Autolysis of cell walls from polygalacturonase-antisense tomato fruit in simulated apoplastic solutions

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A B S T R A C T

Autolysis of cell walls from polygalacturonase (PG)-antisense tomato fruit was studied in a conventional buffer designed to maximize the catalytic activity of PG (30 mM sodium acetate, 150 mM NaCl, pH 4.5), and in solutions mimicking the pH and mineral composition of the fruit apoplast at the mature-green and ripe stages. Autolytic release of uronic acids was very limited under simulated apoplastic conditions compared with the conventional buffer, but minimal differences in the release of reducing groups were observed among the incubation conditions. Autolytic release of uronic acids from active walls was lower than solubilization from enzymically inactive walls. Uronic acids that remained ionic bound to the cell walls during autolysis were subsequently extracted and analyzed by size exclusion chromatography. The elution profiles of ionic bound uronic acids from cell walls incubated under optimal conditions were similar for all ripening stages. In solutions mimicking the pH and mineral composition of the apoplast of mature-green and ripe fruit, uronic acids extracted from pink and ripe fruit cell walls showed a decrease in average molecular mass compared with polymers from mature-green cell walls. The results suggest that the composition of the incubation solution exert strong influence on PG-independent cell wall autolysis and that enzymically active walls restrain PG-independent pectin solubilization.

1. Introduction

Cell wall disassembly is a major determinant of ripening-related textural changes in fleshy fruit. The pectic matrix of ripening fruit undergoes a number of structural changes that lead to pectin solubilization [1] and affect the organ’s final texture. A close correlation between pectin solubilization and the development of a melting texture has been observed among fruit types [2]. Disassembly of the pectic network starts with the hydrolytic removal of neutral sugars from rhamnogalacturonan-I side-chains, leading to pectin solubilization [3,4], and facilitating cell wall hydration. In some fruits, solubilized pectins are subsequently [4–6] depolymerized by endopolygalacturonase (PG, EC 3.2.1.15). This general model, however, is still uncertain.

Assessment of pectin solubilization in ripening fruit is largely based on isolated cell wall materials. Few attempts have been made to assess pectin solubility in vivo or using methodologies that reduce disturbance of the apoplastic environment. Recent approaches to the problem of the solubility status of pectins in the fruit apoplast have included the examination of a pressure-extracted fluid of putative apoplastic origin and the analysis of pectins effluxed from pericarp disks [7]. Results from both approaches suggest that pectin solubility in vivo increases only slightly during ripening and is largely independent on endogenous PG levels [7].

The mineral composition, osmolality, and pH of a pressure-extracted fluid of apoplastic origin have been characterized in tomato fruit [8]. The apoplastic solution of mature-green fruit has a pH near 6.7, 13 mM of K⁺, 12 mM of Mg²⁺, and 4 mM of Ca^{2+}. Apoplastic pH decreases during ripening to 4.4 and K⁺ levels increased to 37 mM in ripe fruit, whereas the levels of divalent cations remain relatively constant during ripening. The conditions prevalent in the apoplast are, therefore, very different from the high ionic strength, calcium-free buffers in which the activity of cell wall hydrolases is typically assayed. For example, PG activity is usually determined in sodium acetate, pH 4.5 containing 150–250 mM NaCl [9].

When cell walls isolated from mature-green tomato fruit are incubated at pH 4.5 the release of pectins is consistently lower in
enzymatically active cell walls than in inactive walls [10]. This effect is much amplified in the presence of added purified PG but is also observed when no PG is added to the cell walls. Although this can be largely explained by the presence of pectinmethyl esterase (PME, EC 3.1.1.11) in active walls [10], the effect of other enzymic activities is not excluded. Non-enzymatic mechanisms for pectin solubilization documented in vegetative tissues [11,12] may also play a role in ripening fruit.

Pectin dissolution and hydrolysis are independent biochemical processes and likely independent in the physiological environment of ripening fruit tissues. Despite the fact that PG activity, under the appropriate catalytic conditions, can have a strong effect of pectin solubilization, the process can occur independently of PG action. Increases in pectin solubility during fruit ripening can occur in the absence of significant PG activity [13–15] and PG can hydrolyze insoluble pectins without concurrent dissolution [10].

Autolysis of cell walls from PG-antisense fruit provides a means for studying PG-independent pectin catabolism during ripening. The aims of this study were to examine the effect of solutions mimicking the mineral composition of tomato fruit apoplast on PG-independent pectin dissolution. Enzymically active and inactive cell walls were used to help distinguish the chemical effects of the incubating solutions on pectin dissolution from those exerted via modulation of endogenous catalytic activity.

2. Results

2.1. Pectin solubilization

The amount of total pectins in the cell wall preparations remained constant during ripening (336 μg g⁻¹) and was not significantly affected by the cell wall isolation protocol (Table 1). The release of uronic acids from cell walls was affected by the ripening stage, the cell wall isolation protocol, and the incubation conditions (Fig. 1). The amount of uronic acid released from cell walls decreased as ripening progressed and was consistently higher in active cell walls than in enzymically active walls. In contrast, the release of reducing groups was consistently higher in active cell walls (Fig. 2).

The incubation solution had a strong influence on pectin solubilization (Fig. 1). Yields of uronic acids from inactive cell walls incubated in the conventional catalysis buffer decreased from 9.9% (33 μg mg⁻¹) of total cell wall uronic acids at the mature-green stage to 6.8% (21 μg mg⁻¹) at the ripe stage (Fig. 1A). In active cell walls incubated under the same conditions yields were slightly lower, 5.7 and 2.3% (19 and 9 μg mg⁻¹) at the mature-green and ripe stages, respectively (Fig. 1A). The simulated apoplastic solutions reduced the autolytic release of uronic acids compared with that obtained in the conventional buffer. Active walls from mature-green, pink, and ripe fruit incubated in a solution simulating the apoplast of mature-green fruit (MG-solution) released negligible amounts of uronic acids, whereas inactive walls yielded 6.2 and 3.3 μg mg⁻¹ of uronic acids at the mature-green and ripe stages, respectively (Fig. 1B). The solution with the apoplastic composition found in ripe fruit (R-solution) enhanced the autolytic release of uronic acids from active walls at all ripening stages (Fig. 1C) as compared with that obtained in the MG-solution. In contrast, inactive cell walls from mature-green fruit released similar quantities of uronic acids in the MG- and R-solution, but at the pink and ripe stages uronic acid release was 20% higher in the R-solution than in MG-solution (Fig. 1B and C).

The release of reducing groups from active cell walls was consistently higher than that of inactive walls (Fig. 2). The release of reducing groups into the conventional buffer was lower than that of uronic acids and was similar at the mature-green and ripe stages (Fig. 2A). The release of reducing groups in the MG-solution remained very low and constant during ripening at 2.5 and 1.6 μg mg⁻¹, for active and inactive cell walls, respectively (Fig. 2B). The release of reducing groups from inactive walls incubated in R-solution was 1.2 and 1.8 μg mg⁻¹, at the mature-green and ripe stages, respectively (Fig. 2C).

2.2. Recovery and molecular mass distribution of CDTA-soluble pectins

After autolytic reactions, the cell walls were heat-inactivated in refluxing ethanol and extracted in 50 mM CDTA, 50 mM Na-acetate,
pH 6.5. The levels of uronic acids extracted by the chelator were affected by the cell wall preparation protocol, fruit ripening stage, and incubation solution (Fig. 3). Inactive walls generally yielded higher amounts of CDTA-soluble uronic acids than did active walls, independently of the incubation solution (Fig. 3). The yield of uronic acids was higher in mature-green than in ripe fruit, the difference being more pronounced in the simulated apoplastic solutions than in the conventional buffer (Fig. 3).

The molecular size distribution of pectins extracted by CDTA following incubation was examined by size exclusion chromatography on Sepharose CL-4B. CDTA-soluble pectins represented 5–7% of total uronic acid from active walls and 22–26% of total uronic acid from inactive cell walls incubated in the conventional buffer (Fig. 3A). Despite the differences in recoveries of uronic acid, CDTA-soluble uronic acid extracted from active and inactive walls showed similar elution profiles at all ripening stages (Fig. 4). CDTA-soluble uronic acids from mature-green fruit cell walls incubated in MG-solution represented 14 (active) or 26% (inactive) of total cell wall uronic acid (Fig. 3B). The high molecular mass fraction eluting in the void volume (Fig. 5A) was progressively reduced during ripening, more so in active than in inactive walls (Fig. 5B and C).

CDTA-soluble uronic acid from cell walls incubated in the R-solution accounted for 11–35% of total wall uronic acid (Fig. 3C) and also showed a reduction in average molecular mass during ripening (Fig. 6).

3. Discussion

PG-mediated solubilization and depolymerization of pectins are major aspects of cell wall metabolism in the pericarp of ripening tomato. However, PG-independent changes in the pectic matrix may affect the solubility of the polymers. In the present study, transgenic fruit with downregulated PG expression were employed to examine the influence of incubation conditions on PG-independent pectin solubilization from cell walls.

The autolytic release of uronic acid from enzymically active tomato cell walls in 40 mM sodium acetate, 150 mM NaCl, pH 4.5 is minimal at the mature-green to the turning stages and increases dramatically in ripe fruit [16], concomitantly with PG accumulation. Adding purified PG to active cell walls from mature-green tomato incubated in a buffer of similar composition resulted in a 77-fold increase in pectin solubilization [10]. Taken together, these results...
indicate that, when present, PG has a strong effect on pectin solubilization from tomato fruit cell walls. The present study, using active walls from PG-antisense fruit, highlights the PG-independent aspects of pectin solubilization.

In active cell walls incubated in the high ionic strength conventional buffer (30 mM sodium acetate, 150 mM NaCl, pH 4.5), uronic acid solubilization decreased from 5.7% (19 µg mg⁻¹) at the mature-green stage to 2.3% (9 µg mg⁻¹) of total wall uronic acid at the ripe stage (Fig. 1A), indicating that wall-associated PG activity was negligible, and consistent with previous studies using the same line [7,17]. These results complement studies with heat-inactivated cell walls [18] suggesting that PG is required for significant release of uronic acid from isolated tomato fruit cell walls. The comparison between enzymically active and inactive cell walls incubated in the same buffer (Fig. 1) also suggests that the combined effect of enzymic activities present in PG-antisense cell walls actually results in reduced pectin solubilization. Hydrolytic activity, however, and despite the very low levels observed, is higher in active cell walls (Fig. 2). The higher hydrolytic activity of active cell walls is likely explained by the effect of exopolygalacturonase (EC 3.2.1.67) and possibly other exoglycanases. Pectate lyase (EC 4.2.2.2), possibly present in tomato fruit [19], does not seem to affect pectin solubilization or depolymerization under these catalytic conditions. Since PME activity is very low at pH 4.5 [20] it is not clear if this enzyme activity can account for the reduced pectin solubility [10].

Pectin solubilization was significantly reduced in simulated apoplastic solutions in comparison with sodium acetate with 150 mM NaCl (Fig. 1). The lower osmolality and the presence of Ca²⁺ (5 mM) in the simulated apoplastic solutions may account for the reduced release of uronic acid. Previous studies have shown that the autolytic release of pectins from pink tomato cell walls is reduced by Ca²⁺ and under low ionic strength conditions [16,21].

Reducing groups from hexuronic acids solubilized from active cell walls in the three incubation conditions showed little variation during ripening (Fig. 2). CDTA-soluble pectins, however, showed molecular mass downshifts in active cell walls from pink and ripe fruit incubated under simulated apoplastic conditions (Figs. 5 and 6). This downshift was not evident in cell walls incubated in the conventional buffer (Fig. 4). Consistent with this observation, chelator-soluble uronic acid from PG-antisense fruit show minimal depolymerization during ripening compared to nontransgenic controls [22].

The composition of the incubation solution exerted a strong influence on autolytic uronic acid release (Fig. 1) and on the subsequent extraction of pectins in CDTA (Fig. 3). The total release of uronic acids during incubation and upon subsequent extraction with CDTA varied widely: it was only 5% of the total uronic acids present in cell walls of pink fruit incubated in MG-solution but
amounted for 37% of the total in cell walls from pink fruit incubated in R-solution (Figs. 1 and 3). The mechanism behind these differences in unknown but preexisting ripening-related changes in the cell wall materials seems to affect their behavior in the incubation solutions. The molecular size distribution of CDTA-soluble uronic acids was also highly dependent on the previous incubation conditions (Figs. 4–6). The pectic matrix is currently envisioned as continuous macromolecular network having specific domains of homogalacturonan, rhamogalacturonan I and rhamogalacturonan II [23,24]. How these domains are organized in vivo is still unknown [24] but noncovalent forces susceptible to dissociation by weak saline solutions (e.g. 50 mM NaCl) have been shown to play a role in the aggregation of pectic polymers in isolated cell wall materials [25]. The differences in solubility (Figs. 1 and 3) and molecular mass distribution (Figs. 4–6) of uronic acid solubilized from inactive cell walls in different solutions suggest a nonenzymic basis for the solubilization observed in this study. Active walls from fruit at all maturity stages showed lower uronic acid solubilization than inactive walls during incubation in each of the three incubation conditions (Fig. 1), possibly due to deesterification by wall-associated PME and increased uronic acid aggregation through calcium bridges; however, active walls also showed reduced levels of CDTA-soluble uronic acid (Fig. 3), indicating that the phenol treatment to inactivate endogenous enzymes may alter the interactions among cell wall polymers.

The mechanisms underlying pectin metabolism in ripening fruit remain unclear and nonenzymic processes cannot be excluded. Levels of soluble uronic acids actually present in the apoplast of tomato fruit are not strongly affected by endogenous PG [7] and PG-mediated solubilization of uronic acids from isolated cell walls is restricted under simulated apoplastic conditions as compared with PG activity in buffers with high ionic strength [10]. The results presented here show that the pH and mineral composition of the incubation solution exert strong influence on PG-independent cell wall disassembly and suggest that nonenzymic mechanisms contribute to the solubilization of uronic acids from inactive cell walls, possibly related to conformational changes in pectic polysaccharides that occur during cell wall isolation [26] and are sensitive to the equilibrium of inorganic ions [27].

4. Materials and methods

4.1. Plant material

Cell walls were isolated from the pericarp of tomato (Solanum lycopersicum L.) fruit containing an antisense construct for PG (Calgene, CA, USA). The fruit were harvested at the mature-green stage, surface sterilized with 2 mM NaOCl, rinsed, air-dried, stored at 15 °C and allowed to ripen. Fruit were sampled during storage at mature-green, pink and red ripe stages [28]. Outer pericarp sections were excised from fruit at these ripening stages and stored at −30 °C until processed.

4.2. Cell wall preparation and total pectin determination

Frozen pericarp tissue was homogenized in cold 80% ethanol for two periods of 1 min using a Kinematica homogenizer (CH–6010, Luzern, Switzerland). The homogenates were stored overnight at −20 °C to facilitate polysaccharide precipitation, filtered through Miracloth (Calbiochem Corporation, La Jolla, CA, USA), and subsequently processed by two different methods, as described [10]. Briefly, enzymically inactive cell walls were obtained by treating the solid residue with Tris-buffered phenol for 1 h at room temperature. After filtration, the residue was suspended in chloroform:methanol (1:1, v/v) for 30 min., filtered through Miracloth, and the residue was washed sequentially with acetone, 80% ethanol, 40 mM HEPES at pH 7.0, distilled water, 80% ethanol, and acetone. The final residue was dried overnight at 40 °C and stored in a dessicator at room temperature. Enzymically active walls were prepared in a similar fashion but the phenol and chloroform:methanol treatments were omitted. Total uronic acids in the cell wall preparations were determined as described [29].

4.3. Autolysis experiments

Autolysis experiments were performed in three solutions with the compositions described below. Cell walls (20 mg) were hydrated in 16 mL of solution in an ice bath for 30 min and subsequently transferred to a water bath for incubation at 34 °C for 2 h.

Solutions simulating the pH and mineral composition of the apoplast of mature-green (MG—solution) and ripe (R—solution) fruit, as previously determined [8], were prepared as described [10]. MG-solution consisted of 5 mM CaCl$_2$, 9 mM MgCl$_2$, 6H$_2$O, 11.5 mM KCl, 6.5 mM NaOH, 1.5 mM KH$_2$PO$_4$, pH 6.5, and had an electrical conductivity of 3.1 dS m$^{-1}$. R-solution consisted of 5 mM calcium acetate, 13 mM MgCl$_2$, 6H$_2$O, 27.4 mM potassium acetate, 6.5 mM NaOH, 4.8 mM K$_2$HPO$_4$, pH 4.5, and 4.4 dS m$^{-1}$. Incubations were also performed in 30 mM sodium acetate, 150 mM NaCl, pH 4.5, referred to as conventional buffer.

Fig. 6. Gel filtration profiles of ionically bound polyuronides from enzymically active (open circles) and inactive (close circles) cell walls from mature-green (A), pink (B) and ripe (C) fruit incubated in a solution simulating the apoplastic conditions of ripe fruit. Tick marks at the top represent the void (left) and total (right) volumes.
4.4. Extraction and chromatography of pectins following autolysis

After autolytic reactions, the suspensions were transferred to an ice bath and filtered through a Whatman GF/C filter. The filtrates were assayed for total uronic acids [30] and reducing groups from hexuronic acids [31].

After filtration, the cell wall residues were suspended in 15 mL 95% ethanol, refluxed for 20 min to inactivate enzymes, and stored at −20 °C until extraction of pectins. Cell wall suspensions were centrifuged at 2000 g for 20 min, the supernatant discarded, and the pellet suspended in 7 mL 50 mM sodium acetate, 50 mM trans-cyclohexane-1,2-diaminetetraacetate (CDTA), pH 6.5 and incubated for 6 h at room temperature (ca. 22 °C) in an alternative shaker. The suspension was filtered (Whatman GF/C) and the filtrate assayed for total uronic acids [30]. CDTA-soluble pectins were separated by size exclusion through a Sepharose CL-4B column (29 cm long, 1.5 cm diameter) operated with a mobile phase of 200 mM ammonia acetate, pH 5.0 [32].

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References