large regions that do not necessarily fall under the auspices of any single county- or district-based research or extension office.

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Extraction and Assay of Tomato Polygalacturonases

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Abstract. **The conditions for extracting and assaying tomato (*Lycopersicon esculentum* Mill.) polygalacturonases (PG I and PG II) have been re-examined. The enzymes were not extracted by water at pH 3, which allowed washing of the cell wall fraction to remove the soluble components that interfere in the PG assay. The extractability of PG in water increased as the pH was lowered or raised from 3, with optima near pH 1.8 and 6.5. Only PG II was extracted by water at pH 1.8, whereas both isoenzymes were extracted at pH 6.5. The extractabilities of the PGs were increased by NaCl, but the amount of total activity extracted by 1.2 M NaCl was independent of pH between 2 and 9. Extracts in 1.2 M NaCl of pH 3 washed cell walls from ripe tomatoes could be assayed without concentration or dialysis. Higher PG activity was recovered when extracts were concentrated by ultrafiltration than by precipitation with $(NH_4)_2SO_4$. The results indicate that the isoenzyme composition and recovery of PG from tomatoes were dependent on extraction and concentration procedures.**

Polygalacturonase (PG) has an important role in tomato softening associated with ripening (3, 6, 7). Tigchelaar et al. (14) suggested that PG also may initiate fruit ripening by releasing wall-bound enzymes involved in various aspects of the process, including ethylene synthesis. Recently, Grierson and Tucker (4) reported that enhanced ethylene evolution preceeds detectable PG activity by about 20 hr and concluded that ethylene actually triggers PG synthesis. Nevertheless, the appearance of PG activity is an early event in tomato ripening, and it is important to have methodology to detect the first traces of the enzyme. Sensitive colorimetric (5) and immunological (2, 15) assays have been developed for tomato PG, but these assays are dependent on the quantitative extraction of the enzyme from the fruit. In our original study on the presence of 2 forms of PG in ripe tomatoes (10), we prepared extracts by blending tomato tissue in 1.0 M NaCl at pH 6. The PG activity in the extract was concentrated by precipitation with $(NH_4)_2SO_4$ followed by dialysis of the enzyme solution against 0.2 M NaCl. This procedure essentially has been adopted to extract and measure PG in tomatoes during ripening (3, 4, 15); however, I have observed that the $(NH_4)_2SO_4$ precipitation step leads to a considerable loss of PG activity. The conditions for extracting and concentrating tomato PG therefore have been examined in detail, and a procedure for maximum yields of PG has been developed.

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PG was analyzed in a 1-ml reaction mixture containing 0.2 M NaCl, 0.5% polygalacturonic acid (pH 4.5), and an aliquot of tomato extract. Blanks were prepared by heating duplicate samples for 5 min in a boiling water bath before the addition of the substrate. The reducing groups formed after 30 min at 37°C were measured by the arsenomolybdate method (8). A unit of PG was defined as the amount that released 1 μmol of reducing groups/30 min.

PG I usually was determined by measuring the residual activity after heating the reaction mixture without the substrate for 5 min at 65°C (15). PG II then was calculated as the difference between total PG and PG I. Some extracts in dilute NaCl solutions were analyzed for the 2 enzymes by HPLC (12), and extracts in 1.0 M NaCl were analyzed on a 2.5 × 45 cm column of Sephadex G-100 equilibrated with 1.0 M NaCl. Pectinesterase was assayed as described previously (9, 11). Ultrafiltration of extracts was conducted in stirred cells using PM-10 membranes (Amicon).

Freshly harvested ripe tomatoes ('Floradade') grown in a greenhouse were used in this study. Slices of fruit (100 g) were added to 100 ml of cold water, and blended 1 min with a VirTis homogenizer and then an additional minute with a Polytron. The homogenate was adjusted to pH 3 with 0.1 M HCl, stirred 15 min, and centrifuged at $8000 \times g$ for 20 min at 2°C. The pellet was washed with 150 ml of cold water at pH 3 by homogenizing, stirring 15 min, and centrifuging. The supernatant solutions from the pH 3 wash step were combined and checked for PG activity after ultrafiltration to 10 ml and dialysis against 1.0 M NaCl. The amount of PG in this solution was only 2% of the total activity in the fruit. The pellet remaining after the washing procedure hereafter is referred to as the cell-wall fraction.

The effect of pH on the extraction of tomato PG by water was determined first. The cell-wall fraction prepared from 200 g of ripe fruit was suspended in 300 ml of cold water by blending 30 sec with a Polytron. Aliquots of the suspension were adjusted to various pH values by the addition of dilute HCl or NaOH and maintained at those values for 30 min. The samples then were centrifuged, and the supernatant solutions were adjusted to pH 4.5 and assayed for PG. The results are presented in Fig. 1. The extractability of PG

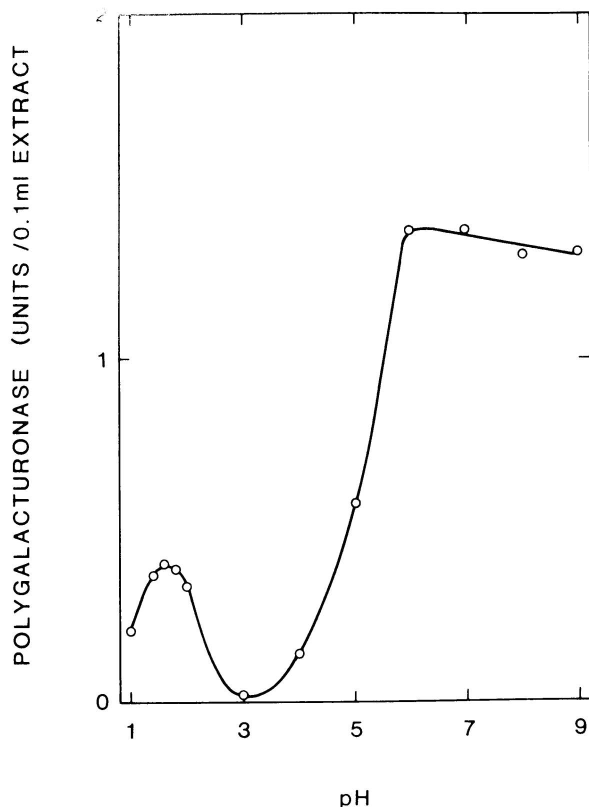

Fig. 1. Effect of pH on the extraction of polygalacturonase activity from the cell wall fraction of tomato.

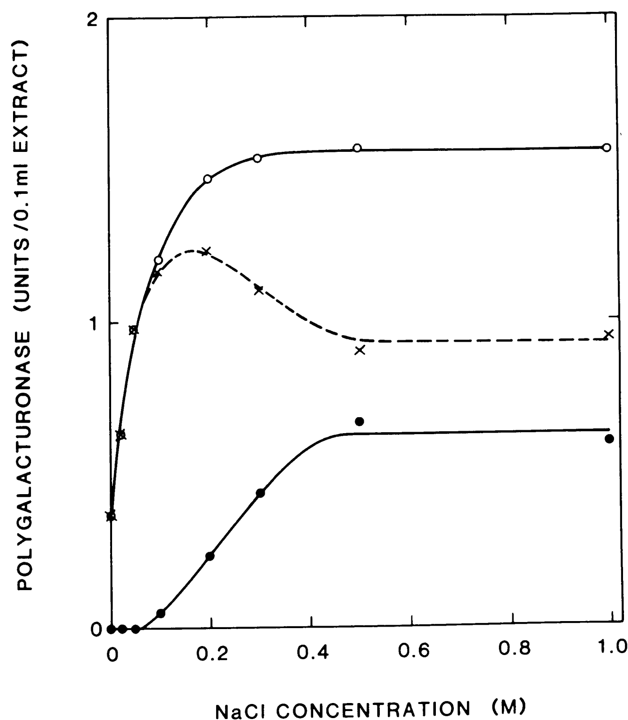

Fig. 2. Effect of NaCl concentration on the extraction of tomato polygalacturonases at pH 1.8. ○, total PG; ● PG I; ×, PG II.

exhibited a minimum near pH 3 but increased as the pH was raised or lowered. The enzyme was most soluble in water at about pH 6.5, with little effect on the solubility between pH 6 and 9. It should be noted that the PG activity extracted by water at pH 6.5 was about 80% of that extracted by 1.2 M NaCl at this pH. Analysis of the pH 6.5 water extract after ultrafiltration showed that it contained 46% PG I and 54% PG II.

The extractability of PG increased also as the pH of the cell walls in water was adjusted below 3 (Fig. 1) although the activity extracted was considerably less than that at pH 6.5. The optimum pH in the acid range was about 1.8, and the activity extracted at this pH was due exclusively to PG II.

It is well known that salts in the extraction medium increase the solubility of cell-wall enzymes (6). However, the effect of NaCl on the solubility of tomato PG was found to be highly dependent on the pH of the solution. At pH 1.8, the solubility of PG increased sharply with increasing NaCl concentration to a maximum at about 0.4 M NaCl (Fig. 2). As in the case of water at pH 1.8, only PG II was extracted by NaCl at low concentrations. PG I was not extracted until the salt concentration exceeded 0.1 M. The level of PG I then increased while PG II decreased with increasing NaCl concentration. The results can be explained in terms of the solubility of PG converter (13), a cell-wall component that combines with PG II to produce PG I. Apparently the PG converter is insoluble in NaCl at low concentrations at pH 1.8 and thus is not available to react with soluble PG II. At higher concentrations of NaCl, the converter is solubilized and it converts PG II to PG I.

At pH 6, increasing concentrations of NaCl in the extraction medium decreased the solubility of total PG, PG II, and especially that of PG I, to minima at about 0.15 M NaCl (Fig. 3). A possible explanation for this reduced recovery of PG activity involves the role of pectinesterase (PE), which accompanies PG in tomato extracts. The level of PE in the extract increased 4-fold when the NaCl concentration was raised to 0.2 M at pH 6 (Fig. 3). The activity of this enzyme also is enhanced by cations (9, 11). Thus, in the presence of 0.15 M NaCl, a high level of PE in the extract rapidly de-esterifies pectin in the cell wall. The negatively charged pectates then adsorb the cationic PG (1) and thus remove the enzyme from solution. The insoluble PG can be extracted from the pellet by raising the NaCl concentration to 1.2 M, but all of the activity usually was not recovered, suggesting that some of the binding between PG and de-esterified cell walls is irreversible.

Extraction of cell-wall fractions at pH 6 with NaCl solutions higher than 0.15 M increased the yields of total PG activity and PG I to maxima at about 1.2 M NaCl (Fig. 3). However, the highest amount of PG II was obtained with 0.5 M NaCl, indicating differences in solubilities of PG II and PG converter in relation to NaCl concentration at pH 6. The amount of total PG activity extracted by 1.2 M NaCl was relatively independent of pH over the range of 2 to 9 (data not shown). The yield of PG decreased only when the pH was lowered to 1, which may reflect instability of the enzyme at high-acid conditions.

There have been numerous reports (1, 10, 15) of concentrating the PG activity in crude extracts of tomatoes by precipitation with $(NH_4)_2SO_4$ as a step in the assay and purification of the enzyme. The re-dissolved material usually is dialyzed against dilute NaCl

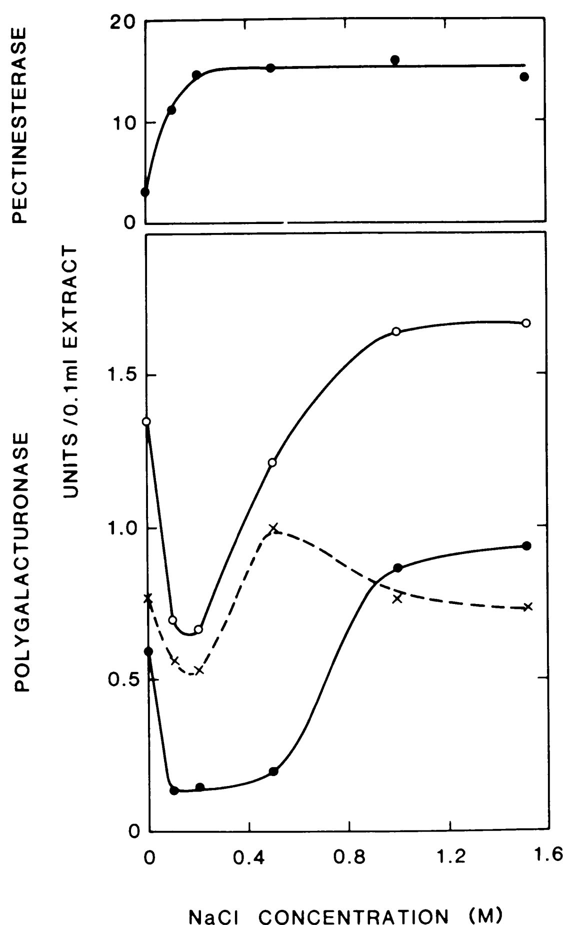

Fig. 3. Effect of NaCl concentration on the extraction of tomato polygalacturonases at pH 6.0. ○, total PG; ● PG I, ×, PG II.

solutions or even water to remove residual $(NH_4)_2SO_4$. The following study was conducted to determine the recovery of PG using this procedure. The cell-wall fraction from 1 kg of tomatoes was suspended in 1.5 liters of 1.0 M NaCl at pH 6 and stirred 30 min. One-third of the supernatant solution obtained by centrifugation was ultrafiltered to 20 ml and dialyzed against 4 liters of 1.0 M NaCl. The remaining two-thirds of the crude extract was treated with $(NH_4)_2SO_4$ at 75% of saturation. The precipitate was collected by centrifugation and dissolved in 40 ml of 1.0 M NaCl. Aliquots of the solution were dialyzed against 0.15 M NaCl and 1.0 M NaCl.

The 3 extracts were clarified by centrifugation and made up to equal volumes. They were assayed for total PG activity and for PG I and PG II after chromatography on Sephadex G-100 in 1.0 M NaCl. Relative to the PG activity in the ultrafiltrate, only 78% and 43% of the activity was recovered in the $(NH_4)_2SO_4$ fractions dialyzed against 1.0 M and 0.15 M NaCl, respectively. The levels of both PG I and PG II were lower in the extracts dialyzed against 1.0 M NaCl. The much lower activity in the extract dialyzed against 0.15 M NaCl was due primarily to the loss of PG I, with only 11% of the level in the ultrafiltrate. A considerable amount of precipitate formed in this extract during dialysis. Some of the PG I was recovered by suspending the precipitate in 1.0 M NaCl followed by centrifugation. It is possible that the precipitate was pectate that would bind PG in dilute NaCl solutions.

The results of this study explain the low levels of PG I in tomatoes reported by Tucker et al. (15). Their procedure for preparing extracts included precipitation with $(NH_4)_2SO_4$ and dialysis of the protein suspension against 0.15 M NaCl. As shown previously, the procedure leads to low recovery of total PG and PG I. They found that only 5% of the activity in 'Ailsa Craig' fruit was due to PG I and only a slightly higher percentage in 'Potentate' (15). In a later study, Crookes and Grierson (3) reported that PG I accounted for 27% of the PG activity in 'Ailsa Craig' tomatoes ripened after harvest, about half of the PG I that I have found in ultrafiltrates of extracts of a number of cultivars.

The procedure for extracting PG from tomatoes usually includes a preliminary step of washing the cell-wall fraction with water at the endogenous pH (10, 15), which is near 4. This study shows that the cell walls should be washed at pH 3 to minimize the loss of PG. Washing the cell walls twice with water at pH 3 reduces the level of soluble sugars in the subsequent salt extract and thus eliminates a time-consuming dialysis step. Maximum PG activity is extracted from the washed cell walls by stirring in 1.2 M NaCl at pH 6.5 for 30 min. The activity in the supernatant solutions obtained by centrifugation of extracts of ripe tomatoes can be assayed without further concentration. A 30-min incubation period of the standard assay mixture containing 0.1 ml of extract is adequate for the arsenomolybdate (8) or cyanacetamide (5) methods for reducing groups. Extracts containing low PG, as from tomatoes beginning to ripen, should be concentrated by ultrafiltration rather than by precipitation with $(NH_4)_2SO_4$.

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