Degradation of Isolated Tomato Cell Walls by Purified Polygalacturonase in Vitro

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ABSTRACT

Cell wall preparations from green pericarp of normal and mutant Never-ripe (N) and ripening inhibitor (rin) tomato (Lycopersicon esculentum Mill.) fruit were all equally degraded in vitro by a cell wall-bound protein extract from ripe normal tomatoes.

Similar cell wall-bound protein extracts from ripe N or rin fruit were not as effective and those from ripe rin fruit gave no cell wall degradation at all in vitro. This was correlated with the absence of polygalacturonase in rin and low activity of N extracts.

Purified polygalacturonase was capable of in vitro cell wall degradation and it seems that this enzyme can account for the cell wall degradation observed with the total cell wall-bound protein extracts from ripe fruit.

During tomato fruit ripening, cell walls show a large loss of galactose residues and a smaller loss of arabinose and uronide residues (2). This is accompanied by an increase in water-soluble pectin (10). Protein extracts from ripe tomatoes have been shown to contain several polysaccharide-degrading enzymes, and to solubilize tomato cell wall material in vitro (13). One of these enzymes, polygalacturonase, has been shown to be absent in green fruit and to increase in activity during ripening (3). This increase in activity is due to de novo synthesis of the enzyme (Tucker, Robertson, and Griers, unpublished). Polygalacturonase is thought to play a role in softening since the softening rates of several tomato cultivars can be correlated to their polygalacturonase activity (4).

Several ripening mutants of tomato are available. Two of these are deficient in polygalacturonase activity. The Never-ripe (N) mutant has reduced levels of polygalacturonase activity (5) due to the synthesis of only one out of two naturally occurring isoenzymes (12). This mutant exhibits a correspondingly slow rate of softening (11). The ripening inhibitor (rin) mutant produces no, or very little, detectable polygalacturonase enzyme (12). This mutant does not soften to any great extent (11). However, it has been shown that after about 6 weeks in storage, rin fruit show the characteristic drop in cell wall galactose residues associated with the ripening of normal fruit (2).

The relationship between polygalacturonase activity and softening in various cultivars and mutants suggests that this enzyme has a key role in fruit softening, and thus, cell wall degradation. However, a direct effect of purified plant polygalacturonase on cell walls has not been demonstrated. It is possible that other polysaccharide-degrading enzymes are also important. Also, changes in cell wall structure may play a role in determining softening. For example, it has been demonstrated that green cell walls of various tomato cultivars show marked differences in their susceptibility to degradation by extracts from ripe tomatoes (13).

The results presented here show that purified polygalacturonase does act on a tomato cell wall preparation and can account for the cell wall degradation observed in vitro. The results also show that the cell wall preparation of normal and mutant fruit are equally susceptible to degradation in vitro.

MATERIALS AND METHODS

Plant Material. Tomatoes (Lycopersicon esculentum Mill.) of the varieties Potentate and Ailsa Craig were grown under greenhouse conditions and harvested just prior to use. Near-isogenic lines of the two mutants N and rin have been developed for Potentate and Ailsa Craig at the Glasshouse Crops Research Institute, Littlehampton, United Kingdom. Seeds of these and the parent cultivars were obtained from Mr. L. A. Darby.

Extraction of Cell Wall-Bound Proteins. Total cell wall-bound proteins were obtained from the pericarp of normal, N and rin fruit by homogenization in water, centrifugation, and then elution of cell wall-bound protein from the pellet into 1 M NaCl (pH 6.0) as described previously (12). Polygalacturonase isoenzyme 2 was purified by chromatography on Sephahex DEAE A-50 and G-100 as described previously (12).

Enzyme Assays. Polygalacturonase was measured by following the production of reducing groups from polygalacturonic acid as previously described (12). Activity units are expressed as mmol galacturonic acid formed min⁻¹. Pectinmethylesterase was assayed in 10 ml 50 mM NaCl, 0.5% citrus pectin (pH 8) at 25°C. The pH was monitored constantly over a period of 2 min and held at pH 8 by the addition of 1 m NaOH using a microsyringe. Results are calculated as μeq min⁻¹. β-Galactosidase was measured by following the release of nitrophenol groups from 1-nitrophenol-β-D-galactopyranoside at pH 4.6 (14). Cellulase was measured by following reduction in viscosity of a 1% solution of carboxymethylcellulose (pH 6 [15]). Protein was measured by the method of Lowry et al. (6) using BSA as a standard.

Preparation of Cell Walls. Crude cell wall preparations were obtained from green pericarp of either normal mature green tomatoes or mature green mutant fruit. The pericarp was homogenized in distilled H₂O and centrifuged at 2,400g for 10 min. The pelleted material was washed with 1 M NaCl (pH 6) at 4°C for 3 h to remove cell wall-bound proteins. The suspension was centrifuged for 10 min at 2,400g. This pellet was resuspended in 0.15 M NaCl, 50 mM sodium acetate (pH 4), and stored frozen at -20°C until used.
Assay for Cell Wall Degradation. Cell wall degradation in vitro was followed by measuring the production of reducing groups by the method of Nelson (8) and that of Milner and Avigad (7), the latter being more specific for hexuronic acids. Cell wall substrate was thawed and washed twice in 0.15 M NaCl, 50 mM sodium acetate (pH 4.0). Assays were performed in this buffer at 25°C. At suitable time intervals, after addition of the test enzymes, 1.5-ml samples were taken, centrifuged at 8,000g for 2 min using a Jobling 300 minifuge, and the supernatant fractions assayed for reducing groups. The amount of cell wall material in each assay was determined by dry weight analysis of an aliquot of the cell wall preparation.

RESULTS AND DISCUSSION

Degradation of Normal and Mutant Cell Walls by Wall-Bound Enzymes from Ripe Fruit. Total cell wall-bound proteins were prepared from normal ripe tomatoes as described. This extract was then used to degrade salt-washed green cell wall preparations of normal, Nr and rin fruit (Fig. 1). Cell wall preparations from green fruit did not show any release of reducing groups when incubated alone at 25°C and pH 4.0 for 60 min. However, in the presence of added wall-bound proteins prepared from ripe fruit, there was an increase in the soluble reducing groups indicating cell wall degradation. This increase was similar using normal, Nr, or rin green cell walls, suggesting that degradation in each case was similar. We were unable to identify the exact nature of the reducing groups released; however, in each case, reducing groups were detected by the method of Milner and Avigad (7) which is specific for hexuronic acids.

It is possible that the cell wall structure was altered during the preparation of the cell wall material, e.g. by the removal of the cell wall-bound proteins, and that this masked differences which existed in vivo. However, experiments using cell walls prepared employing only homogenization in water and then centrifugation, i.e. with cell wall proteins in situ, gave results similar to those presented in Figure 1 using normal and rin green pericarp.

These results suggest that the mutants do not have an altered cell wall structure that renders them resistant to attack by polysaccharide-degrading enzymes.

Comparison of Cell Wall Degradation by Wall-Bound Enzymes from Ripe and Mature Mutant Fruit. Salt-washed cell walls prepared from normal green tomatoes were incubated with cell wall-bound enzymes obtained from normal ripe tomatoes and from rin (mature yellow) and Nr (mature orange) mutant fruit. Similar quantities of protein were included in each incubation. The most active enzyme preparation was that obtained from normal ripe tomatoes. Proteins from Nr fruit were much less active. Those from rin fruit showed almost no cell wall-degrading activity at all (Fig. 2). This suggests that specific cell wall-degrading enzymes may be present in low amounts or are absent in the mutants. Cell wall-bound protein extracts from normal and mutant fruit contain in excess of 20 polypeptides when fractionated by gel electrophoresis under denaturing condition (Tucker, Robertson, and Grieron, unpublished). Thus these extracts can be expected to contain several enzyme activities.

Cell wall-bound proteins from normal and mutant fruit were assayed for cellulase, pectinactase, pectinmethylesterase, and -galactosidase activity (Table I). Each extract contained pectinmethylesterase and -galactosidase. Cellulase was not present in any sample presumably because it is not bound to cell walls using our methods. The only enzyme tested that varied in amount in the different extracts was pectinactase, there being 12% of the normal activity in Nr extracts and no activity in rin extracts. When the results in Figure 2 were expressed per unit pectinactase activity present in each incubation the values for normal

![Fig. 1. Cell wall degradation in vitro by total cell wall-bound proteins of ripe normal fruit. Assays were performed at 25°C in 20 ml buffer containing 0.15 M NaCl, 50 mM sodium acetate (pH 4.0), 1.5 mg cell wall-bound protein (1 unit pectinactase activity), and cell wall material from (C) green normal (0.03 g dry wt ml−1), (E) Nr (0.026 dry wt ml−1), and (D) rin (0.028 g dry wt ml−1) fruit, respectively.](image1)

![Fig. 2. Degradation of cell wall material from green normal fruit by various enzyme preparations. Assays were performed at 25°C in 20 ml buffer containing 0.15 M NaCl, 50 mM sodium acetate (pH 4), green cell wall material (0.03 g dry wt ml−1), and enzyme or protein preparation as shown. Total cell wall-bound proteins from (C) ripe normal fruit: 1.5 mg protein (1 unit pectinactase activity), (E) ripe Nr fruit: 1.5 mg protein (0.0045 unit pectinactase activity), (A) ripin fruit: 1.5 mg protein (no pectinactase activity). (A) Purified pectinactase 2: 0.28 mg protein (1 unit pectinactase activity). (C) Total cell wall-bound proteins from ripe (rin) fruit: 1.5 mg protein plus 0.28 mg protein purified pectinactase 2.](image2)

Table 1. Enzyme Content of Total Cell Wall Protein Extracts of Mature Potentate Fruit Pericarp

<table>
<thead>
<tr>
<th>Fruit Type</th>
<th>Cellulase</th>
<th>Pectinmethylesterase</th>
<th>-Galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol galacturonic acid min⁻¹ g⁻¹ fresh wt</td>
<td>μg min⁻¹ g⁻¹ fresh wt</td>
<td>μmol galactose min⁻¹ g⁻¹ fresh wt</td>
</tr>
<tr>
<td>Normal, red</td>
<td>50</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Nr, orange</td>
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<td>21</td>
<td>4</td>
</tr>
<tr>
<td>rin, yellow</td>
<td>0</td>
<td>15</td>
<td>3</td>
</tr>
</tbody>
</table>

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and Nr extracts were the same. This suggests that polygalacturonase plays a major role in cell wall degradation.

**Cell Wall Degradation by Purified Polygalacturonase Isoenzyme 2.** Polygalacturonase isoenzyme 2 was purified from ripe normal tomatoes as described (12) and its ability to degrade green cell wall preparations was compared with the activities of total cell wall-bound proteins from normal, Nr, and rin fruit (Fig. 2).

The purified enzyme was effective in degrading cell wall preparations, and on the basis of reducing groups released per unit of polygalacturonase, it was as efficient as both the extracts from normal ripe tomatoes and mature orange Nr fruit. This suggests that the release of reducing groups brought about by cell wall-bound proteins is due solely to the polygalacturonase activity in the extracts. Further support for this idea was obtained by testing mixtures of purified polygalacturonase 2 and cell wall-bound proteins from mature yellow rin fruit (Fig. 2). Supplemenenting the rin extract with polygalacturonase 2 resulted in a degree of cell wall degradation similar to that obtained with protein extracts from normal ripe fruit. This suggests that the lack of ability of rin extracts to release reducing groups from cell walls is due to the lack of polygalacturonase activity. Furthermore, rin extracts do not appear to contain any factors which enhance or inhibit cell wall degradation by polygalacturonase 2.

**pH Optimum for Cell Wall Degradation.** The optimum pH for the release of reducing groups from salt-washed cell wall preparations from green tomatoes, using wall-bound enzymes, was found to be pH 4.0.

This was routinely used in all our experiments. No reducing groups were released above pH 6.0 (Fig. 3). Total cell wall-bound proteins from rin fruit had no activity at any of the pH values tested. The pH-activity profile for polygalacturonase 2 was also determined, using the artificial substrate polygalacturonic acid (Fig. 3). In this case, the optimum pH was 3.8 and activity ceased above pH 6.0. The similarities in the pH profiles suggest that the major enzyme present in wall-bound enzyme preparations from normal ripe tomatoes which releases reducing groups is polygalacturonase.

**Possible Role of Polygalacturonase in Vivo.** These results show that purified tomato polygalacturonase 2, which is the main isoenzyme form in ripe fruit (12), is capable of degrading tomato fruit cell wall material in vitro. The results also show that polygalacturonase is the major fruit enzyme in cell wall-bound protein extracts responsible for wall degradation. Some structural changes may have occurred during the preparation of the cell walls, however, and some caution should be exercised in drawing conclusions about the in vivo situation. Other enzymes may also play a role in softening, including some that may have been lost in the water-soluble fraction during preparation of wall-bound proteins. Cellulase activity has been reported in ripe fruit (1) and mature rin fruit (9). The absence of cellulase from our preparations (Table I) suggests that this enzyme may have been lost in the soluble fraction.

Nevertheless, our findings and the correlation between polygalacturonase content and softening of mutant fruit (11) strongly suggests a key role for polygalacturonase in tomato fruit softening during ripening.

**LITERATURE CITED**