetic acid (9:1) to remove borate as its volatile methyl ester. The remaining nonvolatile material was then hydrolyzed in TFA as above and analyzed by paper chromatography in BPW and EPW and by paper electrophoresis in molybdate buffer.

Investigation of Methylesterase Activity of Driselase

α-(1→4)-D-Galacturonotriose ($GalA_3$) was isolated from a pectinase digest of commercial polygalacturonic acid by gel-permeation chromatography on Bio-Gel P-2. It was partially methylesterified by incubation in 50% methanol containing 5% (v/v) pyridine and 5% (w/v) EDC at 25°C for 1 h. Unaltered $GalA_3$ and its mono-, di-, and trimethylesterified derivatives (chromatographic mobility relative to that of GalA in EAW = 0.33, 0.55, 0.75, and 0.85, respectively) were separated by preparative paper chromatography in EAW and eluted from the paper with buffer A. Their stability in Driselase was tested under the conditions described above; the products were analyzed by paper chromatography in EAW and stained with aniline hydrogen-phthalate.

Assay of Radioactivity

Radioactivity on paper strips was assayed at about 60% efficiency by scintillation counting 2 mL of 0.5% PPO:0.05% POPOP in toluene. Radioactive material dissolved in PAW was assayed after drying in vacuo and redissolving in water. Radioactivity in aqueous solutions was assayed at about 75% efficiency after the addition of 10 volumes of 0.33% PPO:0.033% POPOP in toluene:Triton X-100 (2:1, v/v).

RESULTS

Occurrence of *O*-D-Galacturonoyl Esters in Vivo

Cultured cells of the four species tested readily took up the D-[6-^{14}C]glucuronic acid and incorporated radioactivity into the PAW-insoluble (cell wall-rich) fraction. For example, after 48 h of incubation of the spinach culture, only 8% of the ^{14}C remained in the culture medium, and 20% was found in the PAW-insoluble residue. The rest of the ^{14}C was associated with PAW-soluble compounds or was lost as $^{14}CO_2$.

Driselase digestion of the PAW-insoluble fraction from all four species yielded a large amount of free [^{14}C]galacturonic acid and several ^{14}C-labeled digestion products with lower R_F values. A representative autoradiogram of the spinach products is shown in Figure 1. A similar range of ^{14}C-labeled products, some with different R_F values, was observed for the other three species. The formation of the products was not dependent on the PAW treatment, since we obtained identical products from 80% ethanol-insoluble residues as from PAW-insoluble residues (data not shown).

Although [^{14}C]galacturonic acid was a major product, [^{14}C]$GalA_2$ and [^{14}C]$GalA_3$ were almost undetectable in the Driselase digests, indicating that Driselase contains high exogalacturonosidase activity. In the spinach digest, about 5% of the total ^{14}C was present in compounds that migrated slower than galacturonic acid but with $R_F > 0.00$. Typically, about 13% of the ^{14}C was detected at R_F 0.00. The EAW solvent used is capable of shifting oligogalacturonides of degree of polymerization ≤8 away from the origin of the chromatogram within 24 h. Visual inspection showed essentially complete solubilization of the PAW-insoluble material by Driselase. Thus, the ^{14}C-labeled material at R_F 0.00 was water-soluble poly- and/or large oligosaccharides; this could include fragments of [^{14}C]rhamnogalacturonan-II, which is not completely hydrolyzed by Driselase (S. Aldington, personal communication).

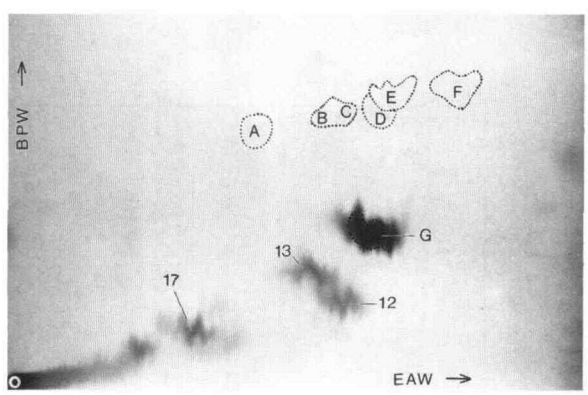

Figure 1. Driselase digestion products of the walls of cultured spinach cells previously ^{14}C-labeled in their uronic acid residues. The photograph shows an autoradiogram of a two-dimensional paper chromatogram; EAW → and BPW → indicate the solvents for the first and second dimensions, respectively (24 h in each dimension); o, origin. The principal radioactive spots investigated are marked (12, 13, 17, and G); some of the minor ^{14}C-labeled compounds were clearly visible only after a longer exposure of the x-ray film. The dotted outlines show the positions of reducing sugars, stained by aniline hydrogen-phthalate on the same chromatogram after autoradiography (A, cellobiose; B, xylosyl-α-(1→6)-glucose; C, xylosyl-β-(1→4)-xylose; D, Gal; E, Glc; F, Ara; G, galacturonic acid). B and C were only partially resolved from each other but were distinguished by color of staining (brown and red, respectively). Galacturonic acid is the only spot that was both radioactive and present in stainable quantities.

A one-dimensional EAW chromatogram of the spinach products was cut into strips, each of which was treated with 50 mM NaOH, reacidified with a small excess of acetic acid, and rechromatographed; the ^{14}C-labeled material in each zone exhibited a substantial decrease in R_F (data not shown), suggesting that alkaline hydrolysis had occurred, and that the compounds contained ester linkages. The fact that the change in R_F was a decrease rather than an increase, despite the fact that the hydrolysis products would have had a lower M_r than the starting material, indicating that in each ester the radioactive ([^{14}C]uronate-containing) moiety was attached to a group with a hydrophobicity higher than that of most sugars. The NaOH-catalyzed decrease in R_F was confirmed for several of the individual spinach compounds (e.g. 12, 13, 17—see Fig. 1) purified by two-dimensional chromatography. In contrast, authentic $GalA_2$ and $GalA_3$ were unaffected by the NaOH treatment, indicating that negligible alkaline peeling occurred under the conditions used (data not shown).

Partial Characterization of Selected Esters by Their Hydrolysis Products

Treatment of compound 17 with cold dilute alkali caused a small increase in $m_{picrate}$ on electrophoresis at pH 3.5 (Fig. 2), showing that the radioactive moiety had acquired a higher charge:mass ratio as a result of alkaline hydrolysis. The ^{14}C-labeled hydrolysis product was found to co-electrophorese and to co-chromatograph in several solvent systems with authentic α-(1→4)-D-galacturonotriose (e.g. Fig. 2), indicating that compound 17 was of pectic origin. Compound 12 also yielded [^{14}C]$GalA_3$, whereas compound 13 gave [^{14}C]$GalA_2$ (data not shown).

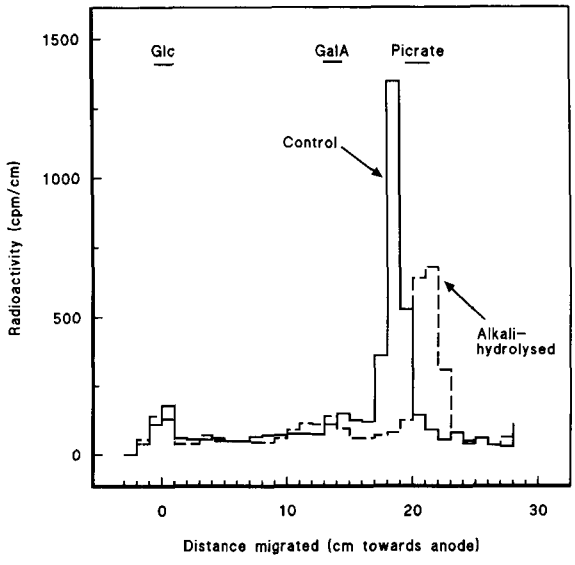

Figure 2. High-voltage electrophoresis of compound 17 and its radioactive saponification products. Compound 17 was electrophoresed before (——) and after (- - - -) hydrolysis in 50 mM NaOH. Horizontal bars indicate the positions of nonradioactive internal markers (of which Glc and GalA were stained after scintillation counting).

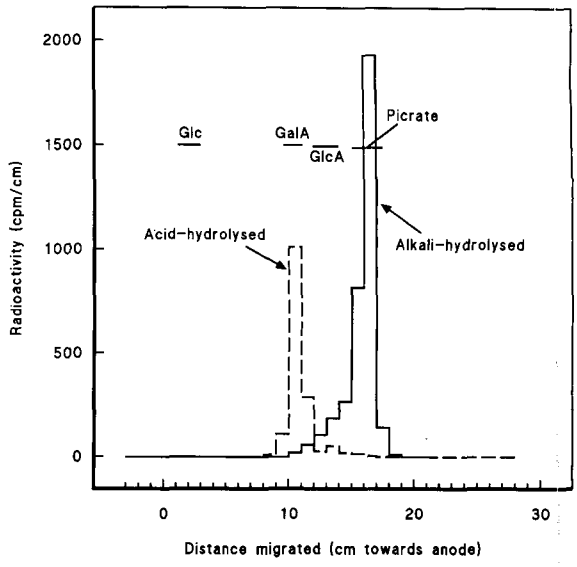

Figure 3. High-voltage electrophoresis of the radioactive acid-hydrolysis products of compound 17. The products obtained by hydrolysis of compound 17 in 2 M TFA (- - - - -) or 50 mM NaOH (——) were subjected to electrophoresis. Horizontal bars indicate the positions of nonradioactive internal markers (of which Glc, GlcA, and GalA were stained after scintillation counting).

Compounds 12, 13, and 17 each yielded [^{14}C]galacturonic acid as the sole radioactive product upon acid hydrolysis (e.g. Fig. 3). No [^{14}C]glucuronic acid was detected.

Gradual alkaline hydrolysis of compound 17 at pH 11 resulted in the formation of only one radioactive product (Fig. 4b), indicating that the $GalA_3$ was monoesterified. The

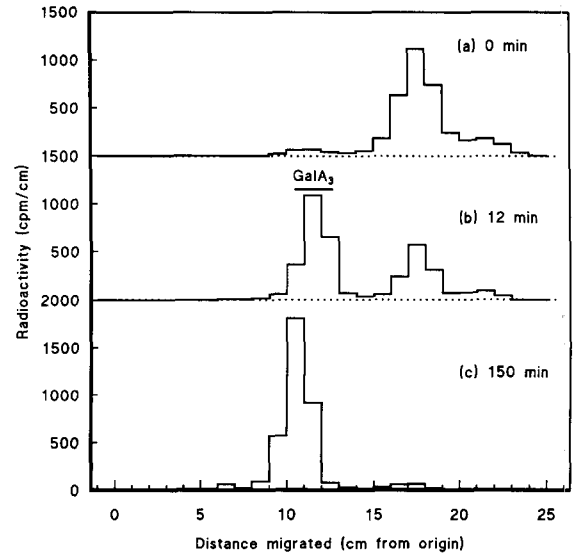

Figure 4. Graded alkaline hydrolysis of compound 17. The ^{14}C-labeled compound 17 was incubated at pH 11 and 25°C for 0 min (a), 12 min (b), or 150 min (c). Products were then chromatographed in EAW. The horizontal bar in b shows the position of nonradioactive internal marker $GalA_3$ stained after scintillation counting.

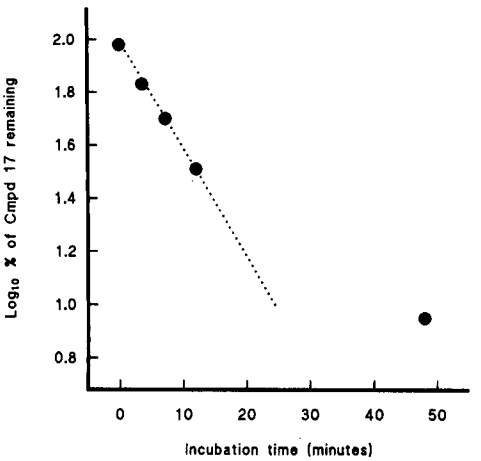

Figure 5. Kinetics of hydrolysis of ^{14}C-labeled compound 17 at pH 11 and 25°C. The disappearance of at least the first 70% of the starting material approximated to exponential decay. Subsequent deviation from the straight line suggests the presence of a small proportion of a more stable contaminant in the starting material. The data are derived from plots like those in Figure 4.

kinetics of production of $GalA_3$ at pH 11 indicated a half-life for the ester bond of compound 17 of 7.2 min (Fig. 5), which is comparable to that of 6-*O*-polygalacturonoyl-D-Glc (Brown and Fry, 1993) and indicates a much greater alkalilability than, for example, an *O*-feroloyl-disaccharide ester bond (Fry, 1982a).

In contrast, the gradual alkaline hydrolysis of compound 12 at pH 11 and 25°C resulted in the transient formation of an intermediate-R_F radioactive product before the end product [^{14}C]$GalA_3$ appeared (Fig. 6), indicating that two hydrophobic ester-linked substituents were hydrolyzed from the [^{14}C]$GalA_3$ moiety. The hydrolysis of the first one appeared to occur with similar kinetics to compound 17 (half-life roughly 10 min), but hydrolysis of the second was considerably slower (half-life roughly 100 min), suggesting that it had a very different structure.

Susceptibility to Borohydride Reduction

Treatment of compound 17 with neutral $NaBH_4$ followed by acid hydrolysis yielded ^{14}C-labeled products that co-chromatographed with Gal and galacturonic acid in the ratio of approximately 1:2.5 (Fig. 7). Presaponified compound 17, on the other hand, yielded only the acid spot (data not shown). The identity of the putative [^{14}C]Gal was confirmed by paper chromatography in EPW (which resolves it from Glc; see Fry, 1988) and by electrophoresis in molybdate (which resolves it from galactitol and glucitol; see Weigel, 1963) (data not shown).

Evidence against Methyl Esters, Phenolic Conjugates, and Lactones

To test whether compounds 12, 13, 17, etc. might be methyl esters of oligogalacturonides, authentic mono-, di-, and trimethylesterified derivatives of $GalA_3$ were treated with Driselase. Each of these compounds was found to be completely digested by Driselase to yield galacturonic acid (data not shown), indicating that Driselase contains enzymes that together are rapidly able to hydrolyze all the ester and glycosidic bonds in mono-, di-, and trimethylesterified derivatives of $GalA_3$. Therefore, the ^{14}C-labeled compounds released from spinach cell walls by Driselase were not methyl esters of $GalA_3$.

To test whether compounds 12, 13, 17, etc. might be

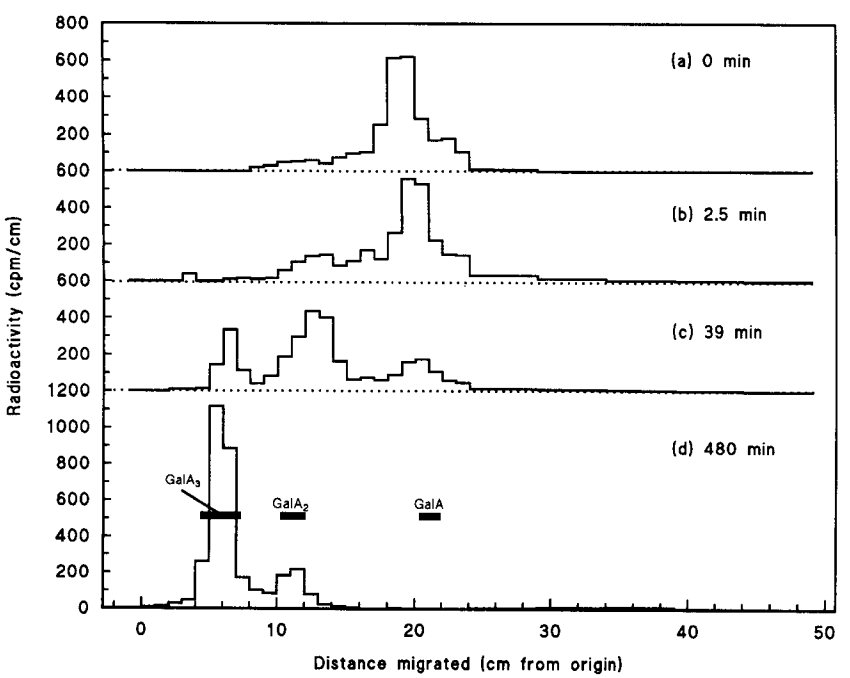

Figure 6. Graded alkaline hydrolysis of compound 12. The ^{14}C-labeled compound 12 was incubated at pH 11 and 25°C for 0 min (a), 2.5 min (b), 39 min (c), or 480 min (d). Products were then chromatographed in EAW. The horizontal bars in d show the positions of nonradioactive internal markers, stained after scintillation counting.

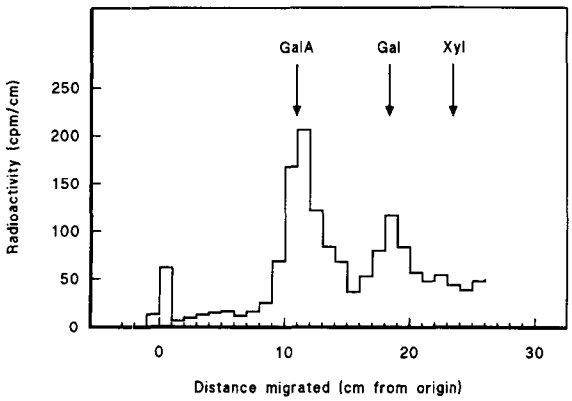

Figure 7. Products obtained when ^{14}C-labeled compound 17 was reduced with neutral $NaBH_4$ and then acid hydrolyzed. Paper chromatography in BPW. ↓, Nonradioactive internal marker monosaccharides, stained after scintillation counting.

phenolic derivatives, cultured spinach cells were fed [U-^{14}C]cinnamate, which is readily taken up and incorporated by spinach cells (Fry, 1984) and is thought to be a precursor of essentially all cell wall phenolics (Bolwell, 1988) except derivatives of gallic acid (Amrhein et al., 1984) and Tyr (Fry, 1982b). Driselase digests of the resulting radioactive cell walls contained several ^{14}C-labeled products, e.g. [^{14}C]feruloyl-arabinobiose and [^{14}C]feruloyl-galactobiose (data not shown), as demonstrated before (Fry, 1987), but none of these co-chromatographed with the *uronate*-^{14}C-labeled products (data not shown). Therefore, compounds 12, 13, 17, etc. were not cinnamate derivatives, and thus probably not phenolics.

In preliminary work, compounds 12, 13, 17, etc. also failed to become radioactive when [^{14}C]mevalonate was fed to the cells, suggesting that they were not terpenoid derivatives.

We also investigated the possibility that compound 17 is a lactone of $GalA_3$. Authentic $GalA_3$ was not converted into material with the R_F in EAW of compound 17 under any of the conditions used in our work, including prolonged treatment with PAW. $GalA_3$ did not yield compound 17 after incubation in an aqueous solution containing 5% EDC and 5% pyridine, conditions designed for the synthesis of *O*-D-galacturonoyl esters (Brown and Fry, 1993). In addition, the drying in vacuo of authentic $GalA_3$ from a solution containing 1 M TFA (conditions that will lactonize glucuronic acid) yielded no compound 17 (data not shown).

DISCUSSION

Driselase digestion of spinach primary cell walls yielded a range of esterified oligogalacturonides, typified by compound 17, which is an ester of $GalA_3$. The R_F of compound 17 in EAW was considerably higher than that of $GalA_3$, indicating that the $GalA_3$ moiety was esterified to a relatively nonpolar substituent (R). The *O*-D-galacturonoyl esters of all the common monosaccharides possess lower R_F values in EAW than free galacturonic acid (Brown and Fry, 1993); thus, R must be substantially less polar than any common monosaccharide. Upon alkaline hydrolysis, compound 17 yielded α-(1→4)-D-[^{14}C]$GalA_3$ as the sole ^{14}C-labeled product, indicating that R did not contain a uronate moiety.

The R group of compound 17 was not an esterified methyl group (which is relatively nonpolar and thus increases the R_F of $GalA_3$), since Driselase readily hydrolyzes mono-, di-, and trimethylesterified derivatives of $GalA_3$. It is nevertheless possible that compound 12, which also yielded [^{14}C]$GalA_3$ upon alkaline hydrolysis, had one galacturonate residue esterified with R and another methylesterified, the R group preventing enzymic access to the neighboring methyl ester bond.

Borohydride reduction indicated that one of the two nonreducing galacturonate residues of compound 17 was esterified at carbon 6 (i.e. was reducible to a Gal residue). This would indicate that compound 17 was either

$$\begin{array}{c} \text{R} \\ | \\ 6 \end{array}$$
$$\alpha\text{-D-GalA-}(1\rightarrow4)\text{-}\alpha\text{-D-GalA-}(1\rightarrow4)\text{-D-GalA,}$$

or

$$\begin{array}{c} \text{R} \\ | \\ 6 \end{array}$$
$$\alpha\text{-D-GalA-}(1\rightarrow4)\text{-}\alpha\text{-D-GalA-}(1\rightarrow4)\text{-D-GalA,}$$

where R—OH is a nonradioactive alcohol. Each of these structures would be converted by $NaBH_4$:acid hydrolysis to D-Gal, D-galacturonic acid, and L-galactonic acid (1:1:1); the two acids are not resolved by paper chromatography in BPW. If the R group had been attached to the reducing terminal galacturonate unit, the predicted reduction product of the latter would have been [^{14}C]galactitol, which was not observed. If R had been an acyl group esterified to an O-2 or O-3 position in $GalA_3$, none of the carboxy groups of the $GalA_3$ would have been activated sufficiently to undergo reduction in neutral $NaBH_4$.

It appeared possible that compound 17 was a lactone of $GalA_3$, which, during saponification, would be converted to the more polar free acid without generating any nonradioactive product. However, we were unable to convert authentic $GalA_3$ into compound 17 artificially. It seems unlikely that compounds 12 and 17, both of which yielded $GalA_3$ upon alkaline hydrolysis, could both have been lactones of $GalA_3$, since they had very different R_F values from each other. Also, compounds 12 and 17 were not detected in Driselase digests of species other than spinach, although apparently related compounds with slightly different R_F values were obtained. The observations indicate that compounds 12 and 17 are neither lactones of $GalA_3$ nor artifacts of our work-up.

Since compound 17 was a monoesterified derivative of $GalA_3$, and since Driselase contains the *exo*-α-D-galacturonosidase activity necessary to hydrolyze $GalA_3$ to galacturonic acid, we conclude that R protected from enzymic hydrolysis two glycosidic bonds in its vicinity. Exoglycosidases are usually stringent in their requirements for a specific, unsubstituted, nonreducing glycosyl terminus but less stringent for a particular aglycone; therefore, R was probably attached to the nonreducing terminal galacturonate residue of $GalA_3$ in compound 17. We propose that during the action of Driselase

on the spinach pectin, the first attacks are catalyzed by endopolygalacturonase (⇑), and then *exo*-α-D-galacturonosidase (↑) further hydrolyzes the oligomers sequentially from the nonreducing terminus until it reaches an R-esterified galacturonate residue:

In conclusion, about 5% of the galacturonate residues in the pectins of cultured spinach cells are ester-linked through the —COOH group to relatively hydrophobic alcohols such as that partially characterized here as R—OH. Related complexes appear to be present in carrot, rose, and fescue cultures, although these compounds have not been examined in detail. Work is in progress to characterize the spinach-derived R—OH, which appears to be neither methanol nor a phenolic, nor any common carbohydrate. If R—OH is a dihydric alcohol, it is possible that it acts as a cross-link between pectin chains; this could account for the solubilization of CDTA-inextactable pectins by cold Na_2CO_3. Alternatively, the —R group could be a side-chain on the pectin molecule, perhaps enabling close contact between the pectin and either the plasma membrane or (when present) the cuticle.

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