In vivo pectin solubility in ripening and chill-injured tomato fruit

Domingos P.F. Almeida [a,b,*], Donald J. Huber [c]

[a] CBQF, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal
[b] Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, 4099-002 Porto, Portugal
[c] Horticultural Sciences Department, Institute of Food and Agricultural Sciences, PO Box 110690, University of Florida, Gainesville, FL 32611, USA

Keywords: Cell wall; Chilling injury; *Lycopersicon esculentum*; Neutral sugars; Polyuronide; Polygalacturonase

Abstract

In vivo pectin solubility was examined in ripening and chill-injured tomato fruit with down-regulated polygalacturonase (PG, EC 3.2.1.15) activity and untransformed wild-type fruit by analyzing a pressure-extracted fluid of apoplastic origin. Pectin concentration in the apoplastic fluid increased threefold during ripening and was not affected by endogenous PG. In contrast, PG strongly affected pectin concentration in a bulk pericarp fluid obtained after tissue disruption. There was a 14-fold increase in bulk pectin levels during ripening of PG-antisense fruit and a 36-fold increase in wild-type. Pectins soluble in the apoplastic fluid decreased slightly during storage of fruit at 5 °C for 14 days but increased considerably upon subsequent transfer to 15 °C. Concentration of monomeric galactose in the apoplastic fluid increased during ripening from 41 to 67 μg mL^{-1}. Galactose levels were threefold to fourfold higher in the bulk than in the apoplastic fluid. Low-temperature storage caused a 50% reduction in the galactose present in the bulk fluid and a 20% reduction in apoplastic concentration of galactose. These results indicate that pectin dissolution in ripening tomato fruit is PG-independent even though the enzyme is catalytically active in ripe fruit. Low-temperature storage reduces in vivo pectin solubility, an effect that is reversed upon transfer to higher temperature following cold storage.

Introduction

Plant cells are encapsulated by a wall, a complex entity composed of polysaccharides, structural proteins, and enzymes. In dicots, pectic polysaccharides account for about one-third of the cell wall material [1]. The pectin matrix plays critical roles in the development of plant organs, determining apoplastic porosity [2], ion-exchange capacity [3], and cell adherence [4]. Some pectic oligosaccharides have signaling properties, the best characterized of which are elicitor and morphogenetic effects [5].

In fleshy fruit, including tomato, ripening is accompanied by cell wall disassembly, a complex process involving both enzymic and nonenzymic mechanisms. The pectic network is particularly targeted during ripening, undergoing deglycosylation, deesterification, dissolution, and depolymerization [6] although synthesis persists [7,8]. Changes in pectin metabolism during low-temperature storage or upon the subsequent ripening period at warm temperature induce abnormal textures in fruit susceptible to chilling injury [9,10].

The ripening-related and the chilling-associated changes in pectic polymers have been ascertained from analysis of isolated cell wall polysaccharides. The isolation procedures involve tissue homogenization, organic solvent precipitation of polysaccharides, and various washing procedures. A portion of the apoplastic Ca^{2+} is likely removed, chelated by organic acids during tissue homogenization, and washed away during isolation of cell wall materials. For example, the use of phenol–acetic acid–water (2:1:1, w/v/v) to inactivate endogenous enzymes displaces as much as 50% of cell wall Ca^{2+} [11], and differences in the calcium content of cell walls isolated by three different procedures can reach 25% [12]. Given the critical role of calcium in determining pectin integration in the cell wall [13], pectin solubility from isolated cell walls likely does not reflect the actual solubility in the apoplast.

Endopolygalacturonase (PG, EC 3.2.1.15) is probably the most widely studied enzyme in relation to cell wall metabolism in fruit. However, how the enzyme functions in vivo under

* Corresponding author at: CBQF, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal. Tel.: +351 22 5580001; fax: +351 22 5090351.
E-mail address: dfalmeida@esb.ucp.pt (D.P.F. Almeida).

physiological conditions is still unclear. Down-regulation of PG expression reduces the amount of water-soluble pectins in ripe tomato fruit [14] but does not affect the proportion of chelator-soluble pectins [15]. However, how these pectic fractions, categorized on the basis of solubility in water and chelators, reflect changes in pectin properties in vivo is unknown. Tomato PG is catalytically restricted in vivo [16], at least in part due to the mineral composition and ionic strength of the fruit apoplastic solution [12]. PG-mediated release of pectins from isolated cell walls is very restricted in solutions mimicking the composition of the apoplastic solution of ripening tomato fruit and is affected by the cell wall isolation protocol [12]. It is also clear that PG activity is enhanced by tissue disruption [17], a condition that increases the ionic strength of the solution [18], and supports the idea that PG action in vivo is limited by apoplastic conditions.

Chilling injury compromises membrane competence and tissue integrity [19], leading to apoplastic conditions that are certainly very different from those of normally ripening fruit. Moreover, PG activity is not detected in tomato fruit during storage at 5 °C but accumulates rapidly upon subsequent transfer to warm temperature [20]. It is thus likely that in vivo pectin dissolution in chill-injured fruit differs from that in normally ripening fruit.

The objective of this study was to characterize pectin solubility in vivo in normally ripening and in chill-injured tomato fruit. We have circumvented the effects of the isolation protocol on pectin solubility by examining a pressure-extracted fluid of apoplastic origin and by analyzing pectins effluxed from pericarp tissue incubated under isotonic conditions. Transgenic fruit with reduced levels of PG and the corresponding wild-type were used to investigate the influence of PG on pectin dissolution in vivo.

Materials and methods

Plant material and storage conditions

Tomato (*Lycopersicon esculentum* Mill.) fruit from line 1436 (Calgene, Davis, CA, USA) containing a PG-antisense construct and corresponding untransformed wild-type were harvested at the mature-green stage, washed in 2 mM sodium hypochlorite for 2 min, rinsed and air-dried. Fruit were stored at 15 °C and sampled at the mature-green, pink, and ripe stages. Other mature-green fruit were stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days to allow the development of chilling injury. Chilling injury was visually assessed. Chill-injured fruit infected with *Alternaria* sp. were discarded and fruit with obvious ripening impairment and yellowing were used for subsequent studies. Symptoms of chilling injury developed similarly in wild-type and PG-antisense fruit.

Extraction of apoplastic and bulk fuids

Apoplastic fluid was extracted with an adaptation of the pressure bomb procedure used by Ruan et al. [21] as previously described [18]. The extractions were performed at 5 °C using a pressure of 0.7 MPa for mature-green and 0.4 MPa for ripe fruit. Fruit stored at 5 °C for 2 weeks were pressurized at 0.5 MPa for fluid extraction whereas 0.3 MPa were required to expel fluid from chill-injured fruit transferred to 15 °C for 6 days after cold storage. The apoplastic nature of the pressure-extracted fluids was ascertained by measuring the osmolality with a Wescor vapor pressure osmometer (model 5500, Logan, UT, USA), as described [18]. Samples with osmolality <50% lower than the bulk fluid extracted from the same fruit were considered from apoplastic origin [18], and further analyzed. The average osmolality of the pressure-extracted fluid was, respectively, 36%, 40%, and 47% of the osmolality of the bulk fluid in mature-green unchilled fruit, fruit stored for 14 days at 5 °C, and chill-injured fruit upon 6 days at 15 °C following low-temperature storage. In pink and ripe fruit, the osmolality of the pressure-extracted fluid was 47% of that of bulk sap, as previously reported [18]. An aliquot of 100 μL of pressure-extracted fluid was added to 400 μL of ethanol and stored at −20 °C until analyzed.

Bulk pericarp sap was extracted from the same fruit used for apoplastic fluid extraction. Approximately 4 g of frozen pericarp tissue were thawed at room temperature and centrifuged at 1250 × *g* for 20 min. The supernatant was collected and used for analyses.

Extraction of proteins and polysaccharides from apoplastic and bulk fluids

Apoplastic and bulk fluid isolates in 80% ethanol were heated at *ca.* 90 °C in a water bath for 15 min in capped polypropylene vials and stored 12 h at −20 °C. The samples were centrifuged for 10 min at 8500 × *g* and the supernatant collected for analysis. The pellets were resuspended in 0.5 mL of 80% ethanol, centrifuged for 10 min at 8500 × *g* and the supernatant discarded. The pellet was then suspended in 1.0 mL of distilled water for subsequent analyses.

Total protein and uronic acid determination

Protein content in apoplastic and bulk fluids was determined by the bicinchoninic acid method [22], with bovine serum albumin (Sigma) used as a standard. Total uronic acids were determined using the *m*-phenylphenol method [23], as modified by Filisetti-Cozzi and Carpita [24], with galacturonic acid as a standard.

Free galactose

The 80% ethanolic supernatants of the apoplastic and bulk fluids were evaporated to dryness under a stream of air in a heating block at 38 °C, and the residues suspended in distilled water. Galactose levels were determined using a galactose oxidase/peroxidase assay adapted from Sturgeon [25]. Briefly, 0.1 mL of sample was incubated for 3 h at 37 °C with 2.9 mL of a solution of 20 U of D-galactose oxidase (EC 1.1.3.9, Sigma), 1250 U of horseradish peroxidase (EC 1.11.1.7, Sigma), and

5 mg of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) per 100 mL of 100 mM phophate buffer, pH 7.0. At the end of the incubation period, the absorbance was measured at 440 nm. D-Galactose was used as a standard.

Neutral sugars in the apoplastic soluble polymers

Ethanol-insoluble pellets suspended in distilled water were dried at 35 °C under a stream of filtered air. Hydrolysis and derivatization procedures were as described by Albersheim et al. [26] with minor modifications. The neutral sugar-containing polysaccharides were hydrolyzed in 2N trifluoroacetic acid at 120 °C for 1 h. The resulting monosaccharides were reduced with 0.66 M sodium borohydride in 1N ammonium hydroxide overnight at 25 °C. The samples were acidified to pH 5 with Dowex 50W. The resin was removed by filtration through a syringe-mounted GF/C filter paper and the samples were dried and subsequently washed three times with methanol and once with ethanol before derivatization. The sugars were converted into acetyl derivatives in the presence of 0.2 mL of acetic anhydride and 0.2 mL of pyridine for 1 h at 100 °C. The samples were then dried, washed with toluene three times, and dissolved in methylene chloride. The sugars were separated on a 25 m crosslinked 5% phenyl substituted methylsiloxane capillary column (HP-5, 0.2 mm internal diameter, 0.33 μm film thickness) using a 5890 series II HP gas-chromatograph equipped with a flame ionization detector. Helium was used as the carrier gas at 60 psi. The oven was held at 210 °C for 5 min, increased to 230 °C at 4°/min, and held at this temperature for 14 min. The quantification of individual sugars was accomplished using *myo*-inositol as an internal standard.

Characterization of pectins effluxed from pericarp disks

Pericarp disks were excised from the equatorial region of fruit with a 15.5 mm diameter cork borer, rinsed with distilled water, blotted dry, and incubated in 300 mM sucrose for 4 h at room temperature (*ca.* 24 °C). Afterward, the bathing solution was filtered through a Whatman GF/C glass fiber filter, and the filtrate diluted to 80% ethanol and stored at −20 °C. The samples were centrifuged at 2000 × *g* for 20 min at 4 °C, the supernatant was discarded and the pellet washed twice in 40 mL of 80% ethanol. The pellet was dissolved in 5 mL distilled water, and total uronic acids were determined.

Pectins released from the pericarp disks were concentrated by rotary evaporation at 35 °C and separated by size on a Sepharose CL-4B column (29 cm long × 1.5 cm diameter) operated in 200 mM ammonia acetate, pH 5.0 as the mobile phase [27].

Results

Soluble protein in the apoplastic and bulk fluids

Apoplastic solutions contained protein at all stages of ripening, ranging from 224 to 475 μg mL^{-1} (Fig. 1A). At the mature-green stage, protein was 20 times more concentrated in the apoplastic than in the bulk fluid. As the fruit ripened, the

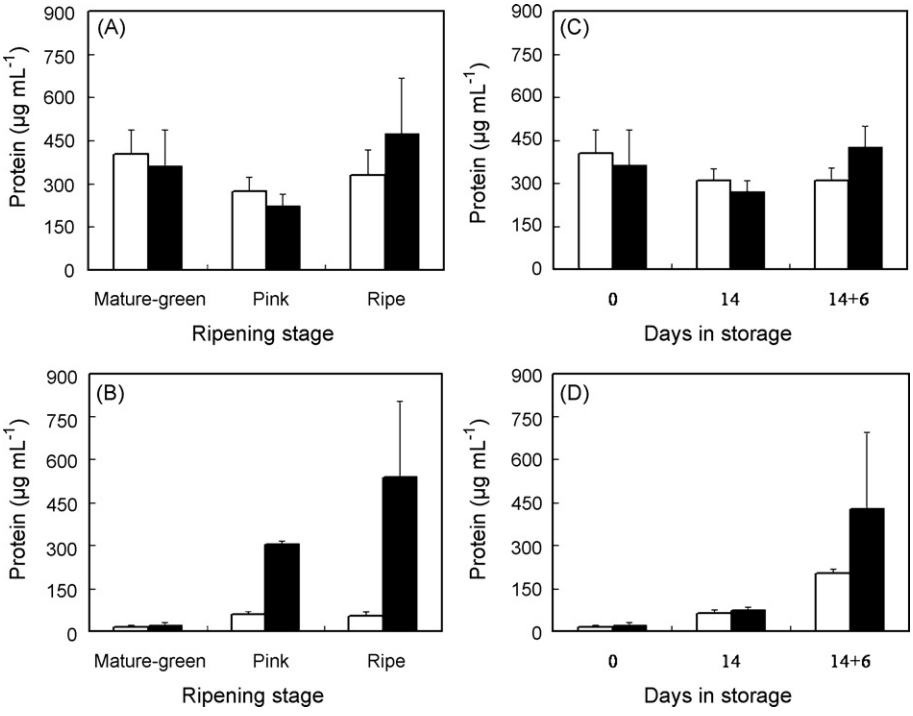

Fig. 1. Protein concentration in the apoplastic (A and C) and bulk (B and D) fluids of tomato pericarp extracted from PG-antisense (white bars) and wild-type (black bars) fruit. Fruit were analyzed during ripening at 15 °C (A and B) or after being stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days (C and D). Values are mean ± S.E. ($n = 4$).

concentration of soluble protein in the bulk fluid increased 3-fold in PG-antisense fruit and 27-fold in wild-type fruit (Fig. 1B). At the ripe stage, wild-type fruit yielded 10 times more soluble protein in the bulk fluid than the PG-antisense fruit (Fig. 1B), whereas the apoplastic solution of both lines had similar protein levels (Fig. 1A).

The concentration of protein recovered from the apoplastic fluid in fruit stored at 5 °C or upon subsequent transfer to 15 °C was within the same range observed in ripening fruit and was not affected by endogenous PG levels (Fig. 1C). Soluble protein in the bulk fluid increased 3.6-fold during storage of the fruit at 5 °C, with no effect of PG levels. Following transfer of fruit to 15 °C for 6 days, protein concentration in the bulk fluid was strongly affected by endogenous PG levels, with the wild-type yielding twice the amount of protein released by PG-antisense bulk fluid (Fig. 1D).

Pectins in the apoplastic and bulk solutions

The concentration of pectins recovered in the apoplastic fluid increased threefold during ripening from 220 μg mL^{-1} at the mature-green stage to about 680 μg mL^{-1} at the ripe stage (Fig. 2A), with no effect of endogenous PG levels. In contrast with the apoplastic fluid, endogenous PG levels strongly affected pectin concentration in bulk fluids (Fig. 2B). The concentration of pectins in the bulk fluid increased 14-fold during ripening of PG-antisense fruit whereas a 36-fold increase was observed in the wild-type.

The concentration of pectins in the apoplastic fluid decreased slightly during cold storage, but increased considerably, in a PG-independent manner, upon transfer to 15 °C (Fig. 2C). In the bulk fluid, pectin concentration changed little during low-temperature storage but dissolution increased dramatically after transfer to 15 °C for 6 days (Fig. 2D).

Galactose levels in apoplastic and bulk solutions

The levels of free galactose in the apoplastic solution were about one-third of those in the bulk solution and increased during ripening in a PG-independent manner in both fluids (Fig. 3). Apoplastic free galactose concentrations were little affected by low-temperature storage (Fig. 3C). In contrast, storage of mature-green fruit at 5 °C for 14 days resulted in a 50% decline in bulk free galactose concentration (Fig. 3D). Additional changes in bulk free galactose were not evident following transfer of chilled fruit to 15 °C.

Polymeric neutral sugars in the apoplastic fluid

Xylose and galactose were the most abundant noncellulosic neutral sugars in the ethanol-insoluble fraction of the apoplastic fluid of ripening fruit, followed by glucose and arabinose (Table 1). The proportion of rhamnose and arabinose increased during ripening, whereas xylose, mannose, and glucose decreased. During ripening, PG down-regulation strongly depressed the amounts of soluble polymers containing xylose and increased those containing galactose (Table 1).

Low-temperature storage induced significant reductions in the levels of rhamnose and glucose, and increases in the proportion of xylose (Table 2). After transfer of fruit to 15 °C

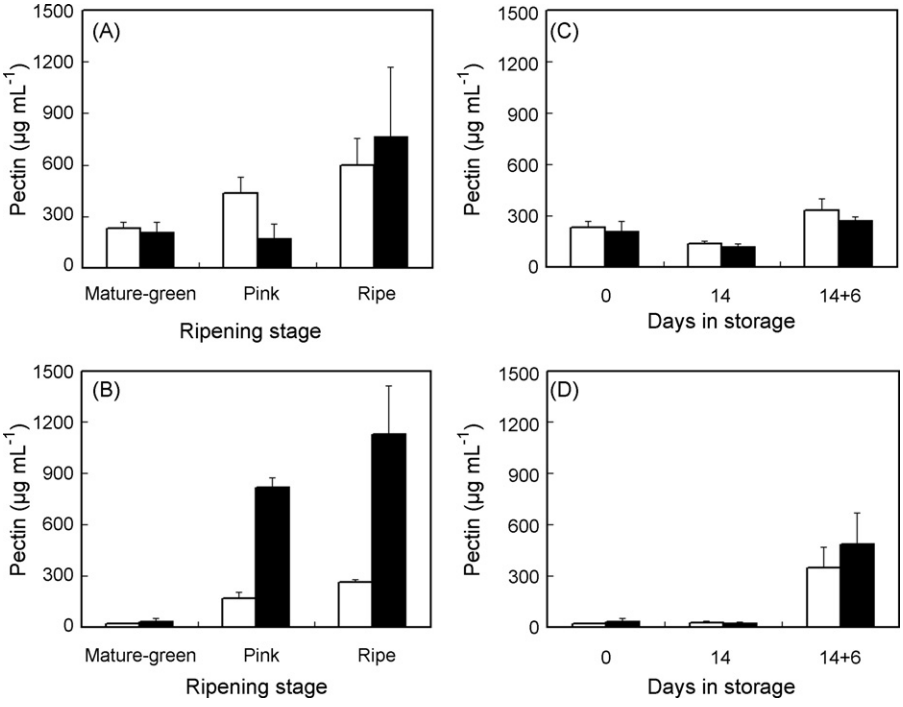

Fig. 2. Pectin concentration in the apoplastic (A and C) and bulk (B and D) fluids of tomato pericarp extracted from PG-antisense (white bars) and wild-type (black bars) fruit. Fruit were analyzed during ripening at 15 °C (A and B) or after being stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days (C and D). Values are mean ± S.E. ($n = 4$).

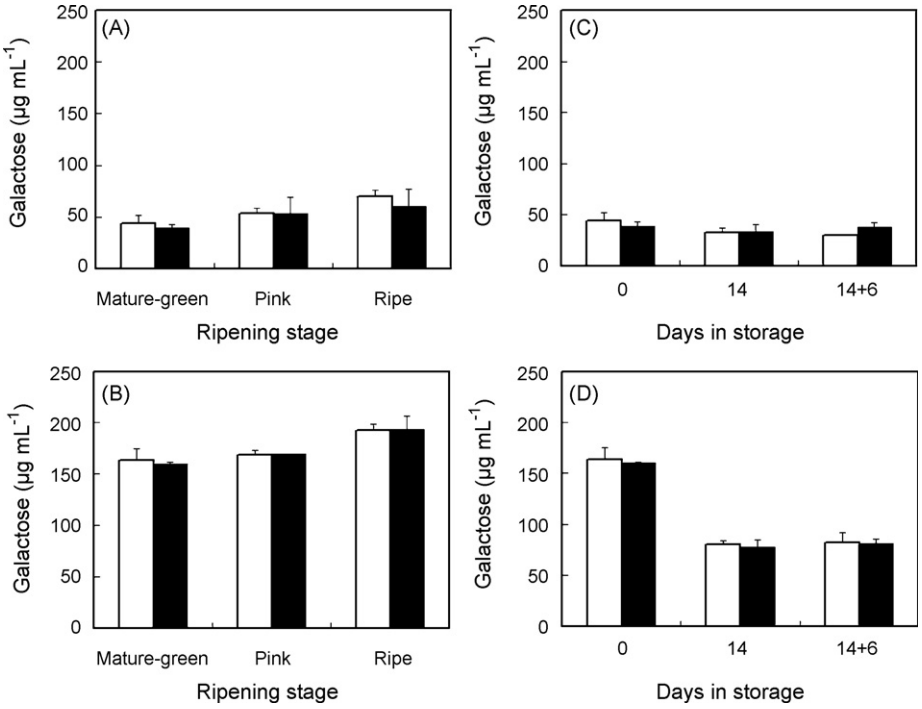

Fig. 3. Concentration of free galactose in the apoplastic (A and C) and bulk (B and D) fluids of tomato pericarp extracted from PG-antisense (white bars) and wild-type (black bars) fruit. Fruit were analyzed during ripening at 15 °C (A and B) or after being stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days (C and D). Values are mean ± S.E. ($n = 4$).

Table 1
Neutral sugars in the ethanol-insoluble polymers from the apoplastic fluid extracted from PG-antisense (AS) and wild-type (WT) ripening tomato (expressed as a mol% of total galacturonic acid)

Line	Stage	Rha (mol%)	Ara (mol%)	Xyl (mol%)	Man (mol%)	Glc (mol%)	Gal (mol%)
WT	MG	2.1 ± 1.3	17.2 ± 5.1	33.1 ± 18.8	7.3 ± 1.2	14.9 ± 5.0	25.4 ± 8.8
	Pink	10.0 ± 5.2	16.8 ± 3.5	33.7 ± 14.2	4.8 ± 1.5	15.1 ± 1.8	19.7 ± 2.3
	Ripe	4.6 ± 1.0	22.2 ± 8.2	27.3 ± 18.3	5.7 ± 1.8	17.2 ± 5.2	23.0 ± 4.1
AS	MG	3.1 ± 2.0	16.9 ± 1.9	24.0 ± 4.6	11.0 ± 1.1	20.9 ± 1.0	24.1 ± 2.7
	Pink	9.8 ± 3.1	25.9 ± 0.5	11.8 ± 1.3	4.0 ± 0.3	21.4 ± 0.6	27.2 ± 1.7
	Ripe	11.9 ± 2.4	25.3 ± 0.9	7.9 ± 0.6	4.6 ± 1.4	16.9 ± 3.0	33.4 ± 3.7

Values are mean ± S.E. ($n = 2$).

for 6 days following cold storage, the proportion of xylose increased whereas arabinose, galactose, and mannose decreased. PG down-regulation in antisense fruit had no effect on the composition of soluble apoplastic polymers in chill-injured fruit (Table 2).

Efflux of pectins from pericarp disks

In a complementary approach to probe the solubility of pectin in the apoplast, the uronic acids passively released from pericarp disks incubated in an isotonic solution were analyzed.

Table 2
Neutral sugars in the ethanol-insoluble polymers from the apoplastic fluid of tomato fruit stored at 5 °C for 2 weeks and subsequently transferred to 15 °C for 6 days (expressed as a mol% of total galacturonic acid)

Line	Sampling date	Rha (mol%)	Ara (mol%)	Xyl (mol%)	Man (mol%)	Glc (mol%)	Gal (mol%)
WT	Before storage	2.1 ± 1.3	17.2 ± 5.1	33.1 ± 18.8	7.3 ± 1.2	14.9 ± 5.0	25.4 ± 8.8
	14 days at 5 °C	ND	14.7 ± 4.1	49.6 ± 6.0	6.2 ± 0.4	10.2 ± 1.4	19.4 ± 3.8
	14 days at 5 °C + 6 days at 15 °C	ND	10.5 ± 0.2	72.0 ± 0.5	ND	6.1 ± 0.7	11.4 ± 0.1
AS	Before storage	3.1 ± 2.0	16.9 ± 1.9	24.0 ± 4.6	11.0 ± 1.1	20.9 ± 1.0	24.1 ± 2.7
	14 days at 5 °C	ND	13.9 ± 2.5	48.2 ± 0.4	7.2 ± 1.7	13.9 ± 2.9	16.8 ± 1.8
	14 days at 5 °C + 6 days at 15 °C	ND	9.2 ± 1.1	71.3 ± 1.4	3.1 ± 0.0	7.0 ± 1.1	10.9 ± 0.9

Values are mean ± S.E. ($n = 2$). ND: not detected.

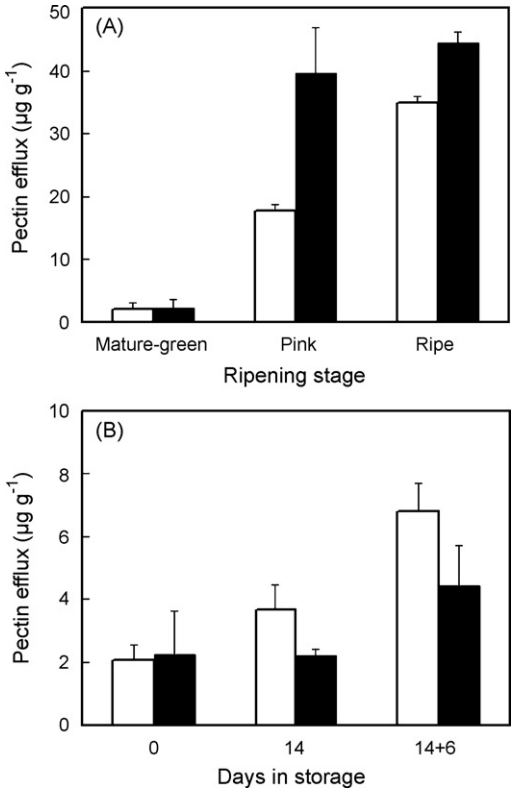

Fig. 4. Pectin released from pericarp disks excised from PG-antisense (white bars) and wild-type (black bars) tomato fruit incubated in an isotonic solution for 4 h. Fruit were analyzed during ripening at 15 °C (A) or after being stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days (B). Values are mean ± S.E. ($n = 4$).

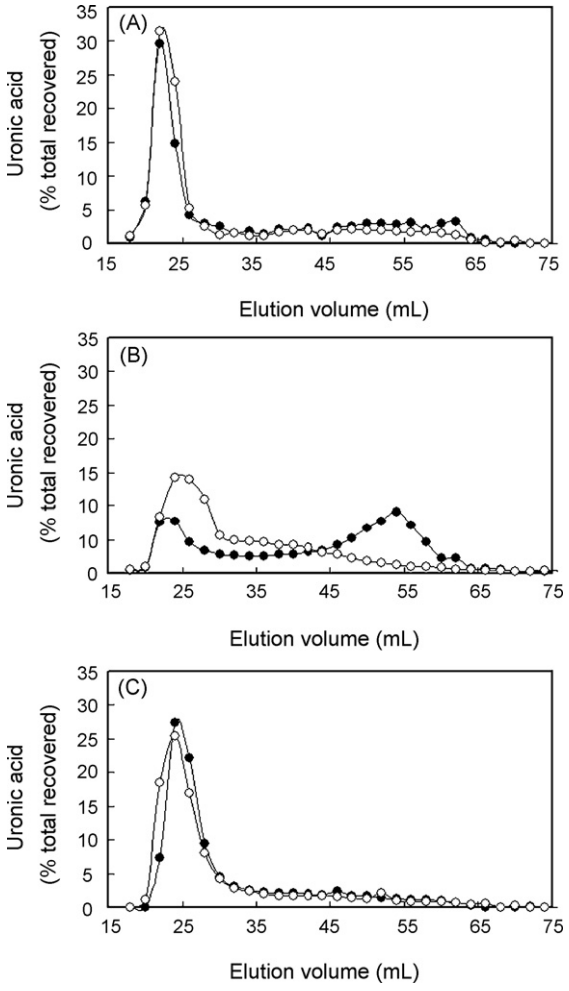

Fig. 5. Molecular size distribution of pectins effluxed from pericarp disks incubated in an isotonic solution. Disks were excised from PG-antisense (○) or wild-type fruit (●) at the mature-green (A) and ripe (B) stages and from chill-injured fruit that had been stored at 5 °C for 14 days and subsequently transferred to 15 °C for an additional 6 days (C).

The release of pectins from pericarp disks at the mature-green stage was similar in both lines (2 μg g^{-1}) and increased dramatically during ripening (Fig. 4A). Although, at the ripe stage, the efflux of pectins from wild-type fruit was slightly higher than the release from antisense fruit, the differences were not significant.

Pectin efflux remained relatively unchanged after 14 days at 5 °C and increased upon subsequent transfer of the fruit to 15 °C for 5 days (Fig. 4B), with little effect of endogenous PG levels.

This methodology allowed the recovery of sufficient amounts of pectins to examine their degree of polymerization. Pectins released from pericarp disks of mature-green fruit had a high degree of polymerization, eluting in the void volume of the Sepharose CL-4B column (Fig. 5A). During ripening, the population of polyuronide molecules became more polydisperse, with a marked downshift in molecular weight distribution of pectins from wild-type fruit, whereas limited depolymerization was observed in the PG-antisense (Fig. 5B). Storage at 5 °C for 14 days and subsequent development at 15 °C for 6 days did not alter mass distribution profile of the eluted polymers (Fig. 5C).

Discussion

Analysis of pectin solubility in fruit is typically performed in preparations obtained by means that are likely to wash away a portion of the apoplastic calcium [11] and significantly alter the physical status of the polymers. A dilute pressure-extracted fluid of putative apoplastic origin, previously used to characterize the apoplastic pH and mineral composition in ripening tomato fruit [18], was analyzed to assess the apoplastic levels of soluble pectin, protein, and free galactose. Fruit from near-isogenic lines containing a PG-antisense construct and the corresponding wild-type were used to evaluate the role of PG on in vivo pectin solubility.

PG is a major protein in tomato fruit, accumulating in the pericarp at the onset of ripening and increasing to 40–50 μg g^{-1} f.w. at the ripe stage [28]. The small difference in apoplastic protein levels between PG-antisense and wild-type fruit (Fig. 1A and C) suggests that PG is largely bound to the cell wall in vivo, a result consistent with findings reported in vitro [29]. The increase in ionic strength resulting from tissue disruption [18] releases bound PG, partially explaining the difference between bulk protein concentration in PG-antisense and wild-type fruit (Fig. 1B). However, since PG accounts for only *ca.* 2% of the protein in a crude extract from ripe tomato

fruit [30] the 10-fold difference in protein concentration observed in the bulk fluid extracted from the two lines cannot be totally explained by differences in the levels of endogenous PG. Presumably, reduced cell breakage and more protein binding to the cell walls of PG-antisense fruit contributed to the large difference observed.

Although the levels of soluble pectins in the apoplast increased during ripening (Fig. 2A) the changes were modest when compared with those occurring in the bulk fluids (Fig. 2B). In contrast with the dissolution in the apoplastic fluid of wild-type and PG-antisense fruit, pectins recovered in bulk tissue isolates differed significantly between the two lines (Fig. 2B). The acceleration of pectin degradation upon tomato tissue disruption, first observed in 1938 [31], has been shown in a number of studies [17,32]. Although the basis for increased activity is unknown, the sensitivity of PG to low pH and elevated monovalent cation levels [33,34], conditions created upon tissue disruption [18], may be involved. Moreover, tissue disruption may reduce the concentration of divalent cations available for cross-linking pectic polymers. This might occur by dilution of the apoplastic solution with symplastic fluid, or by chelation of apoplastic calcium by organic acids released during breakdown of tissue compartmentation.

The results presented here indicate that in vivo dissolution of pectin in ripening tomato pericarp is largely PG-independent (Figs. 2 and 4A), despite the extent of PG-mediated pectin depolymerization (Fig. 5B). PG is involved in pectin depolymerization, but its role on in vivo pectin dissolution is not clear. The water solubility of pectins isolated from tomato pericarp correlates with PG levels [14], but the levels of pectin recovered with the apoplastic solution were unaffected by PG (Fig. 2A). The evidence for the effect of PG on the pectic fraction bound to the cell wall by calcium bridges is contradictory. A 99% reduction of PG activity by antisense did not affect the chelator-solubility of pectins [15], whereas the expression of PG in the *rin* mutant strongly enhanced the chelator-soluble fraction of polyuronides [35].

Since PG is capable of hydrolyzing pectins bound to the cell wall by calcium bridges [12], it is likely that in the equilibrium between the water-soluble and the chelator-soluble fractions in vivo is different from that observed in isolated cell wall materials. The presence of about 5 mM of Ca^{2+} in the fruit apoplast [18] and the possibility for Ca^{2+} removal by cell wall isolation protocols, suggests that in vivo conditions for pectin dissolution may be less than optimal.

Low-temperature storage altered in vivo pectin dissolution in relation to normal ripening. The reduction in the levels of apoplastic pectins observed after 14 days at 5 °C (Fig. 2C) is consistent with the reduction of soluble pectins observed in other chilling sensitive fruit [36], and is presumably related to the demethylation of uronic acid residues by pectinmethylesterase (EC 3.1.1.11) during low-temperature storage [9], and subsequent linkage to the pectic matrix by calcium bridges. The data presented suggest that restricted pectin dissolution (Figs. 2 and 4) and the absence of pectin depolymerization (Fig. 5) explain, at least in part, the abnormal texture of chill-injured tomato fruit [20].

It has been shown that infiltration of galactose into mature-green fruit accelerates the onset of ripening, suggesting that monomeric galactose can play a role in regulating fruit ripening [37]. Galactose loss from apoplastic polysaccharides is a nearly ubiquitous feature of cell wall changes in ripening fruit and several studies have shown that the decrease in cell wall galactosyl residues is accompanied by an increase in free galactose in bulk fluid [38–41]. The results presented here, however, show no correlation between the levels of free galactose in the apoplast and the proportion of galactosyl residues in the polysaccharides recovered in apoplastic fluid. The 63% increase in apoplastic free galactose during ripening (Fig. 3A) occurred in parallel with no change or an increase in the proportion of galactosyl residues in soluble apoplastic polysaccharides (Table 1). During cold storage, a 20% decrease in free galactose (Fig. 3C) was accompanied by a 55% reduction in galactosyl residues in the apoplastic soluble polysaccharides (Table 2). Possibly, removal of galactosyl residues does not increase the overall solubility of polysaccharides under apoplastic conditions, or free galactose originates from polymers distinct from those recovered in the apoplastic exudates.

Previous determinations of free galactose in ripening tomato used bulk fluids extracted after tissue homogenization. This methodology results in the blend of different pools of galactose, mainly derived from the cell wall and galactolipids. When enzymes were inactivated by refluxing ethanol, bulk pericarp fluids showed a fourfold to eightfold increase in free galactose during ripening [38,39]. Using enzymically active homogenates, we observed a 20% increase in bulk free galactose between the mature-green and ripe stages. The amount of free galactose in the bulk pericarp of ripe fruit (192 $\mu g\ mL^{-1}$ = 1.07 mM) is within the range reported elsewhere; however, our mature-green samples contained four to eight times more (161 $\mu g\ mL^{-1}$ = 0.89 mM) galactose than reported for enzymically inactive fluids [38,39]. This possibly indicates that tissue disruption increases the activity of β-galactosidase (EC 3.2.1.23) at the mature-green stage, possibly due to the apoplastic pH.

The increase in free galactose may be a direct result of hydrolysis [41], possibly accompanied by a reduced ability to metabolize free galactose [38]. β-Galactosidase II, a cell wall β-galactanase in tomato fruit, shows a threefold increase in activity during ripening [42]. The enzyme is active between pH 3 and 6.5, with optimum activity around 4.2 and shows a 50% inhibition in the presence of 2 mM galactose [42]. As tomato fruit ripen, the apoplastic pH becomes more favorable for β-galactanase activity [18], and the levels of apoplastic galactose at the ripe stage (0.4 mM) are not likely to strongly inhibit the enzyme.

Net losses of cell wall arabinose and galactose are observed during tomato ripening [42–45]. An increase in the proportion of arabinosyl residues in the polysaccharides soluble in the apoplast during ripening (Table 1) indicates that loss of arabinose reported elsewhere occurs in part due to the dissolution of an arabinose-containing polymer instead of the monomeric sugar. A decrease in galactose in Na_2CO_3-soluble pectins during ripening of PG-antisense and wild-type

tomato fruit has been reported, but the concomitant increase in galactose in water-soluble pectins was observed only in wild-type fruit [14]. In contrast, we observed an increase in the in vivo dissolution of galactose containing-polymers during ripening of PG-antisense fruit and not in the wild-type (Table 1). Moreover, PG-antisense and wild-type fruit showed similar evolution of arabinose-containing polysaccharides (Table 1), where an increase in arabinose levels in water-soluble pectins was observed during ripening of wild-type but not of PG-antisense fruit [14]. In contrast with the trend described in ripening fruit, polymeric arabinose and galactose soluble in the apoplast decreased in chill-injured fruit (Table 2). A decrease in arabinose and galactose content from isolated cell walls is correlated with the severity of chilling injury in nectarines [46].

The analysis of apoplastic soluble polymers reported herein support the notion that pectin dissolution in ripening tomato fruit is PG-independent. However, the limited hydrolytic action exerted by the enzyme in vivo produces significant depolymerization of pectins (Fig. 5B) and affects the composition of the pectic fragments dissolved (Table 1). Pectin dissolution in chill-injured fruit is also independent from PG action and is even more restricted than in ripening fruit. The trends in the polymeric neutral sugars suggest different mechanisms of pectin disassembly in ripening and chill-injured fruit.

References

[1] A. Darvill, M. McNeil, P. Albersheim, D.P. Delmer, The primary cell walls of flowering plants, in: P.K. Stumpf, E.E. Conn (Eds.), The Biochemistry of Plants, vol. 1, Academic Press, New York, 1980, pp. 91–162.

[2] O. Baron-Epel, P.K. Gharyal, M. Schindler, Pectins as mediators of wall porosity in soybean cells, Planta 175 (1988) 389–395.

[3] C. Gillet, M. Voue, P. Cambier, Site-specific counterion binding and pectic chains conformational transitions in the Nitella cell wall, J. Exp. Bot. 49 (1998) 797–805.

[4] M. Knee, Properties of polygalacturonate and cell cohesion in apple fruit cortical tissue, Phytochemistry 17 (1978) 1257–1260.

[5] S.C. Fry, S. Aldington, P.R. Hetherington, J. Aitken, Oligosaccharides as signals and substrates in the plant cell wall, Plant Physiol. 103 (1993) 1–5.

[6] D.A. Brummell, M.H. Harpster, Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants, Plant Mol. Biol. 47 (2001) 311–340.

[7] M. Knee, Fruit softening. II. Precursor incorporation into pectin by pear tissue slices, J. Exp. Bot. 33 (1982) 1256–1262.

[8] M. Huysamer, L.C. Greve, J.M. Labavitch, Cell wall metabolism in ripening fruit. 9. Synthesis of pectic and hemicellulosic cell wall polymers in the outer pericarp of mature green tomatoes (cv XMT-22), Plant Physiol. 114 (1997) 1523–1531.

[9] A.G. Marangoni, R.L. Jackman, D.W. Stanley, Chilling-associated softening of tomato fruit is related to increased pectinmethylesterase activity, J. Food Sci. 60 (1995) 1277–1281.

[10] D.A. Brummell, V.D. Cin, S. Lurie, C.H. Crisosto, J.M. Labavitch, Cell wall metabolism during the development of chilling injury in cold-stored peach fruit: association of mealiness with arrested disassembly of cell wall pectins, J. Exp. Bot. 55 (2004) 2041–2052.

[11] D.J. Huber, Acidified phenol alters tomato cell wall pectin solubility and calcium content, Phytochemistry 30 (1991) 2523–2527.

[12] D.P.F. Almeida, D.J. Huber, Polygalacturonase-mediated dissolution and depolymerization of pectins in solutions mimicking the pH and mineral composition of tomato fruit apoplast, Plant Sci. 172 (2007) 1087–1094.

[13] M.C. Jarvis, The proportion of calcium-bound pectin in plant cell walls, Planta 154 (1982) 344–346.

[14] C.M.S. Carrington, L.C. Greve, J.M. Labavitch, Cell wall metabolism in ripening fruit. VI. Effect of the antisense polygalacturonase gene on cell wall changes accompanying ripening in transgenic tomatoes, Plant Physiol. 103 (1993) 429–434.

[15] C.J.S. Smith, C.F. Watson, P.C. Morris, C.R. Bird, G.B. Seymour, J.E. Gray, C. Arnold, G.A. Tucker, W. Schuch, S. Harding, D. Grierson, Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes, Plant Mol. Biol. 14 (1990) 369–379.

[16] G.B. Seymour, Y. Lasslett, G.A. Tucker, Differential effects of pectolytic enzymes on tomato polyuronides in vivo and in vitro, Phytochemistry 26 (1987) 3137–3139.

[17] D.A. Brummell, J.M. Labavitch, Effect of antisense suppression of endopolygalacturonase activity on polyuronide molecular weight in ripening tomato fruit and in fruit homogenates, Plant Physiol. 115 (1997) 717–725.

[18] D.P.F. Almeida, D.J. Huber, Apoplastic pH and inorganic ion levels in tomato fruit: a potential means for regulation of cell wall metabolism during ripening, Physiol. Plant 105 (1999) 506–512.

[19] M.E. Saltveit, L.L. Morris, Overview of chilling injury of horticultural crops, in: C.Y. Wang (Ed.), Chilling Injury of Horticultural Crops, CRC Press, Boca Raton, FL, 1990, pp. 3–15.

[20] R.L. Jackman, H.J. Gibson, D.W. Stanley, Effects of chilling on tomato fruit texture, Physiol. Plant 86 (1992) 600–608.

[21] Y.L. Ruan, C. Mate, J.W. Patrick, C.J. Brady, Non-destructive collection of apoplast fluid from developing tomato fruit using a pressure dehydration procedure, Aust. J. Plant Physiol. 22 (1995) 761–769.

[22] P.K. Smith, R.I. Kronhn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.

[23] N. Blumenkrantz, G. Asboe-Hansen, New method for quantitative determination of uronic acids, Anal. Biochem. 54 (1973) 484–489.

[24] T.M.C.C. Filisetti-Cozzi, N.C. Carpita, Measurement of uronic acids without interference from neutral sugars, Anal. Biochem. 197 (1991) 157–162.

[25] R.J. Sturgeon, Enzymic determination of D-galactose, in: J. BeMiller, D.J. Manners, R.J. Sturgeon (Eds.), Methods in Carbohydrate Chemistry, vol. X, John Wiley & Sons, New York, 1994, pp. 15–16.

[26] P. Albersheim, D.J. Nevins, P.D. English, A. Karr, A method for the analysis of sugars in plant cell-wall polysaccharides by gas–liquid chromatography, Carbohydr. Res. 5 (1967) 340–345.

[27] A.J. Mort, B.M. Moerschbacher, M.L. Pierce, N.O. Maness, Problems encountered during the extraction, purification, and chromatography of pectic fragments, and some solutions to them, Carbohydr. Res. 215 (1991) 219–227.

[28] G.A. Tucker, D. Grierson, Synthesis of polygalacturonase during tomato fruit ripening, Planta 155 (1982) 64–67.

[29] J.W. Rushing, D.J. Huber, Mobility limitations of bound polygalacturonase in isolated cell wall from tomato pericarp tissue, J. Am. Soc. Hortic. Sci. 115 (1990) 97–101.

[30] Z.M. Ali, C.J. Brady, Purification and characterization of the polygalacturonases of tomato fruits, Aust. J. Plant Physiol. 9 (1982) 155–169.

[31] Z.I. Kertesz, Pectic enzymes. II. Pectic enzymes of tomatoes, Food Res. 3 (1938) 481–487.

[32] N. Errington, G.A. Tucker, J.R. Mitchell, Effect of genetic down-regulation of polygalacturonase and pectin esterase activity on rheology and composition of tomato juice, J. Sci. Food Agric. 76 (1998) 515–519.

[33] J.-P. Chun, D.P. Huber, Polygalacturonase isozyme 2 binding and catalysis in cell walls from tomato fruit: pH and beta-subunit effects, Physiol. Plant 101 (1997) 283–290.

[34] J.-P. Chun, D.P. Huber, Polygalacturonase-mediated solubilization and depolymerization of pectic polymers in tomato fruit cell walls: regulation by pH and ionic conditions, Plant Physiol. 117 (1998) 1293–1299.

[35] J.J. Giovannoni, D. DellaPenna, A.B. Bennett, R.L. Fischer, Expression of a chimeric polygalacturonase gene in transgenic *rin* (ripening inhibitor)

tomato fruit results in polyuronide degradation but not fruit softening, Plant Cell 1 (1989) 53–63.

[36] D.M. Dawson, L.D. Melton, C.B. Watkins, Cell wall changes in nectarines (*Prunus persica*). Solubilization and depolymerization of pectic and neutral polymers during ripening and in mealy fruit, Plant Physiol. 100 (1992) 1203–1210.

[37] J. Kim, K.C. Gross, T. Solomos, Characterization of the stimulation of ethylene production by galactose in tomato (*Lycopersicon esculentum* Mill.) fruit, Plant Physiol. 85 (1987) 804–807.

[38] K.C. Gross, M.E. Saltveit, Galactose concentration and metabolism in pericarp tissue from normal and non-ripening tomato fruit, J. Am. Soc. Hortic. Sci. 107 (1982) 328–330.

[39] K. Gross, Changes in free galactose, *myo*-inositol and other monosaccharides in normal and non-ripening mutant tomatoes, Phytochemistry 22 (1983) 1137–1139.

[40] K.C. Gross, Fractionation and partial characterization of cell walls from normal and non-ripening mutant tomato fruit, Physiol. Plant 62 (1984) 25–32.

[41] J. Kim, K.C. Gross, T. Solomos, Galactose metabolism and ethylene production during development and ripening of tomato fruit, Postharvest Biol. Technol. 1 (1991) 67–80.

[42] R. Pressey, β-Galactosidases in ripening tomatoes, Plant Physiol. 71 (1983) 132–135.

[43] K.C. Gross, Composition of ethanol-insoluble polysaccharides in water extracts of ripening tomatoes, Phytochemistry 25 (1986) 373–376.

[44] S.J. Wallner, H.L. Bloom, Characteristics of tomato cell wall degradation in vitro. Implications for the study of fruit-softening enzymes, Plant Physiol. 60 (1977) 207–210.

[45] R.J. Redgwell, M. Fischer, E. Kendal, E.A. MacRae, Galactose loss and fruit ripening: high-molecular-weight arabinogalactans in the pectic polysaccharides of fruit cell walls, Planta 203 (1997) 174–181.

[46] G.A. Manganaris, M. Vasilakakis, G. Diamantidis, I. Mignani, Changes in cell wall neutral sugar composition and ethylene evolution as potential indicators of wooliness in cold-stored nectarine fruit, J. Food Qual. 28 (2005) 407–416.