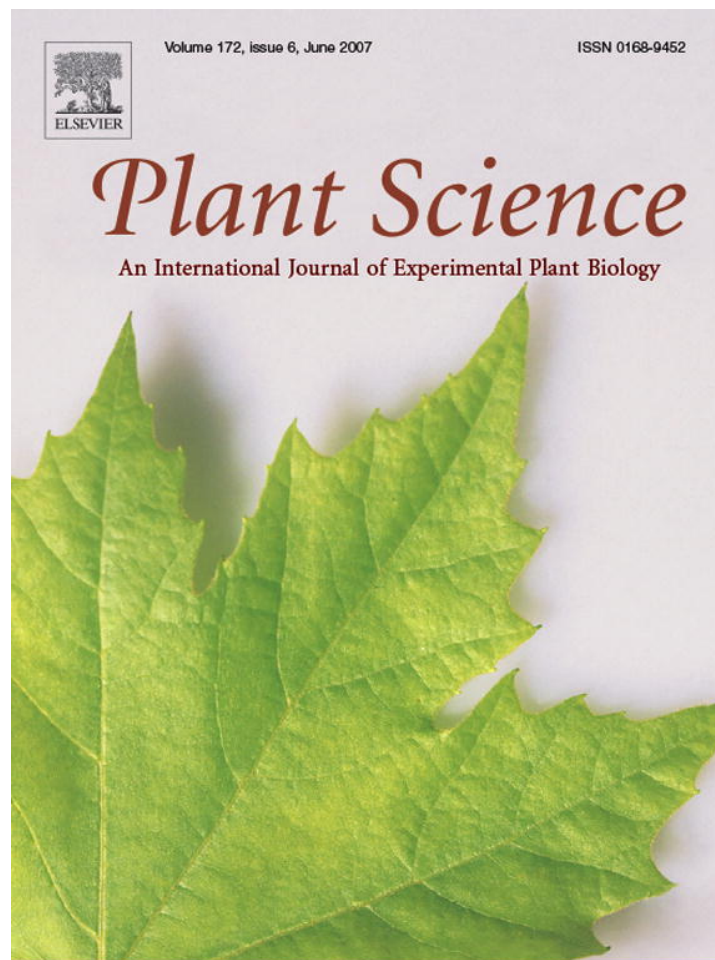


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# Polygalacturonase-mediated dissolution and depolymerization of pectins in solutions mimicking the pH and mineral composition of tomato fruit apoplast

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Received 21 December 2006; received in revised form 14 March 2007; accepted 15 March 2007

Available online 24 March 2007

## Abstract

The effects of polygalacturonase (PG) on pectin dissolution and depolymerization were examined in cell walls from mature-green tomato fruit incubated in a conventional (C) buffer (30 mM sodium acetate, 150 mM NaCl, pH 4.5) and in buffers mimicking the apoplastic solution of mature-green (MG) and ripe fruit (R). Pectin dissolution from cell walls was much higher in C-buffer than in MG- or R-buffers. Buffered phenol inactivated cell walls incubated in C-buffer released  $4.9 \mu\text{g mg}^{-1}$  pectin, which increased to  $86.4 \mu\text{g mg}^{-1}$  when PG was added. In the R-buffer, PG increased the pectin dissolution from inactive cell walls from 0.5 to  $18.3 \mu\text{g mg}^{-1}$ . However, when the assay was conducted in buffer mimicking mature-green fruits, added PG did not increase pectin dissolution. The release of uronic acids from active cell walls in C-buffer and R-buffer was consistently lower than that from inactive walls due to the activity of pectinmethylesterase. Gel filtration profiles of CDTA-soluble pectins extracted from cell walls previously incubated in C-buffer or R-buffer with PG reveal that the enzyme is capable of hydrolyzing insoluble, ionically bound, pectins. These data support the idea that pH and mineral composition of the fruit apoplast provide a means for biochemical regulation of cell wall metabolism.

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**Keywords:** Calcium; Cell wall; *Lycopersicon esculentum*; Pectinmethylesterase; Polyuronides; Polygalacturonase

## 1. Introduction

Cell wall disassembly during fruit ripening proceeds through a number of sequential although partially overlapping steps, involving pectins and hemicelluloses [1,2]. Ripening-related changes in the pectic matrix include loss of neutral sugars, demethylation, increased solubility and decreased molecular mass [3]. These biochemical modifications result in the dissolution of the middle lamella, cell wall swelling and increased porosity.

Endopolygalacturonase (PG, EC 3.2.1.15) is the predominant pectin depolymerase in ripening tomato fruit. Although

PG is neither necessary nor sufficient to bring about fruit softening [4], PG-mediated pectin dissolution and depolymerization are important events in the latter stages of ripening [1], and impact the rheological properties of fruit purees [5]. Despite extensive biochemical and molecular characterization [6–8], a number of issues related with the action of PG under physiological conditions remain unclear. As pointed out by Fry [9], enzyme activity in vitro does not necessarily translate into enzyme action in vivo.

It has been hypothesized that very low levels of PG are sufficient to assure extensive pectin disassembly [4]. However, evidence has shown that the extent of pectin dissolution in ripening tomato fruit is limited in relation with the catalytic potential of PG present [5,10], but the reason for this discrepancy is unknown. In in vitro reactions, the tomato PG isoform 2 (PG2) shows optimum activity at pH 4.5 and 250–300 mM NaCl [6]. Under these assay conditions, PG2 mediates extensive dissolution and depolymerization of pectin from

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isolated cell walls, producing pectic oligomers with a degree of polymerization lower than 10 [11]. Consistent with more restricted pectin hydrolysis *in vivo*, pectic oligomers were not detected in ethanol-insoluble solids from ripe tomato fruit [12].

Although the apoplastic conditions of ripening fruit are not well characterized, they are likely to be substantially different from the conditions used to assay PG and other cell wall enzymes *in vitro*. Ugalde et al. [13] reported that the apoplastic pH of unripe peach was *ca.* 7 and declined to *ca.* 4.2 in ripe fruit. In apricot, apoplastic pH decreased during ripening from values higher than 6 to *ca.* 3.5 [13]. In tomato, a pH of 6 was measured in the apoplastic solution of immature fruit [14], and a pressure-exuded apoplastic fluid acidifies during ripening from pH of 6.7 at the mature-green stage to 4.4 in ripe fruit [15]. In addition to changes in pH, K<sup>+</sup> levels in the apoplastic fluid increase from 13 to 37 mM during ripening [15], leading to an overall increase in ionic strength. Apoplastic Ca<sup>2+</sup> and Mg<sup>2+</sup> levels remain relatively constant during ripening at about 5 and 9–13 mM, respectively [15].

The apoplastic conditions of ripening fruit could provide a means for regulating the activity of cell wall enzymes and might explain the discrepancies between the catalytic potential expressed *in vitro* and enzyme action *in vivo*, as has been observed for PG [5,16]. While the pH of mature-green tomato fruit is clearly not favorable for PG activity, the pH of ripe fruit is near the optimum values observed *in vitro* [6,15].

In addition to pH, the ionic strength and mineral composition of the apoplast may also regulate the catalytic activity of cell wall enzymes *in vivo*. Whereas PG activity *in vitro* is strongly promoted by ionic strength (NaCl) over the range of 0–250 mM [6], the total concentration of major minerals in tomato fruit apoplastic fluid is comparatively low, ranging from 40 to 70 mM [15]. The increase in apoplastic K<sup>+</sup> levels during ripening [15] should promote PG activity, but the high levels of divalent cations present in the apoplast [15] are sufficient to completely inhibit *in vitro* pectin dissolution from enzymically active cell walls [17]. Since PG is thought to hydrolyze previously dissolved pectins [1], the apoplastic levels of Ca<sup>2+</sup> and Mg<sup>2+</sup> are likely to suppress the catalytic activity of pectin-hydrolyzing enzymes *in vivo*. Similarly, in vegetative tissues, cation levels limit the action of pectinmethylesterase (PME, EC 3.1.1.11) [18].

To contribute to the understanding of apoplastic conditions as modulators of the catalytic activity of cell wall enzymes, we have examined PG-mediated dissolution and depolymerization of pectin in cell walls incubated in solutions mimicking the pH and mineral composition of the apoplast of tomato pericarp at the mature-green and ripe stages of development, in contrast with a conventional catalysis buffer.

## 2. Materials and methods

### 2.1. Plant material

Mature-green tomato (*Lycopersicon esculentum* Mill. cv. Solar Set) fruit were harvested, surface sterilized with 2 mM NaOCl, rinsed, and air-dried. Outer pericarp sections were

excised from mature-green fruit and stored at –30 °C until processed.

### 2.2. Cell wall preparation

Frozen peeled pericarp (50 g) 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 and subsequently processed by three different methods. The three protocols were designed to yield enzymically active walls and two forms of enzymically inactive walls to assure that differences between active and inactive walls were due to endogenous enzymes and not to the inactivation procedure. For the preparation of enzymically inactive walls, the ethanolic homogenates were filtered through Miracloth (Calbiochem Corporation, La Jolla, CA, USA) under aspiration. The solid residue was suspended in 100 mL Tris-buffered phenol for 1 h at room temperature [19]. After filtration, the residue (buffered phenol inactivated walls, BP-CW) was suspended in chloroform:methanol (1:1, v/v) for 30 min. The organic solvents were removed by filtration through Miracloth and the residue was washed sequentially under aspiration with acetone (200 mL), 80% ethanol (200 mL), 40 mM HEPES, pH 7.0 (200 mL), distilled water (200 mL), 80% ethanol (100 mL), and acetone (200 mL). The residue was dried overnight at 40 °C and stored in a desiccator at room temperature. Hot-ethanol inactivated cell walls (EtOH-CW) were prepared by refluxing the ethanolic homogenate in a water bath for 20 min and washing the residue as described above. Enzymically active cell wall material was prepared as described for BP-CW, except that the buffered phenol and chloroform:methanol treatments were omitted.

### 2.3. Total uronic acids and mineral content of isolated cell walls

Total uronic acids in the cell wall preparations were determined by the method of Ahmed and Labavitch [20]. For the determination of cell wall mineral content, 20 mg of cell wall material were ashed in a muffle furnace at 550 °C for 10 h. The residue was suspended in 1 mL of 6 M HCl at room temperature (about 24 °C) for 30 min, after which time the volume was brought to 10 mL with ddH<sub>2</sub>O. Ca<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup> and P were determined by inductively coupled argon plasma spectrometry.

### 2.4. PG2 purification, and conditions for PG2-mediated catalysis of isolated cell walls

PG2 was purified from ripe tomato fruit according to the protocol of Ali and Brady [21]. Cell wall-PG assays were performed using a conventional buffer (30 mM sodium acetate, 150 mM NaCl, pH 4.5; [6]) and solutions mimicking the pH and mineral composition of the apoplast of mature-green and ripe fruit [15]. These reaction conditions are hereafter referred to as conventional (C) buffer, mature-green (MG) buffer, and

ripe (R) buffer, respectively. The MG-buffer consisted of 5 mM  $\text{CaCl}_2$ , 9 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 11.5 mM KCl, 6.5 mM NaOH, 1.5 mM  $\text{KH}_2\text{PO}_4$ , adjusted pH 6.5. The R-buffer consisted of 5 mM  $\text{CaCl}_2$ , 13 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 27.4 mM KCl, 6.5 mM NaOH, 4.8 mM  $\text{K}_2\text{HPO}_4$ , adjusted to pH 4.5. The conductivity of the solutions was measured with a YSI conductance bridge (model 31A) equipped with a model 3403 conductivity cell (Yellow Springs, OH, USA) and found to be 3.1, 4.4, and 11.0  $\text{dS m}^{-1}$ , for the MG-, R- and C-buffers, respectively. Osmolality (Wescor vapor pressure osmometer, Model 5500, Logan, UT, USA) was 125, 170, and 335  $\text{mmol kg}^{-1}$  for the MG-, R- and C-buffers, respectively.

For PG assays, cell walls (20 mg) were hydrated in 16 mL of the incubation solutions in an ice-bath for 30 min. Afterward, the suspensions were provided with 3.8  $\mu\text{g}$  of tomato PG2 (in 20  $\mu\text{L}$  of 5 mM sodium acetate, 20 mM NaCl, pH 6.0) and maintained in a circulating water bath at 34 °C for 2 h. Some treatments were designed to simulate the changes in apoplastic pH and  $[\text{K}^+]$  during ripening. Cell walls were hydrated as described above, treated with 3.8  $\mu\text{g}$  PG2 and 17  $\mu\text{g}$  orange peel PME (Sigma), dissolved in distilled water (120  $\mu\text{L}$  added to reaction mixture), and incubated in the MG-buffer at 34 °C for 2 h. After this incubation period the pH was adjusted to 4.5 with dilute HCl, and  $\text{K}^+$  increased with the addition of potassium acetate to simulate the R-buffer conditions. The incubation was prolonged for an additional 2 h. The pH of the suspensions was measured after the initial cell wall hydration and at the end of the incubation period. pH changes during the pre-catalysis incubation period were minimal or absent. During catalysis with PG2, the pH of the cell wall suspensions remained within 0.1 unit of the post-hydration pH in all incubation media and cell wall preparations. Incubation of cell walls in MG-buffer in presence of added PG and PME was accompanied by the release of  $\text{H}^+$ , causing a drop in pH from 6.3 to 5.7 or to 6.1, in inactive and active cell walls, respectively.

Two biological replicates were used for each treatment and the entire experiment was repeated once with similar results.

### 2.5. Post-catalysis extraction and chromatography of pectins

After the incubation periods, the cell wall suspensions were chilled in an ice-bath and filtered through Whatman GF/C filter paper under aspiration into a tube containing 400  $\mu\text{L}$  of

500 mM Tris, pH 7.5 to arrest PG activity. The filtrates were assayed for total uronic acids [22].

After filtration, the cell wall residues were suspended in 15 mL 95% ethanol, refluxed for 15 min to inactivate residual enzymes. The cell walls were recovered by centrifugation at  $2000 \times g$  for 20 min, the supernatants discarded, and the pellets dried at 34 °C. Approximately 10–15 mg of cell walls recovered after catalysis and heat inactivation were suspended in 7 mL of 50 mM sodium acetate, 50 mM CDTA, pH 6.5 [23] and incubated for 6 h at 24 °C with shaking. The suspension was then filtered (Whatman GF/C filter) and total uronic acids determined in the filtrate. CDTA-soluble pectins were separated by size on 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 [24].

## 3. Results

### 3.1. Uronic acid and mineral content of cell wall preparations

The yield of cell walls was 27.5, 23.2, and 26.5% of pericarp dry weight for enzymically active walls, and the inactive BP-CW and EtOH-CW, respectively. Total uronic acid content averaged 288, 241, and 289  $\mu\text{g mg}^{-1}$  for active cell walls, BP-CW, and EtOH-CW, respectively.  $\text{Na}^+$  and  $\text{Ca}^{2+}$  were the most abundant cations in cell walls prepared by each isolation protocol (Table 1). EtOH-CW retained higher levels of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and P, but lower  $\text{Na}^+$  than the BP-CW and active walls (Table 1).

### 3.2. Effect of catalysis medium on PG-mediated pectin release from cell walls

The release of uronic acids from cell walls of mature-green tomato fruit was strongly influenced by the composition of the catalysis medium (Fig. 1). In the absence of PG, enzymically inactive cell walls incubated in the C-buffer released 4.9 (BP-CW) or 12  $\mu\text{g mg}^{-1}$  of uronic acids (EtOH-CW), whereas active cell walls released only negligible amounts of uronic acids (Fig. 1A). The addition of PG strongly increased the release of uronic acids, to 86.4, 72.3, and 45.7  $\mu\text{g mg}^{-1}$ , for BP-CW, EtOH-CW and active walls, respectively (Fig. 1A).

In contrast to cell walls incubated in C-buffer, the dissolution of uronic acid was lower in the R-buffer and negligible in the MG-buffer. Cell walls incubated in MG-buffer did not release more than 1  $\mu\text{g mg}^{-1}$  independently of added

Table 1  
Mineral content of cell wall preparations from mature-green tomato pericarp

Cell wall preparation	Ca ( $\mu\text{g (mg cell wall)}^{-1}$ )	Mg ( $\mu\text{g (mg cell wall)}^{-1}$ )	K ( $\mu\text{g (mg cell wall)}^{-1}$ )	P ( $\mu\text{g (mg cell wall)}^{-1}$ )	Na ( $\mu\text{g (mg cell wall)}^{-1}$ )
Active <sup>a</sup>	4.58 ± 0.03	1.42 ± 0.01	0.26 ± 0.03	0.38 ± 0.01	9.47 ± 1.02
BP-CW <sup>b</sup>	4.83 ± 0.05	1.21 ± 0.01	0.22 ± 0.02	0.39 ± 0.00	12.81 ± 1.97
EtOH-CW <sup>c</sup>	5.73 ± 0.02	1.77 ± 0.02	0.26 ± 0.03	0.67 ± 0.01	5.80 ± 0.05

Values are means ± S.E. of three observations.

<sup>a</sup> Enzymically active cell walls.

<sup>b</sup> Phenol inactivated cell walls.

<sup>c</sup> Hot-ethanol inactivated cell walls.

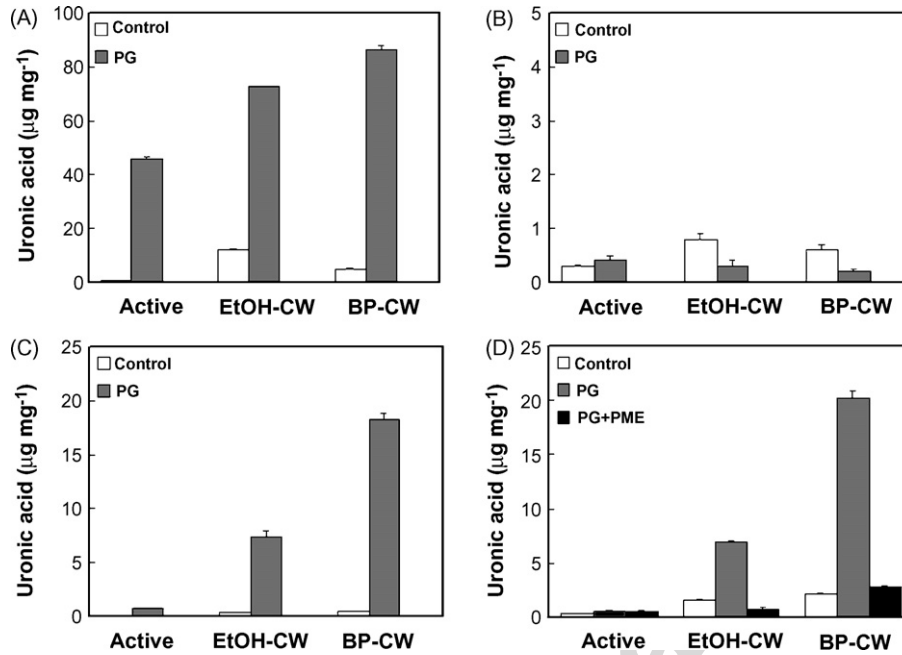


Fig. 1. Uronic acid released from mature-green tomato fruit cell walls incubated in different solutions with and without added enzymes. (A) Incubation in a conventional catalysis buffer (C-buffer) for assaying PG activity consisting on 30 mM NaOAc, 150 mM NaCl, pH 4.5. (B) Incubation in a buffer mimicking the apoplastic pH and mineral composition of mature-green tomato fruit (MG-buffer), with the composition described in Section 2. (C) Incubation in a buffer mimicking the apoplastic pH and mineral composition of ripe tomato fruit (R-buffer), as described in Section 2. (D) Incubation in MG-buffer followed by incubation in R-buffer. Active and inactive (EtOH-CW and BP-CW) cell walls were incubated with and without the addition of purified PG (A–D) and with PG + PME (D). Values are means  $\pm$  S.E. of two observations.

PG (Fig. 1B). The addition of PG to the reaction mixture containing R-buffer strongly increased the release of uronic acids from inactive cell walls but not in active cell walls (Fig. 1C). The amount of uronic acid released from PG-treated active cell walls incubated in R-buffer ( $0.8 \mu\text{g mg}^{-1}$ , Fig. 1C) was similar to the amounts released in the MG-buffer (Fig. 1B).

The lower recovery of soluble uronic acids from PG-treated active walls compared with inactive walls might be caused by increased negative charges generated by PME during the preparation or incubation of active walls, in association with calcium bridges. To test this idea, reactions with tomato PG2, with or without PME, were carried out in MG-buffer for 2 h,

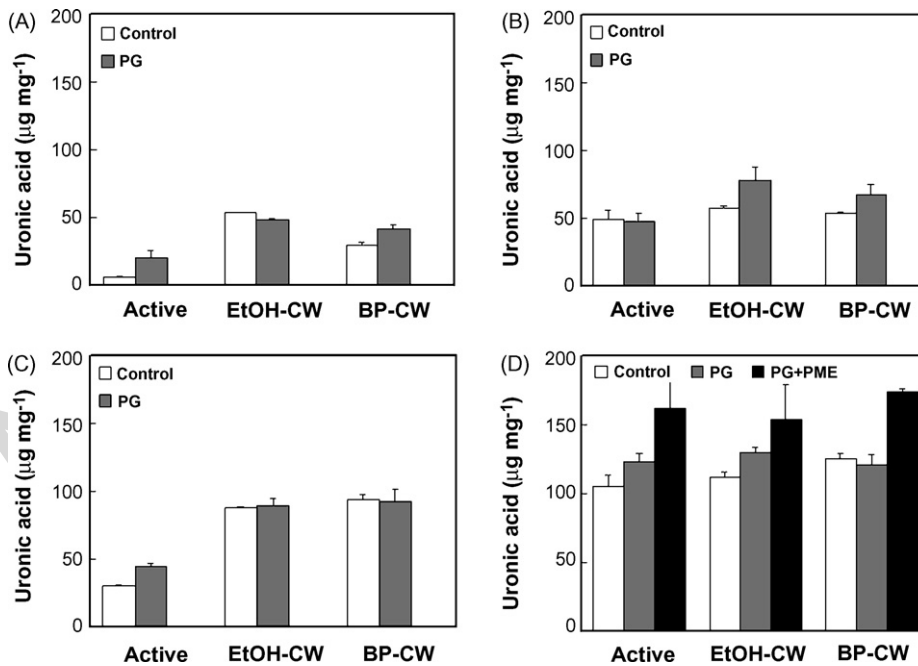


Fig. 2. CDTA-soluble uronic acids extracted from cell walls after incubation in the C-buffer (A), MG-buffer (B), R-buffer (C), and after sequential incubation in MG- and R-buffers (D) with and without added PG (A–D) and with PG + PME (D). Values are means  $\pm$  S.E. of two observations.

after which time the solution pH and  $[K^+]$  were adjusted to those of the R-buffer. As shown in Fig. 1D, PME had no influence on the release of uronic acids from active cell walls but significantly reduced the release of uronates from both phenol (86% inhibition) and hot-ethanol (88% inhibition) inactivated cell walls. The low amounts of uronic acids released by PG in the R-buffer in comparison with the C-buffer are likely due to the presence of relatively high levels of  $Ca^{2+}$  (5 mM) in the former solution. In support of this idea, the addition of 5 mM of  $Ca^{2+}$  (as calcium acetate) to the C-buffer reduced the PG-mediated release of uronic acids from BP-CW from 85 to 13  $\mu\text{g mg}^{-1}$  (not shown), a value similar to the recoveries from walls treated with PG in the R-buffer (18  $\mu\text{g mg}^{-1}$ ).

### 3.3. Gel filtration profiles of pectins extracted post-incubation by CDTA

The low yields of soluble pectins under reaction conditions mimicking the apoplastic composition of mature-green and ripe fruit raised the question of whether catalysis was inhibited or whether hydrolysis was occurring without the release of products. To address this question, cell walls were filtered following catalysis, treated with hot-ethanol to inactivate enzymes and subsequently extracted in 50 mM sodium acetate,

50 mM CDTA, pH 6.5. The yields of CDTA-soluble pectins are presented in Fig. 2.

Gel filtration profiles of the CDTA-soluble pectins recovered post-catalysis are shown in Figs. 3–6. Since profiles from the two inactive cell wall preparations were similar, only those from BP-CW are presented. PG induced a downshift in the molecular mass distribution of CDTA-soluble polymers recovered from inactive cell walls incubated in C-buffer (Fig. 3A), but had no effect on the size distribution of polymers recovered from active cell walls incubated in the same buffer (Fig. 3B).

The molecular size distribution of CDTA-soluble pectins from inactive walls incubated in the MG-buffer presented a large peak eluting in the void volume and was unaffected by PG (Fig. 4A). In contrast, the polymers extracted from active cell walls incubated in the same buffer had a lower average molecular mass and were more polydisperse than those extracted from inactive walls (Fig. 4B).

Depolymerization mediated by PG is evident in polymers released from both inactive (Fig. 5A) and active (Fig. 5B) cell walls incubated in R-buffer, although the effect of PG was less extensive in the active walls.

The most extensive degradation of CDTA-soluble polymers was observed when cell walls were incubated sequentially in the MG- and R-buffers. Under these conditions, PG induced a

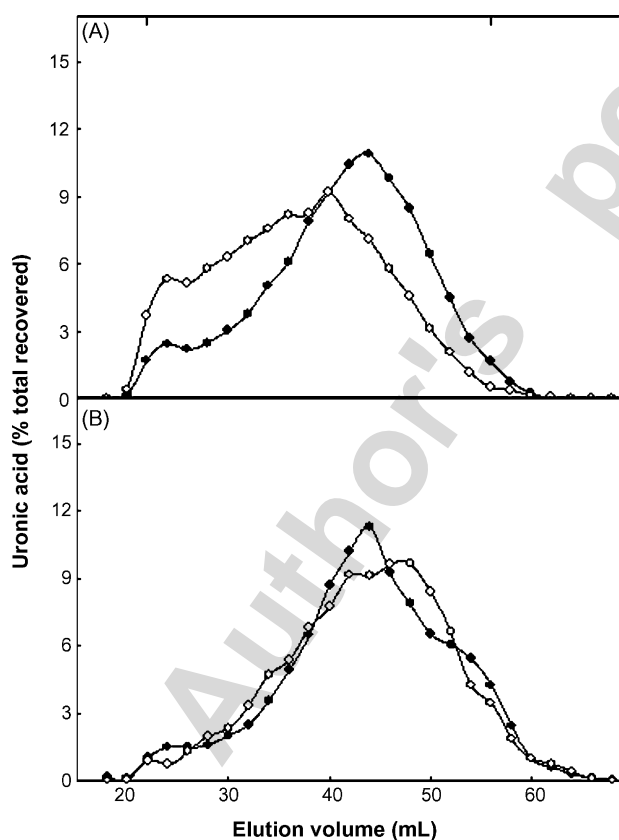


Fig. 3. Molecular size distribution of CDTA-soluble pectins recovered from mature-green cell walls after incubation in C-buffer with (●) or without (○) PG. (A) Buffered phenol-inactivated walls; (B) enzymically active walls. Tick marks at the top of figure represent void (left) and included (right) volumes of the column.

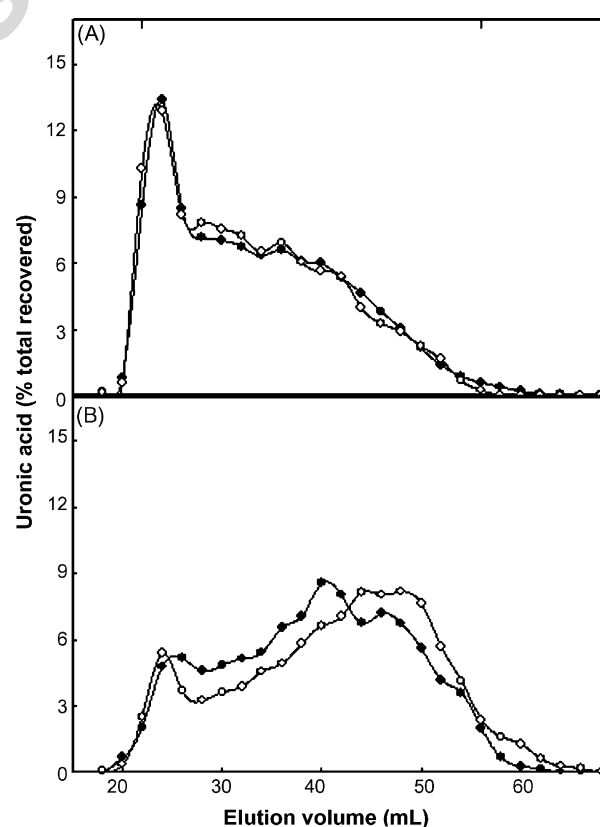


Fig. 4. Molecular size distribution of CDTA-soluble pectins recovered from mature-green cell walls after incubation in MG-buffer with (●) or without (○) PG. (A) Buffered phenol-inactivated walls; (B) enzymically active walls. Tick marks at the top of figure represent void (left) and included (right) volumes of the column.

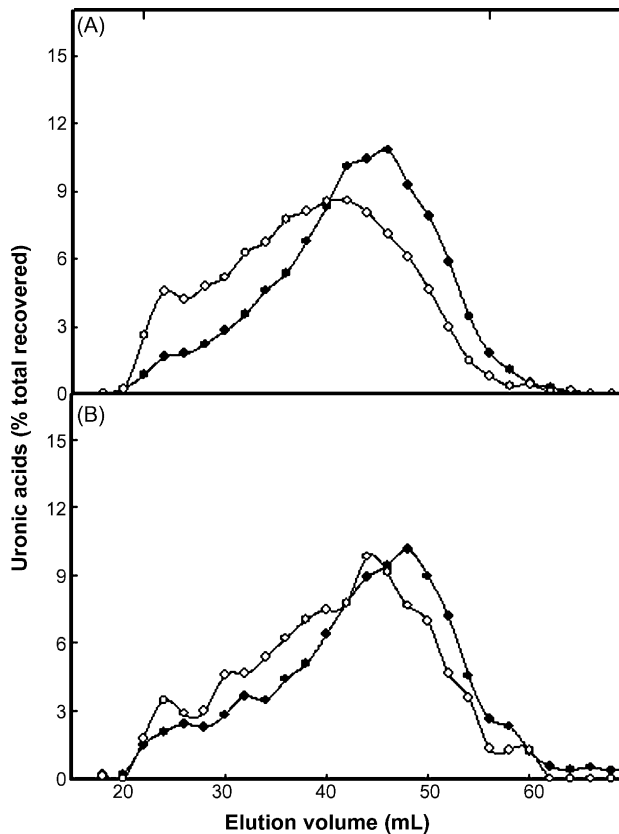


Fig. 5. Molecular size distribution of CDTA-soluble pectins recovered from mature-green cell walls after incubation in R-buffer with (●) or without (○) PG. (A) Buffered phenol-inactivated walls; (B) enzymically active walls. Tick marks at the top of figure represent void (left) and included (right) volumes of the column.

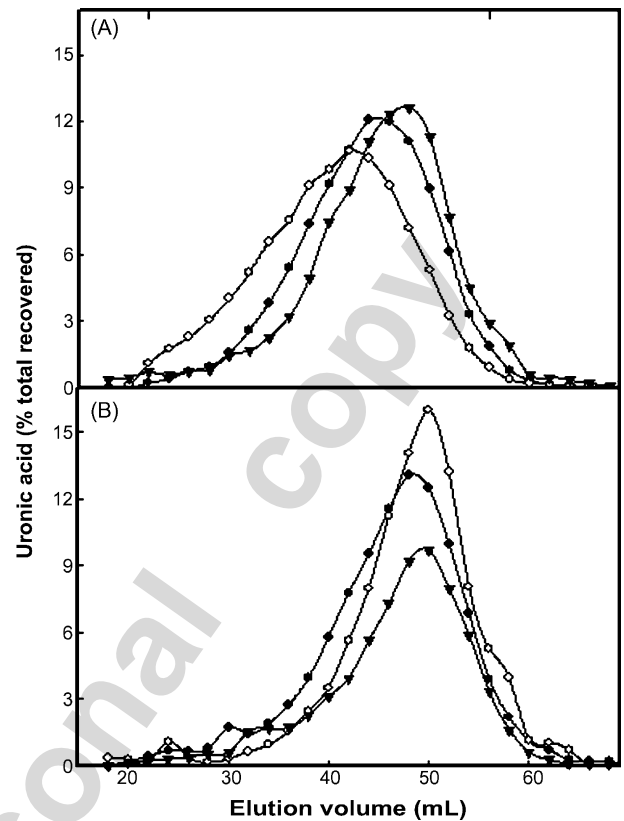


Fig. 6. Molecular size distribution of CDTA-soluble pectins recovered from mature-green cell walls after a sequential incubation in MG- and R-buffers with PG (●), PG and PME (▼) or without added enzymes (○). (A) Buffered phenol-inactivated walls; (B) enzymically active walls. Tick marks at the top of figure represent void (left) and included (right) volumes of the column.

downshift in the molecular size of CDTA-soluble pectins extracted from inactive cell walls, and the combined action of PG and PME decreased even further the average molecular size (Fig. 6A). In contrast, in active cell walls PG and PME did not affect significantly the molecular size distribution of CDTA-soluble pectins (Fig. 6B).

#### 4. Discussion

The present work shows that PG-mediated pectin dissolution is very limited in the conditions anticipated for the tomato fruit apoplast in contrast with the catalytic potential of the enzyme in a buffer optimized for its activity. The restricted release of pectins from mature-green tomato fruit cell walls incubated in solutions mimicking the pH and mineral composition of the tomato fruit apoplast (Fig. 1) can be attributed to a number of factors, namely, the levels of divalent cations, the ionic strength, the mineral composition, and the pH of the solutions.

The levels of  $\text{Ca}^{2+}$  in cell wall preparations (Table 1) account, on a molar basis, for only 8–11.5% of the total uronic acid, since most of the apoplastic calcium is likely lost during cell wall isolation [19]. At the levels present in the apoplast [15] and used in this study (5 mM),  $\text{Ca}^{2+}$  strongly limits the enzymic release of pectins from ripe tomato cell wall incubated under conditions optimized for hydrolysis [17], and certainly

contributes to the restricted pectin release in the simulated apoplastic solutions.

Ionic strength is also a factor in explaining the differences in the catalytic behavior of PG in the conventional buffer and in the simulated apoplastic solution with the same pH (R-buffer). Enzymes with basic *pI*, including PG [21], are tightly bound to the negatively charged cell wall and have restricted mobility [25]. High ionic strength improves enzyme mobility, which can explain the enhancement of PG2 activity with increasing NaCl concentration over the range 0–250 mM [6]. Similarly, the activity of xyloglucan endotransglucosylase (XET, EC 2.4.1.207) from cauliflower is enhanced by increasing the ionic strength to *ca.* 300 mM [26]. Since the osmolality of the apoplastic solution of tomato pericarp, in the range of 65–95  $\text{mmol kg}^{-1}$  for mature-green fruit and 130–140  $\text{mmol kg}^{-1}$  for ripe fruit [15], is much lower than the 335  $\text{mmol kg}^{-1}$  measured in the C-buffer, the apoplastic ionic strength can, *per se*, limit PG action *in vivo*.

In addition to the effects of  $\text{Ca}^{2+}$ , other mineral components play a role on cell wall metabolism.  $\text{K}^+$ , not  $\text{Na}^+$ , is the major monovalent cation present in the apoplast of tomato fruit [14,15] and affects the catalytic behavior of PG. When  $\text{K}^+$  replaces  $\text{Na}^+$  as the major cation, the pH optimum for PG2 activity increases from *ca.* 4.0 to 5.5 [16].

The release of pectin was consistently lower in active than in inactive cell walls (Fig. 1), independently of added PG. The protocols used to inactivate endogenous enzymes affected the mineral content of the cell wall materials (Table 1) and pectin dissolution (Fig. 1). However, the differences in pectin dissolution between the two inactive cell wall preparations are smaller compared with release from active cell walls and can, therefore, be attributed to the activity of cell wall enzymes. The presence in the active cell walls of apoplastic proteins such as the PG  $\beta$ -subunit [27] and polygalacturonase inhibiting proteins [28] could restrict the activity of added PG. However, under the conditions used in this study, the presence of PME in active walls seems to account for the differences in the release of uronic acids from active versus inactive cell walls. In fact, although depolymerization of ionically bound pectins was significantly enhanced by added PME (Fig. 6A), this enzyme inhibited PG-mediated pectin release in reactions performed under apoplastic conditions (Fig. 1D).

These results are consistent with a model in which PME activity, highest at the mature-green stage [29] and active when the apoplastic pH is 6.5–7.0 [30,31], creates during the early ripening stages conditions favorable for subsequent PG activity [32]. PG, however, is inactive at pH 6.0 and above [6] and is unable to hydrolyze pectins under the conditions anticipated for the apoplast of mature-green fruit (Figs. 1B and 4). Under the conditions prevailing in the apoplast of ripe fruit PG increases pectin depolymerization (Fig. 6A), but PG-mediated pectin dissolution is restricted by  $\text{Ca}^{2+}$  binding to negative charges generated by PME (Fig. 1D) [33].

Pectin dissolution and hydrolysis are independent, although often concomitant, events. Although ripening-related pectin dissolution in the absence (or in the presence of low levels) of PG has been documented in a number of situations [34–36], PG is thought to only act upon previously dissolved pectins [1]. However, data presented herein show that PG is capable of hydrolyzing insoluble, ionically bound, pectins from inactive cell walls without concurrent dissolution (Figs. 3 and 5).

The differences in the yields and profiles of CDTA-soluble pectin extracted from inactive walls after incubation in different solutions without added PG (Fig. 2 and panel A in Figs. 3–6) raises the question of whether the solution pH and mineral composition can affect the population of soluble polymers, independently of enzymic activities. A comparison of the yields and profiles of pectins extracted from active versus inactive walls incubated in the same solution without added PG invariably shows that active walls yielded polymers with smaller degrees of polymerization (Figs. 3–6), suggesting that enzymes other than PG might play a role in pectin depolymerization and dissolution.

Discrepancies between in vitro catalytic potential of cell wall enzymes and alterations observed in vivo have been reported for PG [10] and  $\beta$ -galactosidase [37–39] and are probably applicable to other cell wall enzymes assayed under in vitro conditions favoring optimal catalysis. The results presented herein show that PG-mediated pectin dissolution and depolymerization is highly restricted under the conditions anticipated in the apoplast of ripening tomato fruit, and support

the concept that the apoplastic conditions provide a means for biochemical regulation of cell wall metabolism in ripening fruit.

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