Effect of Antisense Suppression of Endopolygalacturonase Activity on Polyuronide Molecular Weight in Ripening Tomato Fruit and in Fruit Homogenates

David A. Brummell and John M. Labavitch*

Pomology Department, University of California, Davis, California 95616

Fruit of tomato (Lycopersicon esculentum Mill.) in which endopolygalacturonase (PG) activity had been suppressed to ≤1% of wild-type levels were slightly firmer than nontransgenic controls later in ripening. Enzymically inactive cell walls were prepared from these ripening fruit using Tris-buffered phenol. When extracted with chelator followed by Na₂CO₃, the amounts of pectin solubilized from cell walls of nontransgenic control or from transgenic antisense PG fruit were similar. Size-exclusion chromatography analysis showed that, relative to controls, in antisense PG fruit polyuronide depolymerization was delayed in the chelator-soluble fraction throughout ripening and reduced in the Na₂CO₃-soluble fraction at the overripe stage. Reduced pectin depolymerization rather than altered extractability thus may have contributed to enhanced fruit firmness. Substantially larger effects of suppressed PG activity were detected in tomato fruit homogenates processed to paste. In control paste the majority of the polyuronide was readily solubilized in water and was very highly depolymerized. In antisense PG paste the proportion of polyuronide solubilized by water was reduced, and polyuronides retained a high degree of polymerization. The suppression of fruit PG activity thus has a small effect on polyuronide depolymerization in the fruit but a much larger effect in paste derived from these fruit. This indicates that in the cell wall PG-mediated degradation of polyuronide is normally restricted but that in tissue homogenates or in isolated cell walls this restriction is removed and extensive pectin disassembly results unless PG is inactivated.

Pectins form a major structural component of plant primary cell walls and are the main constituent of the middle lamella, which is responsible for cell-to-cell adhesion (Carpita and Gibeaut, 1993). Cell wall pectin is broken down during the ripening of many fruit and in tomato (Lycopersicon esculentum Mill.) at least partly due to the action of endo-acting PG (Brady, 1987; Fischer and Bennett, 1991). Tomato fruit ripening and softening are accompanied by massive increases in PG mRNA abundance, immunologically detectable protein, and enzyme activity (Brady et al., 1982; Grierson and Tucker, 1983; DellaPenna et al., 1986). Concomitant with this increase in PG activity is a ripening-related increase in cell wall pectin solubilization and a decrease in polyuronide degree of polymerization (Huber, 1983; DellaPenna et al., 1990; Smith et al., 1990; Huber and O'Donoghue, 1993). In early investigations decreases in polyuronide molecular weight during ripening appeared to be very substantial (Huber, 1983). However, more recent investigations in which Tris-buffered phenol was used to irreversibly inactivate cell wall enzymes (Huber, 1991, 1992; Huber and O'Donoghue, 1993) suggest that the reduction in pectin molecular weight that occurs during ripening is of considerably less magnitude than has been reported previously and may have been overestimated in earlier studies due to incomplete inactivation of PG during cell wall preparation and extraction.

The precise contribution of cell wall pectin disassembly to tomato fruit softening remains unclear. Expression of a chimeric PG construct in fruit of the nonsoftening rin mutant, which normally produces virtually no PG, resulted in polyuronide solubilization and depolymerization but not in fruit softening (Giovannoni et al., 1989; DellaPenna et al., 1990). Suppression of fruit PG mRNA accumulation by constitutive expression of an antisense transgene reduced PG mRNA abundance and enzyme activity (Sheehy et al., 1988; Smith et al., 1988) and largely inhibited the ripening-related reduction in polyuronide molecular weight (Smith et al., 1990), but it did not prevent polyuronide solubilization (Smith et al., 1990) or fruit softening (Smith et al., 1988). However, further analysis of transgenic fruit in which PG activity was reduced to less than 1% of controls indicated that antisense fruit had improved postharvest-handling characteristics (Schuch et al., 1991; Kramer et al., 1992) and remained slightly firmer than controls during ripening, although the effect was small compared with the variability due to different varieties, growth conditions, and postharvest handling (Kramer et al., 1992; Langley et al., 1994). Cell wall separation between cells was reduced in stored antisense fruit, which may be a factor responsible for their slightly increased mechanical firmness (Langley et al., 1994). However, a much more marked difference was observed in processed fruit material; in paste prepared from antisense PG fruit, both total viscosity and serum viscosity were substantially increased relative to controls (Schuch et al., 1991; Kramer et al., 1992).

Abbreviations: CDTA, trans-1,2-diaminocyclohexane-N,N',N''-tetraacetic acid; MG, PK, RR, and OR, mature green, pink, red-ripe, and overripe fruit developmental stages, respectively; PG, fruit endopolygalacturonase; V₀, void volume; Vₜ, total volume.

1 Present address: DNA Plant Technology Corporation, 6701 San Pablo Avenue, Oakland, CA 94608.
2 Corresponding author; e-mail jmlabavitch@ucdavis.edu; fax 1-916-752-8502.
The relationships of cell wall pectin solubility and degree of polymerization to the complex set of factors responsible for fruit pericarp firmness and for processed juice viscosity have not been established. Suppression of PG activity in transgenic fruit appeared to reduce the considerable decline in the average molecular mass that occurred in EDTA-soluble polyuronides during ripening (Smith et al., 1990), but determination of the average molecular mass of pectins provides only a limited amount of information about the population of pectin molecules present in an extract. Furthermore, earlier work has suggested that in cell wall preparations in which endogenous enzymes have not been completely and irreversibly inhibited, autolysis reactions during cell wall preparation and extraction cause a much greater depolymerization of cell wall polysaccharides than occurs naturally in the ripening fruit (Seymour et al., 1987a, 1987b; Huber, 1992; Huber and O'Donoghue, 1993). A commercially significant situation in which cell wall polymer depolymerization may take place in vitro is in fruit homogenates during the manufacture of tomato paste. In this study we prepared cell walls from control and antisense PG fruit using Tris-buffered phenol to irreversibly inactivate cell wall enzymes, and we used size-exclusion chromatography to provide a complete molecular weight profile of the polyuronide molecules present in extracts of pericarp at different ripening stages. Also, polyuronide extractability and molecular weight profiles in extracts of processed tomato fruit paste were examined relative to those occurring in the ripening fruit.

MATERIALS AND METHODS

Fruit was collected at defined ripening stages from plants of tomato (Lycopersicon esculentum Mill., cv C3) grown in a greenhouse. Plants were either nontransgenic controls or suppressed in expression of PG (EC 3.2.1.15) by constitutive expression of an antisense fruit PG transgene (Sheehy et al., 1988), which reduced fruit PG activity to less than 1% of wild-type levels (Kramer et al., 1992). External red-green color was measured (a* on the CIE L*a*b* scale) at four points around the equator of each of three fruit per stage using a Minolta CR200 Chroma Meter (Minolta, Ramsey, NJ). On this scale a* values represent the hue on a green (−) to red (+) scale. Fruit were quartered and seeds and locules removed, then one quarter of each fruit was used for firmness determinations, and the other three quarters were frozen in liquid nitrogen and stored at −80°C. Firmness was measured (three determinations for each of three fruit per stage) on the inside (i.e. locule side) of the pericarp by measuring the force in grams required to insert the slightly convex, 4.5-mm-diameter probe of a penetrometer 2 mm into the pericarp.

Canned fruit pastes from control and antisense PG field-grown fruit were obtained from a commercial pilot plant, and paste was prepared according to a typical hot-break procedure. Briefly, the skinned fruit were subjected to a hot break (93°C, 10 min), pulped, and evaporated at high temperatures to 28°Brix. Pastes were canned at 28°Brix and the sealed cans were sterilized at 93°C.

Cell Wall Preparation

Canned walls were prepared from frozen fruit tissue after the method of Huber and O'Donoghue (1993). Frozen fruit pericarp was homogenized in ice-cold 80% ethanol using a Polytron, and cell wall material was recovered by filtration through Miracloth (Calbiochem). Cell walls were stirred in Tris-buffered phenol for 30 min, precipitated at −20°C by addition of ethanol to 80%, recovered by filtration through GF/C glass fiber filters (Whatman), and washed with ethanol. Insoluble residues were stirred in chloroform: methanol (1:1, v/v) for 30 min, recovered by filtration, and washed with acetone.

Cell Wall Fractionation

Dried acetone-insoluble cell walls (75 mg) were extracted sequentially twice with 30 mL of 50 mM CDTA and 50 mM sodium acetate (pH 6.0) and once with 30 mL of H2O, and all three extracts were pooled (CDTA extract). The insoluble residue was then extracted twice with 30 mL of 100 mM Na2CO3 and 0.1% NaBH4 and once with 30 mL of H2O, and all three extracts were pooled (Na2CO3 extract). Each of the six extractions was for 24 h at room temperature on an orbital shaker, and all solutions contained 0.02% (w/v) NaN3. After each extraction insoluble material was pelleted at 10,000g for 20 min and supernatants were filtered through GF/C glass fiber filters. Na2CO3 extracts were neutralized with acetic acid. Aliquots of the extracts were exhaustively dialyzed (6- to 8-kD molecular mass cutoff) against H2O and the volumes of the dialysates were recorded; then uronic acid content was determined using the assay of Blumenkrantz and Asboe-Hansen (1973) with GalUA as a standard. Uronic acid contents could be determined only in dialyzed samples because of the interference of low-molecular-weight neutral sugars and NaN3 with the assay.

Canned 28°Brix tomato paste (10 g) was resuspended in 65 mL of H2O and stirred at room temperature for 1 h. Insoluble material was pelleted by centrifugation at 10,000g for 20 min and the supernatant (first H2O extract) was decanted. The pellet was resuspended in 50 mL of H2O, stirred for 1 h, and centrifuged, and the supernatant (second H2O extract) was decanted. The pellet was then resuspended in 75 mL of 50 mM CDTA and 50 mM sodium acetate (pH 6.0), stirred for 2 h, and centrifuged, and the supernatant (CDTA extract) was decanted. All extractants contained 0.02% NaN3, and extracts were dialyzed and assayed for polyuronide content as described above.

Determination of amounts of polyuronide in dialyzed extracts were used to calculate the polyuronide contents of total extracts. Extractions of cell walls and pastes were carried out twice with similar results.

Size-Exclusion Chromatography

After dialysis starch was removed from extracts by treatment at room temperature for 24 h with 10 μL of 1 mM CaCl2 containing 25 units of α-amylase (human saliva, type IX-A, Sigma). Aliquots of dialyzed, destarched extracts of
cell wall or paste containing 1 mg of uronic acid were frozen, freeze-dried, and dissolved in 1 mL of 1 M imidazole-HCl (pH 7.0). Samples were applied to a Sepharose CL-2B column (51 × 1.5 cm; Pharmacia) and eluted with 1 M imidazole-HCl (pH 7.0), and 1-mL fractions were collected. Column fractions were assayed for uronic acid content as described above. Size-exclusion chromatography was carried out on two samples obtained from independent extractions with similar results.

GC

Regions of polyuronide profiles from CL-2B chromatography encompassing particular peaks of high- or low-molecular-weight polyuronides were selected for assay of neutral sugar content. Pooled column fractions were dialyzed against H2O to remove imidazole, dried in a stream of air, derivatized to alditol acetates, and analyzed by GC as previously described (Carrington et al., 1993).

Paste Viscosity Determination

Tomato paste canned at a soluble solids concentration of 28°Brix was resuspended in H2O and adjusted to 12°Brix using a refractometer to measure °Brix value. Paste viscosity was measured at room temperature using a Bostwick consistometer (CSC Scientific, Fairfax, VA), recording the mean distance moved (average of minimum and maximum) by the paste front in 30 s.

Paste serum was prepared by resuspending 28°Brix paste in H2O to 9.6°Brix, centrifuging, and decanting the serum. Serum viscosity was determined at 9.6°Brix in crude serum using size 100 Cannon-Fenske viscometers (Fisher Scientific) incubated in a water bath at 20°C. To investigate the relative contributions of dialyzable low-molecular-weight fruit constituents and nondialyzable high-molecular-weight fruit constituents to serum viscosity, serum was dialyzed (6- to 8-kD molecular mass cutoff) exhaustively against H2O to 0°Brix and samples were adjusted to 1.3 mg uronide mL−1. Note that, because of the dilution occurring during dialysis, the uronide content of dialyzed samples was approximately one-half that of nondialyzed samples, in which uronide content could not be determined because of the interference of neutral sugars. The viscosity of the dialyzed serum was determined as described for nondialyzed serum.

RESULTS

Fruit were selected to span a ripening series from MG to OR, showing typical increasing red color and decreasing firmness as ripening progressed (Table I). Fruit of a control and an antisense line in which expression of PG was suppressed were at approximately equivalent ripening stages, as judged by external color. Fruit pericarp firmness declined markedly during ripening in both genotypes, although antisense PG fruit showed a slightly reduced decline in pericarp firmness at the RR and OR stages relative to controls.

Extraction of fruit cell walls with CDTA solubilized increasing amounts of polyuronide as the ripening stage advanced (Fig. 1A). Subsequent extraction of the cell wall residue with Na2CO3 solubilized an additional, smaller amount of polyuronide, which in contrast to CDTA-extractable polyuronide did not increase and declined slightly at the OR stage (Fig. 1B). Relative to control fruit, antisense PG fruit showed slightly elevated amounts of CDTA-extractable polyuronide. The extractions with CDTA and Na2CO3 were sufficient to solubilize most of the cell wall polyuronide, and a subsequent extraction of the residue with 4 M KOH and 0.1% NaBH4 solubilized only 6% as much polyuronide as the combined CDTA and Na2CO3 extractions in MG fruit and lesser amounts from ripened fruit (data not shown).

Size-exclusion chromatography on a Sepharose CL-2B column revealed that in control MG fruit CDTA-soluble polyuronides formed a very-high-molecular-weight peak close to the exclusion limit of the column (20 × 106 for dextran standards), with a tail of lower-molecular-weight material extending throughout the separation range of the column (Fig. 2A). In control fruit at the FK ripening stage the amount of polyuronide in the highest-molecular-weight peak declined relative to MG fruit, concomitantly with an increase in the amount of mid-sized polyuronides (Fig. 2B). In RR fruit the relative amount of high-molecular-weight polymers showed a further decline, and the mid-sized polymers increased in amount and declined in molecular weight (Fig. 2C). By the OR ripening stage the distinction between the peaks of high molecular weight and mid-sized polymers seen in RR fruit began to disappear, with polymers being spread more evenly throughout the separation range of the column (Fig. 2D). In antisense PG fruit the CDTA-soluble polyuronides showed depolymerization during ripening, but this was delayed relative to controls.

Table 1. Color and firmness of fresh harvested fruit

<table>
<thead>
<tr>
<th>Ripening Stage</th>
<th>Control Fruit</th>
<th>Antisense PG Fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Color</td>
<td>Firmness</td>
</tr>
<tr>
<td>MG</td>
<td>-15.3 ± 1.7</td>
<td>348 ± 35</td>
</tr>
<tr>
<td>PK</td>
<td>0.8 ± 2.7</td>
<td>277 ± 44</td>
</tr>
<tr>
<td>RR</td>
<td>20.3 ± 5.7</td>
<td>183 ± 18</td>
</tr>
<tr>
<td>OR</td>
<td>25.9 ± 2.7</td>
<td>73 ± 15</td>
</tr>
</tbody>
</table>

Color was measured as a green to red hue (a* on the L*a*b* scale), and firmness was measured using a penetrometer by measuring force in grams required to insert a probe 2 mm into the pericarp (see “Materials and Methods”). Three fruit were used per ripening stage. Values represent means ± SD of 12 (color) or 9 (firmness) readings.
Figure 1. Polyuronide contents of extracts of cell wall preparations from control (black bars) and antisense PG (striped bars) fruit at four ripening stages. Acetone-insoluble cell walls were extracted twice with 50 mM CDTA followed by H₂O (A) and then twice with 100 mM Na₂CO₃ followed by H₂O (B). After dialysis extracts were assayed for uronic acid content, expressed as milligrams of galacturonic acid equivalents per gram of acetone-insoluble cell wall.

to control fruit. At the MG stage the molecular weight profiles of CDTA-soluble polyuronides from control and antisense PG fruit were virtually indistinguishable. However, whereas in control fruit the CDTA-soluble polyuronides showed a marked depolymerization at the PK stage, this was barely detectable in antisense PG fruit, and the molecular weight profile resembled that at the MG stage. At both the RR and OR stages, CDTA-soluble polyuronides from antisense PG fruit showed depolymerization but less than that in control fruit. Indeed, polyuronides from antisense PG fruit showed depolymerization equivalent to that at the previous ripening stage of controls, suggesting that depolymerization of CDTA-soluble polyuronides in antisense PG fruit occurs but more slowly than in controls.

Polyuronides extracted in the Na₂CO₃ fraction were very different in molecular weight profile from CDTA-extractable polyuronides (Fig. 3). Na₂CO₃-soluble polyuronides were mainly of relatively small size, with a large peak of low-molecular-weight polyuronides near the V₅₀ of the column and a much smaller amount of larger polyuronides extending to the V₅₀ of the column. As ripening progressed there was a limited depolymerization of polyuronides in the major peak near the V₅₀ of the column, plus slightly increasing amounts of very-high-molecular-weight species near the V₅₀ of the column. However, the total amount of polyuronide in the Na₂CO₃-soluble fraction declined during ripening (Fig. 1B). Since equal amounts of uronic acid were loaded on the size-exclusion column, the apparent increase in amount of higher-molecular-weight polyuronides was probably due to a decline in the relative amounts of lower-molecular-weight species with ripening, perhaps because these polymers were lost from this fraction due to increased solubility (Carrington et al., 1993). No effect of suppressed PG activity on polyuronide molecular weight profile was observed in antisense PG fruit relative to controls at the MG, PK, or RR stages (Fig. 3, A–C). In Na₂CO₃ extracts from OR fruit polyuronides from the antisense PG line possessed a larger proportion of species with a higher degree of polymerization than control ex-
Gal/GalUA ratio, particularly in the high-molecular-weight fraction of polyuronides, indicating a loss of Gal from these pectin molecules (Table II). In high-molecular-weight CDTA-extractable polyuronides, the Gal/GalUA ratio declined further in antisense PG fruit than in controls. In Na$_2$CO$_3$-extractable polyuronides the high-molecular-weight polyuronide was associated with a particularly high Gal content, which declined substantially with ripening. In contrast, low-molecular-weight, Na$_2$CO$_3$-extractable polyuronides were associated with a very low ratio of Gal, which changed little with ripening.

No traces of several glycosidase or endo-PG activities were detected in canned tomato pastes (data not shown). Resuspension of these 28°Brix tomato pastes in H$_2$O followed by centrifugation to pellet insoluble particles allowed a serum containing soluble pectins to be decanted. This serum (first H$_2$O extract) contained large amounts of polyuronide (Fig. 4). A second H$_2$O extract removed a much smaller amount of polyuronide, approximately 10 to 20% of that of the first H$_2$O extract. Subsequent extraction of the H$_2$O-insoluble material with CDTA solubilized a further population of polyuronide molecules. In paste prepared from control fruit, the majority (67%) of the total H$_2$O- and CDTA-extractable polyuronide was soluble in the first H$_2$O extract, and only 25% was present in the CDTA-extractable fraction. However, in paste prepared from antisense PG fruit the H$_2$O solubility of extractable polyuronides was markedly lower, and a relatively greater proportion was solubilized by CDTA. The antisense PG paste contained similar quantities (approximately 45%) of polyuronides in the first H$_2$O and in CDTA extracts. Insufficient pectin remained in the insoluble residues of either line to warrant an Na$_2$CO$_3$ extraction. (Subsequent extraction of insoluble residues in 4 M KOH and 0.1% NaBH$_4$ extracted only approximately 5% of the total uronic acid solubilized by the H$_2$O and CDTA extractions combined, data not shown.)

When 1-mg samples of H$_2$O-soluble polyuronides from control and antisense PG pastes were analyzed by size-exclusion chromatography, the polyuronide molecular weight profiles were substantially different (Fig. 5A). The majority of the H$_2$O-soluble polyuronide from control paste was of a relatively low molecular weight and eluted as a large peak at the $V_v$ of the column. In contrast, the majority of the polyuronide from antisense PG paste was of a higher degree of polymerization and greater amounts eluted throughout the separation range of the column. The molecular weight profiles of polyuronides solubilized from control and antisense PG pastes by the second H$_2$O extraction were identical to those removed by the first H$_2$O extraction (data not shown). The molecular weight profiles of CDTA-extractable polyuronides showed two peaks of polyuronide, one of a high molecular weight near the $V_v$ of the column and one of low molecular weight near the $V_i$ of the column (Fig. 5B). In this extract the molecular weight profiles of polyuronides from control and antisense PG pastes were indistinguishable.

The viscosities of tomato paste and serum were strongly influenced by the presence of a transgene suppressing PG expression (Table III). Bostwick analysis, which measures the viscosity of the total paste (the combination of both

![Figure 3](https://plantphysiol.org)
**Table II.** Gal to GalUA ratio in regions of fruit cell wall polyuronide molecular-weight profiles after size-exclusion chromatography

Fruit cell walls were extracted sequentially with the indicated solvents and 1 mg of solubilized polyuronide subjected to gel filtration on a Sepharose CL-2B column (as shown in Figs. 2 and 3). Pooled fractions representing high- and medium-molecular-weight polyuronide from column chromatography, as indicated, were derivatized to alditol acetates and analyzed for neutral sugars by GC. Data are expressed as the molar ratios of Gal (calculated from GC analysis using inositol as an internal standard) to GalUA equivalents (calculated from uronic acid assay against a GalUA standard curve) present in each sample.

<table>
<thead>
<tr>
<th>Extractant</th>
<th>Fractions from CL-2B</th>
<th>Genotype</th>
<th>Ripening Stage</th>
<th>MG</th>
<th>PK</th>
<th>RR</th>
<th>OR</th>
<th>Gal/GalUA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDTA</td>
<td>26-30</td>
<td>Control</td>
<td></td>
<td>0.17</td>
<td>0.14</td>
<td>0.14</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-PG</td>
<td></td>
<td>0.16</td>
<td>0.16</td>
<td>0.11</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50-53</td>
<td>Control</td>
<td></td>
<td>0.09</td>
<td>0.07</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-PG</td>
<td></td>
<td>0.10</td>
<td>0.07</td>
<td>0.06</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>25-31</td>
<td>Control</td>
<td></td>
<td>1.23</td>
<td>0.68</td>
<td>0.87</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-PG</td>
<td></td>
<td>1.06</td>
<td>0.82</td>
<td>0.47</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>62-65</td>
<td>Control</td>
<td></td>
<td>0.05</td>
<td>0.08</td>
<td>0.08</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-PG</td>
<td></td>
<td>0.06</td>
<td>0.05</td>
<td>0.06</td>
<td>0.03</td>
<td></td>
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</tbody>
</table>

H₂O-soluble and H₂O-insoluble components, demonstrated that paste prepared from antisense PG fruit moved a shorter distance in 30 s and, thus, was more viscous than control paste by approximately 18%. In contrast, the viscosity of the serum is due to H₂O-soluble components only, and an increased efflux time indicates greater viscosity. The viscosity of crude, undialyzed serum from antisense PG paste was approximately double that of serum from control paste. Dialysis of the serum to remove small-molecular-weight materials decreased the serum viscosity of both fruit types. Whereas this was presumably mainly due to the dilution involved in dialysis, a contribution of low-molecular-weight solutes, including cell wall oligosaccharides, cannot be ruled out. However, dialysis had no effect on the difference in sample viscosity between fruit genotypes, showing that polymeric materials were mainly responsible for the doubling of viscosity in antisense paste extracts.

**DISCUSSION**

During fruit ripening cell wall pectins are subjected to numerous modifications. Pectins are secreted to the cell wall in a methylesterified form, where they are de-esterified by pectin methylesterase and become available for Ca²⁺-mediated intermolecular cross-linking (Carpita and Gibeaut, 1993). As ripening progresses, unesterified polyuronides are depolymerized (Seymour and Harding, 1987; Smith et al., 1990; Huber, 1992; Huber and O'Donoghue, 1993) and increasing amounts of polyuronide become susceptible to solubilization by chelators (Seymour et al., 1987a; Giovannoni et al., 1989; Smith et al., 1990). In addition, there is a decrease in the content of neutral sugar residues, particularly Gal, associated with polyuronides as side chains (Gross and Wallner, 1979; Gross, 1984).

In tomato at least part of the polyuronide depolymerization and solubilization is due to the activity of PG. PG is
transcriptionally activated close to the beginning of ripening (DellaPenna et al., 1989), and high levels of PG mRNA, protein, and activity accumulate with the ripening process (Brady et al., 1982; Grierson and Tucker, 1983; DellaPenna et al., 1986). In rin fruit, which virtually lack PG activity and in which polyuronide solubilization and depolymerization are very limited, expression of a chimeric PG gene caused both polyuronide solubilization and depolymerization (Giovannoni et al., 1989; DellaPenna et al., 1990). In control, wild-type fruit the amount of chelator-soluble, ionically bound polyuronide increased as ripening progressed, whereas the amount of Na2CO3-soluble, covalently bound polyuronide decreased (Fig. 1). In fruit in which PG activity had been suppressed to less than 1% of wild-type levels by expression of an antisense PG transgene, the amount of polyuronide solubilized by chelator was similar to the wild type in cell walls prepared using phenol/acetic acid/water (Smith et al., 1990) or slightly increased in cell walls prepared using Tris-buffered phenol (Fig. 1). These findings suggest that the low level of PG activity remaining in antisense fruit is sufficient to cause wild-type levels of polyuronide solubilization and that solubilization is not due to a lack of effective PG inactivation in isolated cell wall preparations.

One of the largest changes in the tomato cell wall during ripening is a loss of cell wall Gal, much of which is present in the wall as pectic side chains (Gross, 1984). This loss of Gal occurs in most polymers in the cell wall, based on an analysis of sequential cell wall extracts containing polysaccharides of differing solubility (Gross, 1984; Seymour et al., 1990). The loss of Gal was most marked in high-molecular-weight polymers, and in CDTA-soluble polyuronides appeared to decline more dramatically in antisense PG fruit than in controls. A large decline in the Gal content of a total chelator-soluble polyuronide fraction from antisense PG fruit has been observed previously (Carrington et al., 1993). Why the suppression of PG activity in antisense fruit should be accompanied by an increased loss of pectic Gal is not clear. However, it is possible that the slightly increased chelator solubility of cell wall pectin in antisense PG fruit is related to the relatively greater loss of Gal side chains from these polyuronides.

Chelator-soluble tomato cell wall pectins were of a very high molecular weight in MG fruit, and during ripening a reduction in the amount of the largest-molecular-weight species was accompanied by an increase in the amount of mid-sized polyuronides (Fig. 2). In control fruit the molecular weight profile of polyuronides throughout ripening was very similar to that described for tomato cv Sunny by Huber and O'Donoghue (1993). From a comparison of unripe and ripe control and antisense PG fruit, it was reported that the decline in the average molecular mass of chelator-soluble polyuronides during ripening was largely prevented in antisense PG fruit (Smith et al., 1990). Examination of several ripening stages (Fig. 2) shows that depolymerization of chelator-soluble polyuronides still occurs in antisense PG fruit but is delayed relative to controls. The inhibition of pectin breakdown in antisense PG fruit was relatively modest, and substantial molecular weight shifts occurred in both wild-type and antisense lines. It appears that less than 1% of wild-type levels of PG activity is sufficient to cause substantial polyuronide depolymerization. However, PG is not the only enzyme causing depolymerization of cell wall pectins, which become de-esterified and lose Gal and Ara side chains during development and ripening (Gross, 1984; Carpita and Gibeaut, 1993) and may also be acted on by other enzymes.

The molecular weight profiles of polyuronides in the Na2CO3-soluble fraction (Fig. 3) were very different from those of polyuronides in the chelator-soluble fraction. Na2CO3-soluble polyuronides chromatographed with a much lower apparent molecular weight than chelator-soluble polyuronides, but this may be due to bulk de-esterification and base-catalyzed depolymerization (or cleavage of ester cross-links between pectin chains) of these polyuronides by the Na2CO3 treatment (Cheng and Huber, 1996). During ripening depolymerization of polyuronides in this extract was very limited, and no difference between control and antisense PG fruit was noted before the OR stage. At the OR stage the molecular weight profile showed that the abundance of higher-molecular-weight polyuronides was greater than in controls. Thus, the effects of the suppression of PG activity in antisense fruit is most evident in chelator-soluble polyuronides up to the RR stage of ripening and in Na2CO3-soluble polyuronides at the OR stage. These reductions in polyuronide depolymerization may be responsible for the slightly enhanced firmness of transgenic antisense PG fruit later in ripening (Table 1), as has been noted earlier (Kramer et al., 1992; Carrington et al., 1993; Langley et al., 1994; Fenwick et al., 1996).

Whereas the extractability of polyuronides from fruit cell walls was little affected by the suppression of PG activity, this was not the case in processed fruit paste (Fig. 4). In controls most of the H2O- or chelator-soluble polyuronide was solubilized by a single H2O extraction. Size-exclusion analysis showed that the majority of this polyuronide was highly depolymerized and eluted as a predominant peak of low molecular weight at the V0 of the column (Fig. 5A). The molecular weight profile of these polyuronides resembled that found in isolated fruit cell wall preparations in which PG activity was not inactivated and in which autolytic polyuronide depolymerization has been shown to greatly exceed that occurring in

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bostwick</th>
<th>Serum Viscosity (efflux time)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>cm in 30 s</td>
<td>Undialyzed</td>
</tr>
<tr>
<td>Control</td>
<td>4.4 ± 0.1</td>
<td>197 ± 1</td>
</tr>
<tr>
<td>Antisense PG</td>
<td>3.6 ± 0.1</td>
<td>408 ± 3</td>
</tr>
</tbody>
</table>

Table III. Physical properties of tomato pastes derived from control and antisense PG fruit

Total paste viscosity was measured by Bostwick analysis at 12°Brix. Serum viscosity was measured using viscometry with undialyzed serum at 9.6°Brix and with dialyzed serum at 0°Brix adjusted to 1.3 mg of protein mL−1. Note that, because of dilution during dialysis, viscosities of undialized and dialyzed serum samples cannot be directly compared. Values are the means ± SD of four determinations.
the fruit during normal ripening (Seymour et al., 1987a; Huber, 1992; Huber and O’Donoghue, 1993). In contrast, in antisense PG paste a much smaller proportion of polyuronide was extracted by H$_2$O and a greater proportion of the polyuronide required chelator for extraction. The H$_2$O-soluble polyuronide from antisense PG paste retained a greater range of polydispersity and contained much more high-molecular-weight material than controls. The effect of the virtual lack of PG activity in antisense PG fruit homogenates and the consequent retention of a high degree of polymerization of the polyuronides was to cause an approximate doubling of serum viscosity (Kramer et al., 1992; Table III) and an increase in total paste viscosity (Table III).

The first extraction of control or antisense-PG RR fruit cell walls (with chelator, Fig. 2C) or of antisense-PG fruit paste (with H$_2$O, Fig. 5A) extracted a disperse population of polyuronides that were of a relatively high molecular weight. In contrast, the first extraction of control fruit paste with H$_2$O extracted a much less diverse population of polyuronides containing a large amount of highly depolymerized molecules. Since the most easily extractable polyuronide from fruit cell walls was of high molecular weight, the depolymerization of this polyuronide in paste must have occurred during paste manufacture. Although a typical hot break was used as the first step in paste production, it would seem that this step was inadequate in break temperature, holding time, or temperature homogeneity to inactivate all the PG activity present in ripe fruit. Relative to other species tomato fruit accumulate large amounts of PG activity (Hobson, 1962), which in its PG1 form is quite thermostable (Moore and Bennett, 1994) and difficult to inactivate in tomato pulp by temperature treatments (Luh and Daoud, 1971).

Antisense PG fruit paste, in which PG activity was reduced to less than 1% of that of wild type, did not show this dramatic degradation and increased solubility of the serum polyuronide and consequently possessed enhanced viscosity properties. In laboratory rather than pilot plant experiments, the difference in paste viscosity between control and antisense PG genotypes was observed using a raw cold break but not when using a microwave hot break, which presumably effectively inactivated PG in control fruit homogenates and consequently prevented pectin degradation (Schuch et al., 1991). This suggests that the pectin depolymerization responsible for reducing paste viscosity is occurring after homogenization of the fruit and not during normal ripening. These findings confirm that pectin depolymerization in the cell wall is normally limited in some way (Seymour et al., 1987b; Huber and O’Donoghue, 1993), perhaps by a restriction of PG accessibility to its substrate, but additionally shows that this restriction is removed upon tissue homogenization and not just in acetone-insoluble cell wall preparations. Thus, the present investigation emphasizes the importance of rapidly, completely, and irreversibly inactivating PG activity, both in the production of tomato products and in studies on isolated cell walls.

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**LITERATURE CITED**


Huber DJ, O’Donoghue EM (1993) Polyuronides in avocado (Persea americana) and tomato (Lycopersicon esculentum) fruits exhibit...
markedly different patterns of molecular weight downshifts during ripening. Plant Physiol 102: 473-480


Seymour GB, Colquhoun IJ, DuPont MS, Parsley KR, Selvendran RR (1990) Composition and structural features of cell wall polysaccharides from tomato fruits. Phytochemistry 29: 725–731


