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Polygalacturonases Release Cell-Wall-bound Proteins[1]

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ABSTRACT

Purified polygalacturonases from two fungi released proteins from wall fractions prepared from three plant species. Peroxidase activity was associated with the proteins released from the cell walls, and several of the protein fractions released contained hydroxyproline. Cellulase, purified free of pectic enzyme activity, was ineffective in releasing cell wall proteins. Specific inhibition of endopolygalacturonase activity prevented release of the proteins.

Endopolygalacturonases and endopectate lyases, enzymes that degrade the galacturonate polymers of plant cell walls, cause cell death when applied to excised tissues (3, 4, 13, 20), leaves and cuttings (5, 13), and intact plants (14). Peroxide appears to be involved in the endoPG[2]-induced damage to cotton leaf tissue (13). Peroxidases are associated with plant cell walls (2, 16, 17, 24), and are released from isolated cell walls by purified endopectate lyase (3) and purified endoPG (22). Release of peroxidases from cell walls may be important in the endoPG-induced damage of some plant tissues. In addition, the release of proteins by pectic enzymes is important to our understanding of the localization of proteins in plant cell walls and could be a means of obtaining cell wall proteins in a relatively native state. We have studied the release of wall proteins using cell walls from three plant species and purified pectic enzymes from two fungi.

MATERIALS AND METHODS

Cell Wall Preparation. A modification of Barnett's technique (2) was used to prepare cell walls from potato tuber tissues, carrot xylem parenchyma, and etiolated cotton hypocotyls. Potato tubers and carrots were purchased locally. Cotton hypocotyls were grown in the dark for 8 days in Jiffy-Mix (Jiffy Products of America, West Chicago, Ill.) at 26 to 29 C. Excised tissues were homogenized in a chilled Sorvall Omni-Mixer for 2 min in 0.1 M sodium phosphate, pH 7.4, containing 1% (v/v) 1-octanol. Homogenized material was rinsed on Miracloth with several liters of ice-cold deionized H_2O, suspended in 100 ml of the phosphate buffer containing 2 M NaCl, and allowed to stand for at least 30 min at 4 C. The cold water rinse was repeated, and the osmotically shocked material was passed through a prechilled French pressure cell at 10,000 to 12,000 p.s.i. The homogenization, rinse, salt treatment, and rinse steps were then repeated to produce the final cell wall preparation. Thorough rinsing on Miracloth removed starch grains from the potato tuber cell walls.

Enzyme Purification. EndoPG and exoPG (poly, α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15) from *Verticillium albo-atrum* Reinke and Berth. were purified to homogeneity as described previously (15). Cellulase (β-1,4-glucan glucanohydrolase, EC 3.2.1.4) from culture fluids of *V. albo-atrum* grown on cotton cell walls in a described medium (15) was purified until free of PG and endopectate lyase activity by twice chromatographing on CM-Sephadex and isoelectric focusing in a pH 3 to 10 gradient according to previous methods (15). EndoPG from *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. and Hans. was purified by chromatography on DEAE-Sephadex, CM-Sephadex, and hydroxyapatite (Bio-Rad HTP) according to described techniques (21, 23), then by gel filtration on Sephadex G-75 and isoelectric focusing in a pH 5 to 8 gradient (LKB Ampholine). The *Fusarium* endoPG consisted of a mixture of charge isomers (21, 23). None of the purified cell-wall-degrading enzymes contained peroxidase activity. The purified endoPGs did not release reducing groups (21, 23) from araban or arabic acid, did not reduce the viscosity of carboxymethyl cellulose, had no measurable activity on *p*-nitrophenyl-β-galactoside, and did not solubilize azure blue from a hide powder-azure blue complex.

EndoPG Inhibitor. A protein which specifically inhibits the enzymic activity of endoPG was purified from red kidney bean hypocotyls according to the methods of Anderson and Albersheim (1).

Enzyme Assays. PG activities were quantified viscometrically using 1.5% (w/v) sodium polypectate (Sunkist No. 6024) in 20 mM phosphate buffer, pH 6, for the *Verticillium* endoPG and pH 5.2 for *Verticillium* exoPG and *Fusarium* endoPG. Cellulase activity was quantified viscometrically using 1.0% (w/v) carboxymethyl cellulose (Cellulose Gum type 7MF, Hercules Powder Co.) buffered at pH 5 in 20 mM phosphate. Relative viscometric units were calculated as previously described (13).

Soluble and particulate peroxidase activity were measured using the guaiacol assay reported by Barnett (2). Absorbance at 470 nm was measured with a Beckman model DBG spectrophotometer, continuous recordings being used for the soluble assays. Activity was calculated as nmol H_2O_2 reduced using published extinction coefficients for tetraguaiacol (2) and assuming that tetraguaiacol was the product formed, with 1 molecule of H_2O_2 being reduced for each molecule of guaiacol oxidized (18).

Malate dehydrogenase activity was determined according to the Sigma procedures (Sigma Technical Bulletin No. 340UV). Wall-bound MDH was determined by incubating at 30 C with shaking a known amount of wall material in 0.1 M phosphate, pH 7.4, containing NADH, and measuring A of filtered aliquots at 340 nm after addition of oxaloacetate. Amounts of reagents used were as described for the soluble assay. Both soluble and wall-bound MDH activities were calculated according to Sigma.

Cell Wall Incubation. Cell wall preparations were suspended in 3 ml of 10 mM sodium phosphate for each gram fresh weight of starting tissue; pH 6.0 for incubation with *Verticillium* endoPG and cellulase, pH 5 for incubation with *Verticillium* exoPG and *Fusarium* endoPG. The appropriate enzyme was

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[2] Abbreviations: endoPG: endopolygalacturonase; exoPG: exopolygalacturonase; RVu: relative viscometric unit; MDH: malate dehydrogenase.

added to 5 ml of suspension to establish final enzyme concentrations of 200 RVu/ml for the endoPGs and cellulase, and 20 RVu/ml for the exopolygalacturonase (exoPG). The lower viscosity reducing activity of the exoPG had been determined to represent an amount of activity equivalent to the *Verticillium* endoPG in potential bond hydrolyzing activity (22). Control solutions received appropriate volumes of 20 mM sodium phosphate, pH 5, the buffer solution of enzyme preparations. Suspensions were incubated with shaking for 3 hr at 30 C and filtered with rinsing through sintered glass funnels to remove particulate wall material. Peroxidase activity, protein, and hydroxyproline in the filtrates were quantified and related to the dry weight of the cell walls used in the incubation.

Protein and Hydroxyproline Assays. Soluble protein was measured according to Hartree (6), using BSA as a reference standard. For amino nitrogen and hydroxyproline determinations, filtrates, enzyme solutions, and wall suspensions were hydrolysed in 6 N HCl in tubes sealed after thorough sparging with N_2. Filtrates and enzyme solutions were hydrolyzed for 18 hr, walls for 24, 48, and 72 hr at 105 to 110 C. Amino nitrogen was determined according to Lee and Takahashi (9) with L-leucine as a standard, and multiplied by 6.25 to convert to "protein." Hydroxyproline in the hydrolysates was measured according to Stegemann and Stalder (19).

Electron Microscopy. Cell walls were stabilized in 6% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4 C for 3 hr, then rinsed in the buffer overnight. The walls were then stained with 1% (w/v) OsO_4 for 4 hr at 4 C, rinsed in the buffer, and pelleted at 10,000*g* for 15 min. The pellet was embedded in agar and dehydrated through an acetone series. The agar block containing the cell wall pellet was then embedded in Spurr low viscosity embedding medium. Thin sections of the cell walls were examined using a Zeiss EM-9 2A electron microscope.

RESULTS

Cell Walls. Isolated cell walls from all three plant species contained bound peroxidase activity. Incubation of these fractions with endoPG released peroxidase activity from all three cell wall fractions. Specific inhibition of endoPG activity by a protein from bean hypocotyl cell walls inhibited the release of the peroxidases, but did not affect *in situ* peroxidase activity (Fig. 1).

Electron microscopy was used to check visually the cell wall fractions for cytoplasmic or membrane contamination. None was seen. Malate dehydrogenase activity was used as an enzymic marker to check for cytoplasmic contamination of the wall fractions. Recent results indicated that MDH is not found in plant cell walls (24). Little MDH activity was found associated with the cell wall fractions (Table I), about 1% of the total activity in cotton hypocotyls, and less than 0.1% of the total activity found in carrot xylem parenchyma and potato tuber tissue. There was no correlation between MDH activity and the levels of either bound peroxidase activity or peroxidase activity released by treatment with pectic enzymes. These results indicate that the observed endoPG-induced release of peroxidase activity from plant cell walls was not an artifact related to cytoplasmic contamination of the wall preparations.

Release of Cell Wall Proteins by Pectolytic Enzymes. Results of the release studies are summarized in Figure 2. All three wall preparations contained protein (1-2% of the dry weight), and hydroxyproline (0.05-0.15% of the dry weight). Both protein and peroxidase activity were released from all three cell wall fractions by the endoPGs of *Verticillium* and *Fusarium*. Except for cotton hypocotyl cell walls, the proteins released by both endoPGs contained hydroxyproline. That portion of cotton hypocotyl cell wall protein that is susceptible to release by endoPG is apparently not rich in hydroxyproline, whereas the total wall protein is (Fig. 2). By comparison with the specific activity we obtained for a commercial horseradish peroxidase (Worthington) of 5,000 nmol H_2O_2 reduced/min · μg protein, the proportions of released cell wall protein that are peroxidases would be about 20% from potato tuber walls, about 0.5% from cotton hypocotyl cell walls, and about 0.001% from carrot xylem parenchyma cell walls. This suggests that peroxidases represent a highly variable, but small, fraction of the wall protein released by endoPG.

ExoPG did not release measurable protein, hydroxyproline, or peroxidase activity from cotton or carrot walls. However, this enzyme did release peroxidase activity, without measurable protein or hydroxyproline, from potato tuber cell walls. These results further emphasize the diversity in the composition of the cell walls of higher plants, and the complexity of the specific linkages which position proteins within these walls. Cellulase did not release measurable protein, peroxidase, or hydroxyproline from any of the cell walls.

When the specific inhibitor of endoPG from red kidney bean hypocotyls was added with *Verticillium* endoPG to incubating cell walls, no peroxidase, protein, or hydroxyproline was released from any of the cell walls. The purified inhibitor did not inhibit the ability of *Verticillium* exoPG to release peroxidase from potato tuber cell walls, nor did it inhibit the activity of cell-wall-bound peroxidases (Fig. 1). Addition of the purified endoPG inhibitor at various times to cell walls incubating with endoPG stopped subsequent release of peroxidase activity (Fig. 3), further supporting the conclusion that endoPG activity is responsible for the observed solubilization of peroxidases. Addition of 100 μg histone to incubating potato cell walls did not result in solubilization of peroxidase activity, suggesting that the observed release by endoPG is not due to a charge effect of the highly positively charged *Verticillium* endoPG (15).

DISCUSSION

Release of proteins from particulate plant cell wall fractions by pectic enzymes indicates that galacturonide segments are important links between cell wall protein and the remainder of the particulate cell wall. It cannot be concluded from these studies that galacturonide residues are directly linked to wall proteins, only that rupture of the galacturonide linkages is sufficient to release these proteins from the cell wall matrix. In all cases studied, less than half of the total measured cell wall protein was released by pectic enzyme activity, suggesting that not all cell wall proteins are held in place by a matrix involving galacturonide groups susceptible to pectolytic hydrolysis. The endoPG-solubilized proteins may be related to the uronide-rich fraction of hydroxyproline protein recently isolated from tomato cell walls (12).

Hydroxyproline may not be a component of all wall-bound proteins: the peroxidase activity released from cotton hypocotyl cell walls contained no measurable hydroxyproline, and it has been reported that cell-wall-bound horseradish peroxidase does not contain hydroxyproline (10). The proteins released from the different cell walls varied in their peroxidase activity and hydroxyproline content. The qualitative and quantitative character of the proteins solubilized also varied with the nature of the pectic enzyme used for the digest. This variation in proteins obtained suggests that the linkages which attach proteins to the cell wall matrix vary in either the composition of the linkage carbohydrates, or accessibility of these linkages to pectolytic enzymes.

Solubilization of peroxidase activity from plant cell walls may be important in the endoPG-induced damage to plant cells in systems such as cotton, where peroxide appears to be involved (13). ExoPG, which did not release peroxidase from cotton cell walls, causes no damage to these tissues (13). A highly purified

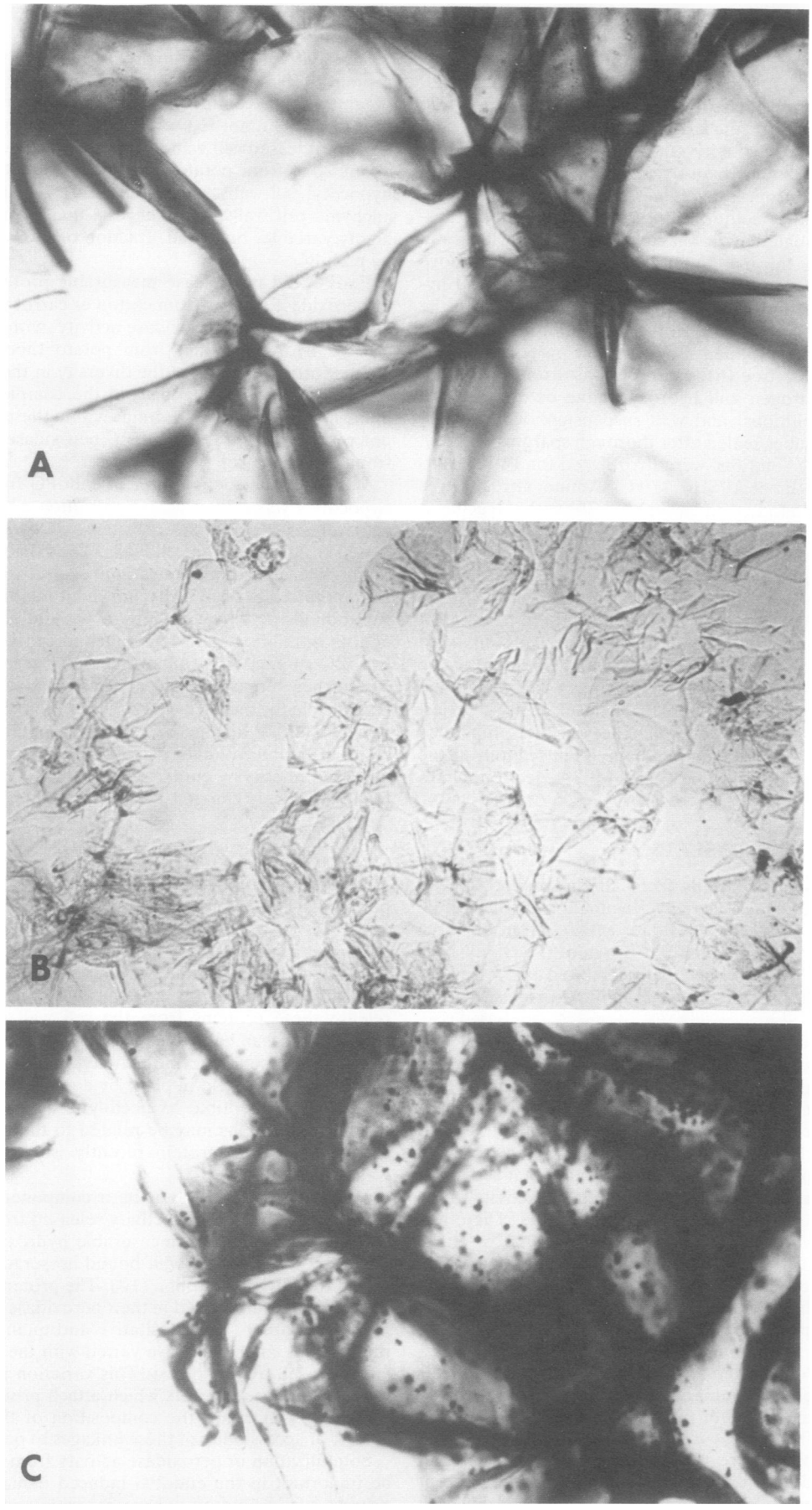

FIG. 1. Potato tuber cell walls stained for peroxidase activity (dark areas on walls). Wall material was incubated with guaiacol assay medium (2) in the dark, centrifuged, and the pellet of stained walls was three times resuspended in H_2O and centrifuged to remove unreacted guaiacol. ×100. A: Walls incubated for 3 hr with buffer; B: walls incubated for 3 hr with *Verticillium* endoPG; C: walls incubated for 3 hr with *Verticillium* endoPG and bean hypocotyl endoPG inhibitor. Particulate material in C is tetraguaiacol.

Table I. Cytoplasmic and Wall-Bound Malate Dehydrogenase (MDH) and Peroxidase Determined During Cell Wall Preparations

Source of Cell Walls	Malate Dehydrogenase[1]		Peroxidase[2]		
	"Cytoplasmic"[3]	Wall-Bound	"Cytoplasmic"[3]	Wall-Bound	Solubilized by endoPG[4]
Potato tuber	1250	0.4	38	800	1390
Cotton hypocotyl	170	2	250	74	49
Carrott xylem parenchyma	450	0.4	9	2.3	0.4

[1]MDH units per mg cell wall.

[2]Peroxidase activity: nmoles hydrogen peroxide reduced per min per mg cell wall

[3]"Cytoplasmic" = total of activities in octanol and salt washes during cell wall preparation

[4]Verticillium endoPG treatment was according to methods in text.

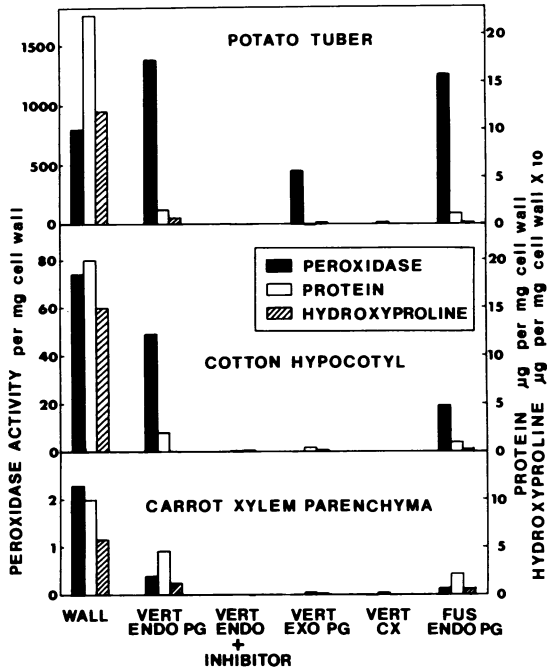

FIG. 2. Release of peroxidase activity, protein, and hydroxyproline from cell walls. Wall protein and hydroxyproline are averages of values obtained after 24, 48, and 72 hr of hydrolysis.

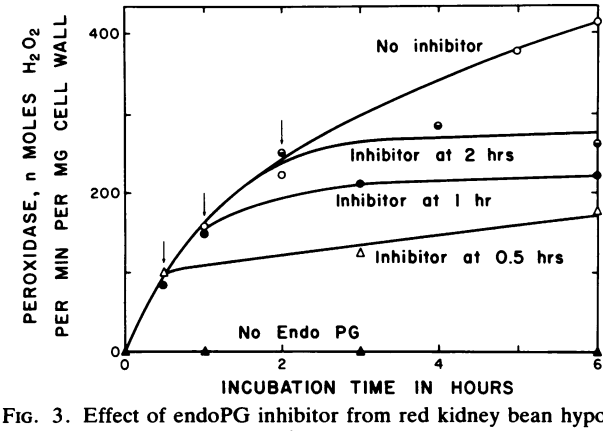

FIG. 3. Effect of endoPG inhibitor from red kidney bean hypocotyl on release of peroxidase activity from potato tuber cell walls by *Verticillium* endoPG. Arrows indicate points of addition of endoPG inhibitor.

endopectate lyase has also been demonstrated to cause cell damage (3). In this system, peroxide does not seem to be involved, although the endopectate lyase does have the ability to release peroxidase from plant cell walls (3, 4). It is also probable that enzymic activities other than peroxidase are released by endoPG activity (11), because the proportion of the protein released that is peroxidase is apparently small.

A cellulase purified to contain no pectic enzyme activity did not release protein or peroxidase activity from any of the cell walls tested. Recent studies have reported the release of peroxidase from cell walls by cellulase (10, 24). These studies used commercial cellulases that often contain contaminating PG activity (Mussell, unpublished). A purified cellulase from *Colletotrichum* would degrade isolated cell walls only after the cell walls were pretreated with endoPG (7).

Recent studies suggest a role for peroxide in rendering cellulose more susceptible to degradation (8). If the peroxidase activity released from cell walls by PG activity affects cellulose similarly to the reported H_2O_2-Fe^{2+} systems, endoPGs could initiate degradation of cell wall polysaccharides far beyond the effects of hydrolysis of uronide polymers. Pectolytic enzymes could, therefore, play a much more important role than expected in the degradation of plant tissues.

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