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Pectic polysaccharides are attacked by hydroxyl radicals in ripening fruit: evidence from a fluorescent fingerprinting method

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• Background and aims Many fruits soften during ripening, which is important commercially and in rendering the fruit attractive to seed-dispersing animals. Cell-wall polysaccharide hydrolases may contribute to softening, but sometimes appear to be absent. An alternative hypothesis is that hydroxyl radicals (·OH) non-enzymically cleave wall polysaccharides. We evaluated this hypothesis by using a new fluorescent labelling procedure to ‘fingerprint’ ·OH-attacked polysaccharides.
• Methods We tagged fruit polysaccharides with 2-(isopropylamino)-acridone (pAMAC) groups to detect (a) any mid-chain glycosulose residues formed in vivo during ·OH action and (b) the conventional reducing termini. The pAMAC-labelled pectins were digested with Driselase, and the products resolved by high-voltage electrophoresis and high-pressure liquid chromatography.
• Key Results Strawberry, pear, mango, banana, apple, avocado, Arbutus unedo, plum and nectarine pectins all yielded several pAMAC-labelled products. GaLA–pAMAC (monomeric galacturonate, labelled with pAMAC at carbon-1) was produced in all species, usually increasing during fruit softening. The six true fruits also gave pAMAC-UA-GaLA disaccharides (where pAMAC-UA is an unspecified uronate, labelled at a position other than carbon-1), with yields increasing during softening. Among false fruits, apple and strawberry gave little pAMAC-UA-GaLA; pear produced it transiently.
• Conclusions GaLA–pAMAC arises from pectic reducing termini, formed by any of three proposed chain-cleaving agents (·OH, endopolygalacturonase and pectate lyase), any of which could cause its ripening-related increase. In contrast, pAMAC-UA-GaLA conjugates are diagnostic of mid-chain oxidation of pectins by ·OH. The evidence shows that ·OH radicals do indeed attack fruit cell wall polysaccharides non-enzymically during softening in vivo. This applies much more prominently to drupes and berries (true fruits) than to false fruits (swollen receptacles). ·OH radical attack on polysaccharides is thus predominantly a feature of ovary-wall tissue.

Key words: Fruit, ripening, cell wall, pectic polysaccharides, hydroxyl radicals, non-enzymic scission, fluorescent labelling, fingerprint compounds.

INTRODUCTION

Ripening: hydrolytic vs. oxidative

Fruit ripening is often accompanied by changes in flavour, odour, colour and texture which are attractive to the animals that will disperse the seeds. In particular, many berries, drupes and pomes soften during ripening owing to changes in cell wall organization. Plant cell walls are complex networks based on cellulose microfibrils, partly tethered by hemicelluloses, with pectic polysaccharides infiltrating the rest of the wall matrix (Albersheim et al., 2010; Fry, 2011a). During fruit softening, the matrix polysaccharides, especially the pectins, often become more readily extractable and/or decrease in molecular weight, indicating depolymerization, e.g. in avocado, plum, mango, banana and tomato (Huber and O’Donoghue, 1993; Prasanna et al., 2003; Ali et al., 2004; Ponce et al., 2010; Basanta et al., 2014). The importance of pectic depolymerization has led to the widely held view that ripening can be regarded as principally a ‘hydrolytic’ process.

Earlier, however, Blackman and Parija (1928) had suggested that ripening involves a loss of ‘organisational resistance’, i.e. fruit cells lose the ability to maintain separate compartments owing to cellular (membrane) degeneration. Although this concept lost popularity, some workers continued to interpret ripening as a form of senescence attributable to oxidation reactions (Brenman and Frenkel, 1977) and more recently to emphasize the (possibly related) decrease in water content that occurs near the onset of ripening (Frenkel and Hartman, 2012). There are indeed similarities between physiological changes (e.g. chlorophyll loss and membrane permeabilization) occurring in a ripening fruit and in a leaf or petal approaching abscission. Lipooxygenases, which often increase during ripening (Ealing, 1994; de Gregorio et al., 2000), generate hydroperoxide groups (>CH–OOH) in unsaturated fatty acid residues, accompanied...
by the formation of reactive oxygen species (ROS). Such lipid oxidation may permeabilize membranes, resulting in the release of certain metabolites, e.g. ascorbate (Dumville and Fry, 2003), into the apoplast (the aqueous solution that bathes the cell wall), and ROS by-products may drive other oxidative reactions.

Viewing fruit ripening as an ‘oxidative’ process is supported by evidence from several quarters. For example, in avocado (Lauraceae; Meir et al., 1991) and serviceberry (Rosaceae; Rogiers et al., 1998), lipid peroxidation was the earliest symptom of ripening, and tomato (Solanaceae) fruit ripening was accompanied by elevated \( \text{H}_2\text{O}_2 \) and the oxidation of lipids and proteins (Jimenez et al., 2002). In the present study, we propose a link between oxidative agents (especially the hydroxyl radical, \( \cdot\text{OH} \)) and pectic polysaccharide degradation in softening fruit.

Wall turnover and enzymes

Primary cell walls control the texture of fruit tissues. Although strong enough to withstand turgor pressure, walls are dynamic structures in which the polymers can be remodelled or degraded, resulting in wall loosening. Many enzymes and expansins have been described that act on cell-wall polymers, and the expression of these proteins has been correlated with fruit softening as well as cell expansion and abscission (reviewed by Franková and Fry, 2013). For example, glycanases and transglycanases cleave cell wall polysaccharides in mid-chain (Taylor et al., 1993; Bewley, 1997; Lazan et al., 2004; Fry et al., 2008; Schröder et al., 2009; Franková and Fry, 2011; Derba-Maceluch et al., 2014), glycosidases release mono- or di-saccharides from non-reducing termini (Fanutti Derba-Maceluch, 2004; Harada et al., 2011), and expansins interfere in polysaccharide–polysaccharide hydrogen bonding (Cosgrove, 2000; Harada et al., 2011; Sasayama et al., 2011).

Several polymer-hydrolysing enzymes have been studied in relation to fruit softening, with tomato as the most extensively studied system (Matas et al., 2009; reviewed by Fry, 2017). Several wall polysaccharide-modifying enzyme activities increase, especially endo-polygalacturonase (endo-PG), cellulase, xyloglucan endotransglucosylase, \( \beta \)-galactosidase, pectin-methyl-esterase and pectate lyase. Although the link between wall-hydrolysing enzymes and fruit softening seems intuitive, tests of this as a functional relationship have often yielded contradictory evidence. A major focus has been endo-PG in tomato. This enzyme is abundant in ripe tomato fruit (Tucker and Grierson, 1990), and suppression of its expression resulted in reduced depolymerization of pectin (Smith et al., 1990). Also, expression of endo-PG in the \( \text{rin} \) (ripening inhibitor) mutant caused an increased degradation of fruit pectins (Giovannini et al., 1989). However, both these studies failed to show a related change in tomato fruit softening; no inhibition of softening was observed in endo-PG antisense fruit, and no effect on softening was induced by the expression of endo-PG in the \( \text{rin} \) mutant. Moreover, other fruits, e.g. strawberry (Pose et al., 2013), persimmon (Cutillas-Illuraide et al., 1993) and kiwifruit (Redgwell et al., 1991), show extensive pectin solubilization and/or a decrease in molecular weight even though they possess very low levels of endo-PG (e.g. Nogata et al., 1993). These observations reinforce the idea that endo-PG is not necessary for fruit softening. The proposed relationship between pectin depolymerization and fruit softening was thus not strongly supported by data, although the excessive softening associated with over-ripening can be prevented by knocking out endo-PG (in the ‘Flavr Savr’ tomato; reviewed by Krieger et al., 2008). Ripening is a robust phenomenon: knocking out any individual player (e.g. endo-PG) often fails to prevent normal softening.

\( \cdot\text{OH} \) cleaves polysaccharides in vitro

In addition to proteins that remodel the wall, the highly reactive hydroxyl radical (\( \cdot\text{OH} \)) can cause polysaccharide chain scission non-enzymically. This phenomenon is readily demonstrated in solutions of purified cell wall polysaccharides upon treatment with ascorbate in the presence of \( \text{O}_2 \) plus traces of \( \text{Cu}^{2+} \) or \( \text{Fe}^{3+} \) (Fry, 1998; Yamazaki et al., 2003; Schweikert et al., 2000, 2002) and in food-related systems (Fauré et al., 2012; Mikkinen et al., 2012; Iurlaro et al., 2014).

\( \cdot\text{OH} \) can also cleave insoluble polysaccharides that are present in situ as structural components of the cell wall: for example, when \( \cdot\text{OH} \) was generated within the cell walls of a frozen/thawed maize coleoptile that was being held under tension, the coleoptile extended in a fashion similar to that induced by certain wall-acting proteins or in response to \( \text{in-vivo} \) auxin treatment (Schopfer, 2002). Likewise, \( \text{in-vitro} \) \( \cdot\text{OH} \) treatment of fruit cell walls of tomato (Dumville and Fry, 2003), banana (Cheng et al., 2008b) and longan (Duan et al., 2011) promoted pectin solubilization and depolymerization.

Proposed beneficial roles of \( \cdot\text{OH} \)

These results indicate that \( \cdot\text{OH} \), if formed in the cell wall \( \text{in vivo} \), could potentially cleave polysaccharides and thereby exert physiological effects. Indeed, it has been suggested that wall loosening induced by ROS (especially \( \cdot\text{OH} \)) contributes to fruit ripening (Brennan and Frenkel, 1977; Fry, 1998; Fry et al., 2001; Dumville and Fry, 2003; Cheng et al., 2008a; Yang et al., 2008; Duan et al., 2011), germination (Müller et al., 2009), cell expansion (Schopfer, 2001, 2002; Rodriguez et al., 2002; Liszkay et al., 2004) and abscission (Sakamoto et al., 2008; Cohen et al., 2014). It is sometimes asserted that \( \cdot\text{OH} \), as a highly reactive ROS, must be biologically detrimental – for example causing mutations, membrane damage and protein denaturation – and that it would be advantageous for cells to prevent \( \cdot\text{OH} \) formation or to scavenge it. However, the half-life of \( \cdot\text{OH} \) within a cellular environment such as a cell wall is estimated at approx. 1 ns, allowing it to diffuse no more than approx. 1 nm (the length of two glucose residues in a cellulose chain) before reacting with some organic molecule (Griffiths and Lunec, 1996); a very short distance in the context of a primary cell wall, which is typically >80 nm thick. Therefore, if produced at an appropriate site (within the cell wall matrix or middle lamella), \( \cdot\text{OH} \) may have little effect on the protoplast. Moreover, in the case of a softening fruit pericarp, the cells involved are shortly destined to die in an animal’s gut, fulfilling their role in promoting seed dispersal. Therefore, any cellular damage caused to the ripe pericarp by \( \cdot\text{OH} \) is irrelevant; likewise in other short-lived tissues such as a lysing abscission zone or a rapidly expanding coleoptile.
How could apoplastic \textsuperscript{*}OH be made in vivo?

The production of \textsuperscript{*}OH in plant cell walls most probably involves a Fenton–like reaction, whereby a transition metal ion in the reduced state reacts with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}):

\[
\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{\textsuperscript{*}OH} + \text{OH}^-.
\]

Two proposals have been considered: (1) the transition metal is the Fe of the haem group in peroxidase, which can be reduced by superoxide in a Haber–Weiss cycle (Chen and Schopfer, 1999; Liszka et al., 2003); and (2) a wall-bound transition metal (Cu and/or Fe) ion is reduced by apoplastic electron donors such as ascorbate (Fry, 1998; Vreeburg and Fry, 2005; Green and Fry, 2005; Kärkönen and Fry, 2006; Lindsay and Fry, 2007; Padu et al., 2005). The H\textsubscript{2}O\textsubscript{2} may be generated by wall-bound oxidases (Lane et al., 1993; Ashtir et al., 2002; Kärkönen et al., 2009) or superoxide dismutase (Yim et al., 1990; Ogawa et al., 1996; Kukavica et al., 2009), or by non-enzymic reduction of O\textsubscript{2} by ascorbate (Fry, 1998). Indeed, Dunville and Fry (2003) showed that the ability of cells in a tomato fruit to secrete ascorbate, and also the tissue’s Cu content, increased during ripening: effects that would be expected to favour \textit{in-vivo} \textsuperscript{*}OH production.

Is apoplastic \textsuperscript{*}OH made in vivo?

Cheng et al. (2008a) showed that, when homogenates of frozen banana pulp harvested at different stages of ripening were incubated for 12 h in phosphate buffer containing deoxyribose, \textit{in-situ} generated \textsuperscript{*}OH (detected by its ability to oxidize the deoxyribose to dialdehyde products) increased in parallel with softening, suggesting that ripening may be associated with \textsuperscript{*}OH production in banana. Yang et al. (2008), who applied a similar method but with a shorter incubation period, also suggested that \textsuperscript{*}OH production increases prior to the initiation of banana fruit softening. However, in both these studies, the source of the \textsuperscript{*}OH in pulp was not clear and it could have been an artefact due to the homogenization, not reflecting reactions that occur \textit{in vivo}.

\textsuperscript{*}OH can cleave wall polysaccharides \textit{in vitro}, but the question of whether \textsuperscript{*}OH is produced in the apoplast of living tissue and actually acts \textit{in vivo} on wall polysaccharides in the manner proposed is still open, a major challenge being to detect such a short-lived free radical as \textsuperscript{*}OH in the walls of living cells. There are two possible experimental approaches: (1) infiltration into the apoplast of a membrane-impermeable ‘reporter’ compound that reacts with \textsuperscript{*}OH to give recognizable products (Kuchitsu et al., 1995; Fry et al., 2002; Schopfer et al., 2002; Miller and Fry, 2004; Müller et al., 2009; for a review, see Vreeburg and Fry, 2005); and (2) detection of the ‘collateral damage’ done to wall polysaccharides \textit{in vivo} when attacked by apoplastic \textsuperscript{*}OH.

The second approach is based on the fact that the \textsuperscript{*}OH radical cleaves polysaccharides by rather indiscriminate oxidative reactions. \textsuperscript{*}OH-driven polysaccharide scission, proposed to contribute to fruit softening, is accompanied by concurrent reactions that introduce relatively stable oxo groups into the polysaccharide (‘collateral damage’; Fig. 1A) without necessarily cleaving it (Zegota and von Sonntag, 1977; von Sonntag 1980; Vreeburg and Fry, 2005; Vreeburg et al., 2014). Such oxo groups can serve as a chemical ‘fingerprint’ revealing recent \textsuperscript{*}OH attack in the cell walls of living cells. A polysaccharide usually has only a single oxo group (its reducing terminus), but \textsuperscript{*}OH attack generates oxo groups in mid-chain sugar residues, converting them to glycosulose residues (Vreeburg et al., 2014). The proportion of such glycosulose residues (non-terminal oxo groups) per 1000 sugar residues would be a valuable measure of the extent of \textsuperscript{*}OH attack \textit{in vivo}. Two methods are currently available for their detection:

\textbf{Radiolabelling to detect glycosulose residues}. We have used reductive tritiation with NaB\textsubscript{3}H\textsubscript{4} to detect oxo group formation in Fenton-treated soluble polysaccharides \textit{in vitro} and in presumptively \textsuperscript{*}OH-exposed cell walls \textit{in vivo} in ripening pears, germinating cress seeds and elongating maize coleoptiles (Fry et al., 2001, 2002; Miller and Fry, 2001; Müller et al., 2009; Iurlaro et al., 2014). In this approach, each mid-chain glycosulose residue, formed by \textsuperscript{*}OH action, is reduced to yield a tritium-labelled simple sugar (aldose) residue – in some cases an aldose that does not frequently occur naturally. For example, \textsuperscript{*}OH attack at carbon-3 of a xylose residue in xyloglucan will introduce an oxo group which, when treated with NaB\textsubscript{3}H\textsubscript{4} and then acid-hydrolysed, yields a mixture of [\textsuperscript{3}H]xylose and [\textsuperscript{3}H]ribose (the 3-epimer of xylose); the latter is not a natural constituent of xyloglucan and is therefore a highly diagnostic fingerprint (Miller and Fry, 2001).

\textbf{Fluorescent labelling to detect glycosulose residues}. As an alternative to radiolabelling, we recently developed a method for fluorensently labelling mid-chain (\textsuperscript{*}OH generated) oxo groups present in polysaccharides by reductive amination with 2-aminoacridone (AMAC) plus NaCNBH\textsubscript{3}, followed by N-isopropylation, to introduce fluorescent 2-isopropylaminoacridone (pAMAC) groups into the polysaccharide (Fig. 1B). The method was developed by model experiments on soluble pectic polysaccharides \textit{in vitro} (Vreeburg et al., 2014). Upon subsequent Driselase digestion (Fig. 1C), the mid-chain glycosulose residues, indicative of recent \textsuperscript{*}OH attack, were released as various products, of particular diagnostic value being pAMAC-disaccharide conjugates. [Note on nomenclature: The designation ‘sugar–pAMAC’ implies that the pAMAC group is linked to the former reducing group (carbon-1 in the case of an aldose) of the sugar, whereas ‘pAMAC-sugar’ indicates that the pAMAC group is attached to a different carbon of the sugar, whose C-1 remains unlabelled.] In contrast, the single reducing terminal oxo group of a poly- or oligosaccharide was released as a monosaccharide–pAMAC product. We now report the application of the pAMAC/Driselase method to demonstrate the changing abundance of \textsuperscript{*}OH-attacked polysaccharides in the cell walls of various contrasting fruits during ripening to give an indication of the involvement of hydroxyl radical attack in fruit softening.

\textbf{MATERIALS AND METHODS}

\textbf{Materials}

2-Aminoacridone was from Fluka (Dorset, UK). Driselase, from Sigma-Aldrich (Dorset, UK), was purified by ammonium sulphate precipitation and gel-permeation chromatography (Fry, 2000). The Luna C\textsubscript{18} high-pressure liquid chromatography
A | B | C
---|---|---
Pectin (homogalacturonan) | Attack on pectin in vivo | In-vitro treatment with AMAC, NaCNBH₃, acetone
---|---|---
OH | | In-vitro Driselase digestion; NaOH
---|---|---
† | | GalA
---|---|---
pAMAC-UA₂ (electrophoresis spot 2A²) | GalA–pAMAC (electrophoresis spot 1A²)
---|---|---

**Key**
- α-β-Galacturonic acid residue, initially (1→4)-linked
- Any mid-chain-cleaving agent (endo-polygalacturonase, pectate lyase, hydroxyl radical)
- Hydroxyl radical (attacking polysaccharide without causing scission)
- Oxo group (ketone)
- Oxo group (aldehyde)
- Reducing terminal α-galacturonic acid moiety (open-chain form)
- Glocosulose (glycosulose-uronate) residue
- pAMAC moiety [2-(iso-propylamino)-acridone]
- Driselase-resistant glycosidic bond
- Indicates a bond between pAMAC and either of two epimers (e.g. galacturonic or guluronic acid)

**Fig. 1.** Schematic view of in-vivo attack on pectins and strategies used to detect it. (A) Part of a pectin (homogalacturonan) chain in the wall of a living fruit cell may be attacked either non-enzymically by a hydroxyl radical (*OH) or enzymically by endo-polygalacturonase or pectate lyase. Any of these three agents can cleave the backbone (e.g. at X), creating a new reducing terminus (shown in its non-cyclic form, and thus possessing an oxo group). In addition, *OH can non-enzymically abstract an H atom (e.g. from C-2 or C-3 of a GalA residue) without causing chain scission; in an aerobic environment, this initial reaction leads to the formation of a relatively stable glycosulose residue possessing a mid-chain oxo group. (B) Wall material (AIR) is treated in vitro with AMAC, NaCNBH₃ and acetone; oxo groups are reductively aminated to form yellow-green-fluorescing pAMAC conjugates. (C) The pAMAC-labelled homogalacturonan is then digested with Driselase, which hydrolyses all glycosidic bonds except any whose sugar residue carries a pAMAC group. The products tend to lactonize and are therefore briefly de-lactonized with NaOH before being fractionated. Further details of the reactions are given in figs 1 and 2 of Vreeburg et al. (2014).

**Preparation of authentic sugar–pAMAC markers**

Fluorescent markers for high-voltage paper electrophoresis (HVPE) and HPLC were prepared as before (Vreeburg et al., 2014). In brief, a reducing sugar (0.4 μmol of dry glucose, GalA, GalA₂, GalA₃ or GalA₄) was suspended in 40 μL of 0-1 M AMAC in dimethylsulphoxide (DMSO)/acetic acid/pyridine (17:2:1, by vol.) followed immediately by 40 μL of fresh aqueous 1 M NaCNBH₃. After incubation of the mixture at 20°C for 16 h, 2 μL of aceton and 40 μL of fresh 1 M NaCNBH₃ were added and the mixture was incubated for another 1 h at 20°C. The mixture was diluted with 5 vols of H₂O and centrifuged (14 000 rpm, 10 min). The sugar–pAMAC product in the supernatant was purified on a C₁₈ cartridge.

**Characterization of fruit softening and preparation of fruit AIR**

Except for strawberry and Arbutus, freshly purchased hard fruits were stored in the dark in a wooden cupboard at room
pAMAC labelling of fruit AIR

All AMAC work was done under subdued red light; ice-cold solvents were used for the washing and precipitation steps. A portion of fruit AIR (alcohol-insoluble residue) suspension (\(\approx 200 \, \text{mg fresh fruit tissue}\)) was thawed and centrifuged. The pellet was washed twice with 75 % ethanol, and resuspended in 261 \(\mu\text{L}\) of water containing 122 \(\mu\text{mol}\) AMAC and 61 \(\mu\text{mol}\) of DMSO containing 8 \(\mu\text{L}\) of 1 % partially purified NaCNBH3 (61 \(\mu\text{mol}\)) and an additional 122 \(\mu\text{mol}\) of fresh NaCNBH3 (61 \(\mu\text{L}\) of a 2 m aqueous solution) were added and the incubation was repeated for 16 h at 20 °C. To remove low molecular weight reagents and by-products, we added 1 mL of 96 % ethanol (to precipitate any water-soluble polysaccharides), pelleted the total polymers (at 12 000 \(\text{g}\) for 5 min), and washed the pellet twice with 1 mL of 75 % ethanol. The pellet was then re-suspended by shaking in 250 \(\mu\text{L}\) of pyridine/acetic acid/water (1:1:98 by vol.) for 10 min at 20 °C. The treatments with 96 and 75 % ethanol were repeated, and the final ethanolic pellet of pAMAC-labelled AIR was blotted to semi-dryness.

Driselase digestion

The blotted pellet of pAMAC-labelled AIR was de-lactonized with 100 \(\mu\text{L}\) of 0.5 M NaOH (50 \(\mu\text{mol}\)) for 5 h at 20 °C, then buffered to pH 4-7 with two molar equivalents (5-75 \(\mu\text{L}\)) of acetic acid, washed with ice-cold 80 % ethanol, pelleted at 12 000 \(\text{g}\) for 5 min, blotted with filter paper and immediately treated with Driselase. [The de-lactonization step facilitated subsequent Driselase digestion.] The blotted, de-lactonized pellet of pAMAC-labelled AIR (equivalent to 200 mg of fresh weight fruit) was digested in 500 \(\mu\text{L}\) of 1 % partially purified Driselase in pyridine/acetic acid/0.5 % chlorobutanol, 1:1:98, by vol.) at 37 °C for 14 d, after which the solution was frozen at −20 °C.

Purification of pAMAC-labelled products on a C18 column

A C18-silica cartridge (500 mg Supelco column; Sigma-Aldrich) was pre-conditioned with 2 vols of methanol then 2 vols of \(\text{H}_2\text{O}\). The soluble components of a whole Driselase digest (approx. 500 \(\mu\text{L}\)) were then loaded and the column was washed with 2 \(\times\) 2 mL of \(\text{H}_2\text{O}\), after which bound solutes were eluted with 2 \(\times\) 2 mL each of 10, 20, 30, 40 and 50 % (v/v) methanol. Each fraction was dried, redissolved in 50 \(\mu\text{L}\) of pyridine/acetic acid/water (1:1:98, by vol., \(\text{pH}\) approx. 4-7, containing 0.5 % chlorobutanol), and stored at −20 °C. Fractions exhibiting the characteristic yellow–green fluorescence of pAMAC groups were pooled for further analysis. Immediately before analysis by HVPE or HPLC, a portion was dried, de-lactonized in dilute NaOH (\(\text{pH} >11\)) at 20 °C for 10 min, and neutralized with acetic acid.

High-voltage paper electrophoresis

Electrophoresis was conducted on Whatman No. 1 or No. 3 paper in a pH 6.5 buffer (pyridine/acetic acid/water, 33:1:300 by vol.) at 4-0 kV for 45–50 min. The papers were cooled with toluene. Methods and apparatus are described by Fry (2011b). After electrophoresis, the papers were dried and viewed under a 254 nm UV lamp and fluorescence was recorded photographically (Camlab DocIt system with LabWorks 4.6 software). Fluorescent spots on paper electrophoretograms were quantified with Image J (http://rsbweb.nih.gov/ij/) as described in the Supplementary Data Fig. S3.

High-pressure liquid chromatography

The HPLC was conducted with a solvent flow rate of 1 mL min−1 at room temperature on a Luna C18 silica column with solvent A (500 mM acetic acid, adjusted to pH 5.0 with NaOH) and acetonitrile. All solvent compositions are given as percentage acetonitrile in solvent A, by vol. The column was pre-equilibrated for 30 min with 10 % acetonitrile. The injected sample (20 \(\mu\text{L}\)) was eluted with: 0–5 min, 10 % acetonitrile, isocratic; 5–15 min: 10–12.5 % acetonitrile, linear gradient; 15–30 min: 12.5 % acetonitrile, isocratic; 30–35 min, 12.5–15 % acetonitrile, linear gradient; 35–40 min, 15 % acetonitrile, isocratic; 40–50 min, 15–25 % acetonitrile, linear gradient; 50–60 min, 25–10 % acetonitrile, linear gradient; 60–65 min, 10 % acetonitrile, isocratic. A fluorescence detector (RF 2000, Dionex) used excitation and emission wavelengths of 442 and 520 nm, respectively.

RESULTS

Application of pAMAC labelling to ripening fruit

Fruit firmness at three empirically defined stages of softening (hard, medium and soft) was measured – in most cases with a
Analysis of total Driselase digestion products of pAMAC-labelled fruit cell walls

**Electrophoresis.** Electrophoresis of the Driselase digestion products of pAMAC-labelled AIR (after de-lactonization) revealed at least two interesting, yellow–green-fluorescing, negatively charged spots (Fig. 3): $1^F$, co-migrating with the labelled monomer GalA–pAMAC; and $2^F$, approximately co-migrating with the labelled dimer GalA$_2$–pAMAC. The anionic nature of $1^F$ and $2^F$ indicates that they were based on Driselase-digestible acidic sugar residues of the fruit cell walls, likely to be mainly GalA. Smaller amounts of putative acidic trimers were sometimes also observed, e.g. in avocado and *Arbutus*. An additional spot ($X^F$), which fluoresced a less yellowish green, was seen in some species, especially in unripe banana.

In addition, strongly yellow–green-fluorescing neutral spots were present in all species; these spots could be based on any neutral, Driselase-releasable cell wall sugar residues. They showed no obvious changes in intensity throughout ripening.

Confirmatory replicate and additional studies, based on essentially the same technique as used for Fig. 3, are shown in Supplementary Data Figs S1 and S2.

**Numerical data.** Table 1 provides a summary of the fluorescent signal of each spot at each stage of ripening for each of the eight fruit species considered. A blank denotes absence of a spot in all replicates.

Despite the approximate co-electrophoresis of $2^F$ with GalA$_2$–pAMAC, spot $2^F$ cannot have contained GalA$_2$–pAMAC itself since this compound is completely digested by Driselase under the conditions used. Instead, it is likely to have a constitution of the type pAMAC-UA-GalA (Fig. 1), a Driselase-resistant ‘fingerprint’ spot diagnostic of *OH attack (Vreeburg et al., 2014).

In pear, mango, avocado and *Arbutus*, spot $1^F$ appreciably increased in intensity (Fig. 3; Table 1), especially when stage 3 and stage 2 (soft and medium soft fruits) are compared with stage 1 (hard fruit). It also increased during softening in plum and nectarine (Table 1; Supplementary Data Fig. S1). On the other hand, the apple and strawberry AIR samples did not show any clear evidence of an increase in $1^F$ at any stage.

The putative ‘fingerprint’ spot, $2^F$, was detected in all species, but was very weak in apple and strawberry. The yield of $2^F$ increased during softening in mango, banana, avocado, *Arbutus*, plum and nectarine, and was always much fainter in hard, unripe fruit (Fig. 3; Table 1; Supplementary Data Figs S1 and S2). In pear, it was detected only at stage 2. The transient appearance of $2^F$ in pear was confirmed in one repeat experiment (Fig. S2); it is possible that a brief period of high $2^F$ yield was missed in an additional experiment (Fig. S1).

Some samples, e.g. of pear and mango, revealed a weak spot that approximately co-migrated with GalA$_2$-lactone–pAMAC (Fig. 3); however, HPLC showed that this specific compound was absent (Fig. 4; see below), as expected because it is Driselase digestible.

**HPLC.** The presence of the reducing-end-labelled monomer, GalA–pAMAC, in the digests was supported by HPLC, which we performed on representative pear, mango and banana digests (stages 3, 1 and 1, respectively; without de-lactonization) both before and after spiking with a mixture of authentic sugar–pAMACs (Fig. 4). About 5–9 fluorescent peaks were detected by HPLC, with some differences between fruit species. In pear and banana, a compound was found (Fig. 4, thick purple arrow) which co-eluted with an internal marker of authentic GalA–pAMAC, supporting the idea that this was its identity. In
mango, GalA–pAMAC partially overlapped with a compound of similar retention time (Fig. 4), which remains unidentified. Thus, in these fruit species, GalA–pAMAC was present, accounting for the 1AF spot seen on electrophoretograms.

Concerning the labelled acidic dimers, HPLC of the pear and mango samples gave no peak exactly co-eluting with authentic internal marker GalA2–pAMAC. The pear sample gave a peak that eluted 1/2 min later than this marker. A small unidentified peak that did approximately co-elute with GalA2–pAMAC was found in the banana digest; however, this cannot have been GalA2–pAMAC itself because this substance is completely digested by Driselase under the conditions used (Vreeburg et al., 2014).

Further characterization of individual fluorescent spots by HPLC

Samples of the material in the 1AF zone were eluted from electrophoretograms and submitted to HPLC analysis, typically giving 2–4 peaks (Fig. 5). In at least four fruits (pear, banana, avocado and Arbutus), a major peak co-eluting with GalA–pAMAC was again detected, whereas in mango, apple and strawberry (the three species which showed the faintest 1AF spots on the electrophoretogram), the corresponding peak was extremely minor. A small peak of GalA-lactone–pAMAC accompanied the GalA–pAMAC in pear, banana, avocado and Arbutus, supporting its identity since lactonization/de-lactonization is reversible and would be expected to occur between the electrophoresis step and the HPLC. Spot 1AF from the electrophoretogram yielded in addition HPLC peaks other than GalA-lactone–pAMAC and GalA–pAMAC (Fig. 5), with some differences between fruit species. In particular, unidentified peaks Y and Z (see Fig. 5) were observed: Y in pear, Z in banana and apple, and both in Arbutus, strawberry and possibly avocado.

Spot 2AF, deduced to be pAMAC-UA-GalA, which co-electrophoresed with authentic GalA2–pAMAC but was Driselase-stable, yielded HPLC peaks that only approximately co-eluted with authentic GalA2–pAMAC and GalA2-lactone–pAMAC in all species tested (Fig. 6). Both the acidic and lactone forms of the proposed pAMAC-UA-GalA were present in these eluates because of their interconversion, which is more rapid than in the case of the GalA2–pAMAC ↔ GalA2-lactone–pAMAC interconversion (Vreeburg et al., 2014).

Electrophoretogram spot X, a greenish-fluorescing compound migrating slightly faster than authentic GalA2-lactone–pAMAC and observed in banana (and possibly Arbutus), was
resolved by HPLC into several small peaks (Fig. 7). These peaks, however, did not match the HPLC peak 'X' found after pAMAC labelling of in-vitro *OH-treated pectin (Vreeburg et al., 2014), even though both have similar migration and fluorescence properties on the electrophoretogram. Both X and X^F remain to be identified.

| Fruit          | Ripening stage | Relative fluorescence of compound | Fruit          | Ripening stage | Relative fluorescence of compound |
|----------------|----------------|----------------------------------|----------------|----------------|----------------------------------|
|                |                | 1A^F | 2A^F                      |                |                | 1A^F | 2A^F                      |
| From Fig. 3    |                |      |                            | From Fig. S1   |                |      |                            |
| Pear (false fruit) | 1             | 87   | 0.82                       | Pear (false fruit) | -            | 1.2  | 0.00                       |
|                | 2             | 202  | 5.6                        |                | -            | 1.7  | 0.00                       |
|                | 3             | 151  | 1.6                        |                | ++           | 2.7  | 0.15                       |
| Mango         | 1             | 47   | 0.16                       |                | ++           | 5.0  | 0.00                       |
|                | 2             | 119  | 0.83                       |                | ++           | 5.1  | 0.74                       |
|                | 3             | 136  | 8.7                        |                | +            | 0.80 | 0.25                       |
| Banana        | 1             | 39   | 3.9                        |                | +            | 0.58 | 0.00                       |
|                | 2             | 115  | 12.1                       |                | ++           | 2.1  | 1.6                        |
|                | 3             | 124  | 18.5                       |                | ++           | 1.8  | 2.2                        |
| Apple (false fruit) | 1           | 43   | 0.54                       | Nectarine      | -            | 1.7  | 0.00                       |
|                | 2             | 76   | 1.0                        |                | +            | 0.87 | 0.45                       |
|                | 3             | 43   | 0.54                       |                | +            | 0.49 | 0.34                       |
| Avocado       | 1             | 133  | 8.4                        |                |               |      |                            |
|                | 2             | 322  | 35.8                       |                |               |      |                            |
|                | 3             | 337  | 32.7                       |                |               |      |                            |
| Strawberry (false fruit) | 1      | 90   | 0.05                       |                |               |      |                            |
|                | 2             | 74   | 0.02                       |                |               |      |                            |
|                | 3             | 74   | 0.00                       |                |               |      |                            |
| Arbutus       | 1             | 232  | 8.3                        |                |               |      |                            |
|                | 2             | 296  | 14.0                       |                |               |      |                            |
|                | 3             | 410  | 47.4                       |                |               |      |                            |

Intensities were quantified on the electrophoretograms of de-lactonized samples as shown in Fig. 3 and Supplementary Data Figs S1 and S2. Relative fluorescence is quoted in arbitrary units of area, as quantified in ImageJ by the method illustrated in Fig. S3.

**DISCUSSION**

A fluorescent fingerprinting method, recently developed for demonstrating hydroxyl radical attack on polysaccharides in-vitro (Vreeburg et al., 2014), has now been applied to the cell wall polysaccharides of several fruit species at different stages of softening, providing useful information on *OH attack.
in vivo. The fluorescent labelling procedure can yield information comparable with a radiolabelling approach used earlier (Fry et al., 2001, 2002; Müller et al., 2009; Iurlaro et al., 2014), and the two approaches are largely interchangeable. However, the fluorescent pAMAC group introduced into the polysaccharides in the new method provides a means of further characterization of these cell wall components by means of a wide variety of accessible chromatography and electrophoresis techniques, including fluorophore-assisted electrophoresis (Goubet et al., 2002). Specifically, pAMAC introduces a pH-dependent charge into the *OH-attacked, plant-derived polymer residue, which facilitates further characterization of the products. Radiolabelling is generally more sensitive and is very straightforward to quantify, but not all laboratories are authorized to use it.

At least two informative fluorescent spots (1AF and 2AF) were visualized on electrophoretograms (Fig. 3). Spot 1AF, including predominantly GaIA–pAMAC (Fig. 1), increased in intensity between stages 1 and 3 of softening in most fruits (Table 1). This would correspond to an increasing number of D-GalA reducing termini during fruit ripening, which could be caused by the pectic polysaccharide-cleaving actions of not
FIG. 5. HPLC of the acidic monomer (1AF) spots from Driselase-digested pAMAC-labelled cell walls of seven fruit species. Each 1AF spot (pooled for all three stages of development for each fruit; de-lactonized) shown in Fig. 3 was eluted from the electrophoretogram and analysed by HPLC. MM, marker mixture containing authentic acidic sugar–pAMAC conjugates. Arrows, putative GalA–pAMAC (and its lactone, which partially re-formed during elution from the electrophoretogram) from fruit cell wall digests. Dashed lines, compounds Y and Z, discussed in the text.
Fig. 6. HPLC of the acidic dimer (2A\textsuperscript{F}) spots from Driselase-digested pAMAC-labelled cell walls of five fruit species. 2A\textsuperscript{F} spots were eluted from a paper electrophoretogram (similar to that shown in Fig. 3 but derived from non-de-lactonized samples; all three ripening stages combined) and analysed by HPLC. MM, marker mixture containing authentic acidic sugar-pAMAC conjugates. Arrows, the proposed fingerprints for OH attack: pAMAC-UA-GalA and its lactone (rapidly reformed during elution from the electrophoretogram) from fruit cell wall digests. Dashed lines, predicted position of authentic GalA\textsubscript{2}-pAMAC and GalA\textsubscript{2}-lactone-pAMAC, deduced from the marker run. The samples in the upper and lower graphs were run on different days, accounting for the slight discrepancy in marker retention times. Strawberry and apple were not included because they did not show any appreciable 2A\textsuperscript{F} spot in Fig. 3.
only $^\cdot$OH (as a result of attack at C-1 or C-4 of homogalacturonan — see fig. 1 of Vreeburg et al., 2014) but also endo-PG and/or pectate lyase. Both endo-PG and pectate lyase, proposed fruit softening agents, can attack a homogalacturonan chain, creating one new reducing terminus per cleavage event and this reducing terminus would become pAMAC labelled. Several studies have reported increases in endo-PG activity (though seldom definitively distinguished from pectate lyase activity) in pear (Pressey and Avants, 1976), banana (Pathak and Sanwall, 1998; Ali et al., 2004) and avocado (Huber and O’Donoghue, 1993). Increasing pectate lyase activity has been measured during ripening in banana (Payasi and Sanwal, 2003; Payasi et al., 2006). In addition, pectate lyase mRNA accumulation was reported in several ripening fruits including banana (Domínguez-Puigjaner et al., 1997; Marín-Rodríguez et al., 2003) and mango (Chourasia et al., 2006). Therefore, spot 1A$^F$ obtained from fruit AIR was not exclusive evidence of $^\cdot$OH attack, but may offer a valuable fingerprint indicating the total pectic chain scission occurring in vivo.

Spot 2A$^F$ was concluded to be a Driselase limit digestion product of the type pAMAC-UA-GaA (Fig. 1), i.e. a ‘fingerprint’ indicating recent in-vivo mid-chain $^\cdot$OH attack. The precise chemical identity of the compound(s) present in spot 2A$^F$ has not been established. 2A$^F$ clearly did not include the reducing-terminus-labelled disaccharide, GaA$_2$–pAMAC, since this compound does not withstand 14 d of Driselase treatment (Vreeburg et al., 2014), and GaA$_2$–pAMAC was not observed in pear and mango by HPLC analysis (Fig. 4). It probably includes pAMAC-GaA-GaA and/or its 2-, 3- and 4-epimers (pAMAC-taluronate-GaA, pAMAC-guluronate-GaA and pAMAC-glucuronate-GaA, respectively). We would expect all these structures to be Driselase resistant because the range of activities present in Driselase probably does not include $\alpha$-taluronidase, $\alpha$-guluronidase and $\alpha$-glucuronidase, and because the pAMAC group would block the action of $\alpha$-galacturonidase.

The intensity of spot 2A$^F$ increased appreciably as hard fruit (stage 1) matured into softer fruit (stages 2 and 3) in mango, banana, avocado and Arbutus. The observation in banana may possibly be related to the ripening-dependent increase in the reported ability of banana fruit homogenates to generate ‘endogenous’ $^\cdot$OH post-mortem (Cheng et al., 2008a; Yang et al., 2008). In pear, the increase in spot 2A$^F$ was transient, peaking in stage 2; this suggests that the glycosulose residues from which 2A$^F$ is generated (Fig. 1) were unstable in vivo. A related observation in pear (increase in $^3$H-labelled products released when fruit cell walls were NaB$_3$H$_4$ labelled and then Driselase digested) was reported by Fry et al. (2001), where the unidentified $^3$H-labelled products were proposed to be ‘fingerprints’ of $^\cdot$OH attack.

The increase in yield of 2A$^F$ during softening depended on the type of fruit under consideration. In true fruits (those whose edible portion is derived from the ovary wall; including mango, banana, avocado, Arbutus, plum and nectarine), there was an increase in 2A$^F$ that correlated with softening. In contrast, it showed little if any increase in apple or strawberry and increased only transiently in pear, which are all false fruits. In false fruits, the edible tissue is derived from the receptacle, not the ovary wall. Therefore, differences in developmental origin of the edible tissue may dictate the mechanism adopted for cell wall modification during ‘fruit’ softening.

Conclusions

It was reported nearly 40 years ago that in pear fruit, endogenous peroxides (and thus potentially also $^\cdot$OH generated from
them) correlate with softening (Brennan and Frenkel, 1977). Later it was found that the polysaccharides of softening pears exhibit radiochemical ‘fingerprints’ diagnostic of recent *OH attack (Fry et al., 2001). Furthermore, of two investigated culti-
vars of muskmelon, the one whose microsomal membranes pro-
duced less *OH in vitro had a longer shelf-life (Lacan and Baccou, 1998). Taken together, the available evidence supports
the view that fruit softening, often viewed as broadly a ‘hydro-
lytic’ phenomenon, is at least partly ‘oxidative’ – a suggestion
raised by Brennan and Frenkel (1977) but often ignored. We
hope that interest in this concept will be revived by the present
study and explored in greater depth. Although several of the
fluorescent ‘fingerprint’ compounds were not fully identified in
the present study and deserve further analysis, our new fluores-
cent labelling method will provide useful information and can
be used in conjunction with other approaches to add to our
knowledge and understanding of the occurrence and rate of
*OH attack relative to endo-PG and pectate lyase action in cell
walls.

SUPPLEMENTARY DATA
Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: electrophoretic
resolution of total Driselase digests of pAMAC-labelled cell
walls from three fruit species. Figure S2: electrophoretic resolu-
tion of total Driselase digests of pAMAC-labelled cell walls
from banana and pear. Figure S3: method for quantification of
fluorescent spots on paper electrophoretograms.

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