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Characteristics of xyloglucan after attack by hydroxyl radicals

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Abstract

It has been proposed that plant cell-wall polysaccharides are subject in vivo to non-enzymic scission mediated by hydroxyl radicals (•OH). In the present study, xyloglucan was subjected in vitro to partial, non-enzymic scission by treatment with ascorbate plus H_2O_2, which together generate •OH. The partially degraded xyloglucan appeared to contain ester bonds within the backbone, as indicated by an irreversible decrease in viscosity upon alkaline hydrolysis. Aldehyde and/or ketone groups were also introduced into the polysaccharide by •OH-attack, as indicated by staining with aniline hydrogen-phthalate and by reaction with NaB^3H_4. The introduction of ester and oxo groups supports the proposed sequence of reactions: (a) •OH-mediated H-abstraction to produce a carbon-centred carbohydrate radical; (b) reaction of the latter with O_2; and (c) elimination of a hydroperoxyl radical ($HO_2^•$). When the partially degraded xyloglucan was reduced with NaB^3H_4 followed by acid hydrolysis, several ^{3}H-aldoses were detected ([^{3}H]galactose, [^{3}H]xylose, [^{3}H]glucose, [^{3}H]ribose and probably [^{3}H]mannose), in addition to unidentified ^{3}H-products (probably including anhydroaldoses). ^{3}H-Alditols were undetectable, showing that few or no conventional reducing termini were introduced. Digestion of the NaB^3H_4-reduced, partially degraded xyloglucan with Driselase released 25 times more [^{3}H]Xyl-α-(1→6)-Glc than Xyl-α-(1→6)-[^{3}H]Glc, suggesting that the xylose side-chains of the xyloglucan had been more heavily attacked by •OH than the glucose residues of the backbone. The radioactive xyloglucan was readily digested by cellulase, yielding ^{3}H-products in the hepta- to nonasaccharide range. A fingerprinting strategy for identifying •OH-attacked xyloglucan in plant cell walls is proposed. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Xyloglucan; Hemicellulose; Ascorbate; Plant cell wall; Non-enzymic scission; Glycosulose residues

1. Introduction

Plant cell walls undergo controlled loosening in vivo, enabling cell expansion and cell separation. These processes are important in germination, primary growth, fruit softening and organ abscission, but are incompletely understood. Wall loosening is widely assumed to be mediated by wall-localised proteins — the scission of structural polysaccharides by hydrolases and transglycosylases, and the rearrangement of hydrogen-bonds by expansins.[1,2] Little attention has been paid to the possibility that polysaccharides in the walls of living plant cells also suffer non-enzymic scission.

Miller[3] found that several plant polysaccharides (e.g., xylan, galacturonan, arabinogalactan and cellulose) undergo gradual non-enzymic scission when incubated in 0.1–

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10 mM H_2O_2. However, much more effective than H_2O_2 is the hydroxyl radical ($^{\bullet}OH$), which readily causes scission of polysaccharides, including hyaluronate,[4,5] chitosan,[6] pullulan[7] and xyloglucan.[8,9] $^{\bullet}OH$ Radicals can be generated by several means, including γ-radiolysis of H_2O,[10] reaction of ozone with H_2O,[11] or Fenton reactions — i.e., the reduction of H_2O_2 by a transition metal ion such as Fe^{2+} or Cu^+:[4,7,9]

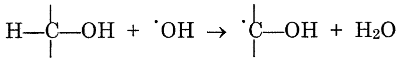
$$Cu^+ + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Cu^{2+}.$$

Cu^+, whose rate constant for reaction with H_2O_2 is 4700 M^{-1} s^{-1}, is 60 times more effective than Fe^{2+}.[12]

Fenton substrates are often present in the apoplast (aqueous solution that permeates the plant cell wall) and there is evidence for the production of extracellular $^{\bullet}OH$ in elicitor-treated rice cells in vivo.[13] In addition, $^{\bullet}OH$ may be generated in the apoplast of healthy cells.[8] The requirements for a Fenton reaction in the apoplast would be (a) O_2; (b) a transition metal e.g., Cu^{2+}; (c) an electron donor to reduce O_2 to H_2O_2 and (d) an electron donor to reduce Cu^{2+} to Cu^+. All these requirements are often fulfilled in vivo. O_2 is ubiquitous in healthy plants. Plant tissues contain Cu at 3–30 mg/kg dry weight, much of it in cell walls.[14] The walls' Cu may be tightly bound to His residues of wall glycoproteins; Cu in this form can, however, still participate in Fenton reactions.[8] An electron donor that can non-enzymically reduce O_2 to H_2O_2 is ascorbate, which is reported to be present at millimolar concentrations in the apoplast.[15,16] Reduction of O_2 to H_2O_2 can also be achieved by oxalate, polyamines, etc., by the action of wall enzymes.[17–19] Dehydroascorbate (a precursor of oxalate[20]) and polyamines[21] are reported apoplast/wall components. In addition, superoxide ($O_2^{\bullet -}$) ion radicals (and/or the corresponding uncharged hydroperoxyl radicals, $HO_2^{\bullet}$), formed in the apoplast by plasmalemmar NAD(P)H oxidase,[22,23] undergo rapid dismutation to form H_2O_2. Whatever its precise source, H_2O_2 is readily detectable in the apoplast of many plant tissues.[19,24,25] $H_2O_2 + HO_2^{\bullet}$ can also react with each other, in the presence of peroxidase, to generate $^{\bullet}OH$ radicals.[26] Ascorbate, dehydroascorbate and $O_2^{\bullet -}/HO_2^{\bullet}$ can each also reduce Cu^{2+} to Cu^+, the fourth requirement for a Fenton reaction.

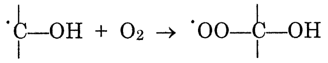
Promotion of polysaccharide scission by apoplastic $^{\bullet}OH$ radicals would be likely to contribute to wall loosening in vivo. Thus, circumstantial evidence is compatible with a role for $^{\bullet}OH$-mediated scission of wall polysaccharides in vivo. These observations invite a reconsideration of the prevailing view that proteins are the sole means of loosening plant cell walls in vivo. There is, however, no direct evidence for or against the formation of $^{\bullet}OH$ radicals in the walls of non-elicited plant cells, and no clear evidence that wall polysaccharides are subject in vivo to non-enzymic scission of the type described in vitro.[8] One approach to obtaining such evidence would be to examine the wall polysaccharides themselves for a chemical 'fingerprint' diagnostic of the action of $^{\bullet}OH$ radicals. These radicals cleave glycosidic linkages by an oxidative process quite distinct from the hydrolyses and transglycosylations catalysed by enzymes such as cellulase and xyloglucan endotransglycosylase. The unique by-products of $^{\bullet}OH$ attack could potentially be used diagnostically in the same way that $^{\bullet}OH$-attacked DNA can be recognised by a 'fingerprint' that includes 8-hydroxyguanine.[27]

The $^{\bullet}OH$ radical, although a reduction product of O_2, is a much more powerful oxidant (standard reduction potential +1.8 to +2.7 V, depending on pH).[28] Studies of the γ-irradiation of monosaccharide solutions have shown that $^{\bullet}OH$ reacts with all sugars exceedingly rapidly (rate-constant, $k = 1–4 \times 10^9$ M^{-1} s^{-1}),[28] abstracting a C-bonded H atom (see also Scheme 1). In many cases, the immediate product is an α-hydroxyalkyl radical[29,30] (Eq. (1)).



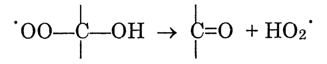
$$H{-}\overset{|}{\underset{|}{C}}{-}OH + {}^{\bullet}OH \rightarrow {}^{\bullet}\overset{|}{\underset{|}{C}}{-}OH + H_2O \quad (1)$$

Such radicals may react with each other; however, under most botanically relevant (aerobic) conditions, they react preferentially with O_2 to form the corresponding peroxyl radical[29] (Eq. (2)).



$$^{\bullet}\overset{|}{\underset{|}{C}}{-}OH + O_2 \rightarrow {}^{\bullet}OO{-}\overset{|}{\underset{|}{C}}{-}OH \quad (2)$$

The latter rapidly eliminates $HO_2^{\bullet}$ (or its ionised form, $O_2^{\bullet-}$, the superoxide anion radical), leaving a relatively stable product containing an oxo group (e.g., a hexosulose, depending on which of the sugar's H atoms was initially abstracted; Eq. (3)).



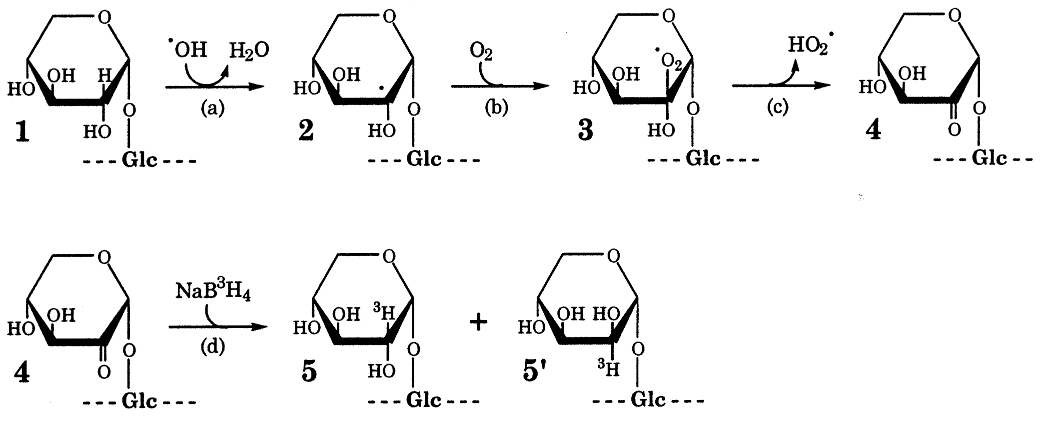
$$^{\bullet}OO{-}C{-}OH \rightarrow C{=}O + HO_2^{\bullet} \quad (3)$$

Less work has been done on the action of $^{\bullet}OH$ on polysaccharides. Model studies on cellobiose suggest that H-abstraction from C1, C4 or C5 of a sugar residue in a (1 → 4)-linked polysaccharide chain leads to scission of the glycosidic bond, whereas H-abstraction from C-2, C-3 or C-6 produces a relatively stable glycosulose residue.[30] Some splitting of C–C bonds within sugar residues also occurs,[29] but this is only prevalent in the absence of O_2. Analysis of the termini of fragments released from hyaluronate by the action of $^{\bullet}OH$ supports this general mechanism of polysaccharide scission.[4] In xyloglucan, the side-chain residues (e.g., xylose) will presumably be attacked in addition to the backbone (glucose) residues (Scheme 1).

One aim of the present work was to develop a simple, effective 'fingerprinting' protocol for detecting the damage done to xyloglucan by $^{\bullet}OH$ radicals. Emphasis was placed on xyloglucan because it hydrogen-bonds to cellulose and may play a particularly important structural role, 'tethering' adjacent microfibrils in the plant cell wall.[31] Scission of polysaccharides obviously results in new termini. Oxidative scission by $^{\bullet}OH$ will produce diverse termini, different from the ordinary reducing termini formed during acid- or enzyme-catalysed hydrolysis. Although in principle it would be possible to look for unusual termini, in practice it appeared more productive to look for the oxo groups of glycosulose residues formed by reaction of $^{\bullet}OH$ at positions that do not lead to scission (Scheme 1). When such oxo groups are treated with NaB^3H_4, the glycosulose residues are predicted to be reduced back to ^3H-aldose residues. Subsequent hydrolysis would then release these as ^3H-aldoses or short ^3H-oligosaccharides. New termini formed in xyloglucan by hydrolysis rather than $^{\bullet}OH$ attack would, in contrast, be predicted to yield ^3H-alditols during this protocol.

2. Experimental

Materials.—Xyloglucan from tamarind seeds was a generous gift of Mr K. Yamatoya, Dainippon Pharmaceutical Co., Osaka, Japan. An oligosaccharide mixture (principally the Glc_4-based structures XLLG, XXLG and XXXG — see Ref. 32 for nomenclature),



Scheme 1. Postulated action of an $^{\bullet}OH$ radical on an α-D-xylose residue (**1**) of xyloglucan. ---Glc--- represents a β-D-glucose residue of the xyloglucan backbone. (a) The $^{\bullet}OH$ radical abstracts any one of the C-bonded H-atoms (the illustration arbitrarily shows H-abstraction from C-2) to form an α-hydroxyalkyl radical (**2**); (b) reaction with atmospheric O_2; (c) elimination of a hydroperoxyl (or superoxide) radical to form a xylos-2-ulose residue (**4**). If **4** is then treated with NaB^3H_4 (d), a mixture of [2-^{3}H]xylose (**5**) and [2-^{3}H]lyxose residues (**5′**) would be expected, which would be released as free ^3H-aldopentoses upon acid hydrolysis.

produced from tamarind xyloglucan by digestion with cellulase, was also kindly provided by Mr Yamatoya. Isoprimeverose was prepared from xyloglucan by digestion with Driselase[33] and purified by preparative paper chromatography. Driselase, from Sigma, Poole, Dorset, UK, was freed from insoluble and low-M_r material as described.[33] *Trichoderma reesei* cellulase was obtained as a suspension in 3.2 M $(NH_4)_2SO_4$ from Megazyme, Ltd., Dublin, Ireland. NaB^3H_4 and D-[U-^{14}C]glucose were from Amersham International, Herts, UK. Authentic monosaccharides were from Sigma.

Chromatography and electrophoresis.—Paper chromatography systems used (all compositions by volume unless stated; all by the descending method on Whatman 3MM paper) were BAW = (12:3:5) butan-1-ol–AcOH–water for 18 h, BPW = (4:3:4) butan-1-ol–pyridine–water for 16 h, ΦW = 80% (w/w) phenol in water for 24 h, EAW = (10:5:6) EtOAc–AcOH–water, EPW1 = (8:2:1) EtOAc–pyridine–water for 24–72 h, EPW2 = (10:4:3) EtOAc–pyridine–water for 24 h, BAB = (9:1:1) butanone–AcOH–(H_3BO_3-saturated water) for 40 h. High-voltage paper electrophoresis was performed in pH 3.5 buffer (10:1:198, AcOH–pyridine–water), typically at 3 kV for 1 h in a white-spirit-cooled tank.[34]

In some experiments, non-radioactive sugars (usually 100 μg for chromatography and 20 μg for electrophoresis) were added to the radioactive sample as internal markers, and stained after detection of radioactivity. External markers are those run alongside the radioactive track but on the same sheet of chromatography paper. Markers were stained with aniline hydrogen-phthalate for reducing sugars or with $AgNO_3$ for non-reducing sugars.[33]

Detection and assay of radioactivity.—For fluorography, the paper was dipped through 7% (w/v) 2,5-diphenyloxazole in Et_2O, dried and exposed to film. For quantitative work, strips of the chromatography paper were assayed for ^{3}H by scintillation counting in 2 mL of 'OptiScint HiSafe' (Wallac Chemicals, Milton Keynes, Bucks, UK). In some cases, radioactive strips were removed from the scintillant, washed with toluene, dried, and eluted with water for further analysis.

Preparation and ^{3}H-reduction of •OH-cleaved xyloglucan: Experiment 1.—Tamarind xyloglucan (6.5 mL; 1.0%, w/v) containing (final concentrations) 50 mM succinate (Na^+, pH 5.7), 20 mM H_2O_2 and 20 mM ascorbate (added last) was incubated in the dark at 20 °C for 16 h in a loosely capped vessel. Catalase was then added to 0.6 mg/mL to ensure that no H_2O_2 remained, and a trace of [^{14}C]glucose was added as an internal marker. A 20-μL portion of the solution was analysed by paper chromatography in EAW. In addition, a 3-mL portion was analysed by gel-permeation chromatography (GPC) on Bio-Gel P-6, equilibrated with pyridine:AcOH:water (1:1:23); fractions were assayed for total carbohydrate by the phenol–H_2SO_4 method.[35] Another 3-mL portion was analysed by GPC on an 80-mL column of Sepharose CL-6B; material of k_{av} 0.5–0.7 was freeze-dried and is referred to as •OH-cleaved xyloglucan.

For ^{3}H-reduction of oxo groups, the •OH-cleaved xyloglucan ($\sim$10 mg) was re-dissolved in 1 mL of 1 M NH_3 solution containing 1 mM NaB^3H_4 (430 TBq/mol) and incubated at 20 °C for 3 days. After acidification with AcOH and removal of the $^3H_2$ gas evolved (fume hood), non-volatile products were de-salted on Bio-Gel P-2 in water, and the void volume (6 mL, containing 3.0 MBq ^{3}H-polymers) was stored frozen.

Preparation and ^{3}H-reduction of •OH-cleaved xyloglucan: Experiment 2.—Tamarind xyloglucan (0.8%, w/v) containing (final concentrations) 50 mM acetate (Na^+, pH 4.7), 10 mM H_2O_2 and 20 mM ascorbate (added last) was incubated in the dark at 20 °C for 8 h in a loosely capped vessel. A control xyloglucan solution received no H_2O_2 and ascorbate. The partially degraded polysaccharide was then recovered by dialysis and freeze-dried.

For ^{3}H-reduction, 6.4 mg of the polysaccharide was incubated in 1.1 mL of 91 mM NH_3 containing 1.15 mM NaB^3H_4 (70 TBq/mol). After 16 h at 20 °C, excess NaB^3H_4 was destroyed with AcOH and polysaccharide was precipitated by addition of 4 vols EtOH and washed 4 × in 70% (v/v) EtOH. The pellet was re-dissolved in water, dialysed against 0.1% (w/v) 1,1,1-trichloro-2-methylpropan-2-ol (a volatile anti-microbial agent), and then freeze-dried.

Effect of saponification and re-acidification on partially degraded xyloglucan.—Eight 6.2-mL aliquots of 0.65% (w/v) xyloglucan [in 40 mM acetate (Na^+), pH 4.7, 2 μM $CuSO_4$] containing 0–968 μM ascorbate (added last) were incubated for 18 h at 20 °C in loosely capped vessels. This treatment relies on the production of $^{\bullet}OH$ from H_2O_2 generated by the non-enzymic reduction of O_2 by ascorbate.[8,12] After the 18 h (when all the ascorbate had been oxidised, as determined by titration with 2,6-dichlorophenolindophenol) the viscosity at pH 4.7 was determined as the flow-time of 0.14 mL of the solution from a 0.2-mL pipette at 25 °C.[8] To a 1-mL aliquot of each solution was added either (a) 10 μL of 10 M NaOH (giving pH $\approx$ 12.7) or (b) 12 μL of 17 M AcOH (giving pH $\approx$ 3.7) and after a further 55 min the viscosity was re-measured. Then the pH of each solution was returned to 4.7 by addition of (a) 12 μL of 17 M AcOH or (b) 10 μL of 10 M NaOH and after a further 75 min the viscosity was measured again. Note that for these final viscosity measurements, the buffer composition of the two solutions was identical. Specific fluidity was calculated as (flow-time of buffer)/(flow-time of xyloglucan solution).

Hydrolysis of ^3H-xyloglucan: Experiment 1.—Four 50-kBq portions of the ^3H-xyloglucan were analysed as follows: (1) the material was heated in 500 μL of 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h, and half the mixture was subjected to paper chromatography in BAW; (2) as (1) but with cold water instead of hot acid; (3) the material was incubated at 25 °C for 4 days in 200 μL of 2.5% (w/v) Driselase (purified) in PyAW/CB [(1:1:198) pyridine–AcOH–water containing 0.25% (w/v) 1,1,1-trichloro-2-methylpropan-2-ol], then formic acid was added to 20% (w/v) and half the mixture was subjected to paper chromatography in BAW, the other half in BPW; (4) as (3) but without any Driselase.

Hydrolysis of ^3H-xyloglucan: Experiment 2.—For acid hydrolysis, 100 μg of ^3H-polysaccharide was heated in 500 μL of 2 M TFA at 120 °C for 1 h; then internal markers (GlcA, Gal, Glc, Man, Ara, Xyl, Rib, Fuc, Rha) were added prior to 2-dimensional paper chromatography [BAW–EPW1]. For cellulase digestion, 800 μL of 0.64% (w/v) polysaccharide was mixed with 200 μL PyAW/CB plus 10 μL (five units) of cellulase suspension. After intervals, 50 μL of the digest was mixed with 10 μL 90% (w/v) formic acid and chromatographed in EAW.

3. Results

Non-enzymic scission of tamarind xyloglucan.—Within 6 min of the addition of ascorbate + H_2O_2 to a solution of xyloglucan at pH 5.7, the viscometer flow-time had decreased from 53 to 2 s, indicating substantial non-enzymic scission of the polysaccharide chains by the $^{\bullet}OH$ radicals generated.[8] Little further change in viscosity occurred over the next 16 h. Paper chromatography (in EAW) of the reaction products showed the presence of reducing groups (stainable with aniline hydrogen-phthalate) at R_f 0.0, indicating that oxo groups had been introduced into the partially degraded xyloglucan by the $^{\bullet}OH$ radicals; no such groups were detectable by this method in untreated xyloglucan (results not shown). No free reducing sugars ($R_f > 0.0$) were detectable. On Sepharose CL-6B, the products eluted as a single broad peak in the k_{av} range 0.30–0.85 (median ≈ 0.6, corresponding to about 30 kDa) (Fig. 1(a)). Undegraded xyloglucan had a median $k_{av} \approx 0.2$, corresponding to 350 kDa (data not shown). On Bio-Gel P-6, most of the reaction products eluted in the void volume ($>$ 6 kDa) (Fig. 1(b)). These results indicate that the $^{\bullet}OH$ treatment had induced substantial chain scission in the polysaccharide, but had not generated appreciable quantities of oligosaccharides.

Effect of saponification and re-acidification on partially degraded xyloglucan.—Ascorbate-generated $^{\bullet}OH$ radicals caused an increase in specific fluidity of tamarind xyloglucan solutions at pH 4.7 (Fig. 2, ○), as reported before.[8] Raising the pH with NaOH caused an immediate additional increase in specific fluidity, even in $^{\bullet}OH$-untreated samples (Fig. 2, ▽), indicating that NaOH diminished xyloglucan–xyloglucan non-covalent bonding. When the pH of the NaOH-treated sample was returned to 4.7, the specific fluidity of the

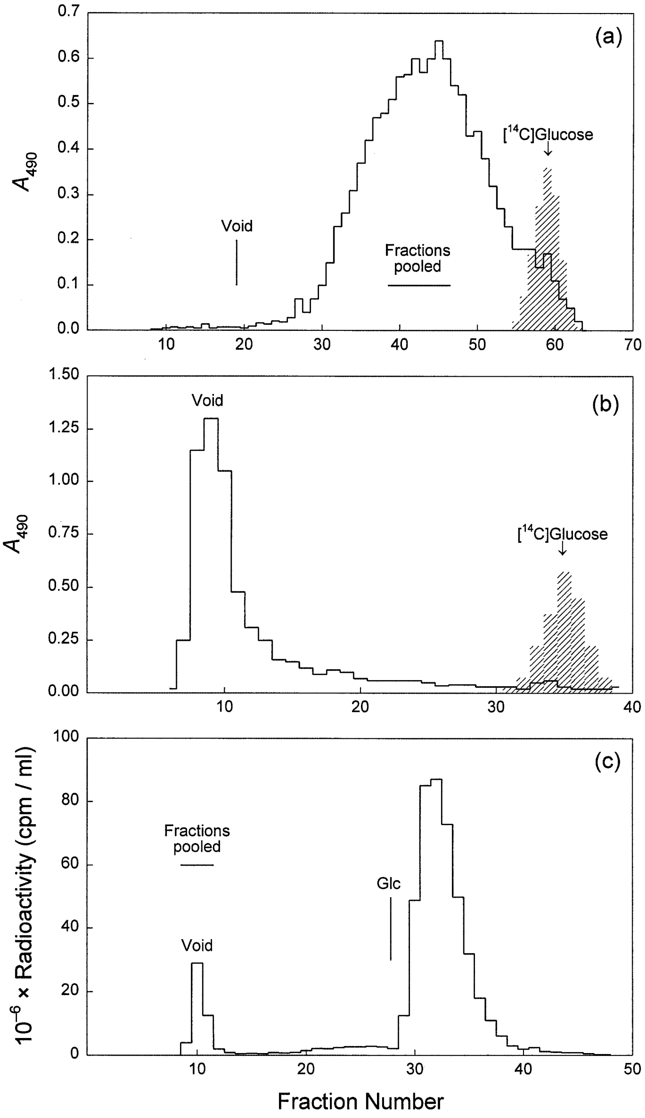


Fig. 1. Gel-permeation chromatography of $^{\bullet}$OH-treated xyloglucan. Tamarind xyloglucan was incubated with a source of $^{\bullet}$OH radicals (ascorbate + H_2O_2; Experiment 1) and chromatographed on Sepharose CL-6B (a) or Bio-Gel P-6 (b). Total carbohydrate in fractions was assayed as A_{490} after addition of phenol–H_2SO_4. The fraction marked (—) in profile (a) was then reduced with NaB^3H_4 and de-salted on Bio-Gel P-2 (c), the void fractions being collected for further analysis.

$^{\bullet}$OH-untreated samples returned to a value close to what it had been before alkalisation (Fig. 2, ◇), indicating that inter-polymeric non-covalent bonds were quickly re-established when the NaOH was neutralised. However, the specific fluidity of the $^{\bullet}$OH-treated samples was found to have been permanently increased by the alkali treatment (Fig. 2, ◇). This indicates that the $^{\bullet}$OH treatment had introduced alkali-labile bonds, most likely ester linkages, into the backbone of the xyloglucan. Treatments with acetic acid followed by NaOH served as a control (Fig. 2, □, △).

Alkali-induced scission of $^{\bullet}$OH-treated xyloglucan could in principle be due to β elimination, in the vicinity of neutral C=O groups, as well as to ester hydrolysis. We made two attempts to demonstrate ester hydrolysis under conditions that would not allow β elimination. (a) Hydroxylamine, which would cleave ester bonds at neutral pH, caused substantial scission even of ascorbate-untreated xyloglucan. For example, hydroxylamine hydrochloride (0.5 M; pH 5–7; 25 °C) caused the viscometer flow-time of a xyloglucan solution to decrease from 11 to 6 s within 5 h; other polysaccharides were similarly affected. This effect may be due to an ability of hydroxylamine, like that of ascorbate, to generate $^{\bullet}$OH radicals in the presence of traces of Cu^{2+}. (b) Pig liver esterase (15 U/mL, pH 7.8) did not mimic NaOH in increasing the specific fluidity of the $^{\bullet}$OH-attacked xyloglucan (data not

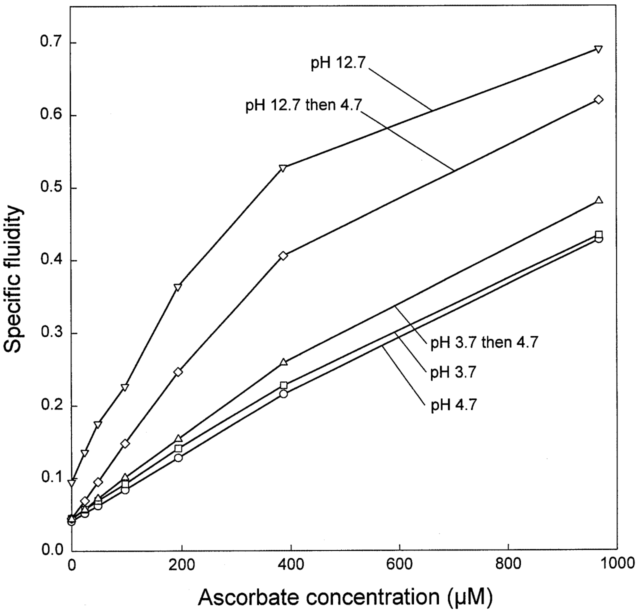


Fig. 2. Reversible and irreversible effects of alkali on the specific fluidity of tamarind xyloglucan after partial degradation by $^{\bullet}$OH radicals. A solution of xyloglucan was treated with the indicated concentrations of ascorbate (as a source of $^{\bullet}$OH) at pH 4.7 for 18 h, and then the specific fluidity was measured (○), which is related to the number backbone scission events. Aliquots of the same solutions were then adjusted to pH 3.7 or 12.7 with acetic acid or NaOH and the specific fluidity was re-measured (□, ▽). These solutions were then re-adjusted to pH 4.7 with NaOH or acetic acid and the specific fluidity was measured again (△, ◇).

Table 1
Chromatographic mobilities of monosaccharides in EPW1

Compound	$R_{\text{Rib–Gal}}$ [a]
Ribose [b]	1.00
Arabinose	0.33
Xylose	0.55
Lyxose	0.63
Allose	0.17
Altrose	0.47
Glucose	0.07
Mannose	0.20
Gulose	0.19
Idose	0.75
Galactose [b]	0.00
Talose	0.61
Sugar acids	[c]

Each monosaccharide was mixed with a small amount of galactose and ribose (as internal markers) and then chromatographed.

[a] Relative mobilities are reported as $R_{\text{Rib–Gal}}$, defined as (distance migrated by compound *minus* distance migrated by Gal)/(distance migrated by Rib *minus* distance migrated by Gal).

[b] Mean absolute distances migrated by the internal markers in 24 h were Gal = 68 mm, Rib = 347 mm.

[c] Glucuronic, galacturonic, mannuronic, galactonic, gluconic and galactaric acids all exhibited $R_f = 0.00$ and showed no signs of lactone formation during development of the chromatogram.

shown). However, this probably reflects the substrate-specificity of the enzyme rather than contradicting the presence of ester bonds.

A mechanism by which glycosidic bonds could be converted into ester bonds in the backbone of a (1 → 4)-linked polysaccharide as a result of •OH-attack at C-1 has been proposed by Schuchmann and von Sonntag.[30]

NaB^3H_4-reduction of •OH-treated xyloglucan.—When the main peak from the Sepharose CL-6B column (k_{av} 0.5–0.7; Fig. 1(a)) was reduced with NaB^3H_4 and re-chromatographed on Bio-Gel P-2, a sharp peak of ^{3}H-polymer was isolated at k_{av} 0.0; in addition, a large peak of (probably inorganic) products had $k_{av} > 1.0$ (Fig. 1(c)). When portions of the ^{3}H-polymer fraction were treated with Driselase-free buffer or with cold water [treatments (2) and (4) — see Section 2], 100% of the radioactivity exhibited R_f 0.00 on all paper chromatography systems tested.

Acid hydrolysis of NaB^3H_4-reduced, •OH-treated xyloglucan.—The material released by acid hydrolysis of the ^{3}H-polymer migrated in BAW as a series of peaks (A–G; Fig. 3(a)). About 6, 23 and 43% of the total ^{3}H ran in zones A, C and D, respectively. Zones C, D and E were eluted from the strips of paper represented in Fig. 3(a) and re-chromatographed in EPW1 (Fig. 3(b–d)). R_f data for all aldopentoses and aldohexoses are presented (Table 1).

Re-chromatography of C (the main ^{3}H-hexose and/or hexitol zone) in EPW1 for 72 h resolved main three components (Fig. 3(b)): galactose, glucose and an unknown in the ratio 64:26:10. The [^{3}H]galactose spot was eluted and shown to be radiochemically pure [^{3}H]galactose by its precise co-chromatography with an internal marker in EPW2, ΦW and BAB (Fig. 3(e)). The [^{3}H]glucose spot was similarly shown to be radiochemically pure (Fig. 3(f)). [^{3}H]Glucitol, which would have been expected in zone C1 or C2, was not detectable (Fig. 3(e, iii)). The ^{3}H-unknown (C3) was resolved into at least three further, unidentified components (Fig. 3(g)), which did not include mannose.

Re-chromatography of D (the main ^{3}H-pentose and/or pentitol zone) in EPW1 for 24 h resolved at least six components (Fig. 3(c)). D0 had R_f 0.00 in EPW1 and was therefore thought to be acidic; upon electrophoresis at pH 3.5 it was shown to contain neutral material plus at least two acidic compounds, neither of which co-migrated with galacturonic or glucuronic acid (Fig. 4(a)). The abundant D1, which approximately co-migrated with arabinose in EPW1 (Fig. 3(c)), was largely resolved from arabinose in three subsequent systems (Fig. 3(i, iii)). D2 co-migrated with xylose in EPW1 (Fig. 3(c)) and was confirmed to be essentially pure [^{3}H]xylose by co-chromatography with an internal marker in three other systems (Fig. 3(j)). D3, a shoulder which approximately co-migrated with lyxose (Fig. 3(c)), was resolved upon re-chromatography into at least two peaks, neither of which was lyxose (Fig. 3(k)). D4, a small peak running with ribose (Fig. 3(c)), was confirmed by co-chromatography to be radiochemically pure [^{3}H]ribose (Fig. 3(l)). Zone D also gave a small peak which approximately co-migrated

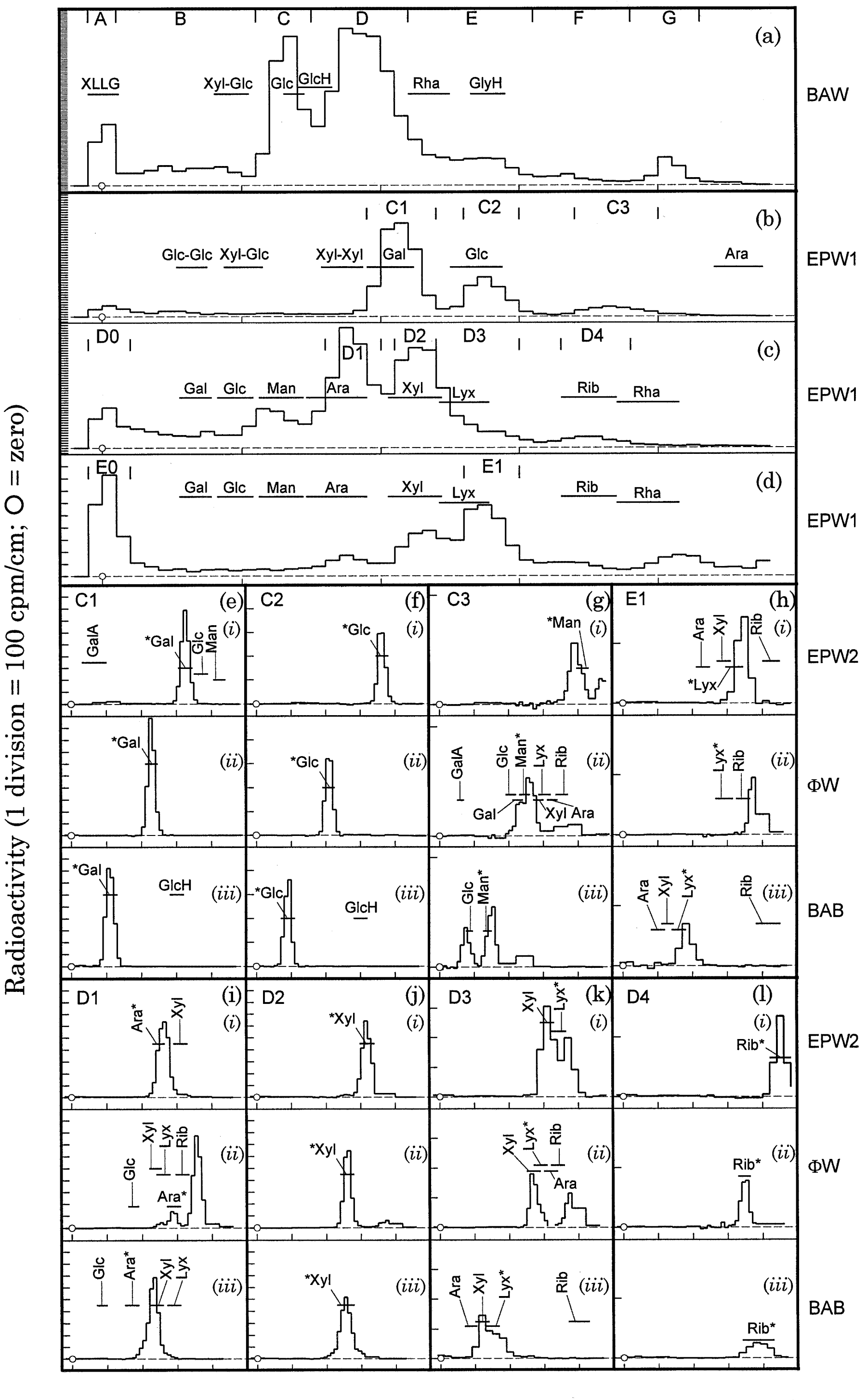


Fig. 3. Paper chromatography of acid hydrolysis products of NaB^{3}H$_4$-reduced, •OH-treated xyloglucan. The profiles show paper chromatograms of: (a) total hydrolysate in BAW; (b) zone C in EPW1; (c) zone D in EPW1; (d) zone E in EPW1; (e) zone C1 in (i) EPW2, (ii) ΦW and (iii) BAB; (f) zone C2 in (i) EPW2, (ii) ΦW and (iii) BAB; (g) zone C3 in (i) EPW2, (ii) ΦW and (iii) BAB; (h) zone E1 in (i) EPW2, (ii) ΦW and (iii) BAB; (i) zone D1 in (i) EPW2, (ii) ΦW and (iii) BAB; (j) zone D2 in (i) EPW2, (ii) ΦW and (iii) BAB; (k) zone D3 in (i) EPW2, (ii) ΦW and (iii) BAB; (l) zone D4 in (i) EPW2, (ii) ΦW and (iii) BAB. (—) Horizontal bars indicate the positions of authentic markers; those marked '*' were internal markers. (|) Vertical bars delineate the strips that were pooled for further analysis. (○ – – –) indicates the origin of each chromatogram and the background count. Non-standard abbreviations used are: Glc–Glc, cellobiose [glucosyl-β-(1→4)-glucose]; GlcH, glucitol; GlyH, glycerol; Lyx, lyxose; Rha, rhamnose; XLLG, xyloglucan nonasaccharide (Glc$_4$·Xyl$_3$·Gal$_2$); Xyl–Glc, isoprimeverose [xylosyl-α-(1→6)-glucose]; Xyl–Xyl, xylobiose [xylosyl-β-(1→4)-xylose].

with mannose (Fig. 3(c)), but this was not analysed further.

Elution of E (which in BAW approximately co-migrated with glycerol; Fig. 3(a)) and re-chromatography in EPW1 for 24 h resolved at least six components (Fig. 3(d)). E0 (R_f 0.00 in EPW1) was shown by electrophoresis at pH 3.5 to consist of several anionic compounds (Fig. 4(b)), and E1 appeared to consist of a single neutral compound which was well resolved from both ribose and lyxose (Fig. 3(h)).

The very high-R_f material in fraction G (Fig. 3(a)) was found to dissolve in scintillation fluid and was thus much more hydrophobic than most sugars.

Acid-stability of authentic monosaccharides.—To investigate the reliability of hydrolysis in 2 M TFA for the analysis of monosaccharides described above, we treated individually each of the four D-aldopentoses and eight D-aldohexoses with hot acid under the conditions used for the ^{3}H-polymer, and monitored the recovery of these monosaccharides semi-quantitatively by paper chromatography in BAW. Recovery was acceptable (> 75%) for ribose, arabinose, xylose, glucose, mannose and galactose, less efficient (25–75%) for lyxose, allose, altrose and talose, and poor (6–25%) for gulose and idose. Thus, the methodology would have underestimated the yield of some of the more exotic monosaccharide residues that may have been generated in xyloglucan by NaB^{3}H$_4$-reduction of $^{\bullet}$OH-generated oxo groups. In addition, all eight aldohexoses, but especially allose, gulose and talose, yielded a by-product (R_f 0.85; stainable by either aniline hydrogen-phthalate or AgNO$_3$), which may have accounted for ^{3}H peak G (Fig. 3(a)). Some of the aldohexoses also gave AgNO$_3$-stainable spots of intermediate R_f (especially allose and gulose, which gave spots of R_f 0.52 and 0.45, respectively), which were probably anhydroaldoses and may have contributed to ^{3}H zones D and E (Fig. 3(a)).

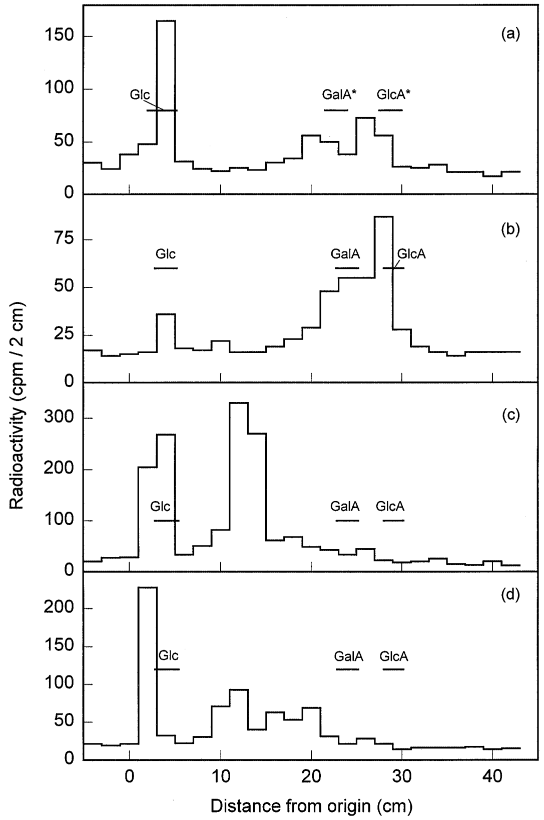


Fig. 4. Paper electrophoresis of acid- and Driselase-hydrolysis products of NaB^{3}H$_4$-reduced, $^{\bullet}$OH-treated xyloglucan. The profiles show paper electrophoretograms (pH 3.5) of: (a) zone D0 (see Fig. 3(c)); (b) zone E0 (see Fig. 3(d)); (c) zone I0 (see Fig. 5(b)); (d) zone J0 (see Fig. 5(c)). (—) Horizontal bars indicate the positions of authentic markers; those marked '*' were internal markers.

Mono- and disaccharides released by Driselase digestion of NaB^{3}H$_4$-reduced, $^{\bullet}$OH-treated xyloglucan.—The material released by Driselase digestion of the ^{3}H-polymer migrated in BAW as a series of peaks (H–K; Fig. 5(a)). About 90% had $R_f > 0.00$, indicating efficient digestion; 38% of the ^{3}H appeared to be associated with oligosaccharides of degree of polymerisation (DP) > 2 (mobile, but lower R_f than the disaccharide, isoprimeverose), 24% co-migrated with isoprimeverose and 25% ran in the hexose zone (Gal, Glc); only 3.6% of the ^{3}H co-migrated with free pentoses (Ara, Xyl, Lyx).

Re-chromatography of peak 'I' in EPW1 for 72 h resolved two main components (Fig. 5(b)). I0 had R_f 0.00 and was thus thought to be acidic. Paper electrophoresis showed that I0 was principally an anionic compound with an electrophoretic mobility (corrected for electro-endo-osmosis) about half that of galacturonic acid (Fig. 4(c)); it is thus likely to be a disaccharide with one neutral and one acidic group. Peak I1, the major fraction of I, co-migrated in EPW1 with isoprimeverose (Fig. 5(b)). Upon re-chromatography in three fur-

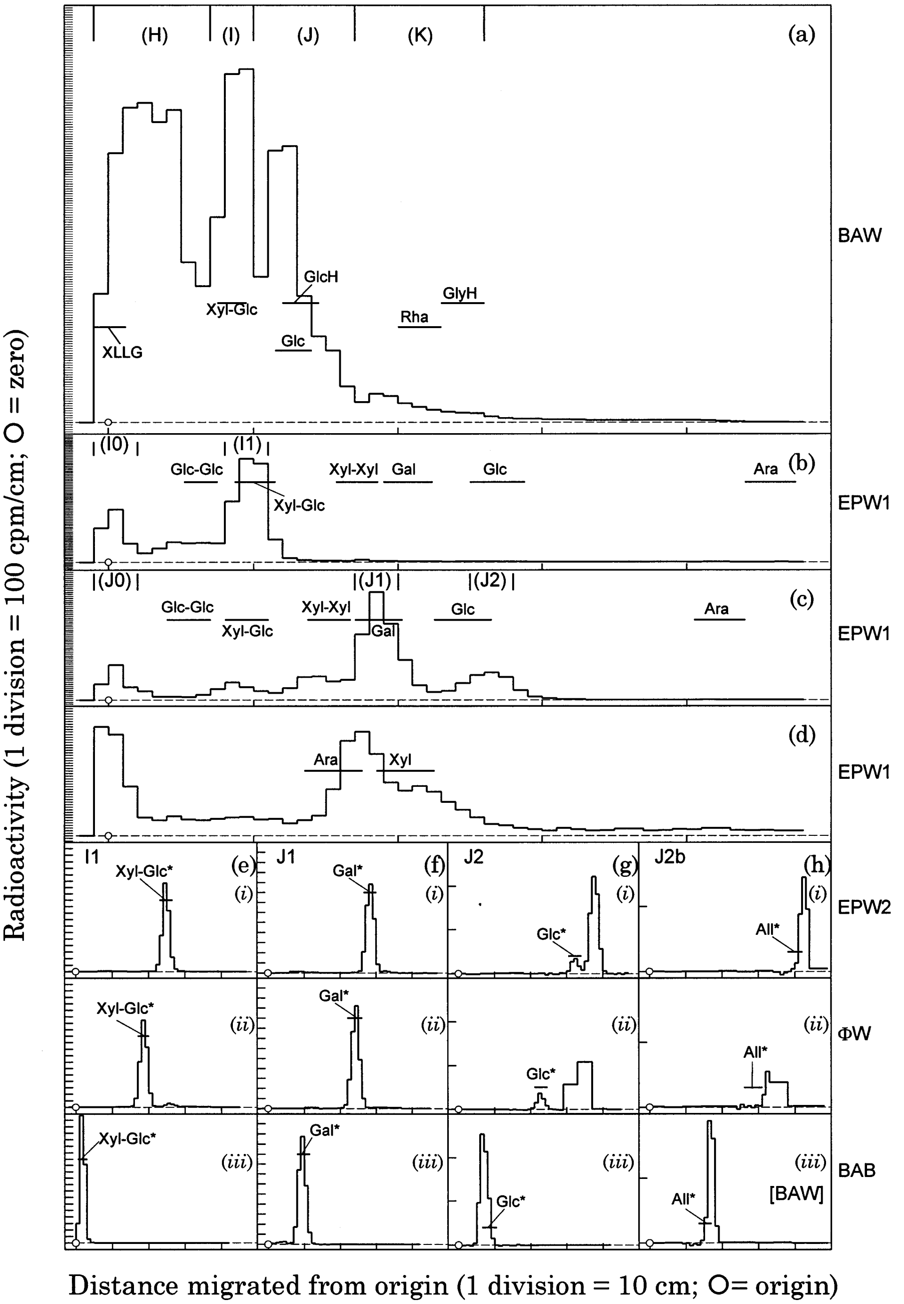


Fig. 5. Paper chromatography of the low-M_r products of Driselase digestion of NaB^{3}H$_4$-reduced, •OH-treated xyloglucan. The profiles show paper chromatograms of: (a) total digest in BAW; (b) zone I in EPW1; (c) zone J in EPW1; (d) zone K in EPW1; (e) zone I1 in (i) EPW2, (ii) ΦW and (iii) BAB; (f) zone J1 in (i) EPW2, (ii) ΦW and (iii) BAB; (g) zone J2 in (i) EPW2, (ii) ΦW and (iii) BAB; (h) zone J2b [= the major peak from Fig. g(i)] in (i) EPW2, (ii) ΦW and (iii) BAW (sic). (—) Horizontal bars indicate the positions of authentic markers; those marked '*' were internal markers. (|) Vertical bars delineate the strips that were pooled for further analysis. (○ – – –) indicates the origin of each chromatogram and the background count. Non-standard abbreviations used are as in Fig. 3 and All, allose.

ther solvent systems, the large majority of the ^{3}H in I1 exactly co-migrated with an internal marker of authentic isoprimeverose (Fig. 5(e)). Acid hydrolysis of I1 followed by re-chromatography in EPW1 yielded [^{3}H]xylose and [^{3}H]glucose in the ratio 25:1 (Fig. 6); thus, I1 was predominantly [*Xyl*-^{3}H]isoprimeverose and contained very little [*Glc*-^{3}H]isoprimeverose.

Re-chromatography of J in EPW1 for 72 h resolved five main components (Fig. 5(c)). The immobile J0 consisted of several acidic compounds (Fig. 4(d)). The major peak, J1, co-migrated with galactose, not only in EPW1 (Fig. 5(c)) but also with an internal marker in three other solvent systems (Fig. 5(f)). Peak J2, which migrated with glucose in EPW1 (Fig. 5(c)), was shown by PC in EPW2 and in ΦW to consist of [^{3}H]glucose and a ^{3}H-unknown in the ratio of about 1:7 (Fig. 5(g, i and ii)). The unknown migrated only slightly slower than a glucose internal marker in BAB (Fig. 5(g, iii)). The purified ^{3}H-unknown, eluted from the chromatogram shown in Fig. 5(g, i), was clearly resolvable from internal marker authentic allose (Fig. 5(h)) and also from glucitol, galactitol and mannose (data not shown).

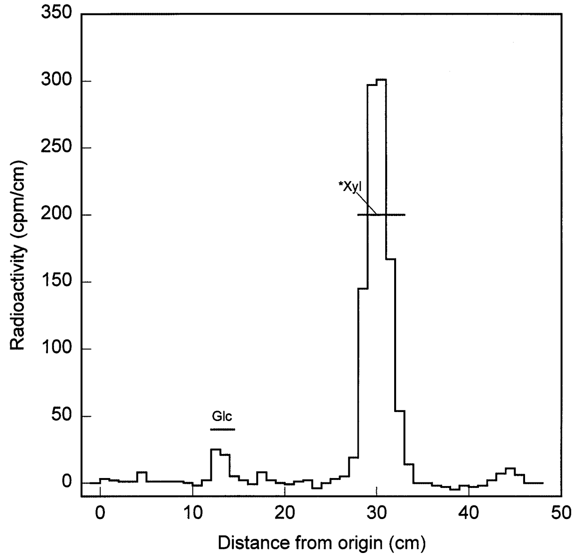


Fig. 6. ^{3}H-Monosaccharide composition of disaccharide I1. Compound I1 (produced by Driselase digestion of [^{3}H]xyloglucan) was eluted from the paper (see Fig. 5(e, ii)) and then subjected to acid hydrolysis. Paper chromatography of the ^{3}H-monosaccharides produced was in EPW1; the xylose marker was internal.

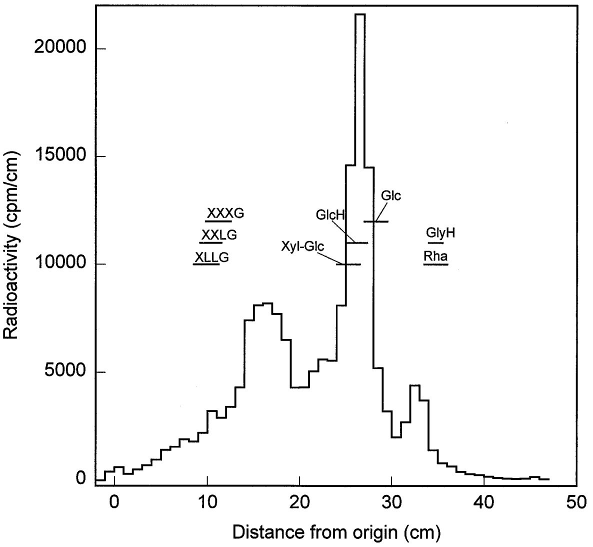


Fig. 7. Paper chromatography of the oligosaccharide products of Driselase digestion of NaB^{3}H$_4$-reduced, •OH-treated xyloglucan. The chromatography solvent was BPW; markers were external. Non-standard abbreviations used are as in Fig. 3.

Re-chromatography of K in EPW1 for 24 h resolved three main components: one immobile (presumably acidic) and two which migrated near external marker pentoses (Fig. 5(d)) but were not definitively identified.

Larger oligosaccharides released by Driselase digestion of NaB^{3}H$_4$-reduced, •OH-treated xyloglucan.—When the Driselase hydrolysate was chromatographed in BPW to provide better resolution of large oligosaccharide products (Fig. 7), 48% of the ^{3}H-products migrated as oligosaccharides of DP ~3–10 and only 0.5% had $R_f = 0.00$. Thus, Driselase was able to convert essentially all the [^{3}H]xyloglucan into chromatographically mobile digestion products, only about half of which were mono- and disaccharides. This contrasts with non-radioactive tamarind xyloglucan, which is almost completely Driselase-digestible to isoprimeverose and monosaccharides (Gal > Glc ≫ Ara). The results indicate that a substantial proportion of the ^{3}H was associated with structurally unusual (thus not completely Driselase-digestible) components of the xyloglucan.

Two-dimensional chromatography as a diagnostic tool for •OH-induced damage.—Two-dimensional chromatography of the products formed from tamarind xyloglucan (treated with, sequentially, •OH, NaB^{3}H$_4$ and TFA; Experiment 2) showed five intense spots and seven weak spots (Fig. 8(a)). By comparison with reference data (Table 1) and more precisely the positions of the internal markers on the same chromatogram stained after fluorography (Fig. 8(c)), three of the major spots were Glc/Gal (not completely resolved from each other; spot **8**), Man (spot **9**) and Xyl (**13**). The two other major ^{3}H-spots (**12**, **14**) were located close to (but resolved from) fucose and may have been anhydroaldoses.[36] The minor spots included ribose (**15**), probably arabinose (**11**) and an acidic compound (**7**;

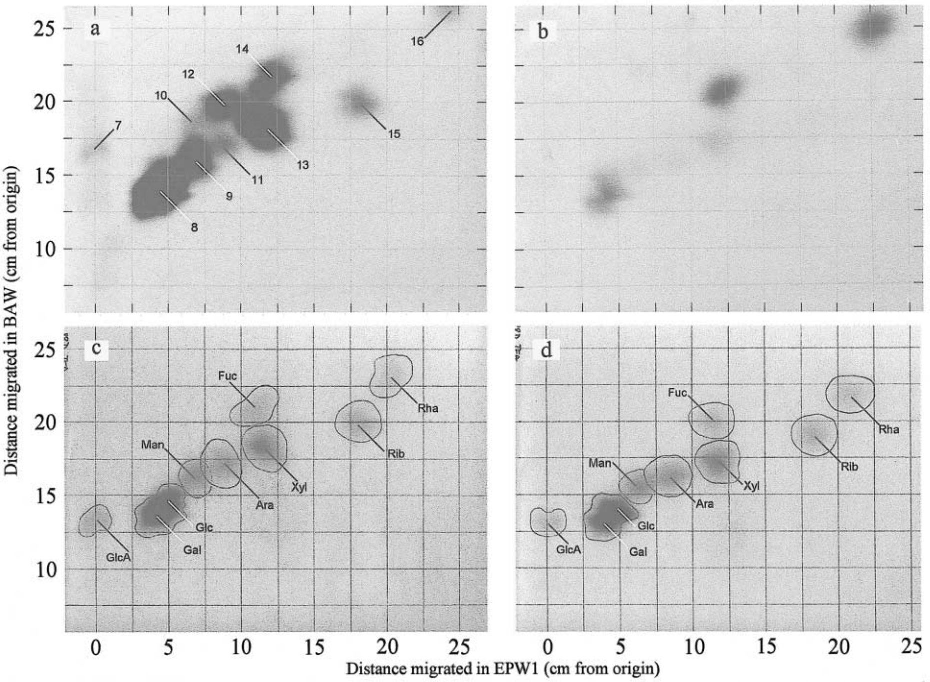


Fig. 8. Two-dimensional chromatography of acid hydrolysis products of NaB^{3}H$_4$-reduced, $^{\bullet}$OH-treated xyloglucan. Tamarind xyloglucan was treated with, sequentially, $^{\bullet}$OH, NaB^{3}H$_4$ and TFA, and the products were subjected to two-dimensional chromatography (BAW, bottom to top; EPW1, left to right) and fluorographed (a). A control omitting the $^{\bullet}$OH step is shown in (b); the mass of hydrolysate loaded, the exposure time and the scanning conditions were identical to those in (a). Panels (c) and (d) show the corresponding internal markers, stained after the fluorography. The grid shows distance migrated in each solvent (cm from origin).

high mobility in BAW, R_f 0.0 in EPW1) and three spots (**16** and two others; not shown) with high R_f values in both solvent systems. The $^{\bullet}$OH-untreated control (Fig. 8(b)) showed three moderately intense spots of ^{3}H, one of which corresponded to Glc/Gal, the others being unidentified; in addition, there were three very weak spots: xylose, mannose and a third that was slightly resolved from arabinose.

The Glc/Gal spot was eluted and re-chromatographed: it contained roughly equal amounts of [^{3}H]glucose and [^{3}H]galactose in both the $^{\bullet}$OH-untreated (Fig. 9(a)) and -treated sample (Fig. 9(b)). It is unclear why the [^{3}H]Glc to [^{3}H]Gal ratio was higher in Experiment 2 than in Experiment 1; the difference in pH during the $^{\bullet}$OH-treatment is one possible explanation.

The $^{\bullet}$OH-untreated sample revealed a shoulder tentatively identified as [^{3}H]glucitol, which would be derived from the reducing terminus of the xyloglucan chain. This small shoulder would have been masked by the large peak of [^{3}H]glucose present in the $^{\bullet}$OH-treated sample.

In conclusion, the most diagnostic TFA-generated ^{3}H spots by which to recognise $^{\bullet}$OH-degraded xyloglucan on a 2-dimensional chromatogram were spot **12** (fairly intense after $^{\bullet}$OH treatment, apparently absent without $^{\bullet}$OH; unidentified), xylose (intense with, very faint without $^{\bullet}$OH), spot **7** (weak with, apparently absent without $^{\bullet}$OH; unidentified) and ribose (spot **15**; weak with, apparently absent without $^{\bullet}$OH).

Cellulase hydrolysis products.—Cellulase was able to digest the majority of the NaB^{3}H$_4$-reduced, $^{\bullet}$OH-treated xyloglucan to produce a peak that migrated in the region occupied by the characteristic Glc$_4$-based oligosaccharides, XXXG, XXLG and XLLG (incompletely resolved from each other; Fig. 10). Thus, the presence of the diverse, ^{3}H-residues within the $^{\bullet}$OH-treated xyloglucan did not prevent cellulase from fragmenting the xyloglucan in the normal way. This pattern of radioactive products would be highly diagnostic of $^{\bullet}$OH-cleaved xyloglucan.

4. Discussion

Our evidence that ester and oxo groups were introduced into xyloglucan after attack by •OH radicals in an aerobic environment supports, in the case of xyloglucan, the type of mechanism proposed[30] for the action of •OH radicals on certain other polysaccharides. Three main consequences of attack by •OH + O_2 may be distinguished, all of which are of physiological significance: (a) scission of glycosidic bonds, which could contribute directly to cell wall loosening; (b) introduction, into the backbone, of ester bonds which, while not immediately leading to chain scission, could potentially provide a novel substrate for specific wall-bound esterases; and (c) introduction of relatively stable oxo groups which would not lead to chain scission but would provide a diagnostic structural feature by which •OH-attacked xyloglucan could be 'fingerprinted'.

When xyloglucan was pre-treated with •OH and NaB^3H_4 the products subsequently isolated from it by hydrolysis were diverse. ^{3}H-Alditols, if present, were extremely minor; therefore the introduction of radioactivity was not principally at the polysaccharide's reducing terminus. Positive identifications included (after acid hydrolysis; Fig. 3) xylose (10.4%), galactose (9.1%), glucose (3.8%) and ribose (1.3%), and (after Driselase hydrolysis; Fig. 5) α-D-[^{3}H]Xyl*p*-(1 → 6)-D-Glc (9.4%), α-D-Xyl*p*-(1 → 6)-D-[^{3}H]Glc (0.38%), galactose (7.4%) and glucose (0.29%). [^{3}H]Mannose was probably also present in the acid hydrolysate (Figs. 3(c) and 8(a)).

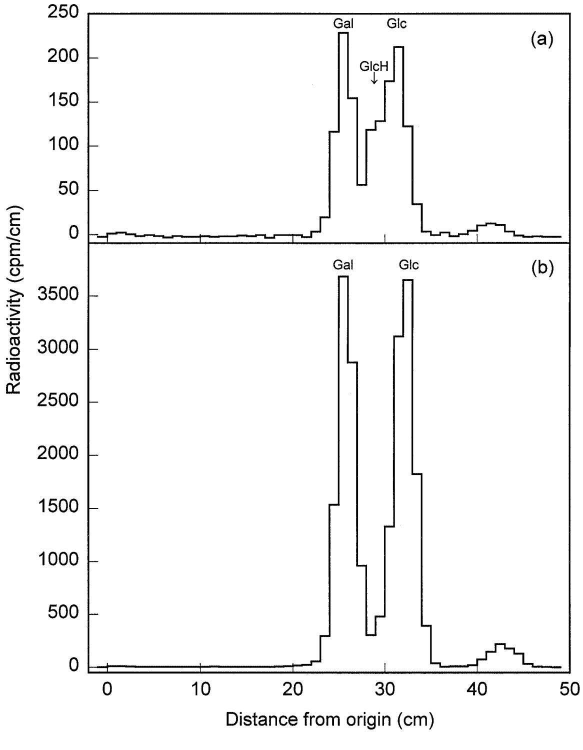


Fig. 9. Re-chromatography of the major ^{3}H-hexose spots released by acid hydrolysis. The 'Glc/Gal' spots from chromatograms identical to those shown in Fig. 8(a and b) (but not stained to reveal the internal markers) were eluted and re-chromatographed in EPW1 for 72 h. GlcH, glucitol.

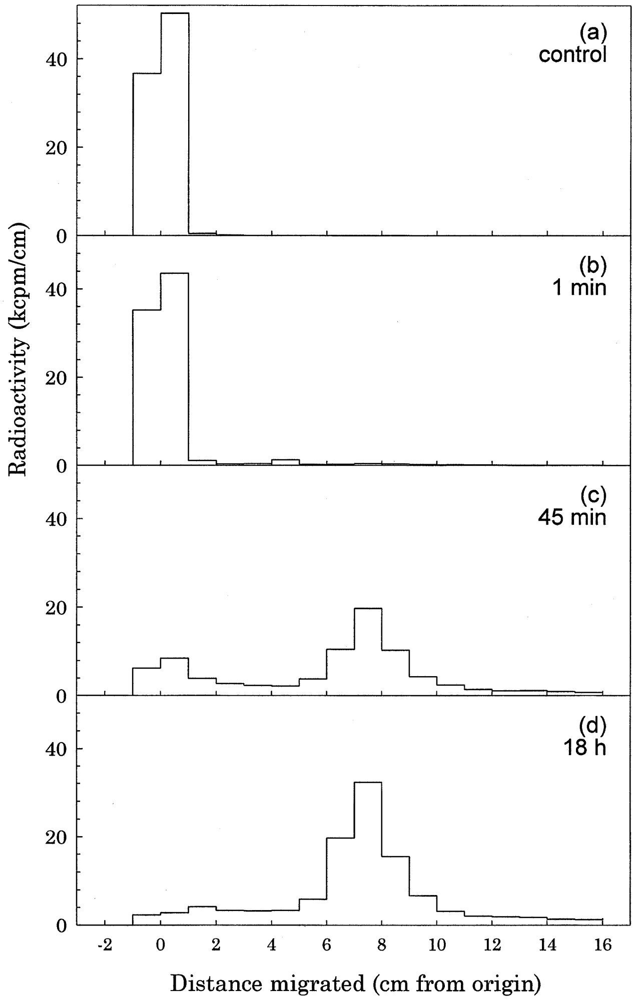


Fig. 10. Cellulase digestion products of ^{3}H-xyloglucan. Tamarind xyloglucan was treated with, sequentially, •OH, NaB^3H_4 and cellulase, and the products were chromatographed in EAW. The incubation in the presence of cellulase was for (b) 1 min; (c) 45 min or (d) 18 h. The control (a) was not treated with cellulase.

The [^{3}H]Gal would be produced when the Gal side-chains of xyloglucan were oxidised by •OH attack, followed by addition of O_2 and elimination of $HO_2^•$, to form various oxo derivatives (galactos-2-ulose, galactos-3-ulose, galactos-4-ulose and dialdehydogalactose residues). These modified residues would be reduced by NaB^3H_4 to [^{3}H]Gal and smaller amounts of various epimers of [^{3}H]Gal depending on which specific H atom was initially abstracted by the $^\bullet$OH radical ([^{3}H]talose, [^{3}H]gulose and [^{3}H]glucose, from galactos-2-ulose, -3-ulose and -4-ulose, respectively). The abundance of [^{3}H]Gal in the 'fingerprint' suggests that the side-chains of xyloglucan are particularly vulnerable to $^\bullet$OH attack. It is less likely that [^{3}H]Gal would arise following abstraction by $^\bullet$OH of H-4 from a Glc residue in the xyloglucan backbone since this is a reaction predicted to lead to backbone scission, producing a sugar derivative which would not be easily recognised.[30]

A second major product of Driselase digestion was [*xylosyl*-^{3}H]isoprimeverose {= α-D-[^{3}H]Xyl*p*-(1 → 6)-D-Glc}. The fact that the Xyl residue of isoprimeverose had a 25 × higher specific radioactivity than the Glc moiety may indicate that the Xyl residues of xyloglucan are more accessible to $^\bullet$OH radicals than are the Glc residues of the backbone. Small differences in $^\bullet$OH-susceptibility between the individual carbon atoms of free glucose have been reported (C atoms 1–6 are attacked in the approximate ratio 4:4:2:2:3:6;[29]). However, the strong preference for xylose over glucose residues reported here suggests a large difference in accessibility to $^\bullet$OH in xyloglucan.

An aim of the present work was to develop a protocol that will allow future analyses of the xyloglucan of plant cell walls for the symptoms of possible in-vivo $^\bullet$OH-attack. The individual ^{3}H-aldoses detected would not permit the unambiguous identification of an attacked polysaccharide as xyloglucan rather than some other Driselase-digestible wall polysaccharide. However, [^{3}H]isoprimeverose appears to be a unique, diagnostic fragment by which xyloglucan can be distinguished from all other known components of the plant cell wall. The most promising, xyloglucan-specific protocol would appear to involve NaB^3H_4 reduction of the xyloglucan followed by digestion with cellulase, which yields a range of oligosaccharides (typically DP 7–9). The presence of modified sugar residues within the oligosaccharide structure clearly does not prevent the normal action of cellulase on xyloglucan. The ^{3}H-oligosaccharides thus generated are amenable to further fingerprinting by acid or Driselase digestion so that the diagnostic products formed after $^\bullet$OH attack on xyloglucan (especially ribose and the unidentified spots **7** and **12**) can be detected and distinguished from the minor ^{3}H-products formed from $^\bullet$OH-untreated control xyloglucan.

It is still unclear whether $^\bullet$OH-attack is a physiologically significant mechanism of wall loosening additional to the more widely discussed protein-mediated mechanisms. Until now it has been difficult to test such hypotheses because no suitable method existed for identifying polysaccharides that had been attacked by $^\bullet$OH radicals. The present work characterises the damage done to xyloglucan by $^\bullet$OH-attack in vitro and provides a relatively simple means by which to search for evidence of endogenous $^\bullet$OH-action in the plant cell wall.

Acknowledgements

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References

1. Fry, S. C. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1995**, *46*, 497–520.
2. Cosgrove, D. J. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1999**, *50*, 391–417.
3. Miller, A. R. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 238–244.
4. Uchiyama, H.; Dobashi, Y.; Ohkouchi, K.; Nagasawa, K. *J. Biol. Chem.* **1990**, *265*, 7753–7759.
5. Hawkins, C. L.; Davies, M. J. *Free Radical Biol. Med.* **1996**, *21*, 275–290.
6. Tanioka, S.; Matsui, Y.; Irie, T.; Tanigawa, T.; Tanaka, Y.; Shibata, H.; Sawa, Y.; Kono, Y. *Biosci. Biotech. Biochem.* **1996**, *60*, 2001–2004.
7. Crescenzi, V.; Belardinelli, M.; Rinaldi, C. *J. Carbohydr. Chem.* **1977**, *16*, 561–572.
8. Fry, S. C. *Biochem. J.* **1998**, *332*, 507–515.
9. Tabbi, G.; Fry, S. C.; Bonomo, R. P. *J. Inorg. Biochem.*, in press.
10. von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor and Francis: London, 1987.

11. Grimes, H. D.; Perkins, K. K.; Boss, W. F. *Plant Physiol.* **1983**, *72*, 1016–1020.
12. Halliwell, B.; Gutteridge, J. M. C. *Meth. Enzymol.* **1990**, *186*, 1–85.
13. Kuchitsu, K.; Kosaka, H.; Shiga, T.; Shibuya, N. *Protoplasma* **1995**, *188*, 138–142.
14. Walker, C. D.; Webb, J. In *Copper in Soils and Plants*; Loneragan, J. F.; Robson, A. D.; Graham, R. D., Eds.; Academic: Sydney, 1981; pp. 189–212.
15. Takahama, U. *Plant Cell. Physiol.* **1994**, *35*, 975–978.
16. Polle, A.; Wieser, G.; Havranek, W. M. *Plant Cell. Environ.* **1995**, *18*, 681–688.
17. Kaur-Sawhney, R.; Flores, H. E.; Galston, A. W. *Plant Physiol.* **1981**, *68*, 494–498.
18. Lane, B. G.; Dunwell, J. M.; Ray, J. A.; Schmitt, M. R.; Cuming, A. C. *J. Biol. Chem.* **1993**, *268*, 12239–12242.
19. Bolwell, G. P.; Butt, V. S.; Davies, D. R.; Zimmerlin, A. *Free Radical Res.* **1995**, *23*, 517–532.
20. Yang, J. C.; Loewus, F. A. *Plant Physiol.* **1975**, *56*, 283–285.
21. Vallée, J. C.; Vansuyt, G.; Negrel, J.; Pedrizet, E.; Prevost, J. *Physiol. Plant* **1983**, *57*, 143–148.
22. Morré, D. J.; Brightman, A. O.; Hidalgo, A.; Navas, P. *Plant Physiol.* **1995**, *107*, 1285–1291.
23. Wojtaszek, P. *Biochem. J.* **1997**, *322*, 681–692.
24. Olson, P. D.; Varner, J. E. *Plant J.* **1993**, *4*, 887–892.
25. Frahry, G.; Schopfer, P. *Physiol. Plant* **1998**, *103*, 395–404.
26. Chen, S. X.; Schopfer, P. *Eur. J. Biochem.* **1999**, *260*, 726–735.
27. Wiseman, H.; Halliwell, B. *Biochem. J.* **1996**, *313*, 17–29.
28. Buxton, G. V.; Greenstock, C. L.; Helman, W. P.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1988**, *17*, 513–817.
29. Schuchmann, M. N.; von Sonntag, C. *J. Chem. Soc. Perkin Trans. II* **1977**, 1958–1963.
30. Schuchmann, M. N.; von Sonntag, C. *Int. J. Radiat. Biol.* **1978**, *34*, 397–400.
31. Fry, S. C. *Physiol. Plant* **1989**, *75*, 532–536.
32. Fry, S. C.; York, W. S.; Albersheim, P.; Darvill, A.; Hayashi, T.; Joseleau, J.-P.; Kato, Y.; Lorences, E. P.; Maclachlan, G. A.; McNeil, M.; Mort, A. J.; Reid, J. S. G.; Seitz, H. U.; Selvendran, R. R.; Voragen, A. G. J.; White, A. R. *Physiol. Plant* **1993**, *89*, 1–3.
33. Fry, S. C. *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*; Blackburn Press: New Jersey, 2000, Reprint edition.
34. Wright, K.; Northcote, D. H. *Phytochemistry* **1975**, *14*, 1793–1798.
35. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
36. Collins, P. M.; Ferrier, R. J. *Monosaccharides: Their Chemistry and Their Roles in Natural Products*; Wiley: Chichester, 1995; p. 89.