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RESEARCH PAPER

Cell wall metabolism during maturation, ripening and senescence of peach fruit

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Abstract

Cell wall changes were examined in fruit of a melting flesh peach (*Prunus persica* L.) allowed to ripen on the tree. Three phases to softening were noted, the first of which began prior to the completion of flesh colour change and an increase in ethylene evolution. Softening in young mature fruit, prior to ripening, was associated with a depolymerization of matrix glycans both loosely and tightly attached to cellulose and a loss of Gal from all cell wall fractions. After the initiation of ripening, but before the melting stage, softening was associated with continuing, progressive depolymerization of matrix glycans. A massive loss of Ara from the loosely bound matrix glycan fraction was observed, probably from side chains of glucuronoarabinoxylan, pectin, or possibly arabinogalactan protein firmly bound into the wall and solubilized in this extract. An increase in the solubilization of polyuronides also occurred during this period, when softening was already well advanced. The extensive softening of the melting period was marked by substantial depolymerization of both loosely and tightly bound matrix glycans, including a loss of Ara from the latter, an increase in matrix glycan extractability, and a dramatic depolymerization of chelator-soluble polyuronides which continued during senescence. Depolymerization of chelator-soluble polyuronides thus occurred substantially after the increase in their solubilization. Ripening-related increases were observed in the activities of *exo*- and *endo*-polygalacturonase (EC 3.2.1.67; EC 3.2.1.15), pectin methylesterase (EC 3.1.1.11), *endo*-1,4-β-glucanase (EC 3.2.1.4), *endo*-1,4-β-mannanase (EC 3.2.1.78), α-arabinosidase (EC 3.2.1.55), and β-galactosidase (EC 3.2.1.23), but the timing and extent of the increases differed between enzymes and was not necessarily related to ethylene evolution. Fruit softening in peach is a continuous process and correlated closely with the depolymerization of matrix glycans, which proceeded throughout development. However, numerous other cell wall changes also took place, such as the deglycosylation of particular polymers and the solubilization and depolymerization of chelator-soluble polyuronides, but these were transient and occurred only at specific phases of the softening process. Fruit softening and other textural changes in peach appear to have a number of stages, each involving a different set of cell wall modifications.

Key words: Cell wall, matrix glycans, pectin, *Prunus persica*, ripening, softening.

Introduction

The softening and textural changes that occur during fruit ripening are characteristic of particular species, and are due to differences in cell wall thickness and composition, cell size, shape, packing, contents, and turgor (Harker *et al.*, 1997). Modification of the cell wall is believed to underlie changes in firmness and texture, but the type and magnitude of the alterations carried out during ripening vary considerably. Some ripening-related cell wall modifications appear to be universal, such as the depolymerization of matrix glycans (see Discussion), but others occur to very different

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Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; CMC, carboxymethylcellulose; PG, polygalacturonase; PME, pectin methylesterase; RG, rhamnogalacturonan.

extents or are absent in certain species. For example, a decline in cell wall Gal and Ara content during ripening is observed in most species, but Gal loss does not take place in plum or cucumber and Ara loss is minor or absent in apple, plum, and apricot (Gross and Sams, 1984). Similarly, the depolymerization of ionically-bound pectin during ripening is almost undetectable in strawberry, banana, and apple (Huber, 1984; Wade *et al.*, 1992; Yoshioka *et al.*, 1992), relatively slight in melon (McCollum *et al.*, 1989; Ranwala *et al.*, 1992; Rose *et al.*, 1998), moderate in tomato (Huber and O'Donoghue, 1993; Brummell and Labavitch, 1997), and dramatic in avocado and watermelon (Huber and O'Donoghue, 1993; Karakurt and Huber, 2002). The extent of pectin solubilization also differs between species, being high in kiwifruit, tomato, and plum and almost absent in apple and watermelon, and is thought to explain variability in cell wall swelling during ripening (Redgwell *et al.*, 1997*a*). The activities of ripening-related enzymes also differ widely between species. These findings suggest that softening and textural changes may be the result of different cell wall modifications in different species, and emphasize that since observations made on one species cannot necessarily be extended to others, each needs to be investigated separately.

In tomato, fruit softening appears to involve the actions of ripening-related expansin (Brummell *et al.*, 1999) and β-galactosidase (Smith *et al.*, 2002) early in the process, whereas the solubilization and depolymerization of pectin mediated by *endo*-polygalacturonase (*endo*-PG) has little effect on firmness (Giovannoni *et al.*, 1989; Smith *et al.*, 1990). However, pectin modification is important in textural changes, since tomato fruit in which *endo*-PG accumulation is transgenically suppressed have improved integrity and extended shelf life during long-term storage (Langley *et al.*, 1994). In bell pepper, pectin depolymerization is undetectable during ripening (Harpster *et al.*, 2002*a*), but unlike wild soft-fruited accessions which soften rapidly, firm-fruited domesticated pepper varieties lack expression of *endo*-PG (Rao and Paran, 2003). Attenuated expression of *endo*-PG and reduced changes in firmness also exist in peach. Softening during ripening in melting flesh peach has two phases, a slow decline in firmness early in ripening followed by a rapid and dramatic softening ('melting') late in ripening (Pressey *et al.*, 1971). The melting flesh phenotype is associated with a large increase in the amount of soluble pectin and progressive pectin depolymerization (Pressey *et al.*, 1971; Dawson *et al.*, 1992; Fishman *et al.*, 1993). In non-melting flesh peach, the final melting phase of softening is absent so fruit remain relatively firm when fully ripe, and pectins undergo little solubilization or depolymerization (Pressey and Avants, 1978; Fishman *et al.*, 1993). Ripening-related *exo*-PG activity is found in both melting and non-melting peach, but *endo*-PG activity accumulates only in ripening melting varieties, coincident with the melting phase (Pressey and Avants, 1978; Orr and Brady, 1993). The lack of a melting phase in non-melting peach varieties appears to be due either to a deletion of *endo*-PG genes or to a truncation of their mRNAs, which causes an absence of immunodetectable *endo*-PG protein (Lester *et al.*, 1994, 1996; Callahan *et al.*, 2004). Thus, *endo*-PG-mediated pectin modification may play an important role in the later stages of softening and textural changes in melting flesh peach. Pectin metabolism is also implicated in the development of the chilling injury leading to mealy flesh (see accompanying paper), again implying the importance of correct pectin modification for normal texture.

Softening in peach begins early in ripening and, presumably, a variety of cell wall modifications are responsible for changes in firmness prior to and during the melting phase. The aim of the present work was to establish the nature and the precise timing of the various modifications of cell wall matrix glycans and pectins during normal ripening of peaches on the tree by comparing many stages in the softening process. Particular aspects of cell wall changes or enzyme activities in ripening peach have been described previously (Pressey *et al.*, 1971; Hinton and Pressey, 1974; Pressey and Avants, 1978; Dawson *et al.*, 1992; Downs *et al.*, 1992; Fishman *et al.*, 1993; Hegde and Maness, 1998; Zhou *et al.*, 2000), but usually in fruit ripened off the tree or after storage, and with only a few (two to four) ripening stages and a limited number of parameters being examined in any one study. Eight developmental stages of peach have been examined. Fruits were harvested directly from the tree, ranging from fruit that were 50% of full size to those that were overripe, and were examined for changes in polysaccharide depolymerization, solubilization, and neutral sugar composition, and these changes were related to softening and enzyme activity measurements from the same tissue. The data also gave a reference point for the determination of which cell wall changes were absent or were taking place incorrectly during chilling injury in stored fruit.

Materials and methods

Plant material

Fruit of peach (*Prunus persica* [L.] Batsch, cv. O'Henry) were collected from trees growing in the Central Valley of California in the summer of 2001. At least five fruit were collected at each of eight developmental stages. 1, Half size (approximately 50% of final full size); 2, immature (full size fruit, flesh predominantly green, no softening); 3, pre-ripe or pre-climacteric (flesh predominantly yellow, basal levels of ethylene production, slight softening); 4, onset (flesh completely yellow, substantial ethylene production detectable, firmness of 60–65 N); 5, early-ripe (ethylene production increasing, 50–55 N); 6, mid-ripe (ethylene production increasing, 35–40 N); 7, fully ripe (climacteric peak, edible firmness, 15–20 N); and 8, overripe (senescent, <5 N). The ethylene evolution of intact fruit was measured by enclosing the fruit in stoppered vessels and withdrawing gas samples after 1 h for ethylene quantification against standards using gas chromatography (separation on a column 2 m in length

packed with alumina, enclosed in an oven at 100 °C, N_2 as carrier gas flowing at a rate of 30 ml min^{-1}, detection by a flame ionization detector). Firmness was measured at two points per fruit (in regions of the mesocarp from which a 1 mm thick section of the skin had been sliced away), using a penetrometer fitted with a convex probe 7.9 mm in diameter which was inserted 10 mm into the mesocarp. Skins were removed and flesh sliced into pieces, which were snap frozen in liquid nitrogen and stored at −80 °C.

Preparation and fractionation of cell walls

Mesocarp material was powdered while frozen and 20 g homogenized in 100 ml of ice-cold 80% ethanol using a Polyton homogenizer. Insoluble material was washed with ice-cold 80% ethanol, stirred in TRIS-buffered phenol, precipitated with ethanol, washed with 95% ethanol, stirred in chloroform:methanol (1:1, v/v), and washed with acetone as previously described (Huber and O'Donoghue, 1993). Aliquots (100 mg) of acetone-insoluble cell walls were sequentially extracted with *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA) (for 24 h, twice) and with Na_2CO_3 containing 0.1% $NaBH_4$ (24 h, twice) as described by Brummell and Labavitch (1997), to produce extracts enriched in ionically bound and covalently bound pectin, respectively. Depectinated cell wall residues were extracted with 4% KOH containing 0.1% $NaBH_4$ (48 h, twice) and 24% KOH containing 0.1% $NaBH_4$ (48 h, twice) as described by Maclachlan and Brady (1994), to produce extracts enriched in loosely bound and tightly bound matrix glycans, respectively.

Size exclusion chromatography

Na_2CO_3 extracts were neutralized with glacial acetic acid, then CDTA and Na_2CO_3 extracts were dialysed extensively against deionized water. After dialysis, uronic acid and neutral sugar contents were quantified using the assay of Blumenkrantz and Asboe-Hansen (1973) and the anthrone reagent (Dische, 1962), respectively, with galacturonic acid and glucose as standards. Volumes containing 1 mg uronic acid were freeze-dried and redissolved in 1.6 ml of 0.4 mg ml^{-1} imidazole then neutralized by the addition of 0.4 ml of 1 M ammonium acetate, pH 5.0. Samples were chromatographed on a column (97×1.5 cm) of Sepharose CL-2B eluted with 0.2 M ammonium acetate, pH 5.0, and fractions of 2 ml collected.

KOH extracts were neutralized on ice with glacial acetic acid, then precipitated with two volumes of ethanol. Pellets were collected by centrifugation, redissolved in 0.1 M TRIS-HCl, pH 7.0, containing 0.02% NaN_3, and treated twice with 26 units of α-amylase (porcine pancreas, Sigma) for 24 h each time to remove starch. Extracts were dialysed extensively against deionized water, then uronic acid and neutral sugar contents were quantified as above. Volumes containing 2 mg neutral sugar were freeze-dried and redissolved in 2 ml of 0.1 M NaOH, and were then chromatographed on a column (97×1.5 cm) of Sepharose CL-6B eluted with 0.1 M NaOH, and fractions of 1.8 ml collected.

Column fractions were assayed for contents of uronic acids and neutral sugars using the assays above.

Neutral sugar composition

The neutral sugar content of pectin extracts was very low, so samples of dialysed unchromatographed extract were assayed directly. For matrix glycan extracts, various peaks were selected from the neutral sugar profile after size exclusion chromatography. Neutral sugar compositions of samples were determined after conversion to alditol acetates (Blakeney *et al.*, 1983) and separation on a Perkin-Elmer 8320 gas chromatograph fitted with a 30 m×0.25 mm DB-23 capillary column (J&W Scientific) operated isothermally at 210 °C. The flame ionization detector signal was integrated by a Total Chrom Workstation (Perkin Elmer) and neutral sugar amount calculated relative to an inositol internal standard.

Degree of pectin methylesterification

Samples of acetone-insoluble cell walls (~1 mg) were assayed for methanol released upon saponification using the method of Wood and Siddiqui (1971). Duplicate samples were assayed for uronic acid content according to Ahmed and Labavitch (1977). Mol of methanol released were related to mol of galacturonic acid equivalents present to calculate degree of methylesterification.

Enzyme activity

Enzyme extracts were prepared similarly to Zhou *et al.* (2000), with modifications. Frozen mesocarp tissue was powdered with a pestle and mortar then stirred in 1 vol. of ice cold 12% polyethylene glycol 3350 containing 0.2% sodium bisulphite. Insoluble material was collected on Miracloth, washed with 2 vols of ice-cold 0.2% sodium bisulphite, and extracted in two different ways. For *endo*- and *exo*-PG, insoluble material was incubated in 0.75 M NaCl/50 mM sodium acetate, pH 5.0, on ice on a shaker for 1 h. Insoluble residues were removed by filtration through Miracloth and the supernatant was clarified by centrifugation at 16 000 *g* for 10 min then assayed for PG activity. Since high salt concentrations are inhibitory to PG activity, a 5-fold dilution of protein extract into the substrate was used to reduce final NaCl concentrations to 0.15 M for both *endo*- and *exo*-PG assays. For *endo*-PG activity, 1 ml of enzyme extract was mixed with 4 ml of 2% citrus pectin (0% methylesterification, Sigma) in 50 mM sodium acetate, pH, 4.4, in size 100 Cannon-Fenske viscometers (Fisher Scientific) submerged in a water bath at 25 °C. Efflux time was measured at 15 min intervals over 2 h and used to calculate the initial rate. One unit was defined as a 1% decrease in viscosity mg^{-1} protein h^{-1}. For *exo*-PG activity, 0.2 ml of enzyme extract was mixed with 0.8 ml of 0.5% citrus pectin (0% methylesterification) in 50 mM sodium acetate, pH 5.5, containing either 1 mM $CaCl_2$ or 5 mM EGTA, and incubated at 30 °C. Samples were removed at 2 h intervals and assayed for reducing sugar content using a cyanoacetamide assay (Gross, 1982) with galacturonic acid as a standard. Calcium-dependent activity was determined after subtraction of activity in EGTA, and one unit defined as 1 μg reducing sugar produced mg^{-1} protein h^{-1}. Activity was linear for at least 16 h.

For other enzymes, after extraction in polyethylene glycol, insoluble fruit material was incubated in 1.25 M NaCl/4 mM EDTA, pH 6.5, for 1 h on ice, filtered, and the supernatant clarified by centrifugation. *Endo*-1,4-β-glucanase activity was assayed viscometrically against carboxymethylcellulose (CMC) by mixing 1.5 ml of enzyme extract with 3.5 ml of 0.25% high viscosity CMC (Sigma) in 0.1 M citrate-phosphate, pH 7.0, in viscometers and measuring viscosity loss as for *endo*-PG activity above. *Endo*-1,4-β-mannanase activity was measured similarly, using 0.2 ml of enzyme extract and 4.8 ml of 0.2% locust bean gum (Sigma) in 0.1 M citrate-phosphate, pH 5.0, and measuring viscosity loss over 2 h. For both enzymes the initial rate of activity was calculated, and 1 unit was defined as a 1% decrease in viscosity mg^{-1} protein h^{-1}. Pectin methylesterase (PME) activity was assayed at pH 7.4 by the gel diffusion assay of Downie *et al.* (1998), using citrus pectin with a degree of esterification of 72% as substrate and incubating at 37 °C for 16 h. Activity in the samples was calculated relative to a standard curve of serial 2-fold dilutions of PME from orange peel (Sigma). α-L-Arabinosidase and β-D-galactosidase activities were measured by mixing 0.1 ml of enzyme extract with 1 ml of 0.1 M sodium acetate, pH 5.0, and 0.2 ml of 10 mg ml^{-1} of the appropriate 4-nitrophenyl-glycoside. Reactions were incubated at 30 °C, and at intervals depending upon the activity, 0.25 ml aliquots of the reaction were withdrawn, mixed with equal volumes of 1 M ammonium hydroxide, and absorbance was measured at 400 nm. Activity was linear for at least 6 h for both enzymes. One unit was defined as 1.0 A_{400} unit mg^{-1} protein h^{-1}. For all activities, reactions using boiled enzyme and lacking enzyme were used as controls.

Protein concentration was determined using a protein assay kit (Bio-Rad) with bovine γ-globulin as a standard.

Results

A broad series of peach fruit development was selected, based on fruit size, ethylene evolution, internal mesocarp colour, and firmness (Fig. 1). Fruit were harvested directly from the tree for measurement and subsequent freezing for biochemical analysis, with no intervening storage period, in order to represent as closely as possible the natural ripening process. Peach is a climacteric fruit (Tonutti *et al.*, 1997), and as such requires ethylene for normal ripening. Nevertheless, fruit firmness began to decline quite early, at stage 3, before mesocarp tissue had turned completely from green to yellow and prior to the large increase in ethylene evolution and the beginning of ripening. Firmness declined rapidly after the onset of ripening at stage 4, when the fruit mesocarp attained complete colour change and substantial ethylene evolution began, generally considered to mark the beginning of ripening. Softening continued progressively through stages 5 and 6. The decline in firmness was very rapid in the melting phase of softening in stage 7, the climacteric peak of ethylene production, and in stage 8, overripe or the beginning of senescence.

The solubility of different classes of cell wall polymers in various extractants changed during fruit development and ripening (Fig. 2). Polyuronides soluble in the chelating agent CDTA, i.e. pectins loosely attached to the wall through ionic calcium bonds (generally assumed to be partially methylesterified homogalacturonans), increased in amount as fruit reached full size, and increased further during ripening in a progressive manner, before a decline at overripe (Fig. 2A). The decline in amount of CDTA-soluble pectin at overripe may be due to the loss of small pectin molecules from the extracts during sample preparation (e.g. either due to their solubility in TRIS-buffered phenol and ethanol or passage through dialysis membranes). The amount of pectin covalently attached to the wall (presumed to be rich in highly-branched rhamnogalacturonan I [RG-I]), and extractable by sodium carbonate (Fig. 2B), was approximately half as much as that cross-linked to the cell wall only via ionic bonds. The amount was fairly constant until early-ripe, then declined in later stages, commensurate with the increase in CDTA-soluble pectin. This suggests that during the ripening process an increasing proportion of the covalent bonds holding some pectin polymers in the wall were broken, so that their only asssociations with the wall were by ionic cross-links. The amounts of matrix glycans in the wall were relatively constant during the first six of the developmental stages examined, with approximately one-third in a loosely attached fraction soluble in 4% KOH, and two-thirds in a tightly attached fraction soluble in 24% KOH (Fig. 2C, D). During the melting

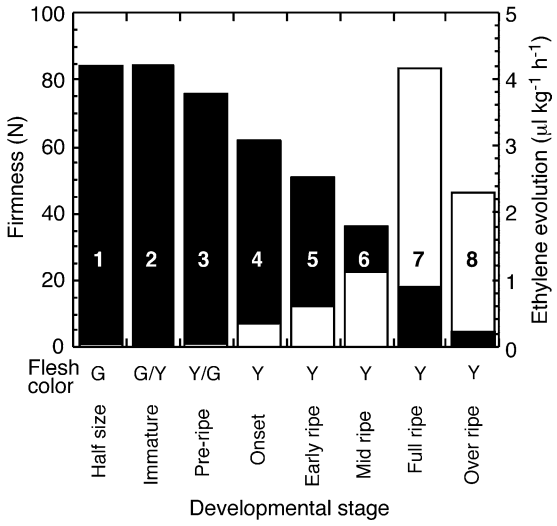

Fig. 1. Fruit softening and ethylene evolution. Fruit were collected directly from the tree at eight ripening stages: 1, half size, ~50% of final size; 2, immature, full size, flesh predominantly green, no softening; 3, pre-ripe, flesh predominantly yellow, little ethylene detectable; 4, onset, flesh completely yellow, ethylene production beginning; 5, early-ripe; 6, mid-ripe; 7, fully ripe, peak ethylene production, melting phase of softening, edible firmness; 8, overripe. Firmness (black bars) was measured using a penetrometer and ethylene evolution (white bars) by gas chromatography. Internal flesh (mesocarp) colour was recorded as green (G) or yellow (Y).

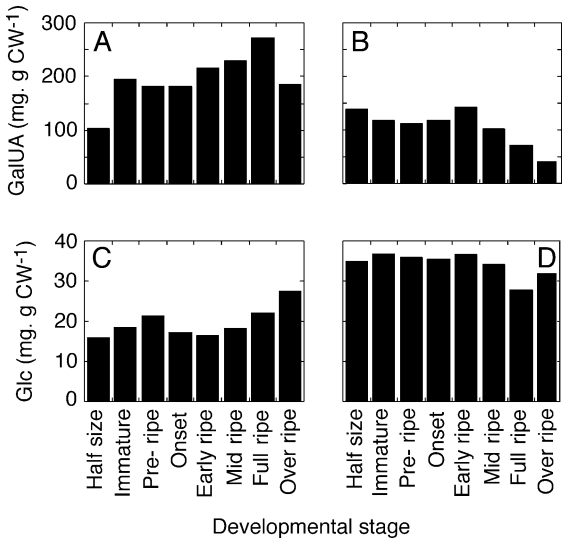

Fig. 2. Amounts of polyuronide and neutral sugar in pectin- and matrix glycan-enriched extracts of cell wall preparations. Preparations of cell walls from fruit at varying ripening stages were extracted with (A) CDTA (to extract ionically bound pectins); then with (B) Na_2CO_3 (covalently bound pectins), and after dialysis the extracts were assayed for uronic acid content. De-pectinated cell wall residues were then extracted with (C) 4% KOH (loosely bound matrix glycans); and with (D) 24% KOH (tightly bound matrix glycans), and after neutralization and precipitation the extracts were assayed for neutral sugar content. Data are expressed as mg of GalUA equivalents g^{-1} cell wall (CW) preparation (A, B), or mg of Glc equivalents g^{-1} cell wall preparation (C, D).

stages of ripening, at fully ripe and overripe, there was an increase in the amount of loosely bound matrix glycans and a decrease in the amounts that were tightly bound. Again, this suggests a proportion of the tightly bound matrix glycans were becoming more loosely bound late in softening.

In the early developmental stages, CDTA-soluble polyuronides were present with a size distribution predominantly of very high molecular weight, in or close to the void volume of the column (20 MDa), with a shoulder of mid-sized molecules and very low amounts of small but still polymeric molecules (Fig. 3). This molecular weight profile did not change substantially during ripening until the fully ripe stage was reached, when a dramatic shift in molecular weight profile occurred consisting of a loss of high molecular weight molecules and an accumulation of much smaller molecules. A further shift away from high molecular weight molecules occurred at the overripe stage. The depolymerization of CDTA-soluble polyuronides thus occurred suddenly and late during peach ripening, when softening was already well advanced. By contrast, the molecular weight profile of Na_2CO_3-soluble polyuronides showed a main peak of low molecular weight resembling that seen from tomato (Brummell *et al.*, 1999), eluting from the column at around fraction 60 (data not shown). This size distribution did not change significantly during development, ripening or senescence.

Matrix glycans loosely attached to cellulose and soluble in 4% KOH possessed a size distribution with two major peaks, one in the void of the column (1 MDa and above) and the other fairly broad with, in half-size fruit, a maximum in fraction 42 (equivalent to dextran markers of ~300 kDa) (Fig. 4). The peak eluting in the void possessed a high uronic acid content, a neutral sugar composition identical to that of the Na_2CO_3 extract and showed similar compositional changes occurring during ripening (data not shown), suggesting that this material is neutral sugar-rich pectin, probably RG-I, quite firmly bound into the wall. The second peak showed a progressive sharpening (becoming less polydisperse) and a small but continued decline in its peak molecular weight during development, until in overripe fruit this was in fraction 48 (equivalent to dextran markers of ~200 kDa). Also, a minor shoulder eluting in fractions 60–63 became more pronounced in riper fruit. As

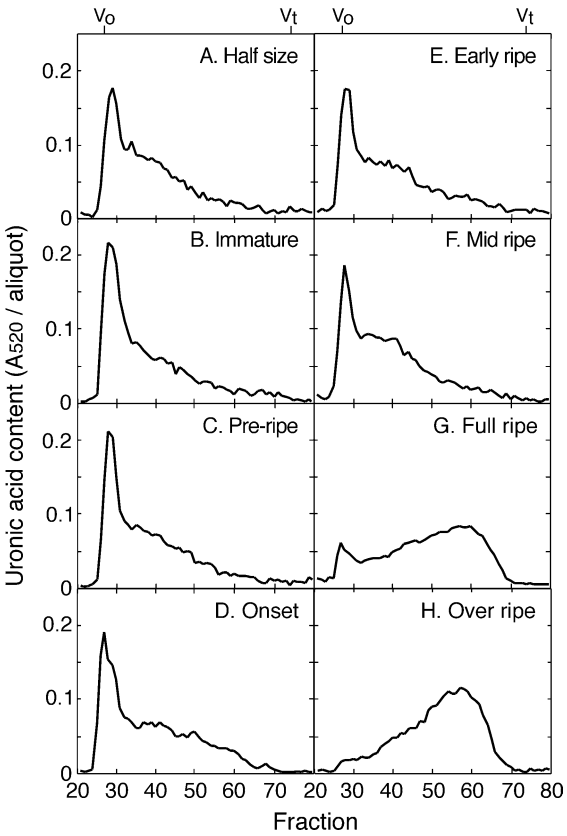

Fig. 3. Size distribution of chelator-soluble polyuronides. CDTA-soluble polyuronides were separated by size exclusion chromatography on a column of Sepharose CL-2B and column fractions assayed for uronic acid content by the meta-phenyl phenol assay. The void (V_o, 20 MDa) and total volumes (V_t, 100 kDa) of the column are indicated. Developmental stages are as in Fig. 1.

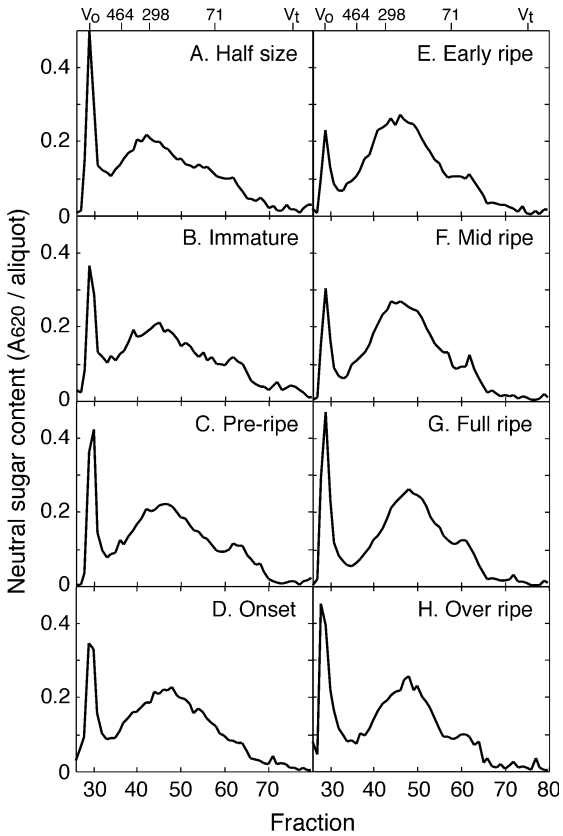

Fig. 4. Size distribution of loosely-bound matrix glycans. Matrix glycans soluble in 4% KOH were separated by size exclusion chromatography on a column of Sepharose CL-6B and column fractions assayed for neutral sugar content by the anthrone assay. The void (V_o, 1000 kDa) and total volumes (V_t, 10 kDa) of the column and the elution points of linear dextran standards (in kDa) are indicated.

determined using an iodine binding assay, approximately half of the polymers making up this extract were xyloglucan (data not shown).

Matrix glycans tightly attached to cellulose and soluble in 24% KOH also possessed a size distribution with two major peaks, one peak in the void of the column and the other, predominant, peak of high molecular weight, with a maximum in half-size fruit in fractions 38 (equivalent to dextran markers of ~400 kDa) (Fig. 5). Between expanding half-size and full-size fruit there was no change in the molecular weight profile, but during subsequent development and ripening there was a progressive loss of the highest molecular weight molecules eluting in and close to the void of the column and a progressive decline in the average molecular weight of the predominant peak, until in overripe fruit this was in fraction 44 (equivalent to dextran markers of ~270 kDa). The largest shift in molecular weight occurred during the melting stage, but matrix glycans remained at moderately high molecular weight even in overripe fruit, and were not depolymerized to small size. Matrix glycans in the 24% KOH extract consisted of ~70% xyloglucan (data not shown) and ~30% other polymers. The molecular weight profiles of xyloglucan in both the 4% KOH and 24% KOH extracts were almost exactly coincident with the predominant peaks of total matrix glycan in the extract, and showed the same depolymerization as total matrix glycans (data not shown). In both extracts, material eluting in the void of the column possessed a high content of uronic acids (similar to the neutral sugar content in the 4% KOH extracts, about half that of the neutral sugar content in the 24% KOH extracts, data not shown), suggesting that much of the highest molecular weight neutral sugar-containing molecules were pectic in nature, as was also observed by Hegde and Maness (1998).

The neutral sugar composition of polyuronide extracts showed that these polymers were rich in sugars typical of pectins, Rha, Ara, and Gal (Fig. 6A, B). Amounts of Fuc,

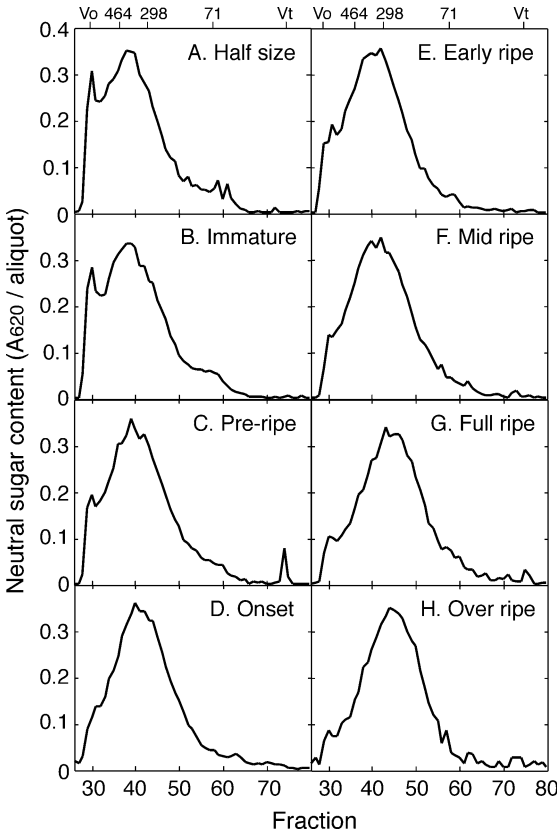

Fig. 5. Size distribution of tightly-bound matrix glycans. Matrix glycans soluble in 24% KOH were separated by size exclusion chromatography on a column of Sepharose CL-6B and column fractions assayed for neutral sugar content by the anthrone assay. The void (V_o, 1000 kDa) and total volumes (V_t, 10 kDa) of the column and the elution points of linear dextran standards (in kDa) are indicated.

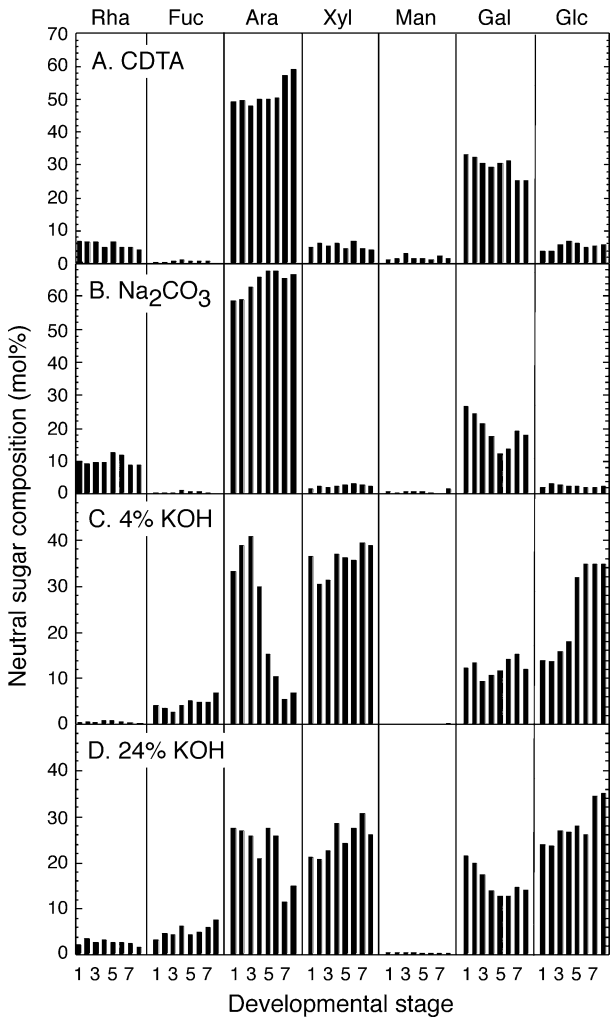

Fig. 6. Changes in neutral sugar composition of cell wall pectins and matrix glycans during ripening. (A) CDTA-soluble pectin extract; (B) Na_2CO_3-soluble pectin extract; (C) loosely bound matrix glycans, main peak after size exclusion chromatography; (D) tightly bound matrix glycans, main peak after size exclusion chromatography. The eight bars for each sugar represent the eight developmental stages described in Fig. 1. For each ripening stage alditol acetate derivatives of neutral sugars were separated on a gas chromatograph and quantified as mol%.

Xyl, Man, and Glc were low, and showed little change during development and ripening. In the CDTA-soluble pectin extract, the amounts of the two most prominent sugars, Ara and Gal, were constant until the fully ripe stage, when amounts of Gal decreased and Ara increased. The converse pattern was seen in Na_2CO_3-soluble polyuronides, where the abundance of Ara increased until ripening began, when levels became constant. The relative abundance of Gal declined during early development, and rose again during ripening.

The matrix glycan extracts were analysed for sugar content after chromatography, selecting the centre of the prominent peak in each case. Loosely bound matrix glycans were rich in Ara, Xyl, Gal, and Glc, with smaller amounts of Fuc (Fig. 6C) and uronic acids (data not shown), suggesting the extract was composed predominantly of glucuronoarabinoxylan, xyloglucan, and pectin, or possibly protein, with highly-branched arabinan and galactan side chains. Firmly bound arabinogalactan-rich pectin is frequently extracted in the matrix glycan fractions from fruit (Redgwell *et al.*, 1997*b*). The content of Ara declined precipitously during development, resulting in apparent increases in other sugars, notably Glc and Xyl.

The neutral sugar composition of the main peak of tightly bound matrix glycans was composed of similar amounts of Ara, Xyl, Gal, and Glc, with substantial amounts of Fuc (Fig. 6D). This was consistent with iodine binding assays showing the presence of high amounts of xyloglucan, presumably with glucuronoarabinoxylan and low amounts of glucomannan or galactomannan. Changes in composition during development were an early decline in Gal content and a dramatic late decline in Ara content, bringing about corresponding relative increases in Xyl and Glc. Man was present only at very low levels in all extracts examined, and was also very low (less than 1.5 mol%) in unfractionated cell walls (data not shown).

The degree of methylesterification of pectins was high in early fruit development and began to decline at stage 5 (Fig. 7). This decline continued during ripening, and methylesterification was quite low in fully ripe fruit. A small increase in the apparent degree of methylesterification of pectin at the overripe stage may have been due to a loss of homogalacturonan fragments of low methylesterification from the wall or during sample preparation at this late stage of fruit development.

All of the cell-wall-modifying enzymes examined showed ripening-related, approximately 3-fold increases in specific activity after ripening began, although the timing and extent of this varied (Fig. 8). The greatest relative rise in activity between unripe and ripe was shown by *endo*-PG, with a ~5-fold increase, whereas the greatest amount of activity in absolute terms was *endo*-1,4-β-mannanase, which increased only ~3-fold between unripe and ripe, but was present at extraordinarily high levels late in ripening, 10 times higher than the maximal levels of *endo*-1,4-β-glucanase (CMCase) and 26 times higher than *endo*-PG. The activity of β-galactosidase was the first to increase, at the immature stage, while PME, CMCase, and α-arabinosidase increased sharply in activity by the early-ripe stage. *Endo*-1,4-β-mannanase activity increased at the mid-ripe stage, but large rises in the activity of *exo*-PG and *endo*-PG did not occur until the fully ripe stage. The timing of the maximum activity observed also varied between enzymes. Some enzymes showed high activity throughout ripening (α-arabinosidase, PME), some increased early in ripening and then declined (*exo*-PG, CMCase), while others continued to increase even at the overripe, senescing stage (*endo*-PG, β-galactosidase, *endo*-1,4-β-mannanase).

Discussion

The ripening-related depolymerization of xyloglucan and other matrix glycans, which together with cellulose make up the major structural network of the wall, appears common to most species. Depolymerization of matrix glycans during softening has been observed in strawberry (Huber, 1984), tomato (Tong and Gross, 1988; Maclachlan and Brady, 1994; Brummell *et al.*, 1999), hot pepper (Gross *et al.*, 1986), melon (McCollum *et al.*, 1989; Rose *et al.*, 1998), kiwifruit (Redgwell *et al.*, 1991), avocado (O'Donoghue and Huber, 1992), persimmon (Cutillas-Iturralde *et al.*, 1994), peach (Hegde and Maness, 1998), and bell pepper (Harpster *et al.*, 2002*a*). Other cell wall changes during ripening frequently include some solubilization, depolymerization, and de-methylesterification of acidic pectins, which are found both as a network in the primary wall and in the middle lamella, and the loss of neutral sugar side chains from numerous polysaccharides (Brummell and Harpster, 2001). How each of these biochemical changes

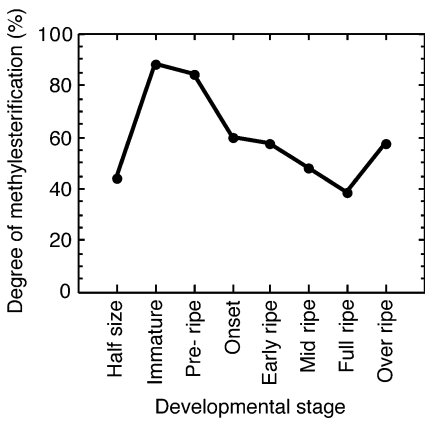

Fig. 7. Degree of methylesterification of pectins in acetone-insoluble cell walls during ripening. Note that values are an average for the whole unfractionated wall, and individual polymers or domains of polymers may possess degrees of methylesterification that are much higher or lower.

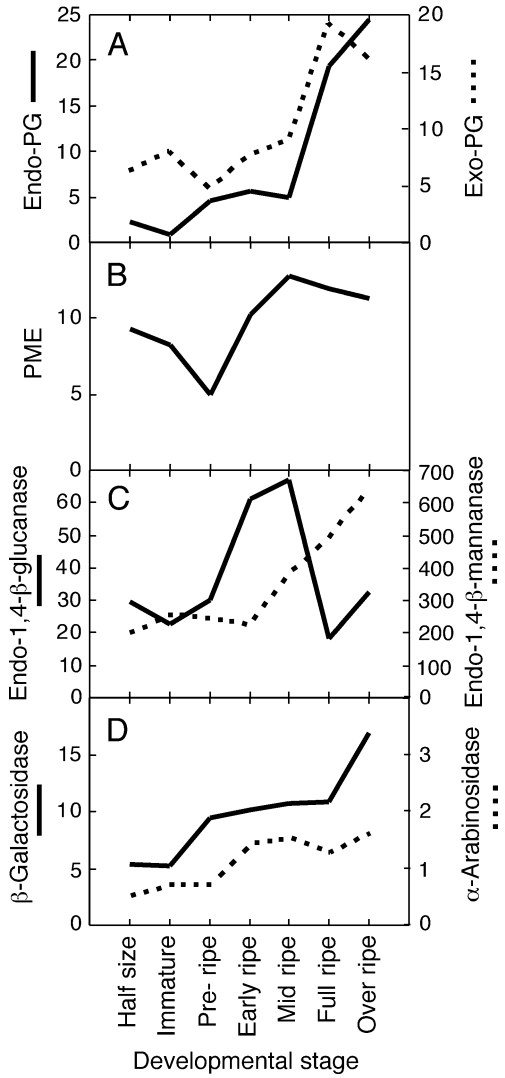

Fig. 8. Specific activities of cell wall modifying enzymes during ripening. (A) *Endo*-PG and *exo*-PG. (B) PME. (C) *Endo*-1,4-β-glucanase and *endo*-1,4-β-mannanase. (D) α-Arabinosidase and β-galactosidase. Activities of *endo*-PG (versus citrus pectin), *endo*-1,4-β-glucanase (versus CMC) and *endo*-1,4-β-mannanase (versus locust bean gum) were estimated by calculating initial rates of viscosity loss in a viscometer. *Exo*-PG activity was assayed by measuring the calcium-dependent rate of release of reducing sugar from citrus pectin. PME activity was determined relative to standards of citrus PME by measuring de-methylesterification of citrus pectin using a plate assay. Activities of α-arabinosidase and β-galactosidase were determined using 4-nitrophenyl derivatives of α-L-arabinofuranoside and β-D-galactopyranoside, respectively. All activities are expressed as units mg^{-1} protein h^{-1}. It was not possible to estimate activities in ripening stage 4 (Onset) due to lack of tissue.

contributes to wall swelling, reduced wall stiffness, reduced intercellular adhesion, and the alterations in different aspects of the physical properties of cell walls and tissues is not known. It is increasingly apparent, however, that softening and textural changes are brought about by the actions of a multitude of cell-wall-localized enzymes acting on specific, potentially highly localized substrates in a prescribed manner.

The different phases of peach development examined can be divided into five periods. The first period (stage 1) is expanding, rapidly growing fruit, the second period (stages 2 and 3) is the initiation of softening in full-size pre-climacteric fruit, the third (stages 4 to 6) is the ripening-related softening period marked by the beginning of ethylene evolution, the fourth (stage 7) is the late softening, melting period, including the climacteric peak, and the fifth (stage 8) is overripe fruit entering senescence. Expanding fruit were very firm, and had CDTA-soluble pectin of very high molecular weight and low solubility. Matrix glycans were also of high molecular weight. As fruit reached full size there were small compositional changes in the 4% KOH-soluble matrix glycans, mainly a reduction in Xyl and increase in Ara, and slight declines in the activities of CMCase and PME. Higher levels of CMCase activity in growing fruit have also been recorded in tomato (Hall, 1964) and Japanese pear (Yamaki and Kakiuchi, 1979).

Softening began early and prior to the beginning of ripening (Trainotti *et al.*, 2003), i.e. moving from stage 2 to stage 3, and cell wall changes were also detected at this time. There was a small increase in the amount of matrix glycan soluble in 4% KOH, and the depolymerization of matrix glycans both loosely and tightly bound to cellulose began. A slight but progressive loss of Gal from polymers in all extracts was observed, corresponding with a doubling in the specific activity of β-galactosidase. A small increase in *endo*-PG activity was also apparent, but depolymerization of CDTA-soluble polyuronides was barely detectable. The relative timing of the beginning of cell wall modifications and the climacteric rise appears to vary between species. In kiwifruit, cell wall changes resulting in wall swelling were observed before the ethylene production associated with the climacteric (Hallett *et al.*, 1992), whereas ultrastructural changes were not observed in avocado until during the climacteric (Platt-Aloia *et al.*, 1980), and in tomato began 3 or 4 d after the onset of ethylene production (Crookes and Grierson, 1983).

Stage 4 marked the beginning of ripening proper, and softening increased substantially throughout the period of the climacteric rise, represented by stages 4 to 6. During this time pectins became more easily extractable, with enhanced amounts of loosely bound pectins at the expense of tightly bound molecules, but this occurred without detectable depolymerization, as reported in nectarine (Dawson *et al.*, 1992). While solubilization of polyuronide from the pectin network can be caused by PG-mediated pectin degradation (Giovannoni *et al.*, 1989), depolymerization is not always necessary for solubilization. In peach, solubilization preceded depolymerization, as in kiwifruit (Redgwell *et al.*, 1992), rather than occurring simultaneously as in tomato (Brummell and Labavitch, 1997). The decline in the Gal content of the tightly bound pectins and matrix glycans ended, while the progressive depolymerization of both loosely bound and tightly bound matrix glycans

continued, and an increased solubilization of matrix glycans began at the end of this period, in stage 6. During this period there was a large increase in the activity of CMCase, and the beginning of a rise in *endo*-1,4-β-mannanase activity. The largest change in neutral sugar composition was in the loosely bound matrix glycans, which lost massive amounts of Ara, consistent with a similar change observed in ripening nectarines (Dawson *et al.*, 1992), and which correlated with a substantial increase in α-arabinosidase activity. Only the mol% of Glc substantially increased, suggesting that some Gal and Xyl may also have been lost. The identity of the polysaccharide(s) from which Ara is lost is unknown, but may include Ara side chains of glucuronoarabinoxylan and the arabinan side chains of firmly bound RG-I, or possibly protein, extracted with the matrix glycan fraction. Polysaccharide purification followed by methylation analysis is necessary to establish this point.

The melting period of softening, stage 7, was marked by a large decline in firmness, increased solubility of both loosely bound pectins and matrix glycans and decreases in the amounts of tightly bound molecules. Depolymerization of CDTA-soluble pectins was dramatic, with a massive loss of large molecules and the accumulation of small polyuronides. This was coincident with large increases in the activities of both *exo*- and *endo*-PG, as noted earlier (Downs *et al.*, 1992; Orr and Brady, 1993). The degree of methylesterification of pectins declined substantially, as PME activity remained high. A decrease in the Gal content of CDTA-soluble pectins occurred, correlated with a large increase in the activity of β-galactosidase. Both loosely bound and tightly bound matrix glycans also showed a marked depolymerization during the melting period, but the activity of CMCase declined, suggesting this enzyme was not the cause of this change. Matrix glycans showed a striking decrease in Ara content, corresponding both with the high activity of α-arabinosidase during this stage and with an increase in the polymeric Ara content of the most soluble pectic extract.

The senescence of fruit, i.e. stage 8 compared with stage 7, was associated with continuing high levels of most cell-wall-degrading enzymes including increases in the activities of *endo*-PG, *endo*-1,4-β-mannanase, and β-galactosidase, and extensive further depolymerization of CDTA-soluble pectins. These changes were correlated with a large decline in firmness, indicating the failure of intercellular adhesive links and the beginning of cellular collapse.

In general, the measured activities of the enzymes correlated reasonably closely with a corresponding modification in cell wall polysaccharides. However, the timing and extent of the increases in activity during ripening varied widely between enzymes, and did not necessarily correlate with ethylene evolution (Trainotti *et al.*, 2003). The two activities that did not correlate with an apparent cell wall modification were CMCase and *endo*-1,4-β-mannanase. *Endo*-1,4-β-glucanase activity of the CMCase type does not appear to be responsible for matrix glycan depolymerization in either pepper or tomato (Harpster *et al.*, 2002*a*, *b*). A very large increase in the activity of *endo*-1,4-β-mannanase was observed, as has been described in several fruit including peach (Bourgault *et al.*, 2001), but all the cell wall extracts contained very low levels of Man. However, *endo*-1,4-β-mannanase, and possibly its substrate, may be localized to specific cell types as in tomato (Bewley *et al.*, 2000). The substrates and functions of both *endo*-1,4-β-mannanase and *endo*-1,4-β-glucanase remain obscure.

The data show that dismantling of the matrix glycan and pectic networks are independent events in peach and both begin early in the softening process, but take different forms. For the pectic network the first change is a progressive removal of galactan side chains from RG-I, followed by a dramatic loss of Ara perhaps from RG-I firmly attached to cellulose and extracted with the matrix glycan fraction. For the matrix glycan network, the first changes are a progressive removal of Gal side chains from tightly bound molecules, probably a loss of the Ara side chains of glucuronoarabinoxylan, and a depolymerization of matrix glycan backbones, including xyloglucan. Softening correlated closely with the depolymerization of matrix glycans, which occurred in both the loosely bound and tightly bound fractions. In this, peach is similar to kiwifruit and Charentais melon (Redgwell *et al.*, 1991; Rose *et al.*, 1998), but differs from tomato where depolymerization occurs only in tightly bound molecules (Tong and Gross, 1988; Harpster *et al.*, 2002*b*). Depolymerization of matrix glycan molecules was limited in extent, as in other species (Redgwell *et al.*, 1991; O'Donoghue and Huber, 1992; Cutillas-Iturralde *et al.*, 1994; Rose *et al.*, 1998; Brummell *et al.*, 1999), and changes in molecular weight were modest with no accumulation of small fragments. This together with the reduction in polydispersity during ripening has been interpreted as showing that matrix glycan depolymerization is a controlled process resulting predominantly from cleavage near the ends of molecules, in the regions attached to cellulose, rather than wholesale cleavage in the regions spanning between microfibrils (Brummell and Harpster, 2001).

Increased solubilization of loosely bound polyuronides began when softening was well advanced, but depolymerization did not occur until late in ripening, coincident with the rapid softening and textural change of the melting stage. Polyuronide depolymerization, therefore, does not appear to be necessary for pectin solubilization or the early stages of softening, but is important in the melting stage, where it may reduce fruit firmness through effects on the integrity of the middle lamella and intercellular adhesion (Langley *et al.*, 1994; Atkinson *et al.*, 2002). Depolymerization of chelator-soluble polyuronides occurs late in peach, as in melon (Rose *et al.*, 1998), rather than throughout ripening as in tomato (Huber and O'Donoghue, 1993; Brummell and

Labavitch, 1997), but is considerable in degree, similar to tomato at the edibly ripe stage but not as extensive as in avocado (Huber and O'Donoghue, 1993). Softening also correlated, but less precisely, with the removal of certain neutral sugar side chains. Loss of particular side chains may be important only for specific stages of the entire softening process, which begins early in fruit maturation and continues throughout ripening. Cell wall disassembly varies during the different periods of fruit maturation, and involves a series of overlapping phases consisting of depolymerization and modifications to various polymers at particular times. However, transgenic experiments in ripening tomato (Smith *et al.*, 2002) show that modification of polymer side chains at early times can affect fruit firmness at later ripening stages.

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