Fingerprinting of polysaccharides attacked by hydroxyl radicals *in vitro* and in the cell walls of ripening pear fruit

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Hydroxyl radicals ($\cdot\text{OH}$) may cause non-enzymic scission of polysaccharides *in vivo*, e.g. in plant cell walls and mammalian connective tissues. To provide a method for detecting the action of endogenous $\cdot\text{OH}$ *in vivo*, we investigated the products formed when polysaccharides were treated with $\cdot\text{OH}$ (generated *in situ* by ascorbate- H_2O_2 - Cu^{2+} mixtures) followed by NaB^3H_4 . Treatment with $\cdot\text{OH}$ increased the number of NaB^3H_4 -reacting groups present in citrus pectin, homogalacturonan and tamarind xyloglucan. This increase is attributed partly to the formation of glycosulose and glycosulosuronic acid residues, which are then reduced back to the original (but radioactive) sugar residues and their epimers by NaB^3H_4 . The glycosulose and glycosulosuronic acid residues were stable for > 16 h at 20 °C in ethanol or buffer (pH 4.7), but were destroyed in alkali. Driselase-digestion of the

radiolabelled polysaccharides yielded characteristic patterns of ^3H -products, which included galactose and galacturonate from pectin, and isoprimeverose, galactose, glucose and arabinose from xyloglucan. Pectin yielded at least eight ^3H -labelled anionic products, separable by electrophoresis at pH 3.5. The patterns of radioactive products form useful 'fingerprints' by which $\cdot\text{OH}$ -attacked polysaccharides may be recognized. Applied to the cell walls of ripening pear (*Pyrus communis*) fruit, the method gave evidence for progressive $\cdot\text{OH}$ radical attack on polysaccharides during the softening process.

Key words: active oxygen species, galacturonan, glycosulose residues, glycosulosuronic acid residues, hemicellulose, scission (non-enzymic).

INTRODUCTION

Copper ions, complexed with plant cell wall polymers, may lead to the production of the hydroxyl radical ($\cdot\text{OH}$), a highly reactive species that can cause non-enzymic scission of polysaccharides. Such scission may loosen the cell wall and thereby promote certain important physiological processes such as plant cell expansion and/or fruit softening [1]. This potential mechanism of wall-loosening is additional to the various proposed protein-catalysed mechanisms, e.g. involving xyloglucan endotransglycosylases [2] and expansins [3].

The $\cdot\text{OH}$ radical is generated in a non-enzymic 'Fenton reaction' between Cu^+ and H_2O_2 [4]:

This reaction may well occur in the walls of living plant cells because the reactants, Cu^+ and H_2O_2 , can both be generated there by either ascorbate or superoxide ($\text{O}_2^{\cdot-}$).

Ascorbate, which has frequently been reported [5,6] to occur in the apoplast (aqueous solution that permeates the cell wall), can non-enzymically reduce wall-bound Cu^{2+} to Cu^+ , and O_2 to H_2O_2 [7], among various other proposed biological roles [8]. Dehydroascorbate can also reduce Cu^{2+} [1]. Thus ascorbate, in the presence of O_2 and catalytic amounts of Cu^{2+} , can generate $\cdot\text{OH}$ [1]. There are several reports of exogenous ascorbate promoting cell expansion [9–11] and of endogenous ascorbate (plus dehydroascorbate) concentrations being correlated with growth rate [6]. It is possible that these observations are attributable to ascorbate's ability to generate wall-loosening $\cdot\text{OH}$.

Alternatively, superoxide (and/or its un-ionized equivalent, the hydroperoxyl radical, $\text{HO}_2\cdot$) can non-enzymically reduce Cu^{2+} to Cu^+ , and superoxide can be enzymically or non-enzymically disproportionated to form H_2O_2 . It is therefore interesting that treatment of plants with growth-promoting doses of auxin appears to promote the activity of plasmalemmar NADH oxidase, which generates apoplastic superoxide [12–14].

Reduction of O_2 to H_2O_2 can also be achieved *in vivo* by the action of amine oxidases, oxalate oxidases and related enzymes [15,16].

It thus seems probable that $\cdot\text{OH}$ production occurs in plant cell walls *in vivo*, whether by the action of ascorbate, superoxide or both. Such $\cdot\text{OH}$ production has indeed been reported in elicitor-treated rice cells [17]. However, the hypothesis that $\cdot\text{OH}$ is generated in the apoplast of a healthy plant cell is difficult to test experimentally. Even if $\cdot\text{OH}$ can be detected in healthy tissue, e.g. by EPR spectroscopy [17] or by their ability to cause aromatic hydroxylation of probes such as phenylalanine and salicylate [18,19], it is not a simple matter to determine whether the radicals were present in the apoplast (and thus potentially able to loosen the cell wall) or in an intraprotoplasmic compartment. Furthermore, in plants, the phenols produced by aromatic hydroxylation are likely to be further metabolized by the action of peroxidases, making quantification difficult.

$\cdot\text{OH}$ cause the non-enzymic scission of polysaccharides *in vitro*, including hyaluronate [20,21], chitosan [22], pullulan [23] and plant cell wall polysaccharides such as xyloglucan and pectin [1,24,25]. Xyloglucan (a hemicellulose) is a key polysaccharide in the primary cell walls of dicotyledonous plants where it is thought to act as a molecular tether, interconnecting adjacent

Abbreviations used: BAW, butan-1-ol/acetic acid/water (12:3:5, by vol.); DP, degree of polymerization; EAW, ethyl acetate/acetic acid/water (10:5:6, by vol.); EPW, ethyl acetate/pyridine/water (8:2:1, by vol.); IP, isoprimeverose [α -D-Xylp-(1 → 6)-D-Glc]; m_{OG} , electrophoretic mobility relative to that of orange G; R_{GAlA} , chromatographic mobility relative to that of galacturonic acid.

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microfibrils and thus restraining cell expansion [2,26,27]. Pectins [polysaccharides based on (1 → 4)- α -D-galacturonan] are also major components of the plant cell wall and middle lamella; pectin degradation is thought to be central to the process of tissue softening during fruit ripening [28].

Damage to polysaccharides by $\cdot\text{OH}$ in an aerobic environment can in principle be recognized by the products of the following sequence of reactions [29]: (a) $\cdot\text{OH}$ acts on a polysaccharide to abstract a carbon-bonded H atom and thereby form a carbon-centred radical, (b) O_2 is added to the latter, and (c) a hydroperoxyl radical ($\text{HO}_2\cdot$; or its ionized form, $\text{O}_2^{\cdot-}$) is eliminated, leaving an oxo group [30]. If reaction (a) involves $\cdot\text{OH}$ attack at position 1, 4 or 5 of a hexopyranose residue, the polysaccharide chain is rapidly cleaved [31]. However, if the $\cdot\text{OH}$ initially attacks at position 2, 3 or 6, the product is expected to be a relatively stable glycosulose (= glycosone) residue, which should be reducible back to the original hexose residue, or an epimer of it, by NaBH_4 [32]. We now report the detection and partial characterization of NaBH_4 -reducible groups introduced into xyloglucan, pectin and galacturonan as a result of treatment with $\cdot\text{OH}$ in the presence of O_2 .

The findings point to a convenient method of looking for the occurrence of $\cdot\text{OH}$ in a polysaccharide-rich sub-cellular compartment such as a plant cell wall. A related application would be the detection of $\cdot\text{OH}$ action in mammalian connective tissues, where $\cdot\text{OH}$ may contribute to the tissue damage associated with diseases such as arthritis [4]. The products of $\cdot\text{OH}$ attack on polysaccharides could potentially be used diagnostically in the same way that $\cdot\text{OH}$ -attacked DNA can be recognized by a 'fingerprint' that includes 8-hydroxyguanine [33].

We also report the application of the fingerprinting strategy in an initial study of the wall polysaccharides of pear (*Pyrus communis*) fruit undergoing tissue softening; a climacteric fruit [34] whose softening has been reported to involve the participation of active oxygen species [35].

MATERIALS AND METHODS

Materials

Tamarind xyloglucan ($M_r \approx 10^6$) was generously given by Mr K. Yamatoya (Dainippon Pharmaceutical Co., Osaka, Japan). Driselase, citrus pectin and (1 → 4)- α -D-galacturonan ('poly-galacturonic acid') were from Sigma Chemical Co. (Poole, U.K.). NaB^3H_4 was from Amersham International (Bucks, U.K.). Driselase was partially purified as described in [36].

Chromatography and electrophoresis

Chromatography was performed on Whatman 3MM paper by the descending method in the following solvents systems: ethyl acetate/acetic acid/water (10:5:6, by vol.; EAW) for 24 h; ethyl acetate/pyridine/water (8:2:1, by vol.; EPW) for 24 h; and butan-1-ol/acetic acid/water (12:3:5, by vol.; BAW) for 16 h [37].

High-voltage electrophoresis was performed on Whatman 3MM paper in acetic acid/pyridine/water (10:1:189, by vol., pH 3.5) at 2.5 kV for 35–45 min with white spirit as coolant (20–30 °C) [38].

Assay of radioactivity

Solutions were mixed with 10 vol. of OptiPhase 'HiSafe' scintillation fluid (Wallac U.K., Milton Keynes, U.K.). Strips of chromatography paper were added to 2 ml of OptiScint scintillation fluid. Both types of sample were assayed for ^3H in a Beckman scintillation counter. The counting efficiency was

approximately 50 % in solutions and 7 % on chromatography paper.

Paper chromatograms and electrophoretograms were fluorographed after dipping through 7 % (w/v) 2,5-diphenyloxazole ('PPO') in diethyl ether [39] and/or cut into 1-cm strips for scintillation counting. For quantitative assay of the detected radioactive spots, the appropriate area of the paper was cut out and placed in scintillation fluid. Alternatively, for further qualitative analysis, the radioactive zones of paper were cut out, washed free of 2,5-diphenyloxazole with toluene, dried, and eluted with water by the method described in [40].

Acid and alkaline hydrolysis

Saponification was by incubation in 150 mM NaOH at 20 °C for 5 min followed by neutralization with acetic acid. Acid hydrolysis was conducted in a sealed tube containing 2 M trifluoroacetic acid at 120 °C for 1 h; cooled trifluoroacetic acid solutions were loaded directly on to chromatography paper.

Formation and stability of glycosulose residues in $\cdot\text{OH}$ -treated polysaccharides (experiment 1)

$\cdot\text{OH}$ were generated *in vitro* by the action of ascorbate plus traces of Cu^{2+} (i.e. a continuous supply of dilute Cu^+) on H_2O_2 . For degradation of xyloglucan or galacturonan with $\cdot\text{OH}$, 80 ml of a 0.8 % (w/v) aqueous solution of the polysaccharide was incubated in the presence of (final concentrations) 1 μM CuSO_4 , 50 mM sodium acetate (pH 4.7), 10 mM H_2O_2 and 20 mM ascorbate (added last), in an open flask (O_2 present), at 20 °C for 8 h. Control polysaccharide solutions received no H_2O_2 and no ascorbate. Each solution was then dialysed against water, and the retained material was freeze-dried. Two portions were re-dissolved at 0.64 % (w/v) in water and immediately frozen at -20 °C. Additional portions were dissolved or re-suspended at 0.64 % (w/v) in (1) pH 4.7 buffer (pyridine/acetic acid/water, 1:1:98, by vol., containing 0.5 % 1,1,1-trichloro-2-methylpropan-2-ol, a volatile, anti-microbial agent), (2) 1 M NaOH, (3) 6 M NaOH, or (4) 80 % ethanol, and incubated for 16 h at 25 °C (37 °C in the case of 6 M NaOH). The alkaline solutions were then acidified with acetic acid and all the samples were dialysed against water. After adjustment to a concentration of 0.246 % (w/v) in water, 100 μl of each sample was added to 10 μl of 12.7 mM NaB^3H_4 (70 TBq/mol) in 1 M NH_3 . After incubation in sealed vials at 20 °C for 16 h, the lids were opened (in a fume hood) and most of the NH_3 was allowed to evaporate; the solution was then acidified by the addition of 100 μl of pyridine/acetic acid/water (1:1:33, by vol.), and after a further 5 h to allow the evolved $^3\text{H}_2$ to disperse (in a fume hood), the solution was chromatographed in EAW. Polymeric material (R_F 0.00) was assayed for radioactivity.

Qualitative analysis of $\cdot\text{OH}$ -attacked polysaccharides (experiment 2)

Each polysaccharide (1 % w/v; tamarind xyloglucan or citrus pectin) was incubated in the presence of (final concentrations) 1 μM CuSO_4 , 50 mM sodium acetate (pH 4.7), 0.5 mM H_2O_2 and 0.6 mM ascorbate (added last), in an open flask (O_2 present), at 20 °C for 16 h. Identical polysaccharide samples were first de-esterified and freed of endogenous reducing groups by incubation for 16 h at 20 °C in 0.5 M NaOH containing 0.25 M NaBH_4 (non-radioactive) and then acidified with acetic acid. A third sample of each polysaccharide (control) was kept in ice-cold distilled water. Each sample was then dialysed against water and freeze-dried. A portion (1 mg) was re-dissolved in 0.75 ml of

water and 0.25 ml of 10 mM NaB³H₄ (17 TBq/mol) in 1 M NH₃ was added.

After 16 h incubation at 20 °C, 0.5 mmol of acetic acid was added to destroy excess NaB³H₄ and the ³H₂ was allowed to escape (in a fume hood). Ethanol (4 vol.) was then added and after 16 h at 4 °C the precipitate of ³H-polysaccharide was collected by centrifugation, washed in 90 % and 100 % ethanol, dried, re-dissolved in 0.5 ml of pyridine/acetic acid/water (1:1:98, by vol., pH ≈ 4.7) containing 1 % (w/v) Driselase (partially purified) and 0.5 % (w/v) 1,1,1-trichloro-2-methylpropan-2-ol (volatile anti-microbial agent), and incubated at 20 °C for 48 h. The solution was then boiled for 20 min to inactivate the Driselase. A 10 µl portion of the solution was assayed for total radioactivity, and replicate 80 µl portions (each derived from 80 µg of polysaccharide) were subjected to chromatography or electrophoresis.

Application of the method to ripening pear fruit

Unripe 'Conference' pear (*Pyrus communis*) pomes ('fruit') were bought from a supermarket and stored at room temperature. At 0, 11 and 22 days after purchase (when the fruit were hard, medium and soft respectively), individual fruit were peeled and cored, and the white flesh from the distal (broad) end of the fruit was diced and transferred into a freezer (−80 °C).

Portions (8 g) of the frozen tissue were thawed into 32 ml of ethanol and then homogenized with an Ultraturrax. The alcohol-insoluble material was thoroughly washed at room temperature in 80 % ethanol followed by methanol, chloroform/methanol (1:1, v/v), methanol again, phenol/acetic acid/water (2:1:1, w/v/v), DMSO/water (9:1, v/v) and water. The final wall residue was freeze-dried.

A portion (approx. 1.5 mg) of each sample of the freeze-dried cell walls was accurately weighed and suspended in water at 0.2 % (w/v); 250 µl of 10 mM NaB³H₄ (17 TBq/mol) in 1 M NH₃ was then added and the suspension was incubated at 20 °C for 16 h. Acidification, ethanol precipitation and Driselase digestion were as described above.

RESULTS

Formation and stability of glycosulose residues in [•]OH-treated polysaccharides

[•]OH-untreated xyloglucan contained one to two NaB³H₄-reacting groups per 1000 sugar residues (Table 1). This is about ten times the number of such groups expected to be contributed by reducing

Table 2 Incorporation of radioactivity from NaB³H₄ into xyloglucan and pectin

See Table 1 for details.

Sample	Pre-treatment	Number of ³ H-labelled groups per 1000 sugar residues
Xyloglucan	NaBH ₄ + NaOH	0.68
	None (control)	0.65
	[•] OH radicals	14.6
Pectin	NaBH ₄ + NaOH	1.10
	None (control)	7.26
	[•] OH radicals	26.9

termini. The NaB³H₄-reacting groups present in untreated xyloglucan were resistant to storage in alkali.

After treatment of xyloglucan with [•]OH, the number of NaB³H₄-reducible groups increased 5–10-fold (Table 1), indicating the formation of glycosulose residues. The newly formed reducible groups withstood storage in buffer (pH 4.7) and ethanol, freezing and dialysis, but were destroyed by alkali (Table 1). This means that conventional hemicellulose extraction protocols [41] would not be useful for investigation of the presence of glycosulose groups in plant cell wall polysaccharides. Inclusion of 3 % (v/v) acetone in the storage solutions did not affect the recovery of polymer-associated oxo groups (results not shown).

[•]OH-untreated galacturonan contained more NaB³H₄-reacting groups than untreated xyloglucan (Table 1). Most of these groups were resistant to alkali, showing that they were not esters. Treatment with [•]OH increased the number of NaB³H₄-reducible groups detectable in galacturonan about 4-fold, again suggesting the introduction of new oxo groups. Like those introduced into xyloglucan, these oxo groups were destroyed by treatment with alkali but withstood treatment with buffer and ethanol, freezing and dialysis (Table 1).

The concentrations of ascorbate (20 mM) and H₂O₂ (10 mM) used in experiment 1 were higher than are likely to occur in the apoplast *in vivo*; the aim was to maximize hydroxyl radical attack in this *in vitro* experiment. In experiment 2 (Table 2), lower ascorbate and H₂O₂ concentrations were used, which are more likely to represent physiological concentrations; polysaccharide

Table 1 Effect of [•]OH treatment and subsequent storage under various conditions on the number of NaB³H₄-reacting groups in polysaccharides

Values are means of duplicate treatments. We assume that the reduction products have 0.25 × the specific radioactivity of the NaB³H₄ and that the mean residue *M_r* of xyloglucan and galacturonan was 150 and 176 respectively.

Storage conditions between treatment ± [•] OH and addition of NaB ³ H ₄	Number of ³ H-labelled groups per 1000 sugar residues					
	Xyloglucan			Galacturonan		
	[•] OH-untreated	[•] OH-treated	Ratio of treated/untreated	[•] OH-untreated	[•] OH-treated	Ratio of treated/untreated
H ₂ O, −20 °C	1.91	10.1	5.4	2.06	8.92	4.3
pH 4.7 buffer, 25 °C	1.27	12.6	9.9	2.39	9.91	4.2
1 M NaOH, 25 °C	1.59	2.29	1.4	1.19	1.69	1.4
6 M NaOH, 37 °C	1.45	1.94	1.4	1.69	0.95	0.6
80% Ethanol, 25 °C	1.16	11.0	9.6	2.18	7.90	3.7

Figure 1 Neutral, low- M , ^3H -labelled products obtained from citrus pectin and tamarind xyloglucan

The polysaccharide was pre-treated with (a) non-radioactive $\text{NaBH}_4 + \text{NaOH}$, (b) water or (c) ^1OH . It was then treated with NaB^3H_4 and digested with Driselase; the products were fractionated by paper chromatography in EPW and detected by fluorography. (d) Control sample with no polysaccharide; (m) marker-mixtures stained with aniline hydrogen-phthalate; X2, xylobiose [β -D-Xylp-(1 $\rightarrow$ 4)-D-Xyl]; X3, xylotriose.

concentrations were kept approximately constant. However, the trend was for higher yields of ^1OH -induced NaB^3H_4 -reacting groups to be produced in experiment 2 than in experiment 1. This apparent anomaly was possibly due the concentrated ascorbate

in experiment 1 acting as a scavenger of ^1OH and thus partially protecting the polysaccharides.

Qualitative analysis of ^1OH -attacked polysaccharides

Pectin

There were more NaB^3H_4 -reacting groups in untreated commercial pectin (experiment 2; Table 2) than in homogalacturonan (Table 1). Nevertheless, ^1OH treatment still caused a 3.7-fold increase in NaB^3H_4 -reducible groups in pectin (Table 2), supporting the idea that exposure to ^1OH in the presence of O_2 creates glycosulose residues. About 85 % of the NaB^3H_4 -reacting groups in untreated pectin were destroyed by pre-treatment with non-radioactive $\text{NaBH}_4 + \text{NaOH}$ (Table 2).

We investigated the possibility that some of the NaB^3H_4 -reducible groups in untreated pectin (Table 2) were methyl-esterified galacturonate residues, which might be reduced to [$6\text{-}^3\text{H}$]galactose residues on treatment with NaB^3H_4 . To investigate the nature of the radiolabelled residues present, we digested the NaB^3H_4 -treated pectin with 'Driselase', a mixture of fungal hydrolases capable of hydrolysing the major pectins (homogalacturonan and rhamnogalacturonan-I) to monosaccharides. However, the yield of [^3H]galactose obtained after Driselase digestion of the NaB^3H_4 -treated pectin was only approx. 6 % of the total incorporated ^3H (Figure 1). Similar yields were obtained after hydrolysis with trifluoroacetic acid (results not shown). ^1OH -treatment caused a 2.6-fold increase in the yield of [^3H]galactose (Table 3), presumably because ^1OH converted a proportion of the galactose residues present in citrus pectin to galactosulose residues.

Most of the radioactive products of Driselase-digestion were immobile on paper chromatography in EPW (a water-poor, high-pH mixture), indicating that they were oligomeric and/or acidic. These products also increased after ^1OH treatment (Figure 1 and Table 3).

Table 3 Relative yield of ^3H -labelled products obtained from variously pre-treated pectin by treatment with NaB^3H_4 followed by Driselase

DP indicates approximate degree of polymerization by comparison with malto-oligosaccharides; 2 and 2' indicate two spots both migrating near maltose; 1 indicates material approximately co-migrating with galactose and galacturonic acid. The DP > 7 spot moved 1–2 cm from the origin. For electrophoretic mobilities of anionic compounds 1–8, see Table 4.

Method of analysis	^3H -labelled Driselase-digestion product	Relative yield from pectin pre-treated with:		
		$\text{NaBH}_4/\text{NaOH}$	None (control)	^1OH
Paper chromatography in EPW	R_f 0 (acidic and/or oligomeric products)	0.15	1.0	3.9
	Galactose	0.18	1.0	2.6
Paper chromatography in EAW	DP > 7	0.15	1.0	3.6
	DP4	0.13	1.0	4.0
	DP3	0.07	1.0	2.8
	DP2	0.14	1.0	3.3
	DP2'	0.20	1.0	2.4
	DP1	0.21	1.0	6.7
Difference*	Acidic monomers	0.18	1.0	9.1
High-voltage electrophoresis at pH 3.5	Neutral compounds	0.25	1.0	3.0
	Anionic compound 1	0.13	1.0	7.9
	Anionic compound 2	0.22	1.0	3.0
	Anionic compound 3	0.19	1.0	5.0
	Galacturonate	0.15	1.0	7.7
	Anionic compound 5	0.12	1.0	6.5
	Anionic compound 6	0.09	1.0	4.3
	Anionic compound 7	0.06	1.0	8.7
	Anionic compound 8	0.24	1.0	25.8

* Total monomers (DP1 in EAW) minus galactose (EPW).

Figure 2 Neutral and acidic, low- to moderate- M , ^3H -labelled products obtained from citrus pectin and tamarind xyloglucan

Details as in Figure 1, except that chromatography was in EAW. M2–M7, maltose to maltoheptaose.

Acidic and oligomeric sugars are mobile in EAW (a water-rich, low-pH mixture). EAW chromatography of the Driselase-digestion products indeed gave mainly mobile ^3H -labelled products, confirming that Driselase efficiently digested all the pectin samples. The major ^3H -labelled spot co-migrated with monomeric hexoses (galacturonic acid and galactose; not fully resolved from each other) (Figure 2). By subtraction of the known (small) $[\text{H}]$ galactose content, the acidic material in this 'monosaccharide' spot was shown to be increased 9-fold by $\cdot\text{OH}$ -treatment (Table 3). In addition, at least six ^3H -labelled oligosaccharides were present in the digest of $\cdot\text{OH}$ -treated pectin (Figure 2). Their R_F values indicated that these ^3H -oligosaccharides had a degree of polymerization (DP) ≈ 2 –5 and > 7 (by reference to marker oligogalacturonides and malto-oligosaccharides), although two spots in the dimer region predominated. The yield of each ^3H -labelled product was increased by $\cdot\text{OH}$ pre-treatment and decreased by pre-treatment with non-radioactive $\text{NaBH}_4/\text{NaOH}$ (Table 3).

High-voltage electrophoresis (pH 3.5) of the pectin digestion products gave at least eight radioactive anions (1–8) in addition to a spot of neutral material (Figure 3; Table 3). Compounds 1–8 were individually eluted from the (unstained) electrophoretogram and subjected to saponification followed by (i) chromatography in BAW or (ii) re-electrophoresis at pH 3.5. The results (Table 4) indicate diverse products, ranging from acidic disaccharides to highly acidic monomers. Compounds 1, 2, 3 and 7 appeared to be disaccharides, as judged by chromatography in BAW. Compound 3 was the only one to change in electrophoretic mobility

Figure 3 Acidic ^3H -labelled products obtained from citrus pectin and tamarind xyloglucan

Details as in Figure 1, except that fractionation was by electrophoresis at pH 3.5 and the markers were stained with AgNO_3 .

after saponification, suggesting that it contained a lactone or ester group. Compound 4 was free galacturonic acid, as shown by exact co-chromatography and co-electrophoresis with internal markers of authentic galacturonate. Compounds 5, 6 and 8 had high R_{GalA} values in BAW (where R_{GalA} is chromatographic mobility relative to that of galacturonic acid), suggesting that they were monomeric. Compounds 7 and 8 had very high electrophoretic mobility, indicating a charge:mass ratio at pH 3.5 greater than that of the dicarboxylic acids, galactaric and glucaric acids.

$\text{L-}[\text{H}]$ Galactonate, the product expected of NaB^3H_4 reduction of the reducing terminal galacturonate moiety of pectin, was undetectable in the Driselase digests [the difference in m_{OG} values (where m_{OG} is electrophoretic mobility relative to that of orange G) of 0.01 between compound 2 and an internal marker of authentic galactonate (Table 4) corresponded to a clear 3–4 mm mis-match of spot centres on the fluorogram and subsequently stained electrophoretogram, and thus a clear demonstration of non-identity]. Thus, NaB^3H_4 -labelling of the reducing terminus of pectin did not contribute to the detected anionic products. All the ^3H -labelled products found after treatment of pectin with

Table 4 Characteristics of eight anionic, ^3H -labelled products obtained after treatment of pectin with $\cdot\text{OH}$, NaB^3H_4 and Driselase

Electrophoretic markers glucose and orange G ran approx. 16 and 362 mm towards the anode respectively. The m_{OG} values are corrected for electro-endo-osmosis by reference to glucose ($m_{\text{OG}} = 0$). n.c., no change; n.d., not determined.

	Initial	After saponification		Proposed nature of product
	m_{OG}^*	m_{OG}^*	$R_{\text{GalA}}^\dagger$	
Compound				
Compound 1	0.350	n.c.	0.34	Disaccharide; not ester/lactone
Compound 2	0.425	n.c.	≈ 0.71	Disaccharide(?); not ester/lactone
Compound 3	0.505	0.690	0.53	Disaccharide; lactone or ester
Compound 4	0.550	n.c.	1.00	Galacturonate
Compound 5	0.605	n.c.	1.39, 1.43‡	Monomers; not ester/lactone
Compound 6	0.680	n.c.	1.41	Monomer; not ester/lactone
Compound 7	0.825	n.c.	0.53	Dimer(?); very high charge:mass ratio
Compound 8	0.870	n.c.	0.95	Monomer; very high charge:mass ratio
Markers				
Glucose	0.000	n.c.		
Galactonate	0.415	n.c.		
Gluconate	0.475	n.c.		
Guluronate	0.520	(n.d.)		
Mannonate	0.520	(n.d.)		
Galacturonate	0.550	n.c.	1.00	
Iduronate	0.645	(n.d.)		
Mannuronate	0.645	n.c.		
Glucuronate	0.710	n.c.		
Galactarate	0.760	n.c.		
Glucarate	0.795	(n.d.)		
Orange G	1.000	(n.d.)		

* Paper electrophoresis at pH 3.5.

† Paper chromatography in BAW.

‡ Two compounds not resolved by electrophoresis.

Table 5 Relative yield of ^3H -labelled products obtained from variously pre-treated xyloglucan by treatment with NaB^3H_4 followed by Driselase

Method of analysis	^3H -labelled Driselase-digestion product	Relative yield from xyloglucan pre-treated with:		
		$\text{NaBH}_4/\text{NaOH}$	None (control)	$\cdot\text{OH}$
Paper chromatography in EPW	R_F 0 (acidic and/or oligomeric products)	1.03	1.0	22.2
	Disaccharides	1.05	1.0	27.5
	Galactose	1.33	1.0	48.8
	Arabinose	0.25	1.0	51.4
Paper chromatography in EAW*	DP > 7	0.63	1.0	35.3
	DP6	1.20	1.0	44.8
	DP4–5	1.32	1.0	25.5
	DP4	1.78	1.0	26.3
	DP3	1.04	1.0	38.3
	DP2	1.12	1.0	15.8
	DP2'	1.33	1.0	31.5
	IP	0.59	1.0	65.5
	DP1	0.74	1.0	22.5
High-voltage electrophoresis at pH 3.5†	Neutral compounds	0.93	1.0	37.2
	Anionic compound A	1.24	1.0	30.9
	Anionic compound B	0.70	1.0	72.9
	Anionic compound C	0.15	1.0	20.1

* For an explanation, see Table 3; note that IP, although a disaccharide, migrates between maltose and galactose.

† Anionic compounds A, B and C had m_{OG} values of approx. 0.21, 0.26 and 0.37 respectively (compare with Table 4).

$\cdot\text{OH}$, NaB^3H_4 and Driselase were also obtained, though in smaller quantities, when the $\cdot\text{OH}$ step was omitted. It thus seems possible that the commercial (control) citrus pectin had already been exposed to a source of $\cdot\text{OH}$ during production or storage.

The highly charged compound 8 was the one which showed the greatest increase in ^3H -labelling after deliberate $\cdot\text{OH}$ -treatment (Table 3), indicating that this compound will be useful in

'fingerprinting' pectin to show whether it has been attacked by $\cdot\text{OH}$.

Xyloglucan

As in experiment 1, untreated xyloglucan already contained a small number of NaB^3H_4 -reacting groups; the number of these

Figure 4 ^3H -labelled products formed from pear fruit cell walls after treatment with NaB^3H_4 followed by Driselase

Soluble products were subjected to paper chromatography in EAW (a), EPW (b) or to high-voltage electrophoresis at pH 3.5 (c). The pear fruits were hard (∇), medium ($\square$) or soft ($\diamond$); data for a blank in which no cell walls were present are also presented ($\circ$). The positions of external markers on the same chromatograms and electrophoretogram are indicated ($\bullet$). The positions of compounds B (see Table 5) and 8 (see Table 4) are indicated on the electrophoretogram. The histograms show radioactivity (released by digestion of 100 μg of NaB^3H_4 -treated cell walls) per cm of chromatography paper.

increased 23-fold upon treatment with $\cdot\text{OH}$. Pre-treatment of xyloglucan with non-radioactive $\text{NaBH}_4 + \text{NaOH}$ did not diminish the number of groups subsequently labelled by NaB^3H_4 (Table 2).

Driselase contains endo- and exo-hydrolases capable of essentially complete hydrolysis of xyloglucan, except that there is no α -D-xylosidase activity and therefore a major product is α -D-Xylp-(1 $\rightarrow$ 6)-D-Glc (isoprimeverose; IP). Driselase digestion of the NaB^3H_4 -treated, $\cdot\text{OH}$ -attacked xyloglucan gave the following products, as resolved by paper chromatography in EPW, in order of decreasing radiochemical abundance: immobile material (acidic sugars and/or products of DP greater than approx. 3) > [^3H]disaccharides > [^3H]galactose > [^3H]glucose > [^3H]arabinose (Figure 1). These products were 20–50 times more heavily radio-labelled in the $\cdot\text{OH}$ -treated xyloglucan than

Table 6 Changes in (non-radioactive) cell wall composition during pear fruit ripening

Pear fruit cell walls were isolated as alcohol-insoluble residues, freed of protein and starch by use of phenol and DMSO respectively, then digested with Driselase. The digestion products were chromatographed in EPW followed by ethyl acetate/pyridine/water (10:4:3, by vol.); final distances migrated (cm from origin) were GalA, 5.2; IP, 18.6; Gal, 23.8; Glc, 28.4; Man, 33.6; Ara, 36.8 (a trace of mannose was present as a Driselase autolysis product). Relative abundance is indicated as the visual score on a paper chromatogram after staining with aniline hydrogen-phthalate [37].

Stage of fruit	Relative abundance in Driselase digest of:				
	GalA	IP	Gal	Glc	Ara
Hard	++++	$\pm$	+	++++	++++
Medium	++	$\pm$	+	++++	+
Soft	+	$\pm$	+	++++	—

in the control; [^3H]arabinose was the spot which showed the largest difference (Table 5). There was little difference between the control and the $\text{NaOH}/\text{NaBH}_4$ -pre-treated xyloglucan; therefore the slight NaB^3H_4 -labelling which occurred in the absence of deliberate $\cdot\text{OH}$ treatment is a process that can occur anew, even a short time after treatment with non-radioactive NaBH_4 . The most likely explanation of this would appear to be ^3H -exchange between the polysaccharide and NaB^3H_4 or a contaminant thereof.

Essentially all the radioactivity migrated away from the origin in EAW (Figure 2), indicating that Driselase had degraded the [^3H]xyloglucan to products of DP < 10. Individual ^3H -oligosaccharides cannot be identified in this analysis, but discrete molecular species were clearly present (Figure 2) which differed from each other in the proportion by which the yield from $\cdot\text{OH}$ -treated xyloglucan exceeded that from controls and would therefore be useful in the diagnosis of $\cdot\text{OH}$ radical damage (Table 5).

Most (approx. 80%) of the ^3H -labelled material from $\cdot\text{OH}$ -treated xyloglucan was electrophoretically neutral at pH 3.5 (Figure 3). The anionic products of xyloglucan were in general slower-migrating than those of pectin, suggesting oligomers in which only a minority of the residues were anionic. The yield of one of these anionic compounds (compound B) was particularly strongly enhanced by $\cdot\text{OH}$ pre-treatment of the xyloglucan (Table 5).

Evidence for $\cdot\text{OH}$ attack in pear fruit cell walls

When cell walls isolated from hard, medium and soft pear fruit were treated with NaB^3H_4 and then Driselase digested, the soluble products contained respectively 0.66, 0.75 and 1.45 ^3H -labelled groups per 1000 wall sugar residues (assuming an average residue has a M_r of 162). Thus there was evidence for a progressive increase in NaB^3H_4 -reducible groups during the process of fruit softening. This supports the hypothesis that, during ripening, glycosulose residues were formed by $\cdot\text{OH}$ radical attack on wall polysaccharides.

Paper chromatography in EAW (Figure 4a) indicated that the radioactive products included monosaccharides and oligosaccharides ranging from DP2 to > 10. Driselase digestion of highly purified cell walls to yield ^3H -labelled monomers and oligomers is strong evidence that the radiolabelled residues were components of cell wall polysaccharides. The yield of

^3H -labelled material of each size-class increased as fruit softening progressed.

Paper chromatography in EPW (Figure 4b) revealed neutral monomers and dimers, most of which also increased in radiochemical yield during fruit softening. Staining of an identical chromatogram demonstrated developmental changes in wall composition; arabinose and galacturonate residues strongly diminished (Table 6), indicating solubilization of pectic polysaccharides associated with fruit softening.

Electrophoresis at pH 3.5 (Figure 4c) showed that the major products were neutral, but that several anionic products were also present: ^3H -labelled species that showed a particularly pronounced increase in yield included anionic compounds that co-migrated with compound B (Table 5) and compound 8 (Tables 3 and 4), two products that showed large increases in yield when xyloglucan and pectin respectively, were artificially treated with $\cdot\text{OH}$.

DISCUSSION

The results show that $\cdot\text{OH}$ treatment of xyloglucan and pectin, two major polysaccharides of the primary cell wall in higher plants, introduces a range of NaB^3H_4 -reducible residues.

In the case of pectins, most of the ^3H -labelled products, released by Driselase, were in the form of at least eight different, low- M_r , anionic products. These were well resolved by high-voltage electrophoresis at pH 3.5. One of these products was identified as $[\text{H}]$ galacturonate. This would be formed after $\cdot\text{OH}$ radical attack on position 2 or 3 of a pectic galacturonate residue followed by the reaction sequence shown (Scheme 1). Driselase contains endo-polygalacturonase and α -D-galacturonidase activities, which together release α -D- $[\text{H}]$ galacturonate residues as the free monosaccharide. $\cdot\text{OH}$ attack at position 2 or 3 would yield not only α -D- $[\text{H}]$ galacturonate residues but also the 2- or 3-epimer, α -D- $[\text{H}]$ taluronate and α -D- $[\text{H}]$ guluronate residues respectively; however, Driselase probably lacks the α -taluronidase and α -guluronidase activities necessary to release these epimers as free monosaccharides. The products would therefore be likely to include Driselase-resistant disaccharides such as α -D- $[\text{H}]$ TalA-(1 $\rightarrow$ 4)-D-GalA and α -D- $[\text{H}]$ GulA-(1 $\rightarrow$ 4)-D-GalA. Such products probably account for some of the 'dimers' reported in Table 4.

Scheme 1 Proposed effect of $\cdot\text{OH}$ radical attack on a pectic galacturonate residue (1) under aerobic conditions

GalA refers to a neighbouring galacturonate residue in the pectin chain. The scheme arbitrarily shows the initial attack occurring at position 2 of the galacturonate residue. The galactosulosuronic acid residue (4) can be reduced by NaB^3H_4 to two epimeric, radioactive products (5 and 5').

No free $[\text{H}]$ glucuronate was obtained. $\cdot\text{OH}$ attack at position 4 or 5 (or 1) of a pectic galacturonate residue would be expected to lead to polysaccharide scission [29] and would not generate D- $[\text{H}]$ glucuronate or L- $[\text{H}]$ altruronate, the 4- and 5-epimers of D-galacturonate respectively.

In the case of xyloglucans, the major products were neutral monosaccharides and a considerable range of oligosaccharides. Although $[\text{H}]$ arabinose was a minor product, it more than the others was increased in yield by $\cdot\text{OH}$ treatment. This may be because arabinose is a minor component of xyloglucan, thus the great majority of the $[\text{H}]$ arabinose would be formed by NaB^3H_4 reduction of $\cdot\text{OH}$ -generated xylos-4-ulose residues rather than by ^3H -exchange with existing arabinose residues.

Small amounts of acidic ^3H -products were generated from xyloglucan, although these had lower charge:mass ratios than those obtained from pectin and therefore appeared to contain a higher proportion of neutral sugar residues. Intact tamarind xyloglucan is neutral [42], so the acidic groups were themselves a result of $\cdot\text{OH}$ attack.

Although many of the ^3H -labelled products in the 'fingerprints' presented here remain to be identified, their electrophoretic and chromatographic mobilities should serve as a valuable diagnostic tool for the recognition of pectin and xyloglucan that had been attacked by $\cdot\text{OH}$ *in vivo*. Experiments on the stability of glycosulose residues show that the polysaccharides to be tested for indications of $\cdot\text{OH}$ attack ought not to be extracted by the alkalis conventionally used for extraction of hemicelluloses.

Application of this methodology in an initial study of pear fruit gave evidence for a gradual increase in NaB^3H_4 -reducible groups within the polysaccharides of the cell wall and/or middle lamella during the process of fruit softening. This suggests that the $\cdot\text{OH}$ -initiated formation of glycosulose residues occurred in the extraprotoplasmic polysaccharides of ripening pears. The introduction of glycosulose residues would not of itself cause scission of the polysaccharide chains, but the formation of such residues accompanies the $\cdot\text{OH}$ -initiated scission that occurs when an $\cdot\text{OH}$ radical abstracts the C-bonded H atom from position 1, 4 or 5 of a (1 $\rightarrow$ 4)-linked pyranose residue [31]. Future work is required to identify the nature of the polysaccharides targeted by $\cdot\text{OH}$ in the cell wall and middle lamella and the precise timing of the radical attack relative to the onset of fruit softening.

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