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PEAR POLYGALACTURONASES

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Abstract—The polygalacturonase activity in extracts of ripe D'Anjou pears is due to two enzymes, which can be resolved by chromatography on Sephadex G-100. One of the enzymes is an endopolygalacturonase that hydrolyzes the pectate chain randomly, with a pH optimum at 4.5. The other enzyme, the major polygalacturonase in pears, catalyzes the step-wise removal of monomer units from the nonreducing ends of the substrate molecules. This exopolygalacturonase has a pH optimum of 5.5 and is activated by Ca^{2+} and Sr^{2+}. It cleaves moderately large substrates most rapidly and exhibits the highest affinity for pectate, the largest substrate.

INTRODUCTION

Earlier workers have reported pectolytic activity in pears. Weurman [1] detected weak activity in ripe pears using both reductometric and viscometric assays with pectin as the substrate. Because pears contain pectinesterase [2], the activity measured may have been due to polygalacturonase, which cleaves deesterified pectin (pectate). Weurman [1] suggested that the actual level of pectolytic activity in pears may be higher than that measured because pear extracts contain a pectinase inhibitor [3]. However, the enzyme inhibitor was detected with commerical fungal pectinase, and it did not appear to influence the activity of the pear enzyme. McCready and McComb [4] and Hobson [5] confirmed that ripe pears contain a low level of polygalacturonase. Also, Nagel and Patterson [6] found weak activity in immature pears during two out of three seasons of testing.

This paper describes the resolution of the activities of two polygalacturonases from pears and includes some of their properties.

RESULTS

Separation of the polygalacturonases

Crude extracts of ripe pears contained low levels of polygalacturonase activity which could be detected by both the reductometric and viscometric methods. Preliminary studies showed that the rate of reducing group formation relative to that of decrease in pectate viscosity was higher than that expected for an endopolygalacturonase. Chromatography of pear enzyme concentrates on Sephadex G-100 revealed two components. The polygalacturonase first eluted with a peak in fraction 17 (PG I), affected both the viscosity and level of reducing groups of pectate solutions whereas the second polygalacturonase (PG II) with a peak in fraction 27, increased the number of reducing groups without greatly affecting substrate viscosity. The fractions corresponding to each peak were combined and concentrated to about 10 ml by ultrafiltration. In all extracts tested, PG II was always the major component on assay at pH 5.

Characterization of PG I

Freshly prepared solutions of PG I required heating to 65° for 5 min for the destruction of half of the activity, but the activity of even the frozen enzyme decreased quite rapidly and usually was lost after a few days. The activity could not be stabilized with sulfhydryl agents. The instability and low levels of this enzyme limited characterization to only the basic properties.

The effect of substrate size on the action of PG I was studied with pectic acid and three polygalacturonic acids (PGA I, II, and III). The polygalacturonic acids were prepared by partial enzymatic hydrolysis of pectic acid and represent fractions in the order of decreasing MWs [7]. At an initial substrate concentration of 0.5%, PG I cleaved PGA I most rapidly. The relative rates of hydrolysis in terms of reducing groups released were 36, 100, 64, and 40 for pectate, PGA I, PGA II, and PGA III, respectively. The pH optimum for hydrolysis was 4.5 according to both the reductometric and viscometric assays and was independent of substrate size. The enzyme was not activated by Ca^{2+} over the concentration range of 0.01–0.5 mM. PG I produced only 80 nmol reducing groups per ml of pectate solution at pH 4.5 while decreasing the viscosity 50% during 2 hr of reaction. These results suggest that PG I is an endopolygalacturonase. It catalyzed the solubilization of pectin from washed cell walls prepared from both pears and tomatoes. The pH optimum for pectin solubilization was 4.5. PG I was more effective in solubilizing pectin from cell walls of tomatoes than of pears.

Characterization of PG II

PG II was active between pH 3.5 and 7, but was optimally active at pH 5.5. The activity of the partially purified enzyme was increased 3-fold by addition of 0.5 mM Ca^{2+} to the reaction mixture. Sr^{2+} was an equally effective activator at a concentration of 1 mM. Other cations tested and found to be ineffective were Mg^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, K^{+}, Na^{+}. At concentrations higher than 0.1 M, Na^{+} was inhibitory.

PG II had a much smaller effect than PG I on the viscosity of pectate solutions. Typically, the viscosity of

Table 1. Kinetic parameters of the hydrolysis of galacturonans by pear PG II

Substrate	K_m ($M \times 10^6$)	Relative maximum velocity¶
Trigalacturonate	47	1.1
Tetragalacturonate	50	2.0
Pentagalacturonate	52	2.8
Hexagalacturonate	52	2.9
PGA III*	23	3.2
PGA II†	14	3.1
PGA I‡	5	2.1
Pectate§	2	1.0

* Assuming D.P. = 13 (D.P.-degree of polymerization). † Assuming D.P. = 20. ‡ Assuming D.P. = 79. § Assuming D.P. = 201. ¶ The absolute maximum velocity for pectate was 0.60 μmol reducing groups per hr per 0.1 ml of PG II. Values are expressed relative to pectate.

Table 2. The products of PG II action on three oligogalacturonates and PGA III

	Substrate			
	OGA IV (mg)	OGA V (mg)	OGA VIII (mg)	PGA III (mg)
Galacturonate	0.90	1.21	1.70	1.83
OGA* II	0.09	0.14	0	0
OGA III	2.53	0.45	0.12	0
OGA IV		3.70	0.14	0.05
OGA V			0.20	0.08
OGA VI			2.60	0.14
OGA VII			5.20	0.30
OGA VIII				0.84
OGA IX†				1.50

* OGA = oligogalacturonate. † This product was eluted after OGA VIII and was assumed to be nonagalacturonate.

0.5% pectate at pH 5.5 in the absence of added Ca^{2+} was reduced 26% with the release of 0.3 μmol reducing groups per ml during 2 hr of reaction. PG II did not release water-soluble pectin from washed pear cell walls. It did catalyze a slow solubilization of pectin from tomato cell walls optimally at pH 5.

The kinetic parameters were determined for PG II acting on a series of substrates ranging from trigalacturonate to pectate (Table 1). The hydrolysis of digalacturonate was too slow to be measured accurately in the presence of the high substrate blank, and those data were therefore not included. The maximum velocity was highest for PGA III, but decreased gradually with increasing or decreasing chain length of the substrate. PG II exhibited highest affinity for pectate, the largest substrate.

The rates of hydrolysis by PG II for sodium borohydride-reduced PGA II and PGA III [9] were identical to those for the normal substrates. This permitted the use of substrates modified at their normally reducing ends to identify the mode of PG II action. A reaction mixture (20 ml) containing 0.5% reduced PGA II, 0.5 mM $CaCl_2$ and PG II was incubated 2 hr at 37°. The solution was heated to stop the reaction and analyzed for reducing groups. The remaining solution was deionized with Dowex 50 (H^+) and acidified to pH 2. Two volumes EtOH were added, and the precipitate that formed was separated from the alcohol solution by centrifugation and dissolved in water. Analyses of the ethanolic and aqueous solutions showed that only 7% of the anhydrogalacturonic acid but most of the reducing groups (92%) were in the alcohol supernatant. The results indicate that PG II produces a low-MW reducing product from reduced PGA II but that the residual substrate remains nonreducing. Thus, the enzyme appears to attack the substrate molecule from the nonreducing end.

The nature of the products released by PG II action was determined by chromatography. Preliminary experiments with PC indicated that the only detectable product from PGA II hydrolysis was galacturonic acid. The quantitative and more sensitive ion-exchange method [8] was then employed with a number of oligogalacturonates and PGA III. The reaction mixtures contained 0.2 ml PG II concentrate and 12 mg substrate (pH 5.5) in a total volume of 6 ml of 0.05 M NaCl and 0.5 mM $CaCl_2$. After 2 hr at 37°, the solutions were heated in boiling water, cooled and applied to a 2.5 × 40 cm column of DEAE-Sephadex A-50 in water (pH 6). The column was eluted with a linear gradient of 0.05 to 0.20 M NaCl. The fractions were analyzed for AGA by the carbazole method. The peaks were identified by comparison with those in the elution patterns of standard galacturonic acid and oligogalacturonates.

The results (Table 2) confirmed that galacturonic acid was the primary product of PG II action. Furthermore, the major fragment of each substrate remaining after 2 hr of reaction was the next lower oligomer. Smaller fragments were formed much more slowly, and digalacturonate was completely absent in hydrolyzates of OGA VIII and PGA III. Clearly, PG II catalyzed the stepwise removal of monomer units from the substrates, and was, therefore, an exopolygalacturonase.

DISCUSSION

Pectic substances are generally assumed to be involved in textural changes associated with ripening of fruits. McCready and McComb [4] presented evidence showing that ripening of pears is in fact accompanied by degradation of these polysaccharides. The extractability of pectin was about two times as great in ripe as in unripe pears. Furthermore, the MW's of the solubilized pectin from ripe pears were lower, based on the difficulty in the precipitation of the pectin with ethanol. They did not detect polygalacturonase in unripe pears, but they found considerable activity in ripe pears and attributed the hydrolysis of the pectic substances to this enzyme.

We have shown that the polygalacturonase activity in pears consists of two enzymes. One of the enzymes cleaves the pectate chain randomly, judging from the rapid reduction of the viscosity of substrate solutions during enzymatic action. The other enzyme hydrolyzes galacturonans one monomer unit at a time from the nonreducing ends of the molecules. This system of endo- and exo-polygalacturonases is similar to that described for peaches [9]. A role for an endopolygalacturonase in pear softening is reasonable because this enzyme has a pronounced effect on the size of pectate molecules and hence their contribution to cell wall rigidity. If pectin molecules are responsible for the integrity of cell wall, their degradation by an endopolygalacturonase, following deesterification by pectinesterase, would lead to tissue softening.

The function of an exopolygalacturonase in fruit softening, on the other hand, is less obvious and has been questioned previously [8, 10]. It is possible that this enzyme simply completes the hydrolysis of pectate initiated by the endopolygalacturonase in a mechanism involving turnover of the pectic substances. The complete degradation and utilization of certain cell wall polysaccharides may accompany the softening process. Evidence against the participation of exopolygalacturonase in such a mechanism is its affinity for large substrates and its slow hydrolysis of short oligogalacturonates. PG II is thus different from microbial exopolygalacturonases which hydrolyze oligogalacturonates much faster than pectate [11, 12]. Furthermore, complete hydrolysis of a macromolecular component of the cell wall should not be necessary for tissue softening. Changes in cell wall structure are difficult to understand, in part, because details such as how pectin is attached to other cell wall components remain unknown. Keegstra *et al.* [13] have provided evidence that, in suspension-cultured sycamore cells, at least some of the pectic polysaccharides are connected to the hydroxyproline-rich cell wall protein. They suggested that the connection involves short chains of arabinogalactan or the tetra-arabinosides that are attached to the hydroxyproline residues. The role of exopolygalacturonase in fruit softening may be involved in cleavage of linkages between the pectic polysaccharides and the protein rather than in degradation of pectin itself.

EXPERIMENTAL

Extraction of polygalacturonase. Because the activity was associated with the cell walls, isolation was facilitated by washing the residue with H_2O before extraction with salt soln. Tissue of ripe D'Anjou pears (100 g) was sliced and blended in 100 ml cold H_2O containing 12 g Carbowax 4000 and 0.2% $Na_2S_2O_5$. Nine other samples were prepared, combined, and blended again. Slurry was centrifuged at 8000 *g* for 20 min at 2°, and supernatant discarded. Sediment was dispersed in 2 l. cold H_2O containing 0.2% $Na_2S_2O_5$ and centrifuged. This washing procedure was repeated once and the insoluble residue was then suspended in 2 l. of 0.5 M NaCl. This mixture was stirred in the cold for 2 hr and centrifuged. The supernatant was concentrated to 30 ml by ultrafiltration with a PM-10 membrane.

Chromatography of pear polygalacturonase on Sephadex G-100. The enzyme concentrate (30 ml in 0.15 M NaCl) was applied to a column of Sephadex G-100 (5 × 90 cm) equilibrated with 0.15 M NaCl. The column was eluted with 0.15 M NaCl, and the fractions (20 ml each) were assayed for polygalacturonase activity.

Polygalacturonase assays. The reaction mixture for the reductometric method consisted of 0.2 ml enzyme in 0.15 M NaCl, 0.2 ml 0.2 M Tris–acetate buffer, 0.1 ml 0.01 M $CaCl_2$ and 0.5 ml 1% substrate (usually PGA I). The pH of the buffer and substrate solns depended on the enzyme being assayed. A blank was prepared for each sample by boiling the reaction mixtures before the addition of substrate. After 1 hr at 37°, the reactions were stopped by heating at 100° for 3 min, and 0.5 ml of each soln was analyzed for reducing groups by the arsenomolybdate method [14]. A unit of activity is defined as that amount which catalyzes the formation of 1 μmol of reducing groups per hr. The reaction mixture for the viscometric method was the same as above except that its total vol was increased to 10 ml, $CaCl_2$ was omitted, and the substrate was pectate. The soln, 5 ml, was transferred to an Ostwald viscometer and flow time was measured at 37°.

Other methods. Pectic acid was purified and the three polygalacturonates (PGA I, II, and III) were prepared as described earlier [7]. The oligogalacturonates were prepared by partial enzymatic hydrolysis of pectate and were purified by chromatography on DEAE-Sephadex A-50 [8]. Washed cell walls were prepared from green tomatoes and firm pears homogenized in 0.1 M NaPi, pH 7 [9]. Anhydrogalacturonic acid (AGA) was measured by the carbazole method [15].

REFERENCES

1. Weurman, C. (1954) *Acta Botan. Neerl.* **3**, 108.
2. Weurman, C. (1954) *Acta Botan. Neerl.* **3**, 180.
3. Weurman, C. (1953) *Acta Botan. Neerl.* **2**, 107.
4. McCready, R. M. and McComb, E. A. (1954) *Food Res.* **19**, 530.
5. Hobson, G. E. (1962) *Nature* **195**, 804.
6. Nagel, C. W. and Patterson, M. E. (1967) *J. Food Sci.* **32**, 294.
7. Pressey, R. and Avants, J. K. (1971) *J. Food Sci.* **36**, 486.
8. Pressey, R. and Avants, J. K. (1975) *Phytochemistry* **14**, 957.
9. Pressey, R. and Avants, J. K. (1973) *Plant Physiol.* **52**, 252.
10. Pressey, R. and Avants, J. K. (1975) *J. Food Sci.* **40**, 937.
11. Mill, P. J. (1966) *Biochem. J.* **99**, 557.
12. Mill, P. J. (1966) *Biochem. J.* **99**, 562.
13. Keegstra, K., Talmadge, K. W., Bauer, W. D. and Albersheim, P. (1973) *Plant Physiol.* **51**, 188.
14. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
15. Rouse, A. H. and Atkins, C. D. (1955) University of Florida Agricultural Experiment Station, Gainesville, Bull. 570.