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Cell wall disassembly events in boysenberry (*Rubus idaeus* L. × *Rubus ursinus* Cham. & Schldl.) fruit development

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Abstract. Boysenberry fruit was harvested at five developmental stages, from green to purple, and changes in pectin and hemicellulose solubilisation and depolymerisation, polymer neutral sugar contents, and the activities of cell wall degrading enzymes were analysed. The high xylose to glucose ratio in the 4% KOH-soluble hemicellulose fraction suggests that xylans are abundant in the boysenberry cell wall. Although the cell wall changes associated with fruit development do not proceed in discrete stages and the cell wall disassembly is a consequence of highly regulated changes occurring in a continuum, the results suggest that the temporal changes in cell wall degradation in boysenberry account for at least three stages: an early stage (green to 75% red colour), associated with metabolism of cellulose and cross-linking glycans; an intermediate period (75 to 100% red colour), characterised by substantial pectin solubilisation without depolymerisation in which α-arabinofuranosidase increases markedly and 50% of the wall arabinose is lost; and a final stage (100% red colour to purple), characterised mainly by a reduction of pectic galactose content and a dramatic increase in pectin depolymerisation associated with higher polygalacturonase, pectin methylesterase, acetyl esterase and β-galactosidase activities. From a biotechnological perspective enzymes involved in pectin matrix disassembly seem to be the better candidates to affect boysenberry fruit late-softening by genetic intervention. A model for cell wall disassembly in boysenberry fruit is proposed.

Additional keywords: cell wall, fruit ripening, hemicellulose, pectin, polysaccharides, softening.

Introduction

The boysenberry (*Rubus idaeus* L. × *Rubus ursinus* Cham. & Schldl.) is an aggregate fruit consisting of a loose collection of drupelets firmly bound to a central column of receptacle tissue (Porter 1988). It shows an extremely high softening rate and its shelf life (2–4 days at 0°C) is, together with raspberries and blackberries, among the shortest described for fruits (Mitcham *et al*. 2002). Two main factors determining the high perishability of boysenberries are the firmness loss and susceptibility to decay, processes directly or indirectly (by reducing the strength of one major barrier against tissue colonisation by plant pathogens) associated with changes in cell wall structure and composition (Vorwerk *et al*. 2004). The pattern and biochemical basis of the ripening fruit cell wall 'organised disorganisation' varies depending on the species considered. In most cases, specific aspects of cell wall metabolism have been investigated in different fruits, but few studies have been performed analysing sufficient number of ripening stages and sufficient number of cell wall changes to permit a description of sequential and interrelated events in cell wall metabolism (Brummell 2006). Various studies have reported the compositional changes of boysenberry during development and ripening (Monro and Lee 1987; Perkins-Veazie *et al*. 2000; Vicente *et al*. 2006). Even though softening is a main trait affecting quality maintenance, the modifications occurring in the cell wall during fruit development have not been analysed yet. Most of the work on 'soft fruits' cell wall disassembly has been done in strawberry (Knee *et al*. 1977; Huber 1984; Rosli *et al*. 2004). Although strawberry is usually grouped operationally with raspberry, boysenberry and blueberry and this classification could be pragmatically useful, the fruits are totally different in terms of their botanical origin and morphology and the only study looking at changes in the cell wall structure of blackberry shows that there are differences in wall thickness, viscosity of aqueous suspensions of cell wall material and swelling compared with that observed in strawberry (Redgwell *et al*. 1997). Even if we assume that some biochemical changes observed in strawberry fruit cell wall during ripening could be, at least in general terms, extrapolated to other soft fruits, the results reported are quite contradictory. For instance, Huber (1984) reported that hemicelluloses of strawberry fruit show depolymerisation and reduction of molecular mass during ripening, whereas only small changes were observed in pectin size. Other authors, analysing ripening-related cell wall changes in three strawberry varieties found that pectin size was reduced, but only slight depolymerisation was observed in hemicelluloses (Rosli *et al*. 2004). Furthermore, Jiménez Bermúdez *et al*. (2002) reported reduced softening and pectin solubilisation in strawberries with antisense expression of the pectate lyase gene.

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Thus, studies that clarify the differences reported for berry fruit cell wall degradation would be of both fundamental (in terms of increasing our understanding of the process of cell wall disassembly in these fruits) and practical interest (e.g. as a guidance to select candidate genes for further studies aimed at trying to reduce softening and decay susceptibility by genetic intervention). In the present work we analyse the changes in cell wall structure and composition and cell wall degrading enzymes during boysenberry fruit development to increase our understanding of the sequence of cell wall metabolism in this highly perishable fruit.

Materials and methods

Plant material

Boysenberry fruit grown at the University of California Davis Student Farm was harvested at five different stages, largely green (G), 25% surface red colour (25%R), 75% surface red colour (75%R), 100% surface red colour (100%R) and purple. The fruit was taken to the laboratory and immediately processed or frozen and stored at –20°C until use.

Fruit weight and water content

One hundred individual fruits were weighed at each developmental stage. For water content measurements, ~20 g of fruit were crushed and transferred to a weighed beaker. Samples were then held in a 70°C oven until no changes in weight were recorded. Fruit water content was expressed as g of water per 100 g of fresh fruit. Measurements were done in triplicate.

Chlorophylls and anthocyanin

Fruit tissue for every developmental stage was crushed in an Ultraturrax (IKA Werke, Janke & Kunkel GmbH & Co KG; Staufen, Germany) with 5 mL of acetone/water (80 : 20), stirred and then centrifuged at 9000*g* for 10 min at 4°C. The supernatant was used to determine the content of chlorophyll according to Costa *et al*. (2002). For anthocyanin measurements frozen fruit was ground in liquid N_2 with a mill and different amounts of the resultant powder (depending on the developmental stage) were poured into 20 mL of HCl-methanol (1% v/v) and held at 4°C for 10 min (Woodward 1972). The slurry was centrifuged at 9000*g* for 10 min at 4°C and the absorbance of the supernatant was measured at 520 nm. For both chlorophyll and anthocyanin measurements, three replicates were done at each developmental stage. Results were expressed as a percentage relative to the maximum value (purple for anthocyanin and green for chlorophylls).

Firmness measurement

Firmness was measured with a texture analyser (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY, USA) fitted with a 2-mm flat probe. Each drupelet was compressed 1 mm at a rate of 0.5 mm s^{-1} and the maximum force developed during the test was recorded. Eighty measurements were done for each stage analysed.

Isolation of cell walls

For cell wall isolation, the endocarp and seeds were removed. For this to occur 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 and was then homogenised in an Ultraturrax with 100 mL of 95% ethanol and boiled for 45 min to ensure the inactivation of enzymes and the extraction of low molecular weight solutes and to prevent autolysis. The insoluble material was filtered through Miracloth (Calbiochem, EMD Biosciences Inc., CA, USA) and sequentially washed with 150 mL of boiling ethanol, 150 mL of chloroform : methanol (1 : 1 v/v), and 150 mL of acetone and dried at 25°C, yielding the crude cell wall extract (alcohol insoluble residue, AIR). After this, the residue was dried overnight at 37°C and weighed. Results were expressed as mg AIR per g of fresh fruit.

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 residue from each sample was suspended in 15 mL of water and stirred at room temperature for 12 h, then centrifuged at 6000*g* and 4°C for 10 min. The supernatant was filtered through glass fibre filters (Whatman International, Maidstone, England) 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 with stirring. The slurry was centrifuged passed through fibre glass filters, as above, and the pellet was washed with CDTA. The filtrate was collected and designated CDTA-soluble fraction (CSF). The CDTA-insoluble pellet was then extracted with 15 mL of 50 mM Na_2CO_3 containing 20 mM $NaBH_4$ at 1°C for 12 h. After filtration (as above) the extracted solution was designated Na_2CO_3-soluble fraction (NSF), it was saved and the pellet was re-extracted with 15 mL of 4% KOH containing 0.1% $NaBH_4$ at room temperature for 12 h, with shaking, and the extracted solution was designated as the 4% KOH-soluble fraction (4KSF). This fraction was filtered, as above, neutralised with glacial acetic acid, and extensively dialysed 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) and leaving a residue of essentially α-cellulose. The tubes containing the α-cellulose were placed in a water-ice bath and 98% (w/w) H_2SO_4 was added slowly to prevent overheating. Water was then added slowly and the samples stirred for 1 h. Two independent serial extraction series were done for each developmental stage analysed. Samples of the different fractions obtained were assayed in triplicate for neutral sugar (NS) and uronic acids (UA) as described below.

UA and NS measurements

Samples from the different cell wall fractions were assayed for total UA and NS. UA were measured according to Blumenkrantz and Asboe-Hansen (1973). A_{520} readings were evaluated on the basis of a galacturonic acid standard curve. Three independent samples were analysed for each developmental stage, measurements were done in duplicate and results were expressed as μg of galacturonic acid per mg of AIR. NS were measured by the anthrone method (Yemm and Willis 1954). A_{620} readings were evaluated on the basis of a glucose standard curve.

Measurements for each independent extraction were done in triplicate and were expressed as μg of glucose per mg of AIR.

Size exclusion chromatography

Aliquots of WSF, CSF, NSF, 4KSF and 24KSF were dialysed (Spectrapor, MW cut-off 8 kD) extensively against water for 1 day at 4°C and lyophilised. Samples from the WSF, CSF and NSF were dissolved in 200 mM NH_4-acetate, pH 5.0, chromatographed on a HW65 (Tosoh Bioscience, Tokyo, Japan) size exclusion chromatography (SEC) column (4 × 30 cm) eluted with 200 mM NH_4-acetate, pH 5.0. Fractions (2.5 mL) were collected at a flow rate of 60 mL h^{-1} and held in a water bath (50°C for 4 h) to volatilise part of the NH_4-acetate, which can interfere with the UA assay. Fractions were assayed for UA and total sugars 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) (Pharmacia, Uppsala, Sweden) and eluting with 0.1 N NaOH. Fractions were collected and aliquots were neutralised with glacial acetic acid before assaying for UA and NS as described above. The totally included and void volumes were determined with glucose and 5 000 000 (M_r) dextran, respectively.

Sugar GC-MS analysis

Dried samples from the WSF, CSF, NSF, 4KSF and 24KSF or 2 mg of AIR for total cell wall analysis were hydrolysed in 2 N trifluoroacetic acid (Albersheim *et al*. 1967), and converted to alditol acetates (Blakeney *et al*. 1983) for gas chromatographic analysis of NS composition. Aliquots of the derivatised samples were injected into a GC (HP Model 6890) fitted with a 30 m × 0.25 mm DB-23 capillary column (J&W Scientific, Agilent Technologies, Santa Clara, CA, USA) and a mass selective detector (HP Model 61098A). Temperature in the injector was 250°C and a linear temperature gradient (initial oven temperature 160°C, oven increase at 4°C per min 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 std), rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc) were prepared and NS amounts were calculated relative to a *myo*-inositol internal standard.

Enzyme assays

Approximately 5 g of fruit were homogenised in an Ultraturrax with 10 mL of 50 mM HAc-NaAc pH 5.5 containing 10 g L^{-1} polyvinylpolypyrrolidone (PVPP), 1 mM cystein and 1 M NaCl. The homogenates were then stirred at 4°C for 3 h and centrifuged at 10 000*g* for 20 min. The collected supernatants were dialysed against 50 mM NaAc pH 5.5 and used for assays of enzymatic activities. For β-galactosidase (β-gal), α-arabinofuranosidase (α-ara), α-rhamnosidase (α-rha) and β-mannosidase (β-man), reaction mixtures containing 200 μL of enzyme extract, 400 μL of 50 mM HAc-NaAc buffer pH 5.5 and 400 μL of 10 mM *p*-nitrophenyl (*p*NP) substrates (Sigma) were incubated at 40°C. Two hundred μL aliquots were taken at intervals, 600 μL of Na_2CO_3 were added and A_{400} was measured to determine the linear reaction rate of each enzyme-substrate combination. In the case of acetyl esterase (AE) the reaction mixture contained 700 μL of NaAc buffer pH 5.5, 100 μL of enzyme extract and 200 μL of 30 mM *p*NP-acetate. Samples were incubated at 40°C and aliquots were taken at different intervals and frozen. When the time-course sampling was finished the samples were thawed, 600 μL of 0.4 M Tris propane pH 7.5 were added and absorbance was immediately measured at 400 nm. For pectin methylesterase (PME), 5 g of fruit were homogenised in an Ultraturrax with 10 mL of unbuffered 1 M NaCl containing 10 g L^{-1} PVPP and 1 mM cystein. The homogenates were then stirred at 4°C for 3 h and centrifuged at 10 000*g* for 20 min. The supernatants were used to assay PME activity according to Hagerman and Austin (1986). The reaction mixture contained 600 μL of 0.15% w/v pectin (degree of esterification 70%), 150 μL of 0.01% bromothymol blue in 0.003 M phosphate buffer pH 7.5, 100 μL of water pH 7.5 and 200 μL of enzymatic extract. A_{620} was measured. Polygalacturonase (PG) activity was measured in reaction mixtures containing 400 μL of 50 mM NaAc-HAc buffer pH 5.5, 400 μL of 0.15% w/v polygalacturonic acid and 200 μL of enzymatic extract. The mixtures were incubated at 40°C. At different times 200 μL aliquots were taken and 1 mL of 1 M sodium borate was added. Reducing sugars liberated were measured with 2-cyanoacetamide according to Gross (1982). Finally, for glucanase/glucosidase activity the reaction mixtures contained 400 μL of 50 mM NaAc-HAc buffer pH 5.5, 400 μL of 0.2% (w/v) carboxymethyl-cellulose (CMC) and 200 μL of enzymatic extract. The mixtures were incubated at 40°C. At different times 200 μL aliquots were taken and assayed for reducing sugars as described for PG activity. In all cases two protein extracts were prepared for each developmental stage analysed and measurements were made in triplicate. One unit of enzymatic activity was defined as a ΔOD of 0.001 per second and per gram for PME, PG, β-man, α-rha, α-ara and β-gal and as a ΔOD of 0.1 per gram per second for AE and glucanase/glucosidase.

Statistical analysis

The experimental design was a randomised block. Data were analysed by ANOVA, and the means were compared by the least significant difference test at a significance level of 0.05.

Results

Fruit weight, pigments, water content, firmness and cell wall yield in ripening boysenberry

Boysenberry fruit was harvested at five fruit ripening stages based on visual assessment of surface color. Fruit weight increased throughout the five stages of the development (Fig. 1*A*). Anthocyanin levels increased dramatically towards the end of ripening, between the 100%R and purple stages (Fig. 1*B*), whereas chlorophyll content dropped at early stages (Fig. 1*C*), accompanying the reduction in fruit firmness. Fruit firmness decreased ~50% between each successive ripening stage (Fig. 2*C*). During early stages of ripening (G to 25%R) fruit water content increased (Fig. 2*A*) but interestingly the ethanol-insoluble residue per gram of fruit (AIR g^{-1}) was not proportionally reduced (Fig. 2*B*) suggesting that a net cell wall synthesis occurred in this period. From the 25%R to the purple stages, the AIR g^{-1} decreased progressively. Some small oligosaccharides could be lost even in ethanol but it is unlikely that the reduction in the AIR yield at the purple stage was due to

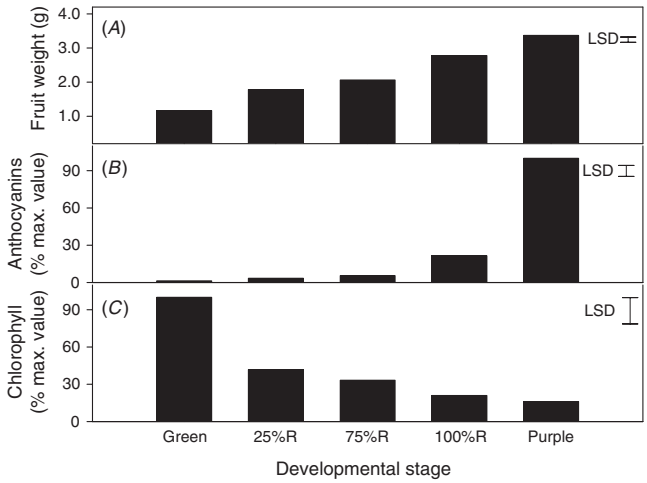

Fig. 1. Changes in (*A*) boysenberry weight, (*B*) anthocyanins and (*C*) chlorophyll content during fruit development. 25%R, 25% surface red colour; 75%R, 75% surface red colour; 100%R, 100% surface red colour. The least significant differences (LSD) at $P = 0.05$ are shown.

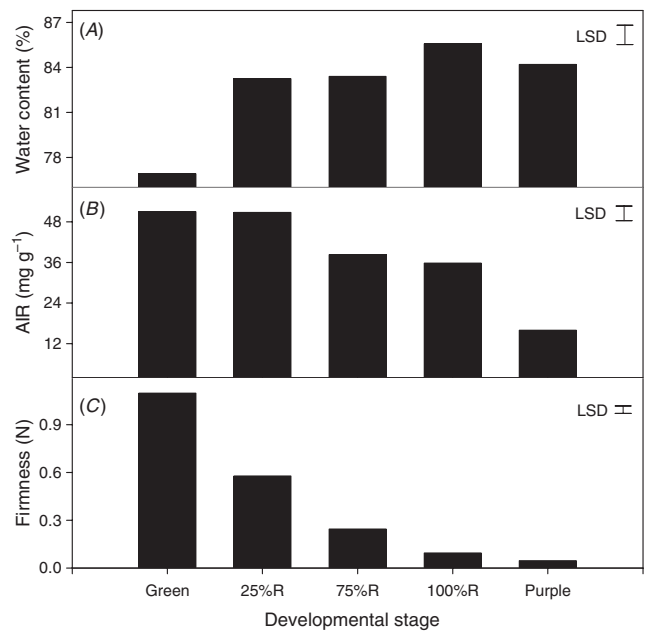

Fig. 2. Changes in boysenberry (*A*) water content, (*B*) cell wall yield (AIR) and (*C*) firmness during fruit development. 25%R, 25% surface red colour; 75%R, 75% surface red colour; 100%R, 100% surface red colour. The least significant differences (LSD) at $P = 0.05$ are shown.

losses of wall-derived oligosaccharides due to their ethanol solubility. Although it is possible that newly synthesised wall components were also being deposited during the last half of the sampling period, this shows that total net cell wall loss was a significant process during these later stages.

Cellulose and cross-linking glycans solubilisation

No changes in total sugar content were observed in the 4KSF during boysenberry fruit development (Fig. 3*A*). In contrast, the

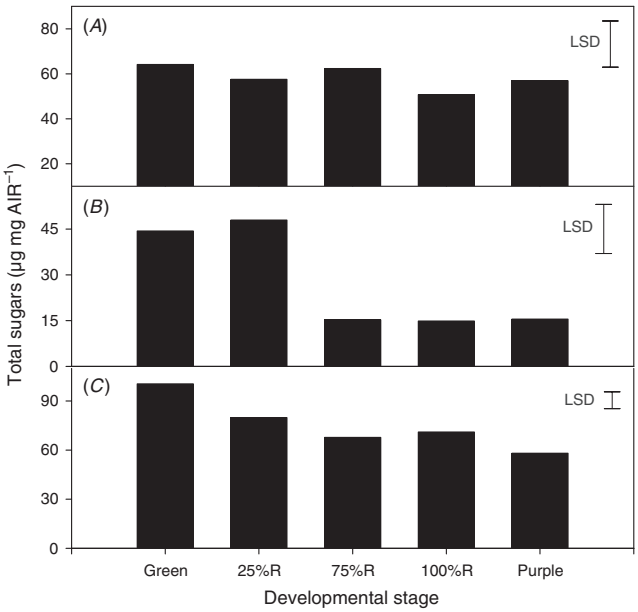

Fig. 3. Changes in cellulose and cross-linking glycans throughout boysenberry fruit ripening. (*A*) 4%KOH soluble fraction. (*B*) 4%KOH soluble fraction. (*C*) Cellulose. AIR, alcohol insoluble residue; 25%R, 25% surface red colour; 75%R, 75% surface red colour; 100%R, 100% surface red colour. The least significant differences (LSD) at $P = 0.05$ are shown.

total sugar content of the 24KSF decreased threefold between the 25 and 75%R stages and remained constant thereafter (Fig. 3*B*), suggesting a more significant role for the metabolism of cross-linking glycans, such as xyloglucan (XyG), in the intermediate expansive phase of ripening, rather than in later stages that are associated with late softening. Finally the cellulosic fraction accounted for 20% of the AIR in green fruit and showed a reduction in early stages of ripening (from G to 25%R stages) remaining then without changes up to the purple stage where cellulose content was reduced again (Fig. 3*C*).

Pectin solubilisation

Loosely-, ionically- and covalently-bound pectins were sequentially extracted with water, CDTA and Na_2CO_3. The UA content in the WSF and CSF increased as boysenberry fruit ripening progressed (Fig. 4*A*, *B*). The clearest increase in pectin solubility was observed between the 25 and 100%R stages. The NSF, enriched mainly in covalently-bound polyuronides, and representing the greatest part of the fruit wall-associated UA, decreased throughout fruit development (Fig. 4*C*), suggesting a progressive disassembly of the pectic matrix. The results from both pectin and hemicellulose content and solubilisation show that even when modifications in both matrices occur during boysenberry ripening, mid and late ripening-associated wall modifications are mainly confined to the pectic matrix, whereas hemicellulose loss occurs in earlier stages.

Pectin and cross-linking glycans depolymerisation

Depolymerisation of wall components has been shown to occur during fruit ripening (Brummell 2006). To determine the extent and timing of wall component depolymerisation

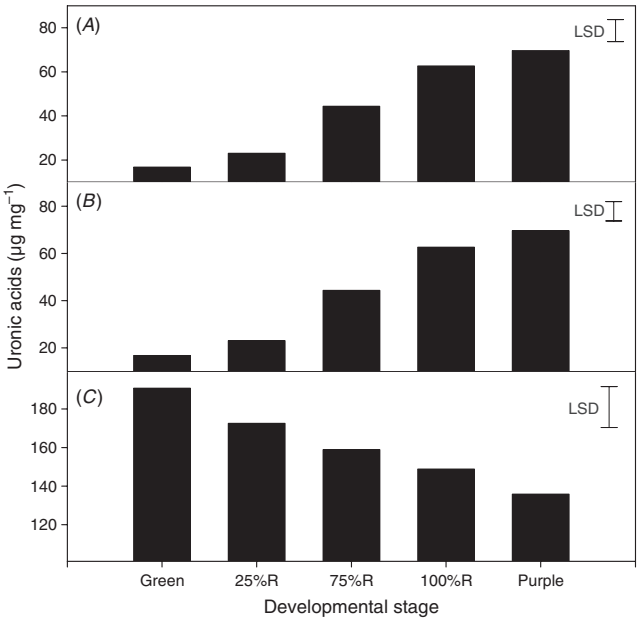

Fig. 4. Pectin solubilisation throughout boysenberry fruit development. (*A*) Water-soluble fraction, (*B*) CDTA-soluble fraction, (*C*) Na_2CO_3-soluble fraction from cell walls prepared from five ripening stages of raspberry fruit were assayed for uronic acids (Blumenkrantz and Asboe-Hansen 1973). The least significant differences (LSD) at $P = 0.05$ are shown. 25%R, 25% surface red colour; 75%R, 75% surface red colour; 100%R, 100% surface red colour.

the hemicellulosic and pectic fractions were analysed by SEC. Reduction in molecular size of matrix glycans has been observed in most species tested (Brummell 2006) and it has been suggested that it might be an important contributor to fruit softening (Brummell 2006). In the case of boysenberry and like what has been reported in raspberry (Vicente *et al*. 2007), no changes in molecular size were observed in the hemicellulosic fractions (4KSF and 24KSF) during ripening (data not shown). In contrast, all pectin fractions showed a dramatic decrease in molecular size during development. Increased depolymerisation of the WSF and NSF pectins was apparent at the 100%R stage (Fig. 5*A*, *C*) and the evidence for substantial pectin breakdown in purple stage (over-ripe) fruits was unmistakable. Although the size distribution of the CSF pectins remained constant between the green and 100%R stages, a clear downshift in pectin size was apparent as fruit became over-ripe (Fig. 5*B*). As indicated before (Fig. 4), higher rates of pectin polymer solubilisation were seen in the earlier stages of fruit ripening, when depolymerisation was low, showing that polyuronide depolymerisation, was not necessary for pectin solubilisation in these stages.

Changes in NS composition

The fact that the clearest increase in pectin solubilisation occurred without substantial reduction in pectin molecular size led us to analyse the modifications in cell wall NS. Significant losses of Gal have been observed as one of the major changes pre-staging firmness loss in fruits such as tomato (Gross and Wallner 1979) and melon (Rose *et al*. 1998). A reduction of Gal was also observed in boysenberry but in this case the changes occurred only in late ripening in the 100%R to the purple stage transition (Fig. 6). In contrast, a 50% reduction in Ara content per gram of AIR was observed at the transition between the 75 and 100%R stages preceding the large polyuronide depolymerisation.

To determine more specifically NS changes, the modifications in the different wall fractions were analysed by GC-MS. In the case of the pectic fractions a clear increase in the proportion of Ara especially in the WSF occurred as ripening progressed (Table 1). Rhamnose levels also increased in the WSF and CSF suggesting together with the increased solubilisation of Ara-rich polymers that rhamnogalacturonan-I (RG-I) might be undergoing extensive degradation. The most abundant sugars in the 24KSF were Glc and Xyl (Table 2). These sugars were present in a proportion that would be expected for XyG, which is often the main hemicellulosic component of dicot primary cell walls. No changes were detected in this fraction's neutral sugar composition throughout boysenberry ripening. Finally the 4KSF NS composition showed some interesting features. Xyl and Glc were also the most abundant sugars, but in this case the proportion of Xyl substantially exceeded that of Glc. XyG has a backbone composed of 1,4-linked β-D-Glc residues. Up to 75% of these residues are substituted at O6 with Xyl, which could be further decorated with Gal or Gal-Fuc chains. Because the ratio of Xyl to Glc for XyG based on its structure is expected to be 3 : 4, the fact that Xyl was much more abundant together with the fact that the 4KSF represented 60% or more of the total hemicellulose at all stages of fruit ripening (Fig. 3) suggests that xylans, are abundant hemicelluloses in boysenberry call walls. Previous work in other fruit such as tomato also showed that xyloglucomannan is a major hemicellulosic polysaccharide (Seymour *et al*. 1990).

Cell wall degrading agents

In general, the measured activities of the enzymes were coincident with the corresponding modifications in cell wall polysaccharides with the exception of glucanase/glucosidase (Table 3). α-ara activity, which is involved in the hydrolysis of terminal, non-reducing arabinofuranosyl residues (Sozzi *et al*. 2002) correlated closely with a loss in cell wall Ara, an increased pectin solubilisation and a corresponding increase in the proportion of Ara in the WSF and CSF pectins. In contrast to what has been reported in other species in which β-gal activity peaks at intermediate stages of ripening, maximum activity in boysenberry was observed in the purple stage, which was coincident with the increased late release of Gal from the cell wall. Cell wall esterases, such as AE, did not show significant changes in early development but increased slightly at the purple stage. PME activity increased from the green to the 100%R stage and decreased afterwards. De-esterification of pectic compounds by AE and PME should increase the availability of preferred substrates for polyuronide depolymerising enzymes in late development and maximal depolymerisation of all boysenberry pectin fractions was observed between the 100%R and purple stages when both AE and PME activities were at their highest levels. Finally PG increased markedly in the later stage of

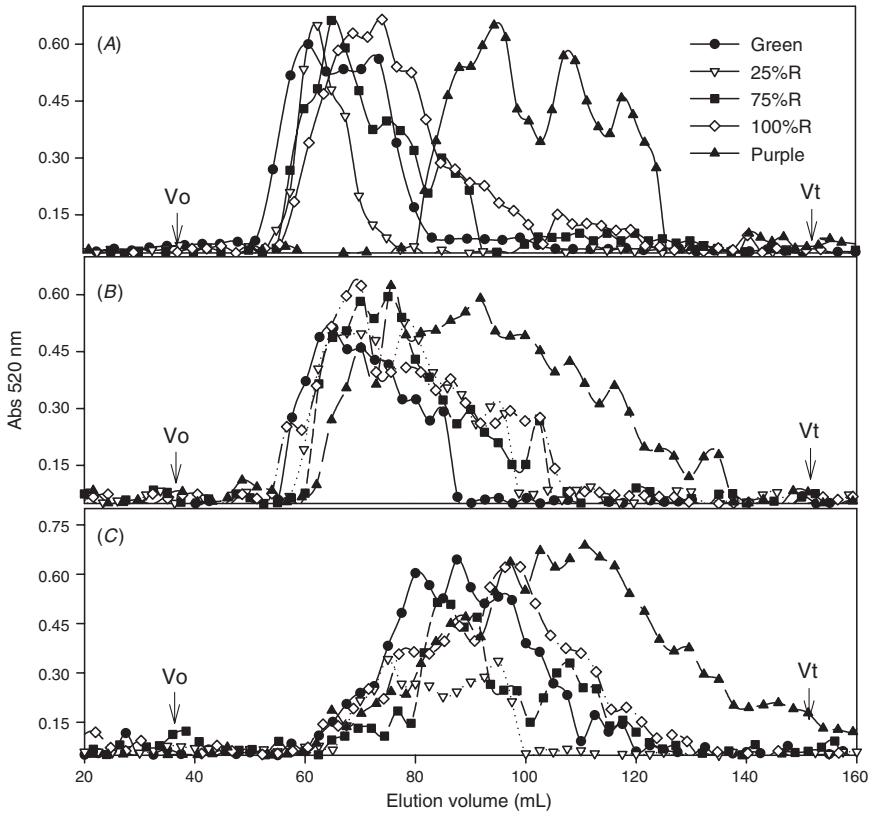

Fig. 5. Size exclusion chromatography profiles from pectins throughout boysenberry fruit ripening, fractionated on HW65. Column fractions (2.5 mL) were assayed for UA content by the *m*-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen 1973). Vo, void volume; Vt, total volume. (*A*) Water-soluble fraction, (*B*) CDTA-soluble fraction, (*C*) Na_2CO_3-soluble fraction.

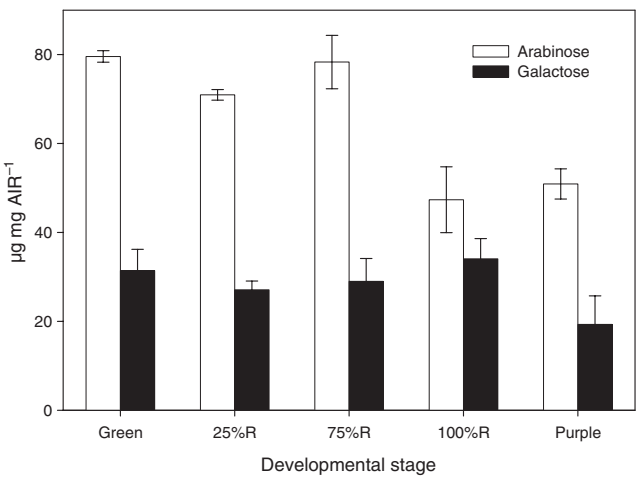

Fig. 6. Changes in galactose and arabinose content of boysenberry cell walls. 25%R, 25% surface red colour; 75%R, 75% surface red colour; 100%R, 100% surface red colour. Error bars are shown ($n = 3$). AIR, alcohol insoluble residue.

ripening, also coinciding with the dramatic depolymerisation of pectins, perhaps in concert with the elevated esterase activities, suggesting that PG action may be associated with softening in terminal stages.

Discussion

Excessive softening and pathogen susceptibility are the main factors limiting quality maintenance of boysenberry fruit. Both processes have been directly or indirectly associated with fruit textural properties. Although tissue firmness is affected by several factors such as turgor pressure (Shackel *et al*. 1991) and cell expansion (Sexton *et al*. 1997), it has been repeatedly shown that progressive dismantling and modification of cell wall polymers play a major role in the mechanical properties of fruit tissues (Carpita and Gibeaut 1993). There have been many studies analysing changes in cell walls of different fruits showing that pectins, hemicelluloses, and possibly the amorphous regions of cellulose undergo structural modifications during fruit development and ripening (Brummell and Harpster 2001). However, few studies have been done in fruits analysing sufficient number of ripening stages and the full set of cell wall changes to allow a sequential interrelation of events (Brummell 2006).

Different from tomato (Gillaspy *et al*. 1993) and similar to strawberry, in boysenberry, fruit elongation continues through the onset of ripening as judged by fruit colour change. Consequently a fine and well orchestrated balance between cell wall synthesis and degradation is needed to generate a wall structure that can support the growth of individual cells (Cosgrove *et al.* 2002), and ultimately permit the dramatic

Table 1. Neutral sugar compositions (mol %) of boysenberry WSF, CSF and NSF pectins throughout fruit ripening

G, green; 25%R, 25% surface red colour; 75%R, 75% surface red colour; 100%R, 100% surface red colour; P, purple; CSF, CDTA-soluble fraction; NSF, Na_2CO_3-soluble fraction; WSF, water-soluble fraction

	Ripening stage	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
WSF	Green	1.5	0.3	18.4	7.4	5.3	9.3	57.8
	25%R	1.6	0.7	22.7	9.3	5.0	12.6	48.1
	75%R	1.3	0.7	35.3	16.5	7.5	20.5	18.2
	100%R	4.0	0.8	35.2	13.3	5.1	16.5	25.1
	Purple	3.1	0.8	43.8	10.6	4.5	16.5	20.7
CSF	Green	2.7	0.6	15.2	2.9	4.3	12.0	62.3
	25%R	2.5	0.6	21.7	6.8	5.3	11.0	52.1
	75%R	2.3	0.6	24.6	7.2	5.3	13.6	46.4
	100%R	2.9	0.8	41.0	10.9	5.0	12.9	26.3
	Purple	13.0	0.3	48.4	4.7	3.5	11.2	18.8
NSF	Green	7.4	1.1	40.0	3.9	1.6	11.9	34.0
	25%R	6.2	0.6	46.5	3.6	1.1	15.5	26.4
	75%R	8.1	1.1	47.9	3.8	1.2	13.8	24.2
	100%R	7.6	0.9	49.8	4.3	1.0	15.7	20.7
	Purple	7.7	1.1	51.9	4.0	1.2	13.3	20.8

Table 2. Neutral sugar composition (mol %) of boysenberry 4KSF and 24KSF throughout fruit ripening

G, green; 25%R, 25% surface red colour; 75%R, 75% surface red colour; 100%R, 100% surface red colour; P, purple; 4KSF, 4% KOH-soluble fraction; 24KSF, 24% KOH-soluble fraction

	Ripening stage	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
4KSF	Green	1.0	0.9	23.5	40.2	3.0	9.7	21.6
	25%R	1.6	1.2	30.5	37.2	2.8	10.0	16.5
	75%R	1.8	1.3	28.6	35.3	3.3	11.0	18.6
	100%R	1.6	0.9	39.9	34.1	2.1	11.0	10.3
	Purple	1.5	0.7	37.8	34.7	2.7	8.9	12.9
24KSF	Green	0.4	2.7	14.0	23.1	8.4	11.6	36.8
	25%R	1.1	4.3	9.9	28.2	6.7	12.5	37.2
	75%R	0.9	3.1	12.7	26.7	8.3	11.9	36.3
	100%R	1.1	3.2	9.3	25.6	8.5	13.0	39.2
	Purple	2.1	2.0	10.2	27.2	8.4	10.8	39.5

Table 3. Changes in cell wall degrading enzyme activities throughout boysenberry fruit ripening

G, green; 25%R, 25% surface red colour; 75%R, 75% surface red colour; 100%R, 100% surface red colour; P, purple. The least significant differences (LSD) at $P = 0.05$ are shown

Fruit ripening stage	Green	25%R	75%R	100%R	Purple	LSD
Acetylesterase (EAU kg^{-1})	8.9	7.9	7.7	9.9	11.2	1.2
α-Arabinofuranosidase (EAU kg^{-1})	10.8	12.4	77.4	82.0	45.0	12.5
β-Galactosidase (EAU kg^{-1})	3.1	2.9	3.2	3.3	59.6	9.8
Pectin methylesterase (EAU kg^{-1})	7.1	29.1	104.6	119.9	109.3	26.5
Glucanase/glucosidase (EAU kg^{-1})	2.9	5.1	7.3	9.7	10.4	2.1
Polygalacturonase (EAU kg^{-1})	3.4	4.7	7.4	21.3	24.9	8.7
α-Rhamnosidase (EAU kg^{-1})	5.5	7.1	21.8	36.0	63.7	17.1
β-Mannosidase (EAU kg^{-1})	0.8	0.6	19.9	78.0	188.4	29.6

disassembly of the structure accompanying the ripening and softening (Rose *et al*. 2004).

The relative cell wall content of cellulose and cross-linking glycans decreased at early stages of boysenberry development but no modifications were observed after the 75%R stage. Depolymerisation of cross-linking glycans during development has been reported in several fruits including, tomato (Maclachlan and Brady 1994), melon (Rose *et al*. 1998), and kiwifruit (Redgwell and Percy 1992). In the case of other soft fruits, such as strawberry, the results reported are quite contradictory. Huber (1984) found that the average molecular size of hemicelluloses extracted from strawberry fruit declines

dramatically during ripening. However, other work analysing ripening-associated changes in cell wall composition in three different strawberry varieties detected only a slight depolymerisation, leading these authors to conclude that the effect of hemicellulosic polymer breakdown on strawberry fruit softening was minor (Rosli *et al*. 2004). No clear depolymerisation of cross-linking glycans was observed for ripening boysenberries. This increased solubilisation without depolymerisation suggests that agents that facilitate cell wall loosening, such as expansins (Cosgrove *et al*. 2002), may be operating. In any case, at least in tomato fruits, XyG metabolism during both fruit expansion and softening is likely influenced by expansin (Brummell and Harpster 2001). The levels of Xyl largely exceeded those of Glc in the 4KSF, which would not be expected if XyG were the main hemicellulosic polymer in this fraction. This suggests then that xylans are an abundant component of boysenberry cell wall. Relatively high levels of Ara and Gal were also found in the 4KSF, suggesting the presence of branched pectins in this fraction, either covalently associated with hemicelluloses or incompletely solubilised with the NSF. More likely is the conclusion that the Ara and Gal represent hemicellulose-associated polymers, because the proportions of these two sugars clearly differ in the NSF and 4KSF.

In some fruits no significant changes in the cellulose content during ripening have been found (Ahmed and Labavitch 1980). This last observation together with the fact that endo-1,4-β-D-glucanase is generally not active on crystalline cellulose has led to the proposition that, although important in determining cell wall physical properties, cellulose changes would not have a role in the textural changes observed during fruit ripening. Stewart *et al*. (2001) found a reduction in the ordered-cellulose in raspberry during ripening and in the present work a clear reduction in the amount of cellulose was observed during boysenberry ripening. So it is not possible to rule out that cellulose metabolism, especially modifications of non-crystalline regions of microfibrils, could contribute, at least in part, to the textural changes observed in boysenberry fruit in early and late stages of development. It is also possible that the increase in overall cell wall weight per fruit that is seen in the green and 25%R stages represents the addition of non-cellulosic polysaccharides to the wall. Thus, the early decrease in relative wall cellulose content could represent an unchanging cellulose content of walls that are increasing in their pectin and cross-linking glycan contents.

Pectins are a major component of the plant cell wall and comprise one of the two major coextensive networks in which cellulose microfibrils are embedded (Carpita and Gibeaut 1993). Increased solubility of pectins during fruit ripening has been shown to occur in an array of fruits (Brummell and Harpster 2001). Reported ripening-associated increases in tomato WSF and CSF often are reflected in a decrease in NSF; perhaps suggesting that metabolism of the pectins in this fraction converts them to a water- or CDTA-soluble form (Carrington *et al*. 1993). The NS components of branched pectins play a critical role in cell wall structure, because removal of neutral side chains has been regarded as an essential part of pectin solubilisation (Dawson *et al*. 1992). Redgwell and Percy (1992) also suggested that pectin solubilisation may result from the loss of neutral sugars from side chains of rhamnogalacturonans. In the case of boysenberry Ara was the major non-cellulosic NS, exceeding Gal by two to threefold, and showed the most dramatic ripening-associated modifications. At intermediate stages of development a large solubilisation of Ara-rich polymers was observed, whereas almost 50% of total cell wall Ara was lost at the 100%R stage. L-arabinans with different degrees of branching at the O-3 and O-2 positions and type I arabino-galactans are attached to the O-4 position of Rha residues of RG I, (Brett and Waldron 1996; Willats *et al*. 2001). Peña and Carpita (2004) found that the loss of the highly branched arabinan pre-staged the loss of texture in apples. Thus the decrease in the highly branched arabinans may be involved in the increased pectin solubility observed in intermediate ripening stages of boysenberry fruit.

Fruit softening usually is accompanied by changes in molecular size of pectins occurring to different extents and at different times depending on the species considered (Brummell 2006). The results for other soft fruits regarding modifications in the pectic matrix have not led to consistent conclusions. Nogata *et al*. (1993) reported that strawberry fruit pectin depolymerisation was limited, whereas Rosli *et al*. (2004) observed clear differences in pectin polymer size, leading them to suggest that strawberry fruit softening was closely linked to pectin metabolism. The report that strawberry fruit with antisense expression of a pectate lyase gene produced ripe fruit with lower cell wall swelling and pectin solubility, and higher firmness (Jiménez Bermúdez *et al*. 2002) also supports the conclusion that pectin breakdown and strawberry fruit softening are mechanistically linked. The extensive pectin depolymerisation found in late ripening in boysenberry when softening proceeded but without marked changes in the cellulose-hemicellulose matrix (Cel-Hem) suggests that pectin size downshifts may be important in softening in terminal stages probably through effects on the integrity of the middle lamella and intercellular adhesion. The Ara loss discussed earlier may be a prerequisite to permit other wall components to become susceptible to enzymatic modification. Furthermore, lateral chain removal may also permit RG-I-associated homogalacturonan to be susceptible to enzymatic hydrolysis, thus influencing the pectin depolymerisation observed later in development (Fig. 5).

Model for cell wall changes accompanying boysenberry fruit development

The data suggest that cell wall changes determining growth and softening in boysenberry fruit are accompanied by modifications in both the Cel-Hem and pectic networks but with the clearest changes occurring in wall polyuronides. The results suggest that temporal changes in cell wall degradation in boysenberry occur in three stages (Fig. 7): an early stage (green to 75%R), associated with relative changes in cellulose and cross-linking glycan content and metabolism, at least in the hemicellulosic glycan component. The changes in cross-linking glycans are characterised by an increase in XyG solubilisation without depolymerisation suggesting the involvement of proteins able to affect the interactions within the Cel-Hem network but without extensive polymer cleavage.

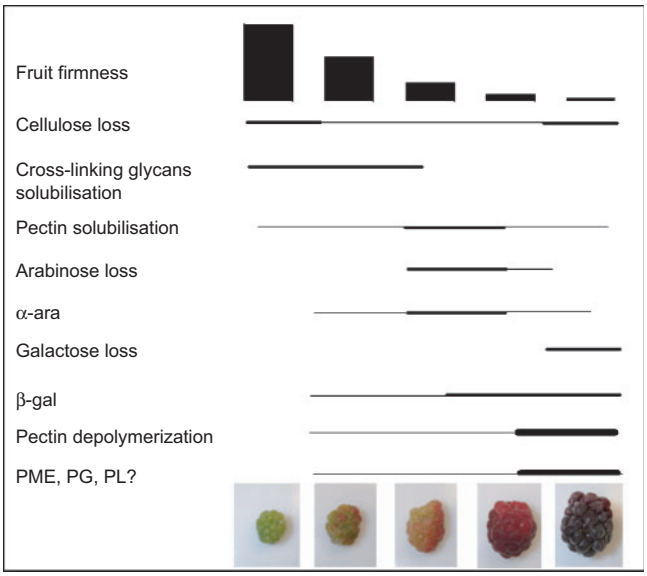

Fig. 7. Proposed model for cell wall changes accompanying boysenberry fruit development and softening. PG, polygalacturonase; PL, pectate lyase; PME, pectin methylesterase.

As development advances, extensive modification of pectin becomes apparent. At intermediate stages of development (75–100%R), the major modifications include a substantial increase in pectin solubilisation without depolymerisation in which Ara is lost. As the fruit reach the 100%R and purple stages several pectin-modifying enzymes, including β-gal causing a loss of Gal from the cell wall, PME and PG, are recruited and substantial pectin depolymerisation occurs.

From a biotechnological perspective enzymes involved in pectin matrix disassembly seem to be the better candidates to affect boysenberry fruit late softening by genetic intervention. Approaches trying to reduce pectin depolymerisation by affecting PG levels should not disregard the potential functional redundancy that pectate lyase might have in such process. Strategies utilising α-ara as a potential target to reduce late softening by directly affecting pectin solubilisation and indirectly pectin depolymerisation should also consider the potential negative side effects of altering arabinan metabolism in a fruit in which enlargement occurs simultaneously with ripening.

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