In Vitro Characterization of Tomato Fruit Softening

THE USE OF ENZYMICALLY ACTIVE CELL WALLS

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ABSTRACT

Cell wall isolated from pericarp of normal tomato (Lycopersicon esculentum Mill. cv 'Rutgers') fruit released pectic polymers in a reaction apparently mediated by wall-bound polygalacturonase that appears with the onset of ripening. Release was negligible in wall preparations from normal green and the ripening mutant rin fruit. Pectin solubilization was most extensive at pH 2.5 with a less significant peak at 5.5. Brief exposure to low (1.5) or high (7.5) pH resulted in reduction of autolytic activity, which was also inhibited by high temperature, Ca++, and treatments employed to dissociate protein from cell wall. Uronic acid solubilization was significantly enhanced by 150 millimolar NaCl and by increasing temperature within the physiological range. These data indicate that the release of polyuronide from isolated cell walls is enzymic and may provide a convenient and reliable system for the study of softening metabolism.

Fruit softening is generally attributed to degradation of the cell wall matrix by a series of enzymic, and perhaps some nonenzymic, mechanisms. Hydrolytic enzymes that have been investigated include polygalacturonases (13), pectinmethylesterases (17, 24), cellulase (4), β-1,3-glucanase (12), β-galactosidase (25), and others that are discussed in a recent review article (16).

The traditional approach to studying the wall metabolism of softening has been to extract the enzymes of interest and assay with a commercially prepared substrate or with some type of cell wall preparation (13, 28). Such work has been informative but is subject to certain limitations. For example, the use of buffers containing citrate in experiments with cell wall preparations should be avoided because the chelation properties of citrate may enhance both enzymic and nonenzymic pectin solubilization (8, 9), thus introducing uncertainty into both the quantitative and qualitative properties of the reaction in question. Secondly, the carbazole method (10) for determination of uronic acid degradation products, still widely employed, is subject to interference by neutral sugars (6). A more specific method for total uronic acid determination employs m-hydroxydiphenyl in chromagen formation and is not affected by the presence of other sugars (6). Finally, the use of a reducing group assay specific for hexuronic acids (22) has been generally ignored.

Perhaps the major criticism of cell wall softening studies is that extraction procedures tend to distort the intimate relationship between the polysaccharide matrix and its bound protein. This problem may be circumvented by isolating cell walls under conditions which minimize the disruption of protein-cell wall interactions, then incubating this preparation under conditions favorable for continued enzymic activity. Several workers have used in vitro autolysis as a tool to characterize the wall metabolism of coleoptile tissue (14, 19, 21). Such an in vitro system seems ideal for study of the enzymic interactions that occur in cell walls of ripening fruits. Gross and Wallner (11) reported a solubilization of uronic acids from cell walls of tomato incubated in distilled H2O. They described this product as water soluble pectin and assumed it was an artifact of extraction, but it seems more likely that they were observing an autolytic solubilization of wall pectins. Knee (20) reported autolytic activity in acetone insoluble residue from apple fruit tissue but the analyses employed were for neutral sugars rather than the acid sugars that predominate in pectins. The objective of this study is to characterize autolytic activity in enzymically active cell wall preparations from tomato fruit through the course of their development.

MATERIALS AND METHODS

Plant Material. Normal tomato (Lycopersicon esculentum Mill. cv 'Rutgers') plants and the ripening mutant rin plants were grown at the University of Florida Horticulture Unit near Gainesville. Fruit were harvested at the following developmental stages: immature-green, mature-green, breaker, turning, pink, red (ripe), and overripe, based on United States Department of Agriculture visual aid for color classification of tomatoes (3). Fruit were surface sterilized with 100 ppm NaOCl and sectioned; seed and placental tissue were removed and pericarp tissue stored at −20°C in sealed polyethylene bags.

Cell Wall Preparation. Cell wall modifications during fractionation may result from mechanical stress, heat, pH extremes, chelators, and wall-bound enzymes. Since our objective was to preserve the integrity of polyuronides and their bound proteins, exposure to heat, chelating buffers, and strong ionic environment were avoided. Approximately 100 g frozen pericarp tissue was partially thawed, peeled, and homogenized in 250 ml distilled H2O (4°C) or in acetate buffer (40 mM, pH 4.5) for 1 min in an Osterizer blender set at maximum speed. The homogenate was transferred to Miracloth and washed with 3 liters distilled H2O (4°C) with continuous stirring. Excess water was squeezed through the Miracloth and the remaining wall material used for autolysis experiments.

Autolysis Experiments. Approximately 100 mg (dry weight) freshly prepared wall material was placed in 15 ml acetate buffer (40 mM, pH 4.5, unless otherwise stated) and incubated 22 h in a shaking water bath at 22°C except in experiments involving temperature as a variable. An antibiotic (Thimerosal, 0.02%) was included to suppress possible microbial interference. NaN3 is often employed but was inappropriate here because of its strong interference with the Blumenkranz assay. In other experiments, buffer contained 150 mM NaCl and 1, 10, or 100 mM CaCl2. Some wall preparations were pretreated by various methods designed to inhibit the autolytic reaction, including boiling

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80% ethanol (20), boiling H$_2$O (24), and phenol:acetic acid:water (2:1:1, w/v/v). The latter technique was employed by Jarvis (18) to inactivate cell walls and is based on a report by Selvendren (26) that such treatment effectively removes protein from extracted cell walls.

Aliquots of 0.5 ml were removed from the reaction mixture at the start of incubation and at intervals throughout the trial. These aliquots were filtered through Whatman G/C glass fiber discs and analyzed for total uronic acids (6) and for hexuronic acid reducing groups (22). Some samples were analyzed by $\beta$-eliminative breakdown as described by Albersheim et al. (1) and modified by Ayers et al. (5). At the end of the incubation period (usually 22 h), remaining walls were transferred to Miracloth, washed with distilled H$_2$O, then placed in 30 ml 100% acetone. This mixture was then filtered through tared glass fiber filters and dried to a constant weight for cell wall dry weight determination. Acid sugars ($\mu$g equivalents) released during autolysis were added to obtain an estimate of initial dry weight. All experiments were repeated at least twice with 2 or 3 replications in each trial.

RESULTS AND DISCUSSION

Homogenization of tomato pericarp in distilled H$_2$O yielded an extract with a pH of 4.3 for green fruit and about 4.2 for ripe fruit. There was no apparent advantage to extracting in acetate buffer in this pH range so the initial grinding was in distilled H$_2$O. All carbohydrate assays at time 0 were negative, indicating that soluble polysaccharides had been removed from the wall preparation.

There was a dramatic increase in the in vitro cell wall autolytic activity as ripening proceeded (Fig. 1). There was virtually no autolytic activity in walls extracted from immature-green fruit, with insignificant increases through the mature-green, breaker, and turning stages. In contrast, walls from pink fruit released up to 35 $\mu$g galacturonic acid equivalents/mg wall dry weight-h for the first 2 h, eventually solubilizing acid sugars constituting from 15 to 20% of the total wall weight in the 22-h incubation period. Slight increases in initial rate were observed in red and overripe tissue, which is consistent with activity values reported by Hobson (13) for PG$^2$ extracts prepared from fruit at these stages of ripeness. He reported a dramatic rise in PG activity as fruit approached the orange-red (pink) stage with a gradual increase through overripeness. However, we observed a decrease in total uronic acid released from walls from overripe tissue (Fig. 1), which may be due to extensive solubilization of polygalacturonans in situ and their removal during the wall isolation procedure.

Cell wall extracted from rin tissue did not autolyze (data not shown), an observation consistent with reports that PG is not present in rin (7). To more fully characterize the features of enzymically-active cell wall, pink tissue was selected for most experiments because its initial rate of cell wall autolysis was rapid and it probably contained more unsolubilized polyuronide than ripe or senescent tissue.

Polygalacturonase activity in vitro is optimally enhanced by 150 mM NaCl (24). We observed approximately a 100% increase in both the initial rate of autolysis and in total product solubilized

![Fig. 2. Characterization of the autolytic reaction in cell wall from pericarp of pink tomato fruit. Reactions were run at 22°C (except in 2B) and in the presence of 150 mM NaCl and 0.02% Thimerosal. Bars indicate standard error and, when absent, fall under the symbol. A, Effect of 150 mM NaCl, (● = pink + 150 mM NaCl, ○ = pink without NaCl, ■ = mature-green + 150 mM NaCl, □ = mature-green without NaCl); B, effect of temperature ($x$ = 4°C, $Y$ = 24°C, $Z$ = 34°C); C, effect of boiling 80% ethanol and hot (90°C) H$_2$O pretreatments (● = cold H$_2$O, ○ = hot H$_2$O, ■ = cold ethanol, □ = hot ethanol); D, effect of phenol:acetic acid:H$_2$O pretreatment (● = water pretreatment control, ▲ = reducing groups for water control, ○ = phenol:acetic acid:H$_2$O pretreatment, △ = reducing groups for pretreated samples); E, effect of CaCl$_2$ (● = no CaCl$_2$, ○ = 1 mM, ■ = 10 mM, □ = 100 mM).](image-url)
when 150 mm NaCl was added to the reaction mixture (Fig. 2A). Similar treatment had no such effect on walls from mature-green fruit, which is consistent with reports that PG is absent at this stage of tomato development (13).

Temperature within the physiological range affected autolytic behavior in a manner consistent with the proposal that the activity is enzymic (Fig. 2B). One might anticipate that exposure to higher temperature could serve as a means of inactivating the walls. However, hot (90°C) water results in solubilization of pectin, possibly via β-eliminative breakdown (1), and therefore is not an appropriate means of inactivating autolytic activity. Knee (20) proposed boiling in 80% ethanol as a means of enzyme inactivation without pectin degradation. We found that both boiling H₂O and 80% ethanol inactivated the wall preparation (Fig. 2C), however the boiling ethanol treatment did not result in release of detectable amounts of wall material. Another method of wall inactivation (26) involves exposure to phenol:acetic acid:H₂O (2:1:1, w/v/v) that presumably removes wall protein. Jarvis (18) used this technique in experiments that presumed the absence of any wall-bound enzyme activity. However, our wall preparations were not fully inactivated by this method, which reduced initial rates and total product solubilized by about 75% (Fig. 2D). The amount of reducing groups relative to total uronic acid, shown in Figure 2D and in Table I, is typical of results obtained throughout all experiments, with reducing groups generally amounting from one-half to two-thirds of the total acid sugars solubilized. Gel filtration analysis of autolytic products confirmed the absence of monomer (data not shown), which is consistent with the concept that the primary mechanism for polyuronide solubilization in tomato fruit involves only endo-PG activity (23).

Polygalacturonase activity in vitro is dramatically inhibited by 10⁻⁷ M Ca (29). Figure 2E illustrates the effect of CaCl₂ on release of pectins from wall from pink tissue. A minimum of 10⁻³ M CaCl₂ was required to achieve 50% inhibition of autolysis, a concentration several orders of magnitude higher than levels required to inhibit the activity of purified enzyme preparations assayed using commercial pectin preparations. We considered the possibility that this discrepancy may be due to the presence of membrane fragments in our wall preparation that have Ca-binding sites, but pretreatment with 100% acetone did not significantly alter the inhibitory effect of Ca. However, cell walls have many such binding sites (18) that may have sequestered the excess Ca and the inhibition may be due to an interaction with substrate rather than to direct effects on the protein. Similar results were obtained when CaSO₄ was substituted for CaCl₂ (data not shown). Tomato PG activity in vitro is also inhibited by 0.001% dextran sulfate (2) but such effects were not observed in our system. This may be explained by the inability of the high mol wt dextran to diffuse through the wall matrix and bind to the enzyme.

Experiments to determine the pH optimum for autolytic activity revealed a peak at 5.5 that probably represents the optimum for enzyme-mediated solubilization. Exposure to low (1.5) or high (7.5) pH extremes resulted in substantial reduction of activity (Table I). Other workers have reported pH optima for PG activity in vitro between 4.0 and 5.0 (13, 24, 27), but we observed at least a 30% increase in polyuronide solubilization when pH was lowered to 2.5. This may be nonenzymic degradation since reducing groups do not appear in comparable quantity. This observation seems reasonable when one considers the involvement of Ca²⁺ in cross-linking of galacturonic acid residues between adjacent pectic polymers. Presuming that increasing H⁺ could result in displacement of Ca, one might predict an increase in polyuronide solubilization by at least two mechanisms. The first is nonenzymic, whereby Ca²⁺ stabilized regions of polygalacturonan are spontaneously solubilized with removal of Ca²⁺, thus accounting for fewer reducing groups at pH 2.5. Second, loosening of wall matrix via Ca²⁺ removal could reduce the physical resistance encountered by enzymes as they migrate to potential sites of hydrolysis, thus increasing the efficiency of enzymic activity. A range of activity was observed up to pH 5.5 and we could not rule out the possibility of β-eliminative degradation. However, all assays for the presence of β-elimination products were negative. One interpretation of the pH effect is that slight changes in cell wall matrix pH may provide an elegant means of regulation of enzyme-mediated fruit softening.

Several distinct advantages are obvious in using enzymically active cell walls for study of softening metabolism. First, it more closely resembles the situation in situ and thus is a more realistic system with which to study the enzymology of softening. Second, it may prove to provide a system for observation of sequential processes during softening, e.g. pectin degradation accompanied by loss of neutral sugars and hemicellulose modifications that, until recently, had not been studied in tomato (15). In addition, it is ideal for study of binding characteristics of protein to the polysaccharide matrix. No single in vitro system reported to date has this potential for characterizing patterns of wall degradation in ripening fruit. Interesting relationships have been observed between pH and ionic strength of the extraction media and subsequent autolytic activity. There seems to be a complex arrangement of ionic and covalent bonds that are disrupted by various means to ultimately lead to softer fruit. We are continuing work on analysis of solubilized carbohydrate and protein products in this system.

**Table 1. Effect of pH on Total Release of Polyuronide from Cell Wall of Pink Tissue**

<table>
<thead>
<tr>
<th>pH</th>
<th>Total uronic acid</th>
<th>Hexuronic acid reducing groups</th>
<th>μg galacturonic acid equivalents/mg wall dry wt</th>
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<tr>
<td>1.5</td>
<td>23</td>
<td>8</td>
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<tr>
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<td>292</td>
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</tr>
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