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RESEARCH PAPER

Cell wall metabolism during the development of chilling injury in cold-stored peach fruit: association of mealiness with arrested disassembly of cell wall pectins

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Abstract

Partially tree-ripened ripe fruit of peach (*Prunus persica* L.) were stored for 1–4 weeks at 5 °C and then ripened at 20 °C for 3 d to induce chilling injury. With increasing cold storage the incidence and severity of mealiness symptoms increased progressively, manifested as reduced quantities of free juice and internal flesh browning. Relative to juicy fruit, tissue of mealy fruit showed altered intercellular adhesion when examined by microscopy and, upon crushing, a higher proportion of cells remained intact and did not release cellular contents. Substantial alterations in the metabolism of cell wall polysaccharides were observed. Chelator-soluble polyuronides from mealy fruit were partially depolymerized during cold storage in a manner dissimilar to that in unripe or ripe juicy fruit, and were not depolymerized further during the ripening period. The solubility of these high molecular weight pectins remained low, and did not show the increase characteristic of juicy fruit. Furthermore, in mealy fruit the dramatic decline in the polymeric Ara content of base-soluble, matrix glycan-enriched fractions occurring during normal ripening was absent, indicating diminished disassembly of an arabinan-rich polysaccharide firmly attached to cellulose. A corresponding rise in the polymeric Ara content of the most soluble pectin fraction was also absent, as was a decline in the Gal content of this extract. The depolymerization of matrix glycans showed only minor differences between juicy and mealy fruit. After cold storage and ripening, the activities of *endo*-1,4-β-glucanase (EC 3.2.1.4), *endo*-1,4-β-mannanase (EC 3.2.1.78), β-galactosidase (EC 3.2.1.23), α-arabinosidase (EC 3.2.1.55), and particularly *endo*-polygalacturonase (EC 3.2.1.15) were lower in mealy fruit than in juicy fruit, whereas pectin methylesterase activity (EC 3.1.1.11) was lower in slightly mealy and higher in very mealy fruit. The data suggest that cold storage affects the activities of numerous cell wall-modifying enzymes, with important consequences for pectin metabolism. These changes alter the properties of the primary wall and middle lamella, resulting in tissue breakage along enlarged air spaces, rather than across cells, which reduces the amount and availability of free juice upon tissue fragmentation.

Key words: Cell wall, chilling injury, matrix glycan, mealiness, pectin, *Prunus persica*.

Introduction

The storage of peaches at low temperatures for prolonged periods can induce a form of chilling injury called mealiness (woolliness), characterized by a lack of juiciness and a mealy texture (Lill *et al*., 1989). In the more advanced stages, the internal flesh also shows tissue separation and discoloration evident as browning. Both peaches and

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

nectarines exhibit the disorder, with variation in susceptibility between different cultivars (Crisosto *et al*., 1999). Plums and apricots can also be affected, although the symptoms are slightly different with a gel-like region forming near the stone (Taylor *et al*., 1993). It is not possible to detect mealiness from the exterior of the fruit, since affected fruit weigh the same, and have a similar firmness to the fingers. However, on biting into the fruit the lack of juice results in an absence of flavour and a dryness which make it inedible, and leads to consumer dissatisfaction. Mealiness is caused by storage of fruit in the 2–8 °C range for a period of at least 2 weeks (Ben-Arie and Lavee, 1971), and is of commercial importance due to the need to ship and store fruit prior to sale.

Numerous studies of the biochemical basis for mealiness have identified factors which may be important in the development of the symptoms, although considerable discrepancy exists between studies. Compared with juicy fruit, mealiness has been found to be associated with a reduction in pectin methylesterase (PME) activity (Buescher and Furmanski, 1978), or with an increase (Ben-Arie and Sonego, 1980), or with unchanged levels (Obenland and Carroll, 2000; Zhou *et al*., 2000*a*). Similarly, *exo*-polygalacturonase (*exo*-PG) activity was reduced in mealy fruit in some studies (Zhou *et al*., 2000*a*), or showed no correlation with mealiness in others (Artes *et al*., 1996). A reduction in *endo*-PG activity during cold storage has commonly been observed (Buescher and Furmanski, 1978; Ben-Arie and Sonego, 1980; Artes *et al*., 1996; Zhou *et al*., 2000*a*), although mealiness develops not in cold storage but during the subsequent ripening period at warm temperatures (Buescher and Furmanski, 1978). If the cold period exceeds a certain critical length (of approximately 2 weeks), or the ripening period is short, no increase in *endo*-PG activity occurs during ripening and, instead of juiciness, the fruit develop mealiness (Ben-Arie and Sonego, 1980; von Mollendorff and de Villiers, 1988*b*). During the ripening period fruit may develop mealiness properties with low extractable juice, but upon extended ripening become juicy (von Mollendorff and de Villiers, 1988*b*; von Mollendorff *et al*., 1989, 1993). However, this apparent restoration of free juice may be due to tissue breakdown and senescence processes.

In mealy fruit the most easily extractable cell wall pectins (soluble in water or chelator) are reduced in amount and are of higher molecular weight and viscosity than in ripened, juicy fruit (Buescher and Furmanski, 1978; von Mollendorff and de Villiers, 1988*b*; Dawson *et al*., 1992; Zhou *et al*., 2000*a*). The degree of methylesterification of pectin may also be altered (Ben-Arie and Lavee, 1971). Cell wall pectin participates in the wall in cell-to-cell adhesion, which is accomplished largely by calcium cross-linking between partially de-methylesterified homogalacturonan in the middle lamella (Jarvis *et al*., 2003; Vincken *et al*., 2003). It has been suggested that changes to pectin metabolism cause mealiness either by cell fluids forming calcium-pectate gel complexes with high molecular weight pectin in the middle lamella (Ben-Arie and Lavee, 1971), or that the decreased intercellular adhesion in mealy fruit reduces cell rupture during biting and chewing, preventing the release of cellular contents (King *et al*., 1989).

The above studies have been carried out using different peach or nectarine cultivars and different lengths and extents of temperature treatment to examine particular aspects of cell wall polymer metabolism or enzyme activity. The aim of the present work was to examine cell wall polymer depolymerization, cell wall polysaccharide sugar composition, enzyme activity, and tissue physical characteristics comprehensively in the same study. Increasing time of cold storage was used to induce increasing severity of mealiness symptoms in order to examine which changes in cell wall polymers or enzyme activities precede or accompany the development of a mealy texture.

Materials and methods

Plant material

Fruit from peach trees [*Prunus persica* (L.) Batsch, cv. O'Henry] growing in the Central Valley of California were harvested when they reached a firmness of 55–65 N (pre-climacteric but physiologically mature, equivalent to developmental stage 4 in the accompanying study, Brummell *et al*., 2004). O'Henry is a mid-season peach variety moderately susceptible to the development of chilling injury storage disorders (Crisosto *et al*., 1999). Fruit were placed into storage at 5 °C (90% relative humidity), and batches were removed from storage after periods of 1, 2, 3, or 4 weeks. Upon removal from cold storage, two plugs 1 cm in diameter were excised from the same side of each fruit and frozen in liquid N_2, recording from which fruit they had been taken. The holes in the fruit from which the plugs had been removed were treated with fungicide, and the fruit incubated at 20 °C for 3 d to ripen. After this ripening period, fruit were cut into quarters, and the quarter from which the plugs had been taken was discarded. Two of the remaining quarters were used to estimate free juice by the method of Crisosto and Labavitch (2002), and the remaining quarter frozen in liquid N_2 for later analysis of cell wall polysaccharides and enzyme activities. Discoloration (flesh browning) of the mesocarp tissue was estimated visually by comparison to an arbitrary 5-point reference scale, consisting of 0, 25, 50, 75, and 100% of the maximal extent of internal browning typically seen.

After cold storage for 1 or 2 weeks, fruit were divided into those which became juicy after ripening, and those which became mealy. After storage for 3 or 4 weeks, all fruit became mealy or leathery after ripening. For cell wall analysis, plugs were pooled from fruit which would become juicy upon subsequent ripening (predominantly 1 week of storage), and from fruit which would become mealy upon subsequent ripening (predominantly 3 and 4 weeks of storage). For enzyme activity measurements, plugs from fruit stored for 1 or 2 weeks were kept separate, and then pooled into groups based on whether the fruit from which they had been excised subsequently became juicy or mealy after ripening. This approach was used to reduce sample variability, and to ensure the distinction between what would become juicy or mealy tissue. Only a few fruit became juicy after 2 weeks of storage followed by ripening; the small amount of tissue plugs obtained was sufficient solely for assay of enzyme activities, and the tissue of the rest of these fruit after ripening was used only for measurement of free juice content and enzyme

activities. Other than this category, tissue from at least 15 fruit was pooled into each of the various categories examined.

Preparation and fractionation of cell walls

Powdered mesocarp tissue was homogenized in 80% ethanol and treated with TRIS-buffered phenol then chloroform:methanol (1:1, v/v) and washed with acetone as previously described (Brummell *et al*., 2004). Aliquots (100 mg) of acetone-insoluble cell walls were sequentially extracted as described (Brummell *et al*., 2004) with *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA) then with Na_2CO_3 containing 0.1% $NaBH_4$, to produce extracts enriched in ionically-bound and covalently-bound pectin, respectively. Depectinated cell wall residues were extracted with 4% KOH and 24% KOH (both containing 0.1% $NaBH_4$) to produce extracts enriched in loosely-bound and tightly-bound matrix glycans, respectively.

Size exclusion chromatography

Pectic extracts were dialysed against deionized water, and pectin 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. Matrix glycan extracts were neutralized on ice with glacial acetic acid and then precipitated with ethanol. Pellets were redissolved and treated with α-amylase to remove starch as described by Brummell *et al*. (2004), then dialysed and pectin and neutral sugar contents were quantified as above. Volumes containing 2 mg neutral sugar were freeze-dried. Freeze-dried samples were redissolved and separated by size exclusion chromatography on columns of CL-2B (for pectic samples) or CL-6B (for matrix glycan samples) as described (Brummell *et al*., 2004). Column fractions were assayed for contents of uronic acids and neutral sugars using the assays above.

Neutral sugar composition

Samples of dialysed unchromatographed pectic extracts and various peaks of matrix glycan extracts after size exclusion chromatography were converted to alditol acetates, separated on a gas chromatograph and quantified relative to an inositol internal standard as described (Brummell *et al*., 2004).

Degree of pectin methylesterification

Samples of acetone-insoluble cell walls (~1 mg) were assayed for methanol released by the method of Wood and Siddiqui (1971) and for uronic acid content according to Blumenkrantz and Asboe-Hansen (1973). Methanol content was related to galacturonic acid equivalents on a mol basis to calculate degree of methylesterification.

Microscopy

Mesocarp tissues from samples described above were fixed in 4% gluteraldehyde in citrate-phosphate buffer, pH 7, and 4% sucrose. After dehydration in a graded series of increasing concentrations of ethanol they were embedded using a Leica Historesin Kit (Leica Instruments GmbH, Heidelberg, Germany). Sections 200 μm thick were cut with a microtome (model 2050, Reichert-Jung, Vienna, Austria) using a glass knife. For light microscopy, sections were stained for 5 min with 10-fold diluted MultiStain (0.61 g toluidine blue and 0.225 g basic fuchsin in 100 ml of 30% ethanol), then washed with water and examined using a light microscope.

For immunostaining, sections were blocked with 1% non-fat dried milk in PBS, then reacted with LM5 or LM6 monoclonal antibodies at dilutions of 1:200 at 4 °C overnight. The sections were washed three times in PBS and then exposed to rabbit anti-rat IgG antibody conjugated to fluorescein isothiocyanate (Sigma) at a dilution of 1:1000 at room temperature for 1 h. The sections were washed three times in PBS, once in water and then examined in a light microscope using fluorescent light. Images were recorded as below.

To examine cell separation during tissue rupture, a cylinder of mesocarp tissue cut from juicy or mealy fruit was placed into a 5 ml plastic syringe (without needle) inside which four layers of cheesecloth had been placed over the exit aperture. The tissue was pressed through the cheesecloth and luer aperture with the plunger, and the juice passing through the aperture was collected. Large pieces of tissue did not pass through the cheescloth, and the collected juice contained only single or small clumps of cells. The juice was stained with fluorescein diacetate, and examined under the light microscope using fluorescence and visible light. Images were recorded using an Opthonics 750 digital camera (Opthonics Inc., Goleta, CA) on a Nikon E600 microscope (Nikon USA, Stanford, CT).

Enzyme activity

Powdered frozen mesocarp tissue was stirred in 12% polyethylene glycol then washed and extracted with low or high salt buffer as described by Zhou *et al*. (2000*a*). For PG activity, insoluble residues were extracted with 0.75 M NaCl/50 mM sodium acetate, pH 5.0, and clarified soluble protein extracts used for enzyme activity determination. *Endo*-PG activity was measured by viscometry versus citrus pectin (0% methylesterification) in 50 mM sodium acetate, pH, 4.4. Calcium-dependent *exo*-PG activity was assayed using 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 measuring increases in reducing sugar content. One unit of *exo*-PG activity was defined as 1 μg reducing sugar produced mg^{-1} protein h^{-1}. In assays for both *endo*- and *exo*-PG, NaCl was at a final concentration of 0.15 M in the reaction mixture.

For other enzymes, after extraction in polyethylene glycol, insoluble fruit material was extracted with 1.25 M NaCl/4 mM EDTA, pH 6.5, and the filtered supernatant clarified by centrifugation. *Endo*-1,4-β-glucanase activity was measured versus carboxymethylcellulose (high viscosity, Sigma) in 0.1 M citrate-phosphate, pH 7.0, and *endo*-1,4-β-mannanase activity was measured against locust bean gum in 0.1 M citrate-phosphate, pH 5.0, both by viscometry. For viscometry, 1 unit was defined as a 1% decrease in viscosity mg^{-1} protein h^{-1}. 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 comparing with a standard curve of PME from orange peel. α-L-Arabinosidase and β-D-galactosidase activity were measured in 0.1 M sodium acetate, pH 5.0, against the appropriate 4-nitrophenyl-glycoside. One unit was defined as 1.0 A_{400} unit mg^{-1} protein h^{-1}. Protein concentration was determined using a protein assay kit (Bio-Rad) with bovine γ-globulin as a standard. For further experimental details see Brummell *et al*. (2004).

Results

Partially ripened fruit were stored at 5 °C for 1–4 weeks then ripened at 20 °C for 3 d and, based on the texture of the mesocarp after cutting open the fruit, were divided into those which had become juicy and those which had become mealy. Mealiness was observed as a lack of juice on the cut surface, a grainy texture to the flesh and a degree of discoloration. Quarters of fruit were used for determining the amount of free juice in the tissue (Fig. 1A), and discoloration was assessed as the degree of flesh browning (Fig. 1B). Some fruit stored for 1 or 2 weeks developed

a juicy texture, with no internal browning and high levels of free juice. Other fruit became mealy after 1 or 2 weeks of storage, and after 3 weeks of storage all fruit developed mealy or leathery symptoms. The degree of mealiness increased substantially with increasing length of cold storage, and was minor after 1 week of storage with slight flesh browning and free juice almost as high as in similarly stored juicy fruit. As the severity of mealiness symptoms increased, free juice declined from over 50% after 1 week of storage to only 13% after 4 weeks of storage. The degree of flesh browning also increased, but was minimal after 1 or 2 weeks of storage, suggesting that changes in free juice occur early in the mealiness process and browning occurs later. After 3 or 4 weeks in cold storage, some fruit developed a leathery texture. This was similar to mealiness in that free juice was low and flesh browning was high, but the texture of the flesh was firm rather than grainy.

The extractability of pectin loosely attached to the wall by ionic bonds and solubilized by chelating agents was relatively high in fruit stored for 1 week and ripened to a juicy texture, but was substantially less in similar fruit which had developed a slight mealiness (Fig. 2A). With increasing length of cold storage and increasing development of mealiness, the extractability of chelator-soluble polyuronides declined further, and fruit stored for 4 weeks then ripened had only half as much chelator-soluble pectin as juicy fruit. Fruit developing leatheriness also had reduced amounts of chelator-soluble pectin. In plugs taken from fruit after cold storage but prior to the ripening period, moderate amounts of chelator-soluble pectin were observed, with slightly higher amounts from fruit which subsequently would become mealy. Amounts of covalently bound pectin extracted by treatment with Na_2CO_3 (Fig. 2B) were lower than those of ionically bound pectin (Fig. 2A), with which they showed an approximate inverse correlation. Juicy fruit possessed pectin of greater extractability than mealy fruit, with high amounts of loosely bound polyuronides and low amounts of tightly bound pectin. The extractability of matrix glycans showed less difference between juiciness and mealiness than did pectin. For both loosely bound (Fig. 2C) and tightly bound matrix glycans (Fig. 2D), amounts were higher in plugs taken from fruit after storage than in fruit subsequently ripened to a juicy or slightly mealy texture. After ripening, loosely bound matrix glycans showed little difference in amount after any period of storage up to 4 weeks, but with increasing mealiness the amounts of tightly bound matrix glycans increased. Leathery fruit also had greater amounts of tightly bound matrix glycans. Mealiness thus appears to be correlated with

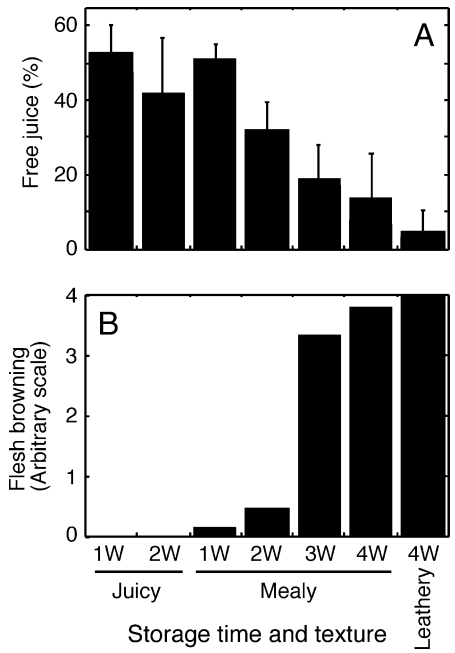

Fig. 1. Development of mealiness symptoms in stored peach fruit. (A) Free juice. (B) Flesh browning. Fruit were stored at 5 °C for 1–4 weeks (W) then ripened at 20 °C for 3 d. Fruit were divided into classes based on the texture of the flesh (juicy, mealy or leathery), and means calculated from at least seven fruit per category. Free juice was determined using the method of Crisosto and Labavitch (2002), and flesh browning was assessed visually.

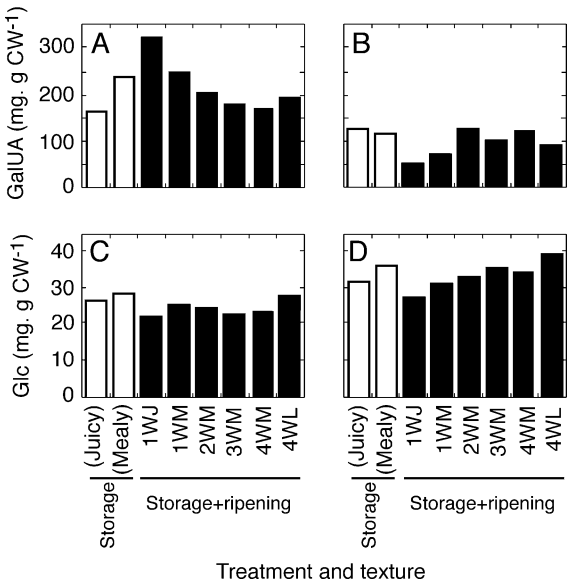

Fig. 2. Amounts of polyuronide in pectic extracts and polymeric neutral sugar in matrix glycan extracts of cell wall preparations. Fruit were stored at 5 °C for 1–4 weeks (W) then ripened at 20 °C for 3 d (solid bars), and fruit categorized as juicy (J), mealy (M), or leathery (L) texture. Plugs, taken from fruit after storage but prior to ripening (open bars), were divided into those that came from fruit which subsequently became juicy and those that came from fruit which became mealy after ripening. Acetone-insoluble cell walls were extracted sequentially with CDTA (A) and Na_2CO_3 (B) to produce extracts enriched in ionically bound pectin and covalently bound pectin, respectively. Residues were extracted with 4% KOH (C) then 24% KOH (D) to produce extracts enriched in loosely bound matrix glycan and tightly bound matrix glycan, respectively. Data are expressed as mg GalUA equivalents g^{-1} of acetone-insoluble cell walls (A, B), or mg Glc equivalents g^{-1} of acetone-insoluble cell walls (C, D).

reduced pectin solubility and increased amounts of tightly bound matrix glycans.

In plugs taken from fruit after cold storage and prior to ripening, chelator-soluble polyuronides were of a broad molecular weight distribution, but were of predominantly large size with the majority of the molecules eluting between fractions 27 and 42 (Fig. 3A, B). There was no obvious difference in size distribution between fruit which would subsequently become juicy (Fig. 3A) and those which would subsequently become mealy (Fig. 3B). In fruit stored for 1 week and then ripened, chelator-soluble polyuronides became depolymerized to low molecular weight in fruit which developed juiciness or a low level of mealiness (Fig. 3C, D). In fruit developing a high level of mealiness (Fig. 3E–G), polyuronides exhibited only minor depolymerization during the 3 d ripening period (compare Fig. 3E–G with Fig. 3A, B), with a slight increase in the amount of polyuronides eluting between fractions 42 and 65. Mealiness is thus associated with a reduced depolymerization of chelator-soluble pectin and an accumulation of large to mid-sized molecules. The development of leatheriness (Fig. 3H) showed a similar lack of polyuronide depolymerization, with no detectable depolymerization relative to pre-ripening and a greater arrest of depolymerization than in mealy fruit. Polyuronides extractable in Na_2CO_3 showed a predominant peak in fraction 60 with a minor shoulder of higher molecular weight, similar to that in unripe fruit (Brummell *et al*., 2004), and showed little change during the development of mealiness (data not shown).

Matrix glycans loosely bound to the wall showed a size distribution with two main peaks, one of high molecular weight in the void of the column and one broader peak eluting close to fraction 50 (Fig. 4). The peak eluting at the V_o of the column contained uronic acids and was composed mainly of neutral sugars associated with pectin (65–75 mol% Ara, 13–20 mol% Gal, data not shown). In fruit which had been stored, but prior to ripening, the main peak of loosely bound matrix glycans eluted with a peak in

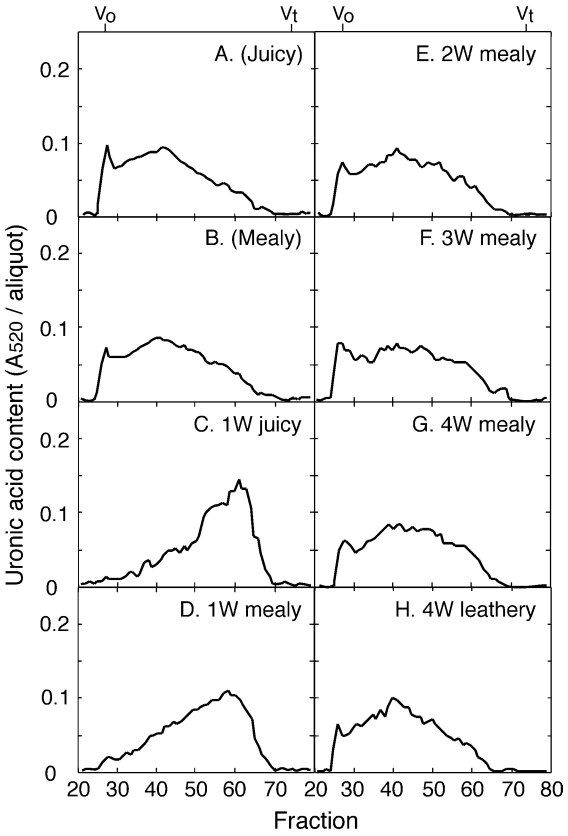

Fig. 3. Size distribution of chelator-soluble polyuronides. Polyuronides soluble in CDTA were separated by size exclusion chromatography on a column of Sepharose CL-2B and column fractions assayed for uronide content by the meta-phenyl phenol assay. The void (V_o, 20 MDa) and total (V_t, 100 kDa) volumes of the column are indicated. (A) Plugs taken after storage at 5 °C, would become juicy upon ripening; (B) plugs taken after storage at 5 °C, would become mealy upon ripening; (C) 1 week at 5 °C + ripening, juicy; (D) 1 week at 5 °C + ripening, slightly mealy; (E) 2 weeks at 5 °C + ripening, mealy; (F) 3 weeks at 5 °C + ripening, mealy; (G) 4 weeks at 5 °C + ripening, very mealy; (H) 4 weeks at 5 °C + ripening, leathery.

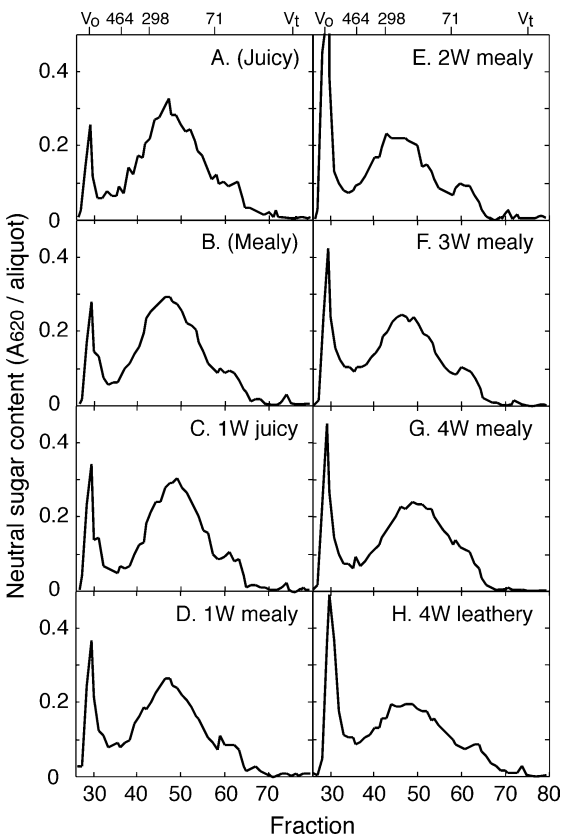

Fig. 4. Size distribution of loosely-bound matrix glycans. Polymeric neutral sugars soluble in 4% KOH were separated by size exclusion chromatography on a column of Sepharose CL-6B and column fractions assayed for neutral sugar content by the anthrone assay. The void (V_o, 1000 kDa) and total (V_t, 10 kDa) volumes of the column and elution points of linear dextran standards (in kDa) are indicated. Panels show eight temperature treatments and textures as Fig. 3.

fraction 46 or 47 (equivalent to linear dextran standards of ~210 kDa) (Fig. 4A, B). In fruit stored for 1 week and then ripened, this molecular weight profile did not change in fruit which had developed a low extent of mealiness, but in fruit which became juicy the peak eluted in fraction 49 (equivalent to dextran standards of ~160 kDa) (Fig. 4C, D), suggesting juicy fruit have slightly enhanced depolymerization of loosely bound matrix glycans. However, with increasing time of storage and increasing display of mealiness symptoms, loosely bound matrix glycans also exhibited a progressive depolymerization of the major peak (Fig. 4E–G), suggesting that this was due to physiological ageing of the fruit rather than to mealinesss. Leathery fruit (Fig. 4H) displayed a molecular weight profile resembling that of similarly-aged mealy fruit (Fig. 4G). Older mealy or leathery fruit (Fig. 4E–H) possessed a greater proportion of high molecular weight, pectic-associated neutral sugars eluting in the void of the column. Increasing amounts of pectin extracted with the matrix glycans is perhaps related to the reduced solubility of pectin in mealy fruit (Fig. 2A).

Matrix glycans tightly bound to the wall showed slight depolymerization during the development of mealiness symptoms (Fig. 5). After storage, but prior to the ripening period, the single major peak eluted with a maximum in fraction 40–42 (equivalent to dextran standards of ~310–370 kDa) (Fig. 5A, B). After 4 weeks of storage followed by ripening to a mealy or leathery texture, the major peak eluted with a broad maximum in fractions 41–44 (equivalent to dextran standards of ~270–330 kDa) (Fig. 5G, H). In both loosely and tightly bound matrix glycan fractions, increasing mealiness appeared to be accompanied by a broadening of the major peaks, indicating an increase in the polydispersity of matrix glycans, the opposite of the situation in normal ripening, although the difference was slight.

CDTA and Na_2CO_3 extracts contained Rha and relatively high contents of Ara and Gal (Fig. 6A, B), sugars typical of pectic molecules, together with smaller amounts of Xyl and Glc. CDTA extracts of fruit stored for 1 week and then ripened to either a juicy or slight mealy texture exhibited a slight loss in the mol% of Gal, and a slight increase in Ara (Fig. 6A). This change in Gal and Ara content did not occur during the development of mealy symptoms. The neutral sugar composition of pectins in the Na_2CO_3 extract showed little change during the development of mealiness symptoms (Fig. 6B). Matrix glycans were rich in Glc, Xyl, Gal, Ara, and Fuc (Fig. 6C, D), consistent with the major matrix glycan polymers consisting of fucosylated xyloglucan and an arabinoxylan. In both loosely and tightly bound matrix glycans, the development of mealiness was associated with a progressive increase (or lack of decrease) in the content of Ara, accompanied by slight declines in the mol% of Fuc, Xyl, and Glc. In the tightly bound matrix glycans a large decline in Ara content occurred after 1 week of storage followed by ripening, but this failed to occur in fruit stored for a longer time which

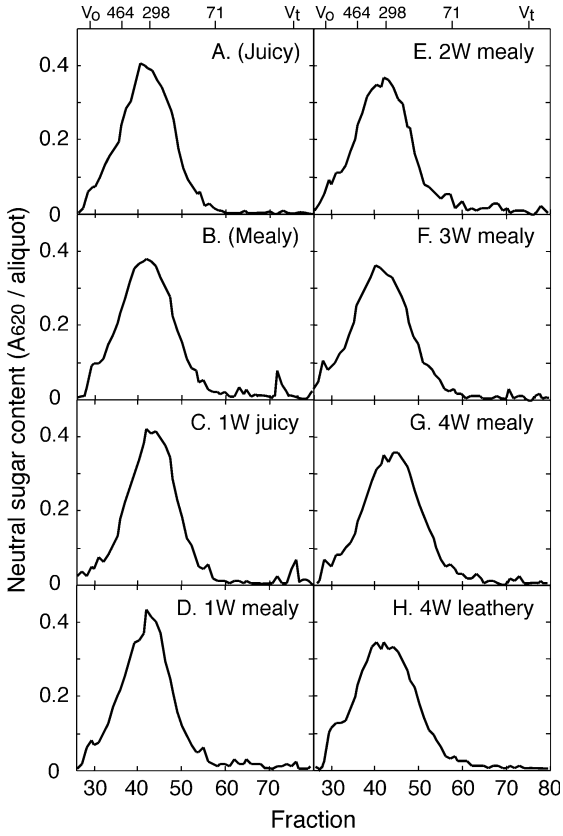

Fig. 5. Size distribution of tightly-bound matrix glycans. Polymeric neutral sugars soluble in 24% KOH were separated by size exclusion chromatography on a column of Sepharose CL-6B and column fractions assayed for neutral sugar content by the anthrone assay. The void (V_o, 1000 kDa) and total (V_t, 10 kDa) volumes of the column and elution points of linear dextran standards (in kDa) are indicated. Panels show eight temperature treatments and textures as Fig. 3.

developed a high degree of mealiness or leatheriness. The large decrease in Ara content was associated with a reciprocal but slightly smaller change in the mol% of Glc and Xyl. The content of Gal did not change in either extract, regardless of age or development of mealiness symptoms.

Methylester groups in the wall are thought to be present on pectin homogalacturonans, which are initially synthesized with a high degree of methylesterification that declines during development (Vincken *et al.*, 2003). Immediately after storage, cell walls from tissue plugs which would subsequently become juicy had a low level of methylesterification, similar to walls from ripened juicy fruit (Fig. 7). Plugs which would become mealy had a higher level of pectin methylesterification, which declined during subsequent ripening if cold storage was short and, consequently, the degree of mealiness was low. Increasing cold storage and decreasing free juice (Fig. 1) was approximately correlated with higher levels of methylesterification.

Mesocarp taken from juicy or mealy fruit was embedded in resin and thin sections examined by light microscopy (Fig. 8A–E). At harvest or after cold storage for 1 week tissue appeared similar, with strongly staining cell walls,

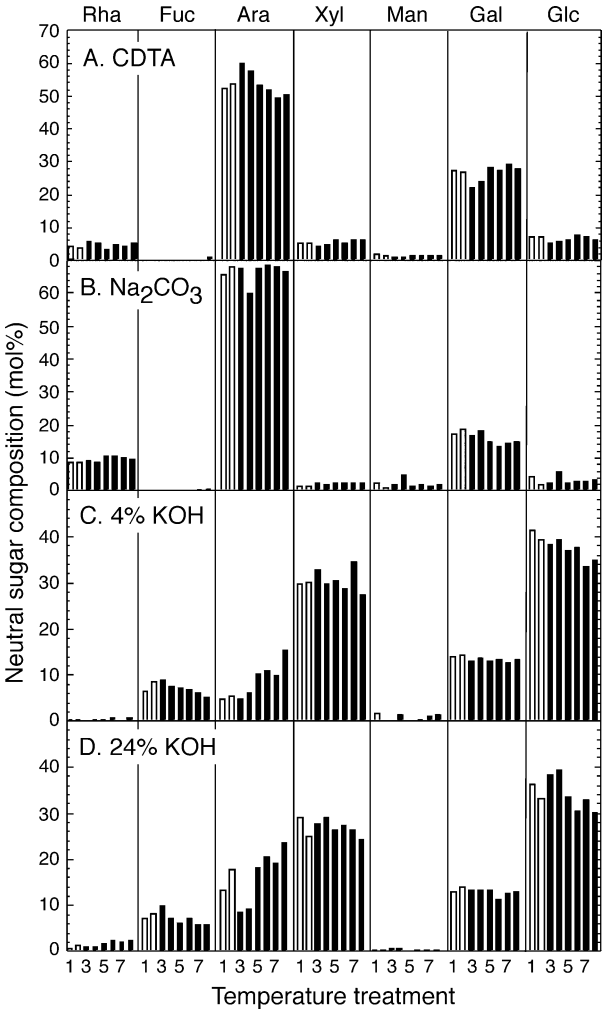

Fig. 6. Neutral sugar content of cell wall pectins and matrix glycans. Cell wall preparations were obtained from fruit stored for 1–4 weeks at 5 °C (open bars) or the same storage period followed by a ripening period at 20 °C (solid bars), after which fruit were divided into juicy, mealy, or leathery textures. (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. Alditol acetate derivatives of neutral sugars were separated by gas chromatography and quantified as mol%. Temperature treatments and textures were: 1, plugs taken after storage at 5 °C, would become juicy upon ripening; 2, plugs taken after storage at 5 °C, would become mealy upon ripening; 3, 1 week at 5 °C + ripening, juicy; 4, 1 week at 5 °C + ripening, slightly mealy; 5, 2 weeks at 5 °C + ripening, mealy; 6, 3 weeks at 5 °C + ripening, mealy; 7, 4 weeks at 5 °C + ripening, very mealy; 8, 4 weeks at 5 °C + ripening, leathery.

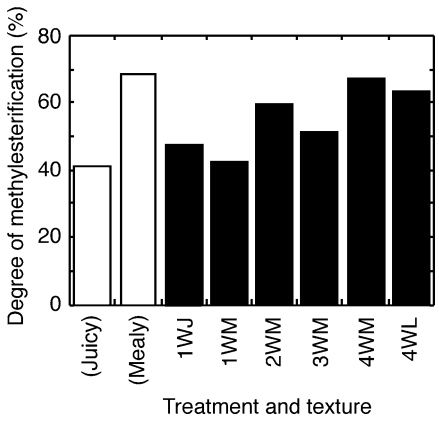

Fig. 7. Degree of methylesterification of pectins in acetone-insoluble cell walls. Cell wall preparations were obtained from fruit as Fig. 2, after 1–4 weeks cold storage (open bars) or after 1–4 weeks cold storage followed by ripening (solid bars), when fruit were divided into juicy, mealy, or leathery textures. Degree of methylesterification was calculated after conversion of methylester groups to methanol. Note that values are an average for the whole wall and individual polymers or domains of polymers may possess much higher or lower degrees of methylesterification.

tight adhesion between cells and small intercellular air spaces (Fig. 8A, B). In juicy fruit (1 week storage plus ripening), staining of the cell walls was lighter, and there was more separation between cells giving cells a slightly rounded appearance (Fig. 8C). After cold storage for 3 weeks, cells still had relatively good adhesion, but intercellular spaces were larger (Fig. 8D). Ripening after storage for 3 weeks produced mealy fruit, in which cell separation was evident (Fig. 8E). The region of contact between cells was reduced and cells assumed a more spherical form with less attachment to their neighbours, and intercellular spaces were enlarged. Sections were probed with two antibodies specific for side chains of rhamnogalacturonan I (RG-I). Immunostaining cell walls with LM5, an antibody specific for 1,4-β-D-galactans (Jones *et al.*, 1997), showed the most intense presence of the epitope adjacent to the plasma membrane and much more faintly in older regions of the wall and the middle lamella. There was little difference in the intensity of the staining between tissue from juicy (Fig. 8G) or mealy (Fig. 8H) fruit. The epitope for LM6 antibody, which is specific for 1,5-α-L-arabinans (Willats *et al.*, 1998), was also most intense next to the plasma membrane, but its distribution extended throughout the primary wall and middle lamella. Staining in tissue from juicy fruit (Fig. 8I) was weaker than in tissue from mealy fruit (Fig. 8J), suggesting that mealy fruit have a higher content or increased accessibility of RG-I arabinan side chains.

After tissue from juicy or mealy fruit had been crushed by pressing through cheesecloth and the aperture of a disposable syringe, the resulting juice contained varying numbers of whole cells and cell fragments (Fig. 8K–N). Cells in the juice were stained with fluorescein diacetate, which enters only whole viable cells (Fig. 8K, M). Juice collected from crushed juicy fruit contained almost no intact cells (Fig. 8K), whereas in juice collected from mealy fruit approximately 30% of the cells in the juice were viable, as shown by their ability to take up fluorescein diacetate stain (Fig. 8M).

The specific activities of various cell wall enzymes were examined in fruit tissue after cold storage for 1 or 2 weeks, and after cold storage for varying periods followed by a ripening period in which fruit developed either a juicy texture or increasing severity of mealiness or leatheriness

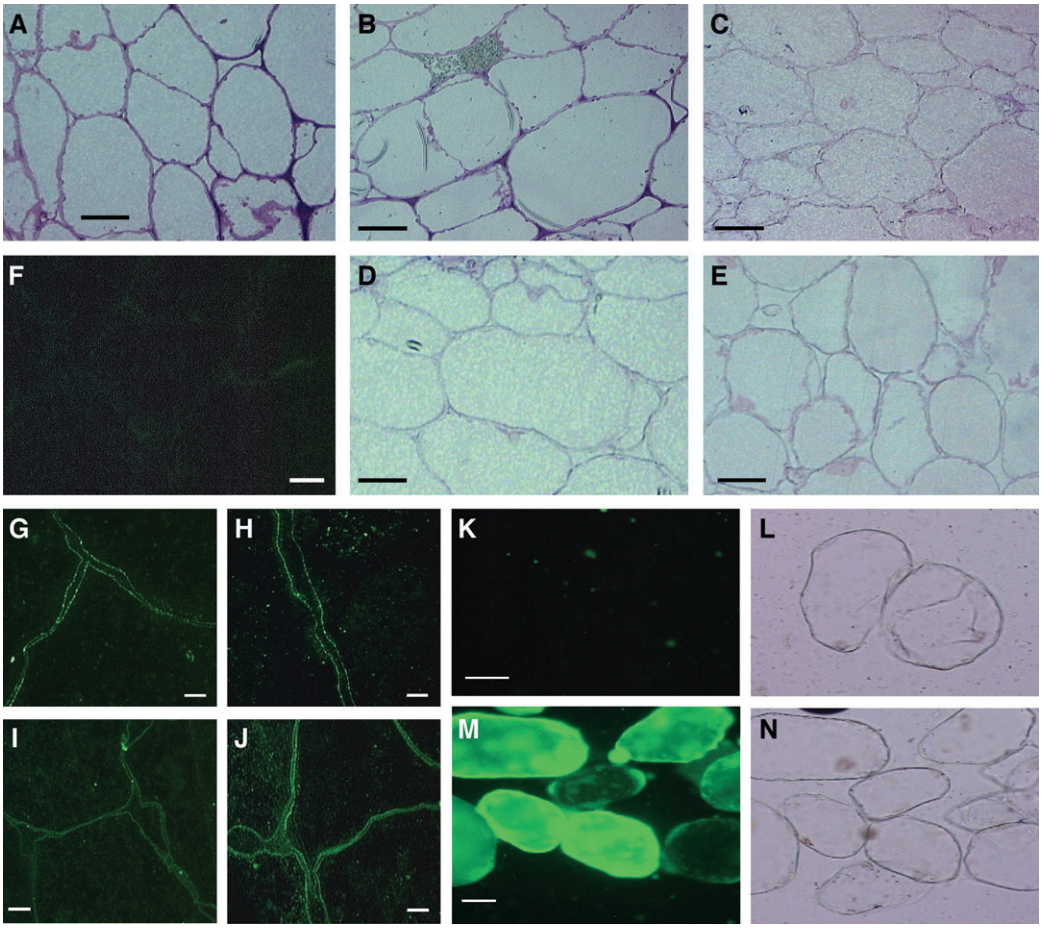

Fig. 8. Appearance of juicy and mealy fruit examined by light microscopy. (A–E) Sections of mesocarp tissue at varying stages of storage and ripening stained with toluidine blue and basic fuchsin. (G–J) Indirect immunofluorescence of juicy and mealy tissue probed with monoclonal antibodies specific for galactan (LM5) and arabinan (LM6) side chains to RG-I. (K–N) Cells in juice from crushed tissue of juicy and mealy fruit. (A) At harvest, no storage or ripening; (B) cold storage for 1 week; (C) storage for 1 week plus ripening (juicy); (D) cold storage for 3 weeks; (E) storage for 3 weeks plus ripening (mealy); (F) autofluorescence control; (G) juicy fruit as (C) immunostained with LM5; (H) mealy fruit as (E) immunostained with LM5; (I) juicy fruit as (C) immunostained with LM6; (J), mealy fruit as (E) immunostained with LM6; (K) cells in juice from crushed juicy fruit stained with fluorescein diacetate and examined under fluoresence; (L) same cells as (K) under visible light; (M) cells in juice from crushed mealy fruit stained with fluorescein diacetate and examined under fluoresence; (N) same cells as M under visible light. Scale bars represent 10 μm in (A–E) and (K–N) and 1 μm in (F–J).

symptoms (Fig. 9). Plugs taken after cold storage were developmentally more immature than cold-stored fruit which were subsequently ripened. In plugs taken from fruit which subsequently would become juicy, an increase in activity between 1 and 2 weeks of storage was seen for all the enzymes except *endo*-1,4-β-mannanase. In plugs taken from fruit which would become mealy, these activities were similar or lower than in fruit which would become juicy, and the increase was reduced or absent. After a ripening period, fruit which attained juiciness developed high activities (after either 1 or 2 weeks of storage, or both) for all the enzymes examined except PME. In mealy fruit, enzyme activities showed two patterns of accumulation. For *endo*-PG, *endo*-1,4-β-glucanase, *endo*-1,4-β-mannanase, β-galactosidase, and α-arabinosidase, activities were reduced relative to juicy fruit by half or more, even after only 1 week of cold storage when mealiness was not pronounced, and this was not exacerbated by extending cold storage for up to 4 weeks. *Endo*-PG activity was particularly low. For *exo*-PG and PME, a short cold period had a great effect on activity, which was very low in the early stages of mealiness, but increased with longer times of cold storage and greater severity of mealiness. *Exo*-PG activity did not correlate with mealiness, since both juicy and very mealy fruit had similar levels. For PME, activity eventually increased to levels higher than in juicy fruit. Relative to mealy fruit of the same length of storage, leathery fruit possessed lower activities of the enzymes examined, except for PME and *endo*-1,4-β-glucanase which were similar.

Discussion

Fruit firmness declines slowly during prolonged cold storage (von Mollendorff *et al*., 1992), and the development of mealiness symptoms is accompanied by reduced

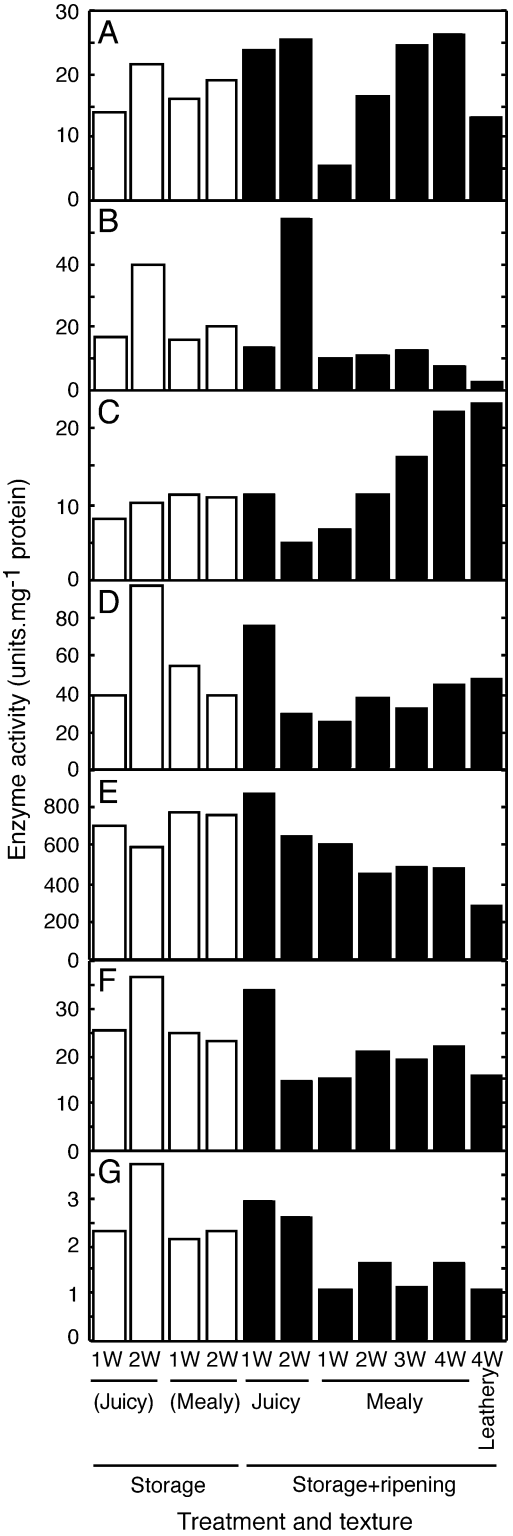

Fig. 9. Specific activities of cell wall enzymes after cold storage (open bars) and after cold storage followed by ripening (solid bars). Fruit stored at 5 °C for 1 or 2 weeks (W) had tissue plugs removed and frozen (for each time period these were subsequently separated into two groups depending on the texture of the fruit after the ripening period). Fruit stored at 5 °C for 1–4 weeks were then ripened for 3 d at 20 °C and divided into those which became juicy, mealy or leathery. Cell wall protein extracts were assayed for activites of (A) *exo*-PG; (B) *endo*-PG; (C) PME; (D) *endo*-1,4-β-glucanase; (E) *endo*-1,4-β-mannanase; (F) β-galactosidase; (G) α-arabinosidase. For definitions of units and experimental details see Materials and methods.

rates of respiration, very low ethylene evolution (von Mollendorff and de Villiers, 1988*a*; Zhou *et al*., 2001), and reductions in extractable protein of over 50% (Obenland and Carroll, 2000). These findings indicate that the normal metabolism associated with ripening is partially suspended. The synthesis of many cell-wall-modifying enzymes, including *endo*-PG and *endo*-1,4-β-glucanase, is regulated by ethylene, although the accumulation of others such as PME may be controlled by different factors (Brummell and Harpster, 2001). Plugs taken from fruit before a ripening period showed that, except for PME and *endo*-1,4-β-mannanase, increases in enzyme activity occurred between 1 and 2 weeks of cold storage if fruit would become juicy upon ripening, but that this increase was absent in fruit which would become mealy. After subsequent ripening, juicy fruit accumulated high levels of all enzymes examined, except for PME. The increase was greater after 1 week of storage for some enzymes, and after 2 weeks of storage for others. Since some enzymes increase during ripening and others increase then decrease (Brummell *et al*., 2004), the 'normal' activity of a particular enzyme is dependent on developmental stage. Juicy fruit thus retain the ability to synthesize or activate numerous enzymes both during cold storage and upon ripening, but this capacity is impaired in mealy fruit. Mealy fruit possessed low levels of all activities examined except *exo*-PG, but high levels of PME. The lack of correlation of *exo*-PG activity with mealiness (Artes *et al*., 1996) and the decline then rise in PME activity with increasing cold storage (Ben-Arie and Lavee, 1971) are consistent with previous studies. The advanced stage of mealiness was correlated with low levels of *endo*-PG and high levels of PME activity (Ben-Arie and Lavee, 1971; Buescher and Furmanski, 1978; Ben-Arie and Sonego, 1980; von Mollendorff and de Villers, 1988*b*; Artes *et al*., 1996), but also with reduced activities of *endo*-1,4-β-glucanase, *endo*-1,4-β-mannanase, β-galactosidase, and α-arabinosidase. Expansin protein amount is also reduced in mealy fruit (Obenland *et al*., 2003). These observations suggest cold storage causes a reduction in ethylene-regulated enzymes, including *endo*-PG, which are required for normal ripening in melting flesh varieties. Melting flesh peach varieties are much more susceptible to mealiness than the more firm-fruited non-melting flesh varieties (Brovelli *et al*., 1998; Crisosto *et al*., 1999) which lack *endo*-PG activity (Lester *et al*., 1996).

Altered cell wall enzyme activity during cold storage will affect the metabolism of cell wall polysaccharides. The largest difference observed was in the depolymerization of chelator-soluble polyuronides (Dawson *et al*., 1992; Zhou

et al., 1999, 2000*a*). This fraction consists predominantly of homogalacturonan from the middle lamella of the wall (Jarvis *et al*., 2003; Vincken *et al*., 2003). During normal ripening, chelator-soluble polyuronides are rapidly depolymerized from a very large to a small size during the final melting stage of ripening (Brummell *et al*., 2004). When fruit emerged from cold storage, chelator-soluble polyuronides were predominantly large (Fig. 3A, B), but were partially depolymerized relative to unripe or early ripe normal fruit, showing that some limited breakdown of polymers had occurred during storage. When fruit were ripened to a juicy texture or a slight degree of mealiness (Fig. 3C, D), the polymers were depolymerized in a manner similar to normal ripe fruit. However, when fruit became mealy after ripening, further depolymerization of these molecules was slight (Fig. 3E–G). Polyuronides from mealy fruit had a molecular weight profile that was not seen in normal ripening, and did not resemble either unripe or ripe fruit but was in between the two, suggesting a substantial alteration in pectin metabolism. The increase in solubility of pectic polymers observed in normally ripening fruit also did not occur, and solubility remained low. Most of these differences can probably be explained by the very low levels of *endo*-PG activity in mealy fruit, although the role of pectate lyase in the pectin depolymerization associated with peach ripening (Trainotti *et al*., 2003) and mealiness is unexplored. The degree of pectin methylesterification declines during normal ripening (Brummell *et al*., 2004), and was lower in juicy fruit than in mealy fruit. This suggests that de-methylesterification is retarded during the development of mealiness, consistent with the lower levels of PME activity observed early in storage. The degree of pectin methylesterification has been reported to be lower in the advanced stages of mealiness (Ben-Arie and Lavee, 1971), and it is possible that this would have been observed with longer storage, since PME activity increased to very high levels.

The depolymerization of matrix glycan molecules during the development of mealiness has been little studied previously, but reports based on single ripening stages suggest mealy and juicy fruit have similar molecular weight profiles (Dawson *et al*., 1992; Zhou *et al*., 1999, 2000*a*). A slight and progressive depolymerization of both loosely and tightly bound matrix glycans was observed during the development of mealiness, similar to that observed during normal ripening (Brummell *et al*., 2004). This suggests that mealiness does not prevent the normal depolymerization of matrix glycans. However, a slight increase in the polydispersity of matrix glycans with maturation, rather than the normal decrease, may be a consequence of the inhibitory effect of cold storage on certain enzyme activities.

Mealiness in peaches and nectarines has been associated with a reduced Gal loss from CDTA-soluble polyuronides, and a loss of Ara from most or all cell wall fractions (Dawson *et al*., 1992, 1995; Lurie *et al*., 1994; Zhou *et al*., 2000*a*). Altered pectic Ara metabolism has also been correlated with mealiness in apple (Nara *et al*., 2001). A loss of Gal from the CDTA-soluble extract observed in stored, ripened juicy fruit (Fig. 6A) and in normally ripened fruit (Brummell *et al*., 2004) was absent in mealy fruit. Mealiness was also associated with a lower Ara content in the CDTA-soluble pectin, and a higher Ara content of matrix glycan fractions. During normal ripening, the Ara content of the loosely bound matrix glycans declines dramatically throughout the early stages, followed during the melting phase, when juiciness develops, by a substantial decline in the Ara content of the tightly bound matrix glycans, coincident with a corresponding rise in the Ara content of the CDTA-soluble extract (Brummell *et al*., 2004). These changes were also observed in fruit ripened after 1 week of cold storage to a juicy texture, suggesting that an Ara-containing molecule tightly attached to the wall is metabolized to a more soluble form in juicy fruit. This change was reduced or did not occur in mealy fruit. The identity of the Ara-containing molecules involved is unknown, but may be a population of firmly-attached RG-I with arabinan side chains since increased reaction with LM6 antibody specific for such side chains was observed in mealy fruit. High molecular weight pectic arabinogalactans very strongly associated with cellulose have been found in most fruit species (Redgwell *et al*., 1997).

Ultrastructural observations show that chilling-injured mealy fruit possess greatly enlarged intercellular air spaces (King *et al*., 1989; Luza *et al*., 1992; Harker and Sutherland, 1993; Brovelli *et al*., 1998), and that while little disassembly is evident in the cellulose-containing region of the wall, there are extensive changes in the middle lamella (King *et al*., 1989; Luza *et al*., 1992). Cell separation from tissue discs incubated on a shaker found that, in normally ripened fruit, cells were typically released individually, whereas from mealy fruit cells were released in small clumps (Brovelli *et al*., 1998). Extensive cell separation is a normal part of the ripening process in melting flesh fruit, but this process differed in mealy fruit with clumps of cells splitting off at the weak points across large air pockets (Brovelli *et al*., 1998). The increased separation of clumps of cells appears to reduce cell fracture during fragmentation of the fruit (Fig. 8). A large increase in the content of pectin positive for the coriphosphene stain was detected in the middle lamella of mealy fruit (Luza *et al*., 1992), and this pectin may incorporate free juice from ruptured cells into a calcium-pectate gel (Zhou *et al*., 2000*b*), giving a dry appearance to a fracture surface of the fruit rather than the visible free juice in normally ripened fruit (Harker and Sutherland, 1993).

Highly branched RG-I pectins with arabinan and galactan side chains are firmly bound into the wall structure, and are co-extensive with homogalacturonans which, after de-methylesterification, can be cross-linked by calcium into calcium-pectate gels (Vincken *et al*., 2003). The

water-holding capacities of calcium-pectate gels are affected strongly, not only by the total degree of methylesterification but also by the random or blockwise esterification pattern (Willats *et al*., 2001). Arabinans may function in the wall by modulating the properties of homogalacturonans (Jones *et al*., 2003; Vincken *et al*., 2003), affecting water binding and cell wall characteristics. In tobacco callus, a mutant lacking arabinan side chains on RG-I had pectins which were no longer strongly associated with cellulose-matrix glycan complexes, and intercellular attachment was reduced (Iwai *et al*., 2001). Interestingly, in the *Cnr* mutant of tomato, cell wall arabinan deposition is disrupted and homogalacturonan solubilization and de-methylesterification are reduced, resulting in weakened intercellular adhesion, extensive intercellular air spaces and a mealy texture (Orfila *et al*., 2001, 2002). Compared with normal juicy peach fruit, mealy peaches had a decreased solubilization and depolymerization of middle lamella homogalacturonans, and a reduced mobilization of polymeric arabinan from molecules strongly associated with cellulose. These cell wall changes alter the properties of the cell wall and middle lamella, and tissue fragments into clumps across enlarged air spaces. It seems likely that mealiness is caused by a combination of altered tissue properties preventing the normal breakage of cells and release of juice during biting and mastication, combined with the presence of calcium-pectate gels containing high molecular weight polyuronides that can absorb or prevent the release of the contents of the few cells that are ruptured.

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