

Temporal Sequence of Cell Wall Disassembly Events in Developing Fruits. 1. Analysis of Raspberry (*Rubus idaeus*)

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Raspberry fruits were harvested at five developmental stages, from green to red ripe, and the changes in cell wall composition, pectin and hemicellulose solubilization, and depolymerization were analyzed. Fruit softening at intermediate stages of ripening was associated with increased pectin solubilization, which occurred without depolymerization. Arabinose was found to be the most abundant noncellulosic neutral sugar in the cell wall and showed dramatic solubilization late in ripening. No changes in pectin molecular size were observed even at the 100% red stage. Subsequently, as fruit became fully ripe a dramatic depolymerization occurred. In contrast, the hemicellulosic fractions showed no significant changes in content or polymer size during ripening. The paper discusses the sequence of events leading to cell wall disassembly in raspberry fruit.

KEYWORDS: Raspberry; *Rubus idaeus*; cell wall degradation; pectin; hemicellulose; fruit ripening

INTRODUCTION

The “soft fruit” grouping includes several different commodities such as strawberries, raspberries, blueberries, blackberries, and their hybrids (1, 2). This grouping has no strictly botanical basis and includes species from different families and with very diverse fruit structures. However, these fruits have several characteristics that make the grouping useful from a postharvest technological perspective. All are characterized by a rapid ripening rate and a very short shelf life (3). Controlled softening is desirable to attain optimum maturity for consumption, but excessive loss of firmness is one of the main factors limiting their storage, transport, and marketability. Fruit firmness is affected by several factors. For example, firmness may change due to altered hydrostatic pressure (turgor) within fruit cells (4). Membrane damage and dehydration and mesocarp cell enlargement could be involved in textural changes in some fruits (5). However, fruit textural changes are thought to be, at least in part, a consequence of changes in the composition and architecture of the cell wall (6). The second factor that makes soft fruit postharvest management difficult is the high susceptibility of the ripe fruit to decay caused primarily by *Botrytis cinerea* (7). This fungus produces several cell wall degrading enzymes at early stages of colonization, and some *Botrytis* polygalacturonases (PGs) are virulence factors. For instance, mutant *Botrytis* strains lacking *Bcpg1* were shown to be less virulent than wild-type strains (8). Furthermore, reduced susceptibility to *Botrytis* was observed in plants overexpressing a polygalacturonase-inhibiting protein (PGIP) (9). Consequently, both excessive softening and postharvest decay, the two main

problems limiting soft fruit postharvest life, seem to be associated with cell wall modifications. Plant cell walls are highly complex, dynamic, and organized structures composed of polysaccharides, proteins, and phenolic compounds, as well as some ions (10). The pattern and biochemical basis of the “organized disorganization” of cell walls that accompanies fruit ripening varies depending on the species considered. Many studies have analyzed cell wall changes of different fruits during ripening (6). However, so far, few detailed studies have been done to evaluate cell wall disassembly in soft fruits other than strawberry. Therefore, the objective of this work was to characterize the temporal sequence of cell wall biochemical modifications during raspberry fruit development.

MATERIALS AND METHODS

Plant Material. Raspberry fruit was harvested at five different stages: large green (G), 25% surface red color (25%R), 75% surface red color (75%R), 100% surface red color (100%R), and red ripe (RR; fully ripe). Harvested fruits were taken to the laboratory and immediately processed.

Firmness Measurement. Firmness was measured using a texture analyzer (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY) fitted with a 2 mm flat probe. Each drupelet was compressed 2 mm at a speed of 0.5 mm s⁻¹, and the maximum force developed during the test was recorded. Eighty measurements from 40 different fruits were done for each stage analyzed.

Fruit Weight and Isolation of Cell Walls. One hundred individual fruits were weighed at each developmental stage. For cell wall isolation, the endocarp and seeds were removed. To do that, the individual drupelets were cut with a scalpel and the seeds were removed with forceps. The sample was immediately placed in 95% (v/v) ethanol to limit the action of cell wall modifying enzymes isolated with the tissue. Approximately 30 g of tissue (exocarp plus mesocarp) for each developmental stage was homogenized in an UltraTurrax (IKA Werke,

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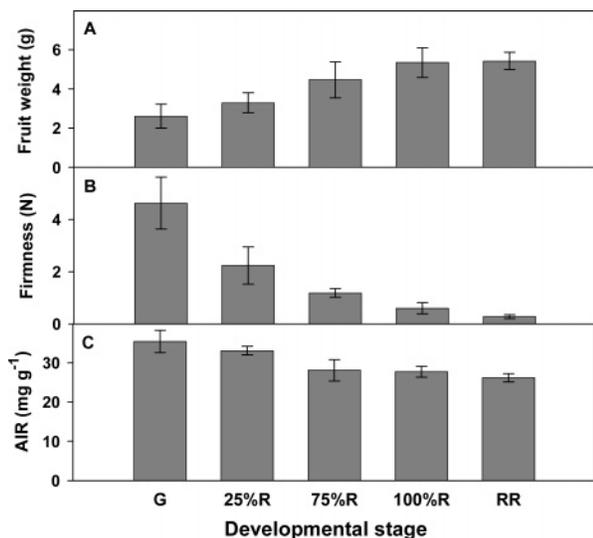


Figure 1. Changes in raspberry fruit weight (A), firmness (B), and cell wall yield (AIR) (C) during ripening. G, green; 25%R, 25% surface red color; 75%R, 75% surface red color; 100%R, 100% surface red color; RR, red ripe. The standard deviations are shown.

Janke & Kunkel GmbH & Co. KG, Staufen, Germany) with 75 mL of 95% ethanol and boiled for 45 min to ensure the inactivation of enzymes, thus preventing autolytic activity, and to extract low molecular weight solutes. The insoluble material was filtered through Miracloth (Calbiochem, EMD Biosciences, Inc., San Diego, CA) and sequentially washed with 150 mL of boiling ethanol, 150 mL of chloroform/methanol (1:1 v/v), and 150 mL of acetone, yielding the crude cell wall extract (alcohol insoluble residue, AIR). The AIR was dried overnight at 37 °C and weighed. Results were expressed as milligrams of AIR per gram of fresh fruit.

Uronic Acids (UA) and Neutral Sugar (NS) Measurements. Three milligrams of AIR was solubilized in H₂SO₄ (11), and aliquots of the AIR solution were subsequently assayed colorimetrically for UA (12) and NS (13). Absorbance readings were evaluated against a standard curve of galacturonic acid (uronic acid assay) or glucose (total sugar assay). Three independent samples were analyzed for each developmental stage, measurements were done in duplicate, and results were expressed as micrograms of galacturonic acid or glucose per milligram of AIR.

Cell Wall Fractionation. Fractions of different cell wall components were obtained by sequential chemical extraction of the cell wall material (AIR). Approximately 200 mg of AIR from each sample was suspended in 15 mL of water and stirred at room temperature for 12 h. The samples were then centrifuged at 6000g and 4 °C for 10 min, the supernatant was filtered through glass fiber filters (Whatman GF/C), and the pellet was washed with water. The filtrate and water washings were combined and designated the water-soluble fraction (WSF). The residue was then extracted with 15 mL of 50 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA), pH 6.5, and for 12 h at room temperature with stirring. The slurry was centrifuged and passed through fiber glass filters, as above, and the pellet was washed with CDTA solution. The combined filtrates were collected and designated the CDTA-soluble fraction (CSF). The CDTA-insoluble pellet was then extracted with 15 mL of 50 mM Na₂CO₃ containing 20 mM NaBH₄ at 4 °C for 12 h. After filtration (as above) the extraction solution was designated the Na₂CO₃-soluble fraction (NSF). The pellet was then extracted with 15 mL of 4% KOH containing 0.1% NaBH₄ at 4 °C for 12 h, with shaking, and the filtered extract was neutralized with acetic acid and designated the 4% KOH-soluble fraction (4KSF). This fraction was extensively dialyzed against water. The 4% KOH-insoluble residue was then subjected to an extraction using 24% KOH (steps as above) to produce the 24% KOH-soluble fraction (24KSF). Two independent serial extraction series were performed on separate AIR isolates for each developmental stage analyzed. Samples were assayed in triplicate for NS and UA as described above.

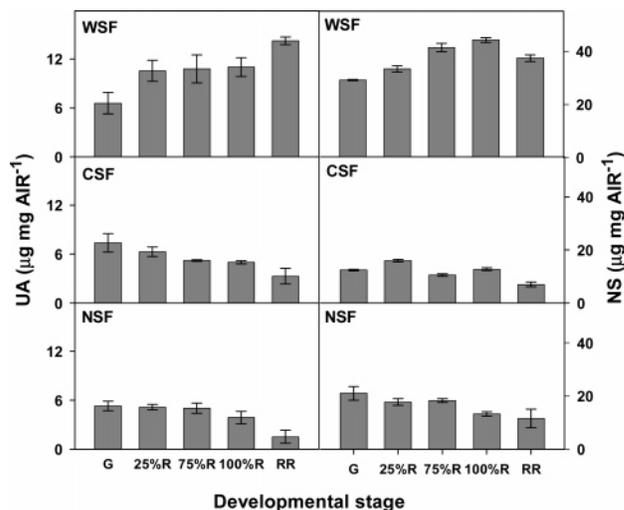


Figure 2. Content of uronic acids (left) and neutral sugars (right) in raspberry fruit WSF, CSF, and NSF during ripening. Developmental stages are defined as in the caption of Figure 1. Two independent extractions were done for each stage, and measurements were done in triplicate. The standard deviations are shown.

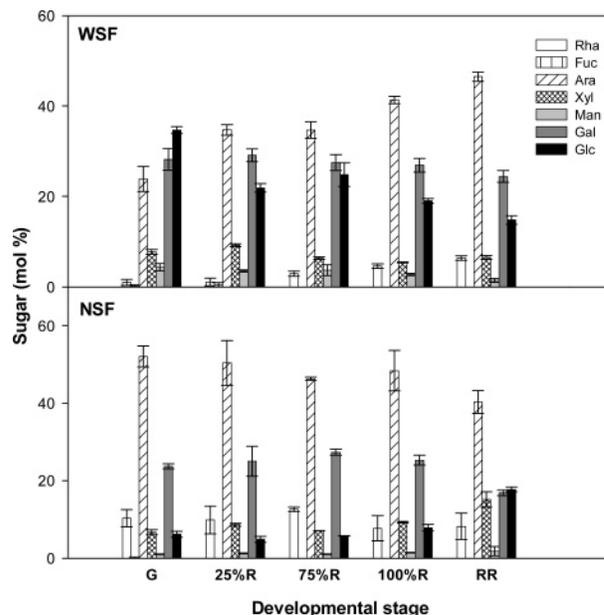


Figure 3. Neutral sugar composition (mole percent) of raspberry cell wall WSF and NSF throughout ripening. Developmental stages are defined as in the caption of Figure 1. The standard deviations are shown.

Size Exclusion Chromatography (SEC). The WSF, CSF, NSF, 4KSF, and 24KSF were dialyzed (Spectrapor 1, SpectrumLabs, Rancho Dominguez, CA; MW cutoff 8 kDa) against water for 1 day at 4 °C and lyophilized. Samples from the WSF, CSF, and NSF were dissolved in 200 mM ammonium acetate, pH 5.0, chromatographed on an HW65F (fractionation range = 1×10^4 to 1×10^6 Da; Tosoh Bioscience, Tokyo, Japan) SEC column (3×30 cm) eluted with 200 mM ammonium acetate, pH 5.0. Fractions (2 mL) were collected at a flow rate of 60 mL h⁻¹ and held in a water bath (50 °C for 4 h) to volatilize the NH₄OH, which can interfere with colorimetric assays. Fractions were assayed for UA as described above. The size distributions of polymers in the 4KSF and 24KSF samples were examined by fractionating the extracts on a Sepharose CL-4B column (1.0×90 cm) (fractionation range = 7×10^4 to 2×10^6 Da; Pharmacia, Uppsala, Sweden) and eluting with 0.1 N NaOH. Fractions were collected, and aliquots were neutralized with acetic acid prior to assaying for NS, as described above.

GC-MS Analysis. Dried samples from the WSF, CSF, NSF, 4KSF, and 24KSF or 2 mg of AIR for total cell wall analysis were hydrolyzed

Table 1. Changes in the Content of Raspberry Cell Wall Uronic Acids, Neutral Sugars, and Noncellulosic Neutral Sugars during Ripening (Standard Deviations Are Shown)

	green	25% red	75% red	100% red	red ripe
UA ($\mu\text{g mg}^{-1}$)	210 \pm 31	217 \pm 29	321 \pm 46	323 \pm 24	264 \pm 21
NS ($\mu\text{g mg}^{-1}$)	593 \pm 23	616 \pm 56	572 \pm 4	536 \pm 41	535 \pm 42
Rha (mol %)	5.85 \pm 0.07	5.10 \pm 0.67	6.74 \pm 1.00	5.08 \pm 1.58	4.86 \pm 0.84
Fuc (mol %)	1.20 \pm 0.41	0.82 \pm 0.59	1.39 \pm 0.21	1.21 \pm 0.03	0.87 \pm 0.80
Ara (mol %)	35.31 \pm 3.78	36.60 \pm 3.26	32.87 \pm 2.36	32.66 \pm 3.44	39.61 \pm 0.68
Xyl (mol %)	23.74 \pm 4.39	23.44 \pm 2.12	20.04 \pm 1.63	20.76 \pm 0.28	26.40 \pm 2.16
Man (mol %)	3.76 \pm 0.12	3.83 \pm 0.19	5.38 \pm 0.48	4.18 \pm 0.38	4.08 \pm 0.15
Gal (mol %)	17.34 \pm 3.20	16.81 \pm 3.50	17.93 \pm 3.04	18.26 \pm 0.77	12.57 \pm 3.41
Glc (mol %)	12.80 \pm 1.44	13.40 \pm 2.21	15.65 \pm 1.96	17.85 \pm 2.00	11.61 \pm 0.66

in 2 N trifluoroacetic acid (TFA) (14) and converted to alditol acetates (15) for gas chromatographic analysis of neutral sugar composition. Aliquots of the derivatized samples were injected into a GC (HP model 6890) fitted with a 30 m \times 0.25 mm DB-23 capillary column (J&W Scientific, Folsom, CA) and a mass selective detector (HP model 61098A). Temperature in the injector was 250 °C, and a linear oven temperature gradient (initial temperature = 160 °C, 0 min; the oven increased at 4 °C min⁻¹ to 250 °C) was used to improve separation. The different alditol acetates were identified on the basis of their MS spectra and also by comparison with standards containing *myo*-inositol (internal standard), rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal), and glucose (Glc). Neutral sugar amounts were calculated relative to the *myo*-inositol internal standard.

Statistical Analysis. Experiments were performed according to a factorial design. Data were analyzed using ANOVA, and the means were compared by the LSD test at a significance level of 0.05.

RESULTS AND DISCUSSION

Fruit Weight, Firmness and Cell Wall Yield, and Total Cell Wall Composition. Raspberry fruit weight increased through the first three stages of ripening, with no increase after the 100% red stage had been reached (Figure 1A), and fruit firmness decreased with each ripening stage (Figure 1B). Cell wall (AIR) yield expressed as a proportion of fresh fruit mass also decreased markedly from the green to the 75%R stages, with no changes observed afterward (Figure 1C).

When the total cell wall contents of sugars and uronic acids were measured by colorimetric analysis of the H₂SO₄ solutions of AIR samples, no significant changes in the proportions of these general categories of wall constituents were found at the five stages of development analyzed (Table 1). This could indicate that no changes occurred in these components or that an equal degradation of both pectic and nonpectic cell wall components had occurred, leading to no apparent change in the overall proportions of the wall's components. However, this does not mean that there were no changes in the cell wall structure and/or composition. For instance, modification in polymer size and ramification will profoundly affect the cell wall organization but would not be revealed in these analyses. To determine whether these subtle cell wall changes occur in ripening raspberries, qualitative and quantitative analyses of fractionated cell wall material were performed.

The proportions of individual neutral sugars in the noncellulosic wall were measured by GC-MS of alditol acetate derivatives of sugars released from wall polymers by acid hydrolysis with 2 N TFA, a treatment that does not hydrolyze cellulosic glucans. Ara was found to be the most abundant sugar present in the noncellulosic cell wall (Table 1). This is similar to what has been reported in apple and pear (16), but differs from other fruits such as tomato (16), pepper (16), and melon (17) in which Gal is the predominant noncellulosic sugar. It also contrasts with earlier studies of raspberry cvs. Glen Clova

and Glen Closen (18), which identified Xyl as the predominant noncellulosic neutral sugar component. The proportions of the different sugars did not change substantially during development, although toward the end of fruit ripening a reduction in the proportion of Gal was detected. This correlated with an increase in β -galactosidase (β -gal) activity observed from the 100%R stage (data not shown). In tomato, a decline in wall Gal and an increase in β -gal activity occur, but much earlier in ripening, preceding or accompanying the increase in pectin solubilization (19).

The Xyl/Glc ratio was higher than 1 at all stages of development analyzed. Plant cell wall Xyl could be present either in glucuronarabinoxylans (GAXs), which consist of a backbone of β -(1,4)-linked Xyl residues associated with side chains of 4-*O*-methylglucuronic acid and Ara, or in the short branches of xyloglucans. GAXs are the most abundant hemicellulosic components of wood tissue and grass cell walls, where they play a major role in the organization of lignified cell walls as they can cross-link with lignin. However, in the primary cell wall of most dicot species Xyl is thought to be associated with xyloglucan (20). The xyloglucan polymer has a backbone composed of 1,4-linked β -D-Glc residues. Up to 75% of these residues are substituted at O6 with xylosyl residues, which could be further substituted with galactosyl or galactofucosyl chains. Because the Xyl/Glc ratio for xyloglucan based on its structure is expected to be 3:4, the fact that the Xyl/Glc ratio was much higher suggests that xylans or GAXs, which have usually been considered only as minor hemicellulosic polymers in fruit tissues, might be abundant in the cell wall of raspberry.

Pectin Solubilization, Depolymerization, and Composition. Even when the proportion of uronic acids in the AIR did not change dramatically during ripening (Table 1), there were modifications in the relative solubility of the pectic polymers. A clear increase in the proportion of pectin in the WSF was observed as fruit ripening progressed (Figure 2). A reciprocal decrease was seen with the proportions of CSF and NSF (Figure 2) in that the amount of uronic acids remaining in these fractions decreased at more advanced stages of development. When the individual sugar compositions of the WSF and NSF were analyzed by GC-MS, the clearest change observed was an increase in the proportion of Ara in the WSF (Figure 3), an increase reflected by a reduction in the Ara content of the NSF (Figure 3). The relatively high amount of Glc in the WSF was unexpected (Figure 3).

To determine the modifications to the molecular sizes of extracted pectins, the WSF, CSF, and NSF were analyzed by SEC (Figure 4). The CSF polymers did not change in size until late in ripening when a slight decrease was observed. More obvious size changes were observed in the NSF, where the peak eluting at 90 mL was substantially reduced in amount as the RR stage was reached. Changes in WSF polymer size were even

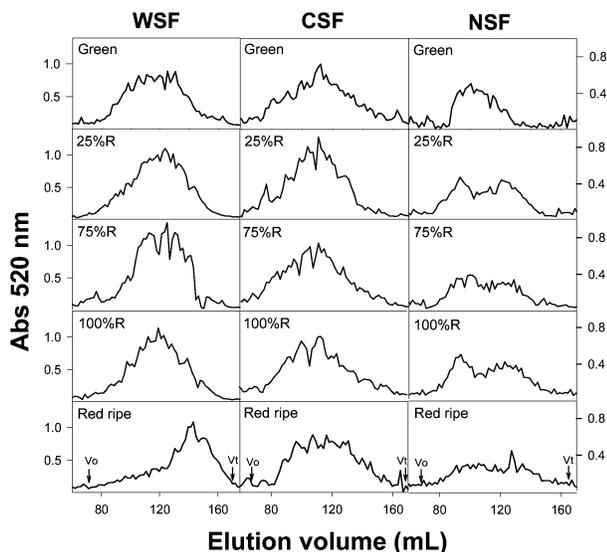


Figure 4. Size exclusion chromatography profiles of raspberry pectic WSF, CSF, and NSF throughout ripening. The samples were fractionated on an HW65F column. The collected fractions (2 mL) were assayed for UA, as described (11). V_o , void volume; V_t , total volume.

more dramatic, and an extremely large pectin molecular size downshift was observed at the RR stage. Previous work evaluating raspberry cell wall-degrading enzymes showed that PG activity markedly increases in late ripening stages (21). Thus, PG might be involved in the dramatic pectin depolymerization observed after the 100%R stage. Other pectin-depolymerizing enzymes such as pectate lyases (PLs) have received much less attention than PGs but might also be important contributors to pectin metabolism in ripening fruits (22). In the case of strawberry, down-regulation of a PL resulted in higher firmness retention, suggesting that the product of this gene might be crucial in fruit softening (23). It is clear that enzymes in addition to PG must be studied to obtain a more complete picture of possible factors causing pectin depolymerization in raspberries and other soft fruits.

Hemicellulose Solubilization, Depolymerization, and Composition. In contrast to the findings for the pectin-rich fractions, the changes in the neutral sugar contents of the polymers in the 4KSF and 24KSF were slight, although some small decreases were observed in late ripening (Figure 5). The sugar composition data for the two hemicellulose extracts strengthen the arguments that both xylans and xyloglucans are abundant wall components, with xylans being preferentially extracted in the 4KSF and xyloglucans in the 24KSF (Figure 6). The extraction data are consistent with reports for tomato (17), although the abundance of the xylan component of a tomato cell wall is much less. The sugar composition data also support the earlier contention that the xylan and xyloglucan polymers change little as raspberry fruits ripen (Figure 6). In the case of the 4KSF, relatively high levels of Ara and Gal were found, suggesting the presence of arabinoxylans and/or branched pectins, presumably associated with hemicelluloses. Many cell wall models of dicot species presume non-covalent interactions between polymers and suggest the existence of a cellulose–xyloglucan network embedded in a matrix of pectic polysaccharides, with no covalent associations between these two polymer networks (24). However, recent findings support the existence of xyloglucan–RG-I conjugates in plant cell walls (25). Putative xylan–pectin complexes have been reported in ripening tomato fruit (26), and covalent associations between glucuronoxylans and xyloglucan were also found in olive pulp (27). Results from

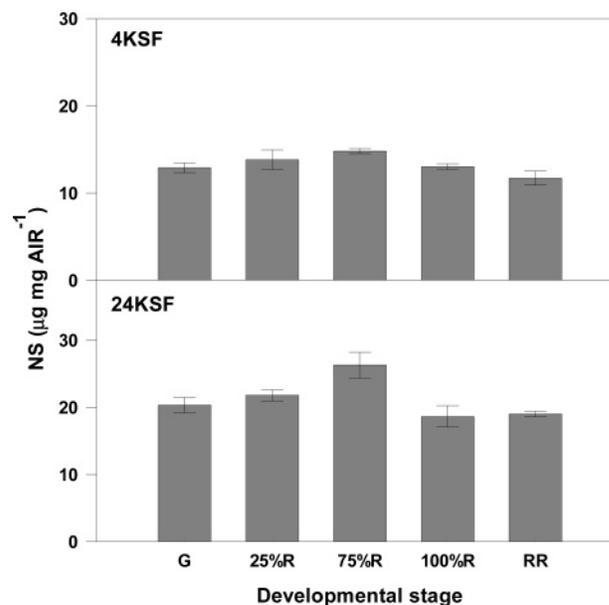


Figure 5. Content of neutral sugars in raspberry fruit 4KSF and 24KSF throughout ripening. Developmental stages are defined as in the caption of Figure 1. Two independent extractions were done for each stage, and measurements were done in triplicate. The standard deviations are shown.

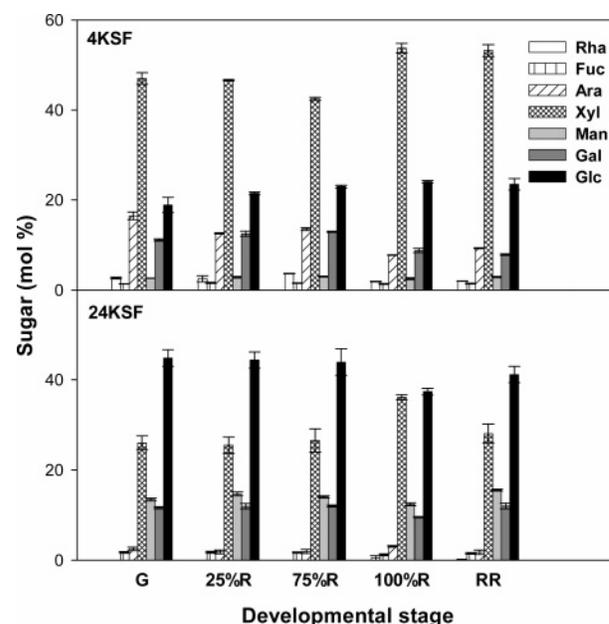


Figure 6. Neutral sugar compositions (mole percent) of raspberry 4KSF (A) and 24KSF (B) throughout fruit ripening. Developmental stages are defined as in the caption of Figure 1. The standard deviations are shown.

the SEC analysis of the 4KSF and 24KSF showed that there were no clear modifications in polymer size throughout development (Figure 7). A reduction in hemicellulose size during fruit ripening has been described in many fruits (28). In strawberry, early work by Knee et al. (29) reported that the cell wall became swollen during fruit development, and this coincided with changes in the cell wall neutral sugars. They proposed that this reflected degradation of hemicellulose and cellulose; with the cellulose–hemicellulose network thus relaxed, remaining polymers might be more readily hydrated, allowing the wall to swell. The average molecular size of hemicellulose extracted from strawberry fruit declines dramatically during ripening (30). Our results show that raspberries do

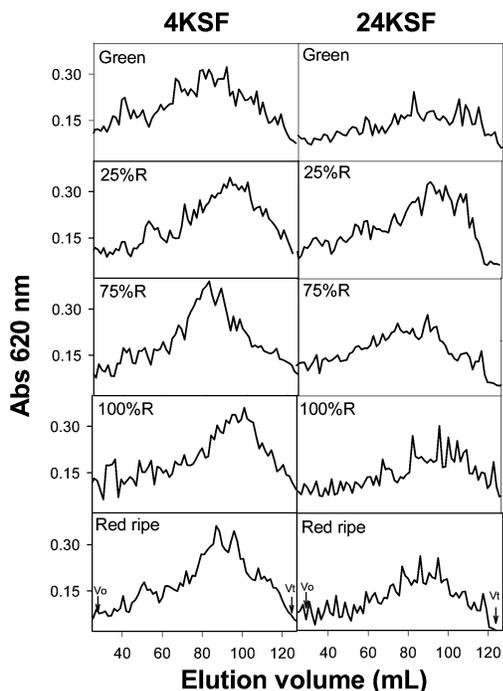


Figure 7. SEC profiles of 4KSF and 24KSF from five raspberry fruit ripening stages and fractionated on a Sepharose CL-4B column. The collected fractions (1.5 mL) were assayed for neutral sugars using the anthrone method (12). Vo, void volume; Vt, total volume.

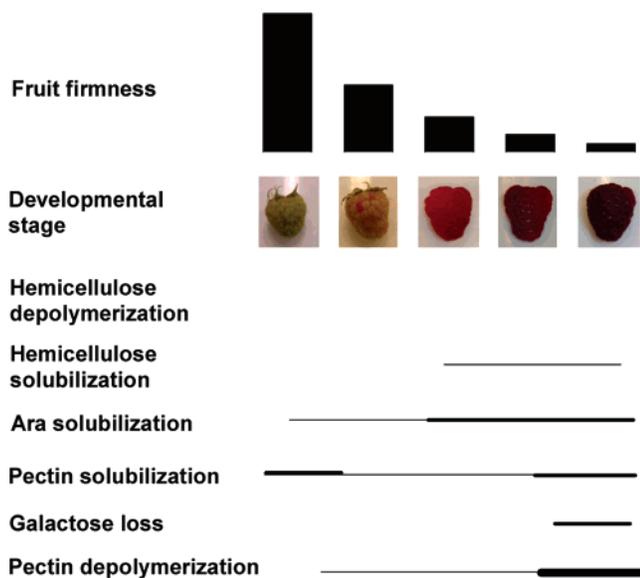


Figure 8. Proposed model for cell wall changes accompanying raspberry fruit ripening and softening.

not undergo extensive changes in the hemicellulosic polymers during development and are significantly different from those in strawberry, which has been usually used as a model for soft fruit ripening. Thus, whereas substantial changes in raspberry fruit cell walls do occur as ripening proceeds, these are limited to the pectic polysaccharides.

Model for Cell Wall Changes Accompanying Raspberry Development. The results improve our understanding of the biochemistry of cell wall degradation in raspberry, a fruit with extremely high cell wall metabolism and a high softening rate. Raspberry fruits have the additional peculiarity of having overlaps between fruit growth and ripening and for which there

have not been prior detailed studies. Ara was the most abundant cell wall noncellulosic sugar in raspberry followed by Xyl and Gal. An additional peculiarity of raspberry fruit walls is the relative abundance of xylans (or arabinoxylans) in the base-soluble hemicelluloses. However, the absence of change in the hemicellulosic polymers suggests that the metabolism of these polymers is not a key contributor to fruit softening. Late in ripening, the solubility of cell wall polyuronides increased and was accompanied by a dramatic depolymerization (**Figure 8**) and a shift of Ara-rich polymers from the NSF to the WSF. Thus, pectic compounds seem to be the cell wall polymers undergoing the most extensive modifications during raspberry fruit development.

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