

Separation and Characterization of Endopolygalacturonase and Exopolygalacturonase from Peaches

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ABSTRACT

Two polygalacturonases (PG I and PG II) have been separated from extracts of ripe peaches (*Prunus persica*) by chromatography on Sephadex G-100. PG I hydrolyzes polygalacturonic acid from the nonreducing ends of the molecules, releasing galacturonic acid as the product. It functions optimally at pH 5.5, requires Ca^{2+} for activity, and hydrolyzes low molecular weight substrates most rapidly. In contrast, PG II cleaves the molecular chain of the substrate randomly with a pH optimum at about 4. This enzyme is most reactive with substrates of intermediate molecular weight. It catalyzes the release of water-soluble, but 70% ethanol-insoluble, pectin from washed peach cell walls.

MATERIALS AND METHODS

Substrates. Pectic acid, purchased from Sigma Chemical Company,¹ was purified by precipitating a 1% solution in water at pH 5 with 2 volumes of 95% ethanol. The precipitate was collected on Miracloth, dissolved in water, and precipitated with ethanol. This procedure was repeated three times, and the pectic acid was finally homogenized with 95% ethanol and acetone and dried under vacuum.

The polygalacturonic acids were prepared by enzymatic hydrolysis of pectic acid as described earlier (15). PGA I is a fraction that is insoluble at pH 2 and 25 C, while PGA II is insoluble at pH 2 and 3 C. PGA III is soluble at pH 2 and 3 C, but its calcium salt is insoluble at pH 6, and the free acid is insoluble in 63% ethanol at pH 2.

Reduced polygalacturonic acids were prepared by treating with sodium borohydride, according to the procedure of McCready and Seegmiller (8). This reaction changes the D-galacturonic acid unit on the reducing end of each chain to L-galactonolactone.

Polygalacturonase Assays. The reaction mixture consisted of 0.5 ml of enzyme preparation in 0.15 M NaCl and 0.5 ml of 0.1 M acetate-tris buffer. Duplicate samples heated in boiling water for 3 min served as controls. The reactions were started by adding 1 ml of 1% substrate adjusted to the pH of the reaction mixture. The samples were incubated at 37 C for 1 hr. After heating the tubes for 3 min in boiling water to terminate the reactions, the reducing groups were measured by the arsenomolybdate method (11). A unit of enzyme activity is defined as that amount of polygalacturonase which releases 1 μmole of reducing groups per hour from the substrate PGA I.

The viscometric method for polygalacturonase was conducted using pectic acid as substrate and the above conditions in a total volume of 45 ml. Five-ml aliquots were withdrawn at frequent intervals and heated for 3 min in boiling water. The viscosity of the solutions was then measured with an Ostwald viscometer immersed in a water bath at 37 C. The reducing groups in the solutions were determined as above.

Preparation of Washed Cell Walls. Firm ripe Elberta peach (*Prunus persica*) tissue (100 g) was blended 2 min with 100 ml of 0.1 M sodium phosphate, pH 7, 12 g of Carbowax 4000, and 2 ml of 10% sodium bisulfite in a VirTis homogenizer. The insoluble material was collected by centrifugation at 8000g for 20 min and suspended in 200 ml of 0.1 M phosphate, pH

Changes in polysaccharides leading to the destruction of the cell wall structure are probably responsible for the marked softening of fruits during ripening. The softening of freestone peaches, for example, has long been associated with the conversion of protopectin to soluble forms (13, 19-21). Although changes in other polysaccharides may be involved, the solubilization of pectin has received the most attention because of the occurrence of this polysaccharide in the middle lamella, as well as in the cell wall. Elucidation of the mechanism of pectin solubilization has been hindered by the complexity of protopectin and by the failure to identify the enzymes involved. In a recent study (16), we found that polygalacturonase was not detectable in unripe peaches, but activity appeared when the fruit began to soften and then increased sharply as ripening proceeded. Development of the enzyme paralleled the formation of water-soluble pectin. Furthermore, the molecular weights of the solubilized pectin decreased during fruit ripening. The results suggested a key role for polygalacturonase in pectin solubilization and, therefore, in textural changes accompanying peach ripening.

The very low level of polygalacturonase in peaches, compared to that in tomatoes, presents problems in studying this system. Because the activity is associated with the particulate fraction of homogenates, we originally conducted our assays with aliquots of washed cell fragments incubated for long periods. We have now developed a procedure for solubilizing and concentrating the polygalacturonase activity in ripe peaches. This paper describes the resolution of the activity into two components and some of their properties.

¹ Reference to a company or product name does not imply approval or recommendation of the product by the United States Department of Agriculture to the exclusion of others that may be suitable.

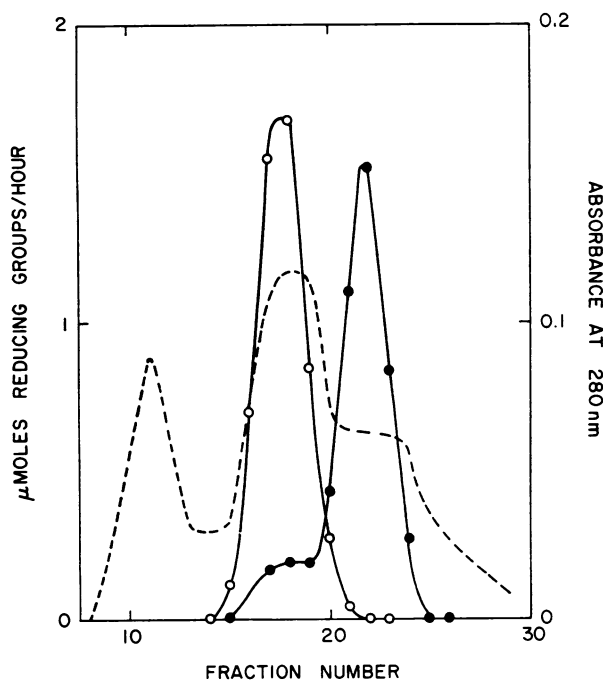


FIG. 1. Gel filtration of peach polygalacturonase activity on Sephadex G-100. A crude extract (10 ml) was applied to the column (2.5×90 cm) and eluted with 0.15 M sodium chloride. Fractions of 10 ml each were collected and assayed for polygalacturonase activity and protein. ●—●, polygalacturonase assayed at pH 4.5; ○—○, polygalacturonase assayed at pH 5.5 in the presence of 0.5 mM Ca^{2+} ; -----, absorbance at 280 nm.

7, with a Polytron homogenizer. After centrifuging, the insoluble fraction was washed twice with water, then with ethanol and acetone, and dried under vacuum. All steps were carried out at room temperature.

Extraction of Peach Polygalacturonase. The peaches were obtained from orchards at Byron and Watkinsville, Georgia during the summer of 1972. Soft ripe fruit were selected and stored in a freezer. The frozen peaches were thawed by immersing in water containing 0.1% sodium bisulfite, pH 6. All subsequent steps were conducted at low temperature using cold solutions. About 1 kg of peach slices was added to 1 liter of water containing 0.1% bisulfite. The tissue was disintegrated with a Polytron homogenizer (Brinkmann Instruments, Inc.) set on high speed for 2 min. The suspension was centrifuged at 8000g for 30 min, and the supernatant was discarded. The sediment was washed twice with 2 liters of water containing bisulfite with the aid of the Polytron homogenizer followed by centrifugation. The washed residue of peach tissue was then resuspended in 2 liters of 0.2 M NaCl and stirred 3 hr. The supernatant obtained on centrifugation of this suspension at 8000g for 30 min was concentrated to about 65 ml by ultrafiltration using a PM-10 membrane (Amicon Corp.) and dialyzed against 0.15 M NaCl for 16 hr.

Separation of Two Forms of Polygalacturonase. The dialyzed extract was adjusted to pH 6, and 10 ml of the solution were applied to a column (2.5×90 cm) of Sephadex G-100 equilibrated with 0.15 M NaCl. The fractions obtained on elution with 0.15 M NaCl were assayed for polygalacturonase activity according to the standard assay at pH 4.5 and also at pH 5.5 in the presence of 0.5 mM CaCl_2 . A typical elution for an extract prepared from Cardinal peaches is presented in Figure 1. Similar results were obtained with extracts of Elberta

and Dixie Red peaches. The enzymes were designated PG I and PG II in the order of elution from the column. The fractions between the two peaks were discarded to ensure separation of the enzymes. Fractions 16 and 17 were saved for PG I and fractions 22 and 23 for PG II. The solutions were concentrated to about 3 ml by ultrafiltration.

RESULTS

Effects of Cations on the Polygalacturonases. In contrast to the activation of tomato polygalacturonase by monovalent cations (7, 12, 15), neither of the peach enzymes responded to the addition of low levels of NaCl to the assay solutions. Both enzymes were inhibited by NaCl at concentrations higher than 0.15 M. We discovered that PG I was strongly activated by Ca^{2+} ions. Activation was maximal at about 0.4 mM CaCl_2 . The reaction rate with PGA I as the substrate at this level of Ca^{2+} was 1.55 μmoles reducing groups/hr compared to 0.28 $\mu\text{mole/hr}$ for the control solution. The low level of activity for the control may have been due to endogenous Ca^{2+} in the enzyme or substrate solutions. This possibility was confirmed by addition of EDTA or citrate to the reaction solution. Both of these chelating agents reduced the activity to zero, indicating that a divalent cation may be required for enzymatic activity. Sr^{2+} also activated PG I, but it was only about 25% as effective as Ca^{2+} . At 0.5 mM, Mg^{2+} , Pb^{2+} , Hg^{2+} , Cd^{2+} , Ni^{2+} , and Cu^{2+} had no effect, and Ba^{2+} was inhibitory. The cations tested were acetate or chloride salts. PG II was not activated by Ca^{2+} at low concentrations and was inhibited at concentrations higher than 0.5 mM.

Substrate Specificity. PG I did not hydrolyze citrus pectin (Sigma Chemical Co., 70% methyl esterified). A slow reaction was obtained with PG II, but the rate was only 15% of that for pectic acid as the substrate. The hydrolysis of pectin probably indicates that a sufficient number of de-esterified monomer units are present within the chain to render it susceptible to polygalacturonase action.

Effects of Substrate Size and pH. Our studies on tomato polygalacturonase (15) showed that the molecular weight of the substrate can markedly affect the initial rate of cleavage. Of the two peach enzymes, PG I was more dependent on substrate size, with a gradual increase in the rate of reducing group formation as the molecular weight of the substrate decreased (Table I). Because the rate of cleavage was still increasing for PGA III (degree of polymerization about 18), PG I may be most effective on oligogalacturonides. PG II, on the other hand, cleaved PGA I most rapidly and was less effective on both smaller and larger substrates.

The two enzymes further differed in their responses to changes in pH. PG II hydrolyzed pectic acid most rapidly at

Table I. *Effect of Substrate on the Rate of Hydrolysis*

PG I was assayed at pH 5.5 in the presence of 0.5 mM Ca^{2+} . PG II was assayed at pH 4.5. The final substrate concentrations were 0.5%, and the solutions were incubated at 37 C for 1 hr.

Substrate	Rate of Formation of Reducing Groups	
	PG I	PG II
	$\mu\text{mole/ml}\cdot\text{hr}$	
Pectic acid	0.39	0.34
PGA I	0.72	0.70
PGA II	1.03	0.56
PGA III	1.10	0.53

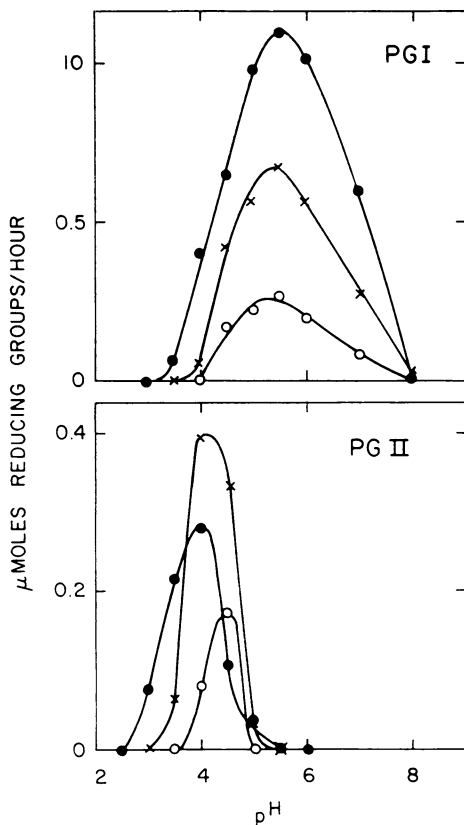


FIG. 2. Effects of substrate size and pH on the activities of PG I and PG II. PG I was assayed in the presence of 0.5 mM Ca^{2+} . ○—○, pectic acid; ×—×, PGA I; ●—●, PGA III.

about pH 4.5, and the activity decreased sharply to zero at about pH 3.5 and 5 (Fig. 2). The pH optimum shifted to the acid side as the molecular weight of the substrate decreased. For the smallest substrate, PGA III, the optimum was slightly below pH 4, and the activity extended to nearly pH 2.5. PG I exhibited activity over a broader range of pH with an optimum at pH 5.5, regardless of substrate size. Its activity was also extended to the acid side as the substrate size decreased, but not as much as for PG II. The exclusion of Ca^{2+} from the reaction solution did not affect the pH optimum nor the range of activity.

Patterns of Action. An approach that is commonly employed for determining the mode of polygalacturonase attack involves measuring the viscosity and reducing power of the substrate during reaction. Random cleaving enzymes have a much greater effect on the viscosity of pectate than terminal cleaving enzymes at comparable rates of reducing group formation. This method was applied to the peach polygalacturonases, and the results are presented in Figure 3. PG I was assayed at pH 5.5 and PG II at pH 4.5, both in the presence of 0.05 M NaCl. Regardless of whether PG I was assayed at 0, 0.1 and 0.5 mM Ca^{2+} , it decreased the viscosity of pectate very slowly, while the reducing groups increased linearly. In the absence of Ca^{2+} , the viscosity decreased only 8% after 2 hr, and about 1% of the glycosidic bonds were cleaved. PG II, on the other hand, decreased the viscosity of the substrate 50% after only 10 min. The number of reducing groups formed during this time is estimated to be 0.01 $\mu\text{mole/ml}$ or about 0.03% of the total glycosidic bonds in the substrate.

Paper chromatographic examination of the reaction products confirmed the above mechanisms for the peach polygalacturonases. The reactions were conducted with PGA I as the

substrate at the optimum conditions for each enzyme and using 1 unit of enzyme/ml. Aliquots of the solution were withdrawn at hourly intervals, heated 3 min, and chromatographed on Whatman No. 3 MM paper using the solvent ethyl acetate-acetic acid-water (10:5:6) and the descending method (14). Twenty-hour development of the chromatograms was sufficient to resolve galacturonic acid and the first 4 oligogalacturonides which were detected by a mixed indicator dip solution (14). Galacturonic acid was not detectable in the reaction solutions containing PG II, but after 8 hr some of the products moved a short distance from the origin. These products were considerably larger than pentagalacturonic acid. In contrast, PG I produced galacturonic acid after 1-hr incubation. Galacturonic acid increased during hydrolysis of PGA I and was the only mobile product after 8 hr.

It is clear that PG I is an exopolygalacturonase that cleaves off monomer units. The question then arises as to which end of the polymer molecule is attacked. Reducing the substrates with sodium borohydride did not affect the rates of their cleavage by PG I. For example, the rates of PGA II and reduced PGA II were 1.85 and 2.05 μmoles reducing groups/hr, respectively. The nature of the reducing end of polygalacturonate is either not critical or the enzyme hydrolyzes the molecule at the nonreducing end.

Further evidence for PG I action at the nonreducing end of polygalacturonate was obtained in the following experiment. Twenty-one ml of reaction solution containing 0.5% reduced PGA II, 0.5 mM CaCl_2 , and PG I were incubated for 1 hr at 37 C. After heating in boiling water for 4 min, 1 ml of the

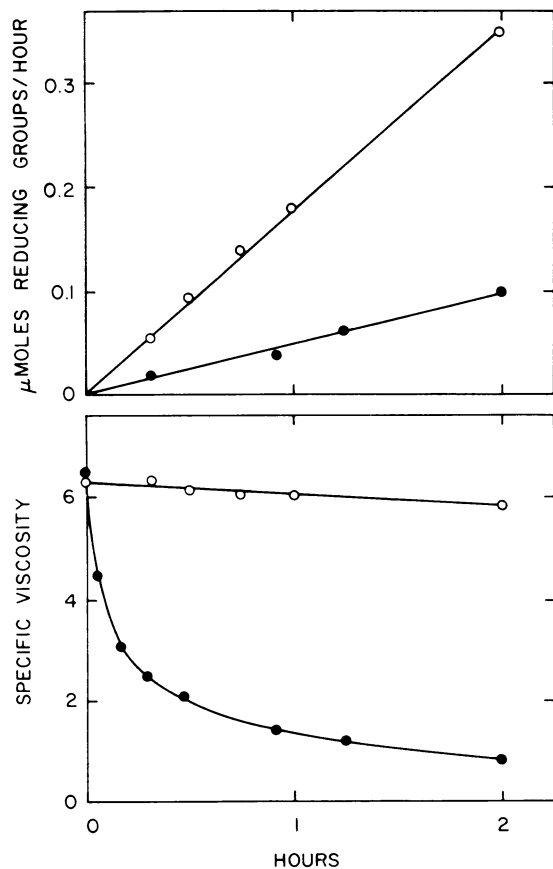


FIG. 3. Changes in the viscosity and reducing groups of pectic acid during degradation by peach polygalacturonases. PG I was assayed at pH 5.5 and PG II at pH 4.5. ○—○, PG I; ●—●, PG II.

solution was analyzed for reducing groups (2.04 μ moles/ml). The remaining solution was deionized with Dowex 50 (H^+), adjusted to pH 2, and 2 volumes of ethanol were added. This fractionation with ethanol separated soluble products such as galacturonic acid from the unhydrolyzed substrate. The precipitate was collected by centrifugation and dissolved in 20 ml of water. This solution of partially hydrolyzed polygalacturonate contained only 0.03 μ mole reducing groups/ml. In contrast, the alcohol supernatant, corrected for dilution of the original 20 ml, contained 1.98 μ moles reducing groups/ml or 97% of the reducing groups formed during the reaction. The reducing groups formed during hydrolysis therefore appear to be associated with the galacturonic acid released, and the unhydrolyzed substrate remains nonreducing. A mechanism that could account for these results is that hydrolysis proceeds from the nonreducing end of the polygalacturonate chain. Had cleavage occurred at the reduced end, the partially hydrolyzed substrate would have been reducing.

The possibility remains that PG I attacks a given substrate molecule and, after releasing a monomer unit, continues to degrade the same molecule. The polygalacturonate molecule could be completely hydrolyzed before the enzyme proceeds to another substrate molecule. This mechanism has been proposed for a fungal endopolygalacturonase that releases mono-, di-, and trigalacturonic acids (3). If this were the case for peach exopolygalacturonase, the data of the earlier experiments would not be proof for attack at the nonreducing end. The number of partially hydrolyzed substrate molecules would be too few to detect, and it would not be possible to determine which end of the molecule is attacked.

The progress of enzymatic hydrolysis was therefore followed by gel chromatography. The substrate selected for this study was PGA II because it can be chromatographed on Sephadex G-100. Aliquots of the reaction solutions were heated and diluted to a level of 0.05% anhydrogalacturonic acid. Two ml

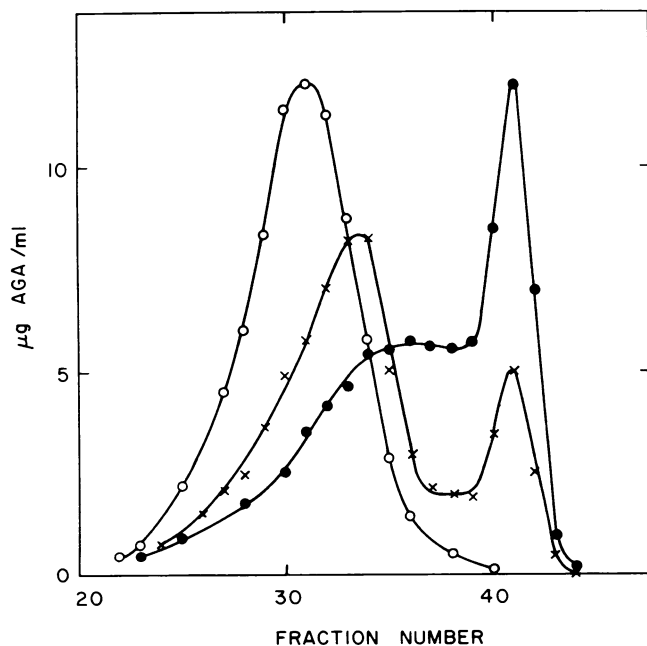


FIG. 4. Degradation of PGA II by PG I as determined by gel filtration on Sephadex G-100. The products were chromatographed in 0.15 M NaCl on a 2.5×90 cm column, and 10 ml fractions were collected. The fractions were analyzed for uronic acids by the carbazole method. \circ — \circ , control sample for PGA II; \times — \times , products after 4-hr reaction; \bullet — \bullet , products after 8-hr reaction.

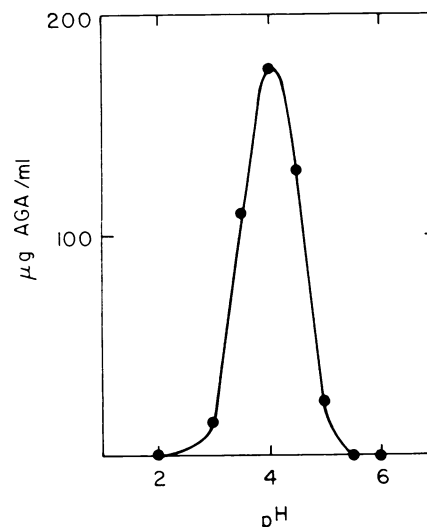


FIG. 5. Effect of pH on the solubilization of pectin from peach cell walls by PG II.

of solution were applied to a column (2.5×85 cm) of Sephadex G-100 previously equilibrated with 0.15 M NaCl. The fractions were analyzed for anhydrogalacturonic acid content according to the carbazole method (18). Had the enzyme degraded an individual substrate molecule completely before proceeding to the next molecule, the change in the elution pattern would have been a simple decrease in the substrate peak while galacturonic acid accumulated. Instead, the substrate peak not only decreased but also shifted progressively to the right as the reaction proceeded (Fig. 4). This indicates that the enzyme does not remain associated with the first substrate molecule it attacks but rather degrades all of the substrate molecules gradually.

The above solutions were also chromatographed on paper. As in the studies with the substrate PGA I, the only mobile product after 8 hr of reaction was galacturonic acid. Oligogalacturonides did not appear until after 24 hr of reaction.

Solubilization of Peach Pectin. The abilities of the polygalacturonases to solubilize protopectin were determined by incubating the enzymes with washed cell walls prepared from peach tissue. Fifty mg of the cell wall preparation were suspended in 10 ml of water by stirring for a few minutes. The reaction mixture was then prepared by adding 1 ml of the suspension to a solution containing 0.5 ml of 0.1 M tris-acetate buffer and 1 ml of diluted enzyme in 0.15 M NaCl. The samples were incubated at 37 C, and the reactions were terminated by cooling in ice water followed by centrifuging at 0 C. The supernatant was then analyzed for galacturonides by the carbazole method (18).

PG I did not release soluble forms of pectin over the pH range of 2 to 8. In contrast, PG II was very effective in solubilizing the pectin. The reaction occurred optimally at about pH 4 (Fig. 5). The pH activity plot is broader than that for the hydrolysis of any one polygalacturonate substrate (Fig. 2) but does not exceed the range established with the substrates of variable size. The rate of soluble pectin formation was linear with respect to both enzyme concentration and incubation time when the enzyme level was in the order of 0.05 unit. Higher concentrations of PG II and longer incubations resulted in leveling off of soluble pectin formation. The pectin solubilized by PG II was completely precipitated by 2 volumes of ethanol, indicating relatively high molecular weights.

Other Properties. The dependence of polygalacturonase activity on substrate concentration was determined at the opti-

mum conditions for each enzyme and using approximately 1 unit enzyme/ml reaction solution. Rates of reaction were maximum at 0.36 and 0.11% PGA I for PG I and PG II, respectively. Substrate concentrations of 0.5% were not inhibitory.

The molecular weights of the enzymes, as determined by gel filtration on Sephadex G-100, were 68,000 and 41,000 for PG I and PG II, respectively. The values were calculated from the elution volumes for cytochrome *c*, ovalbumin, and bovine serum albumin according to the method of Whitaker (22).

DISCUSSION

The necessity to demonstrate that the enzymes detected with model substrates are actually able to catalyze degradation of cell walls before assigning physiological roles to them in the process has been pointed out by Karr and Albersheim (6). The polygalacturonases, for example, have been detected in many ripe fruits, but it is not clear whether they initiate cell wall degradation. The basis for the insolubility of the pectin in unripe fruit may be either high molecular weights or bonding of the pectin chains to other polysaccharides. Solubilization of the pectin could, therefore, involve cleavage of the pectin chains by polygalacturonases. The requirement for a wall-modifying enzyme before cell wall polysaccharides are rendered susceptible to enzymatic attack has been demonstrated (6).

The polygalacturonases found in higher plants are usually random-cleaving enzymes (1, 7, 12, 17). They are specific for the de-esterified pectate, and therefore their action must be preceded by that of pectinesterase. The endopolygalacturonase in peaches is similar to the enzymes characterized earlier, but we have demonstrated that it is able to catalyze the solubilization of protopectin in peach walls. Its action coupled with that of the exopolygalacturonase, which also is present in peaches, provides a system for the complete hydrolysis of the pectin. Free galacturonic acid has been detected in ripe peaches (2), indicating that extensive degradation of pectin actually occurs.

The discovery of an exopolygalacturonase in peaches represents only the second such enzyme in higher plants. Hatanaka and Ozawa (5) have isolated and characterized an end-group cleaving polygalacturonase from carrots. Numerous exopolygalacturonases have been identified in microorganisms (4, 9, 10). As in the case of peach PG I, these enzymes cleave the polygalacturonate chains from the nonreducing ends. The possible usefulness of exopolygalacturonases in structural studies on pectin has already been mentioned (5) and is based on the assumption that neutral sugars in the pectin chain or branching of any kind would block degradation beyond such features.

The extent of hydrolysis by an exopolygalacturonase would be a measure of the heterogeneity of the pectin chain.

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