Inhibition of *Penicillium expansum* Polygalacturonase Activity by Increased Apple Cell Wall Calcium

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We acknowledge the valued assistance of G. A. Brown and J. N. Livsey, Biological Laboratory technicians.
Accepted for publication 29 February 1988.

ABSTRACT

Conway, W. S., Gross, K. C., Boyer, C. D., and Sams, C. E. 1988. Inhibition of *Penicillium expansum* polygalacturonase activity by increased apple cell wall calcium. Phytopathology 78:1052-1055.

Apples were pressure infiltrated at harvest with solutions of $CaCl_2$ and stored at 0 C. After 6 mo, fruit were removed from storage and cell walls were extracted and analyzed for Ca content. Polygalacturonase was purified from the decayed area of nontreated apples that had been inoculated with *Penicillium expansum*. Cell wall Ca content was positively correlated with the percent Ca used in the infiltration solutions. Extracted walls with varying Ca content were then used as substrate for *P. expansum* polygalacturonase to test the effect of wall Ca on in vitro enzyme activity. Approximately 60% less product was formed when high Ca cell walls were used as substrate compared with low Ca cell walls. Since Ca is known to stabilize the cell wall, decay in apples with high levels of Ca may be decreased because maceration by polygalacturonase is reduced.

Increasing calcium (Ca) content of apples with preharvest sprays reduces postharvest losses attributed to decay (16). More recent investigations have shown that when the Ca content of apples was increased by postharvest infiltration of Ca solutions, postharvest decay caused by *Penicillium expansum* Link ex. Thom was reduced (4,6). These postharvest treatments not only increased total Ca in the fruit, but the cell wall-bound Ca increased as well (5). In earlier work with *Rhizoctonia solani* Kühn in bean hypocotyl tissue, it was found that young, susceptible bean hypocotyls had a low Ca content, while older, more resistant hypocotyls had a high Ca content. It was concluded that the increased number of salt bridges in maturing hypocotyls could account for resistance of older tissue to maceration by fungal polygalacturonase and for the observed resistance to the pathogen (3). More recently, increases in the concentration of Ca in nutrient solutions supplied to potato plants resulted in tubers with increased cell wall Ca concentration. High Ca tubers, when injected with pectolytic enzyme preparations from *Erwinia carotovora* pv. *atroseptica* (van Hall) Dye were more resistant to tissue breakdown than low Ca tubers. These results also indicated that since Ca improved the structural integrity of the cell wall, Erwinia soft rot may be reduced in high Ca tubers because maceration by pectolytic enzymes is reduced (13).

Because increased Ca in plant tissues, resulting from either natural maturation or nutrient regimes, is involved in resistance to maceration by pectolytic enzymes, the objective of this investigation was to determine if Ca applied exogenously to apples would induce resistance in fruit tissue to polygalacturonase (PG) produced by *P. expansum*.

MATERIALS AND METHODS

Calcium infiltration and inoculation of fruit. Apples of the cultivar Golden Delicious (*Malus domestica* Borkh.) were harvested from a commercial orchard in Pennsylvania. The fruit were randomized and infiltrated under 68.95 kPa of pressure for 2 min with distilled water or calcium chloride ($CaCl_2$; 99%) made up as 1, 2, or 4% solutions in distilled water. After treatment, fruit were allowed to drain for 2 hr before storage at 0 C. After 6 mo, the length of time this cultivar might be typically kept in cold storage, fruit were removed from storage and wound inoculated by dipping in a conidial suspension (104 spores per milliliter) of *P. expansum* as previously described (4).

Cell wall extraction and Ca determination. Ca content of the apple cell walls from similarly infiltrated but uninoculated fruit was determined after removing the peel and outer flesh to a depth of 2 mm with a mechanical peeler. The next 3 mm of flesh was then removed, immediately frozen in liquid nitrogen, and lyophilized. This layer of tissue was used for Ca analysis because this was the depth to which the apples were punctured for inoculation. Cell walls were extracted according to a modification of a procedure previously described (11). Lyophilized apple tissue was homogenized in 80% ethanol for 1 min with a polytron homogenizer (Brinkman Instruments). After we filtered the homogenate through Miracloth (Calbiochem), the residue was suspended in 20 mM HEPES-NaOH (pH 6.9), mixed well, and filtered twice through Miracloth. The resulting residue was then suspended in a solution of 20 mM HEPES-NaOH (pH 6.9) containing 1 mg/ml of α-amylase (Sigma Type I-A). One drop of toluene was added, and the mixture held at 37 C for 18–24 hr with constant shaking. The solution was filtered through Miracloth and the residue washed with 20 mM HEPES-NaOH (pH 6.9). The residue was suspended in phenol/acetic acid/H_2O (2:1:1, w/v/v) for 10 min with occasional stirring to inactivate and extract endogenous wall-associated enzymes (12,15). This suspension was filtered through a sintered glass filter and the residue suspended in chloroform-methanol (1:1 v/v) for at least 10 min. During this period, the suspension was homogenized gently for 30 sec with a polytron homogenizer and then filtered through a sintered glass filter. The residue was washed with chloroform-methanol and suspended in acetone for 10 min, filtered, and washed with excess acetone. Cell wall material was dried over P_2O_5 in vacuo at 25–37 C to a constant dry weight. Dried cell walls (125 ± 5 mg) were ashed at 500 C overnight and the residue dissolved in 5 ml of 2 N HCl and analyzed for Ca content with an Instrumentation Laboratory spectrophotometer (Wilmington, MA). All Ca values are reported on a dry-weight basis. Four samples were analyzed for each treatment. Galacturonic acid content was determined by dissolving 10 mg of wall material in concentrated H_2SO_4 (1) and assaying aliquots for galacturonic acid using carbazole (8). Both pathogenicity and cell wall experiments were repeated.

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Enzyme preparation. PG was extracted from nontreated Golden Delicious apples inoculated with *P. expansum*. All extraction procedures were carried out at 4 C. Fruit were wound inoculated with a conidial suspension of *P. expansum* as previously described (4). When the surface diameter of the decayed area reached 30 mm, the peel over the lesion was removed, the center 15 mm in diameter of the decayed area was removed with a cork borer and discarded, and the outer area of the lesion was the portion collected for enzyme extraction to ensure relatively high PG activity. The decayed tissue was homogenized for 1 min in 1 N NaCl containing 5% (w/v) polyvinyl-polypyrrolidone (Sigma Chemical Co.) in 5 mM MES buffer (pH 6.0) with a polytron homogenizer. The homogenate was adjusted with 2 N NaOH and maintained at pH 6.0 for 30 min with constant stirring. It was subsequently filtered through four layers of cheesecloth with the filtrate further clarified by centrifugation, and the supernatant was collected. Solid ammonium sulfate was added to the stirred supernatant, and the fraction that precipitated between 30 and 90% saturation was collected by centrifugation. This pellet was dissolved in 0.15 N NaCl and loaded onto a Sephacryl S-200 column (2.5 × 52 cm) equilibrated with 0.15 N NaCl. The enzyme was eluted with the equilibration buffer at 30 ml/hr, and 3.75-ml fractions were collected. Following this elution, those fractions exhibiting PG activity were combined and dialyzed overnight against 20 mM MES buffer (pH 6.0). This enzyme solution was fractionated further by CM-Sephadex (Pharmacia C-25, cation exchanger) equilibrated with 20 mM MES buffer (pH 6.0). The PG solution was placed on the column (1.6 × 9.8 cm) and washed with the equilibration buffer. The PG was eluted with a linear gradient (30 ml/hr) of 20 mM MES to 20 mM MES plus 0.7 M NaCl, pH 6.0, and collected in 4.5-ml fractions.

Enzyme assay. PG activity was assayed by measuring the increase in reducing sugars with 2-cyanoactamide (10). Reaction mixtures contained 100 μl of 0.4% citrus polygalacturonic acid (Sigma Chemical Company) (washed with 80% methanol before use) in 100 mM sodium acetate (pH 5.5), 50 μl of distilled water, and 50 μl of the enzyme preparation. For quantifying released reducing groups with 2-cyanoacetamide, reactions were terminated after a 20-min incubation period at 30 C by adding 1 ml of cold 100-mm borate buffer (pH 9.0). Then, 200 μl of 1% 2-cyanoacetamide (Aldrich Chemical Co.) were added, and the samples were mixed and immersed in a boiling water bath for 10 min. After equilibration to 25 C, the absorbance at 276 nm was determined.

Optimum buffer and pH determination. Optimum buffer and pH for enzyme activity were determined by preparing 0.4% citrus polygalacturonic acid in the following buffers: 100 mM sodium acetate, pH 4.0–7.0; 100 mM sodium citrate, pH 4.0–7.0; and 100 mM MES, pH 4.5–7.0. The amount of reducing sugar released was determined using 2-cyanoacetamide according to the protocol described above.

The protein content of the enzyme was determined using bicinchoninic acid (17). Bovine serum albumin was used as a standard.

Molecular weight determination. The molecular weight of the PG was estimated using Sephacryl S-200 gel filtration. The molecular weight standards, ribonuclease A (13,700), chymotrypsinogen (25,000), bovine serum albumin (67,000), catalase (232,000), and thyroglobulin (669,000), were obtained from Pharmacia Fine Chemicals.

Cell wall maceration. The effect of increasing apple cell wall Ca content on enzyme activity was determined by using cell walls, prepared as above, and containing various amounts of Ca, as a substrate for PG produced by *P. expansum*. A reaction mixture containing 5 mg of cell walls, 1 ml of sodium acetate buffer, pH 5.5, and 1 ml of PG (20 units of enzyme activity; one unit of enzyme activity releases 1 nmol of galacturonic acid per minute) was allowed to proceed for 10 min with constant shaking at 30 C. The reaction was terminated by filtering the mixture through a Millex-HV 0.45-μm filter (Millipore) into a test tube partially immersed in a boiling water bath. A 250-μl aliquot was then assayed for galacturonic acid content using carbazole to determine the amount of product formed as a result of PG activity. Four samples were analyzed per treatment, and the experiment was repeated.

RESULTS

The area of decay resulting from inoculation of fruit with *P. expansum* and its relationship to cell wall Ca concentration is shown in Figure 1. As cell wall Ca content increased, the resulting percent reduction in area of decay increased. Fruit treated with a 4% solution of $CaCl_2$ had approximately 60% less decay area than nontreated fruit, while fruit treated with 1 or 2% $CaCl_2$ solutions had significant but lesser reductions in area of decay.

The Ca content of the extracted cell walls (Table 1) is assumed to be mainly that bound to the cell wall since the extensive extraction procedure would probably remove any Ca not bound tightly to the wall. The Ca concentration of the nontreated fruit, representing the native cell wall Ca, was about 350 μg/g, which increased to about 1,900 μg/g in fruit treated with a 4% solution of $CaCl_2$. There was a definite increase in Ca bound to the cell wall as the Ca concentration of the solutions was increased.

Similar amounts of uronic acid were present in the cell walls of fruit treated with a 4% solution of $CaCl_2$ and in the nontreated fruit (Table 1). There were 337 mg/g of uronic acid in the cell walls of nontreated fruit and 371 mg/g in fruit treated with 4% $CaCl_2$, but the difference in uronic acid content was not significant.

PG was purified with ammonium sulfate, Sephacryl S-200, dialysis, and CM-Sephadex (Table 2). A single PG peak was separated on Sephacryl S-200, the molecular weight of which was estimated to be 47,000 daltons. After dialysis, a single peak was also separated on the CM-Sephadex column, which eluted at a concentration of 0.1 N NaCl. The purification procedure resulted in a 40% recovery of PG activity.

The optimum pH for PG activity in all buffers was 5.0–5.5; however, the optimum buffer-pH combination for greatest PG activity was the sodium acetate buffer at a pH of 5.5.

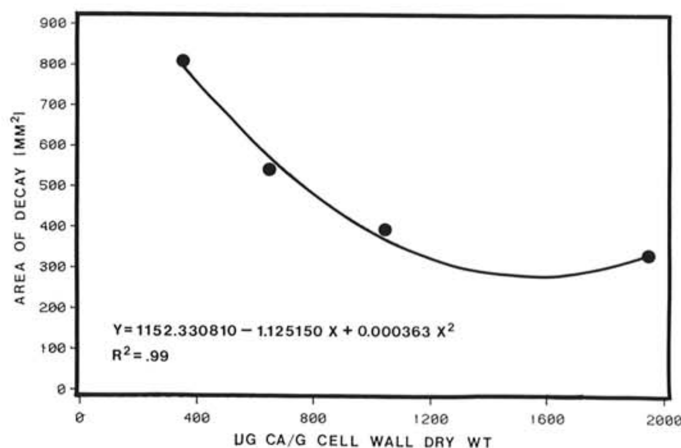

Fig. 1. Relationship between area of decay and calcium concentration of apple cell walls. Data points indicate the concentration of $CaCl_2$ solutions (0, 1, 2, or 4%) from left to right, respectively, with which the fruit were pressure infiltrated (68.95 kPa).

TABLE 1. Cell wall calcium and uronic acid content of Golden Delicious apples pressure infiltrated (68.95 kPa) with calcium chloride ($CaCl_2$) solutions at harvest[a]

Treatment (% $CaCl_2$)	Cell wall calcium content (μg Ca/g dry wt)	Cell wall uronic acid content (mg/g dry wt)
0	355 ± 31[b]	337 ± 20
1	652 ± 40	346 ± 20
2	1,043 ± 88	351 ± 20
4	1,944 ± 110	371 ± 30

[a] Samples were taken after 6 mo of storage at 0 C.
[b] Numbers represent the mean ± SE.

TABLE 2. Purification of polygalacturonase from apple inoculated with *Penicillium expansum*

Purification step	Activity (units/ml)[a]	Volume (ml)	Protein (μg/ml)	Specific activity (units/μg protein)
30–90% $(NH_4)_2SO_4$ Sat.	904	6.0	60.0	15.0
Sephacryl S-200	250	46.0	15.0	16.7
Dialysis	288	45.0	14.5	19.9
CM-Sephadex C-25	385	25.6	1.1	350.0

[a] One unit of enzyme activity releases 1 nmol of galacturonic acid per minute.

TABLE 3. Amount of uronic acid released by polygalacturonase (produced by *Penicillium expansum*) from cell walls extracted from Golden Delicious apples pressure infiltrated with calcium chloride ($CaCl_2$) solutions at harvest[a]

Treatment (%) $CaCl_2$)	Uronic acid released from cell wall by polygalacturonase[b] (μg)	% Reduction in product
0	39.33 ± 6.73[c]	...
1	28.91 ± 3.61	26
2	27.29 ± 3.37	31
4	14.63 ± 3.07	63

[a] Samples were taken after 6 mo of storage at 0 C.
[b] A reaction mixture containing 5 mg of cell walls, 1 ml of sodium acetate buffer, pH 5.5, and 1 ml of polygalacturonase (20 units of enzyme activity) was allowed to proceed for 10 min with constant shaking at 30 C.
[c] Numbers represent the mean ± SE.

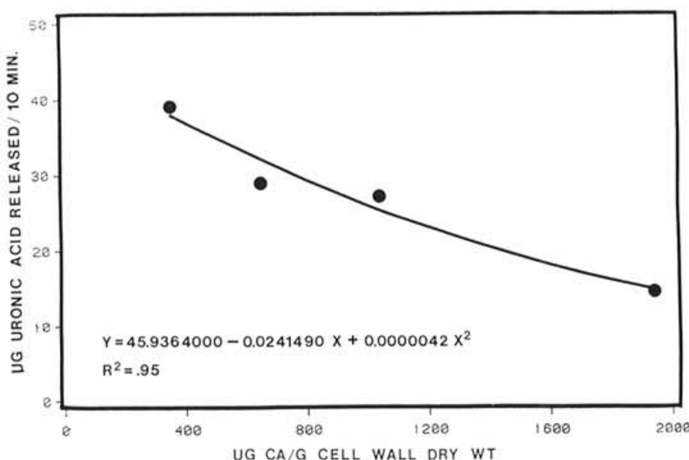

Fig. 2. Relationship between the calcium concentration of apple cell walls and the amount of uronic acid released by polygalacturonase (produced by *Penicillium expansum*) from cell walls extracted from Golden Delicious apples pressure infiltrated (68.95 kPa) with $CaCl_2$ solutions. Data points indicate the concentration of $CaCl_2$ solutions (0, 1, 2, or 4%) from left to right, respectively.

The results of the effect of cell wall Ca content on PG activity are given in Table 3. PG activity released 40 μg of uronic acid from the cell walls of the nontreated fruit, but only 15 μg from the cell walls treated with a 4% solution of $CaCl_2$. This was a reduction of approximately 60%. There is, then, a very good correlation ($R^2 =$ 95) between the Ca content of the cell walls and the amount of uronic acid released from these cell walls by PG activity (Fig. 2). There is also an excellent correlation ($R^2 = 96$) between the area of decay of the intact fruit, and the amount of uronic acid released from the cell walls by PG activity (Fig. 3).

DISCUSSION

The PG extracted in these studies was produced in vivo rather than in vitro because previous work showed a definite variability between enzymes produced by *P. expansum* in vivo compared with

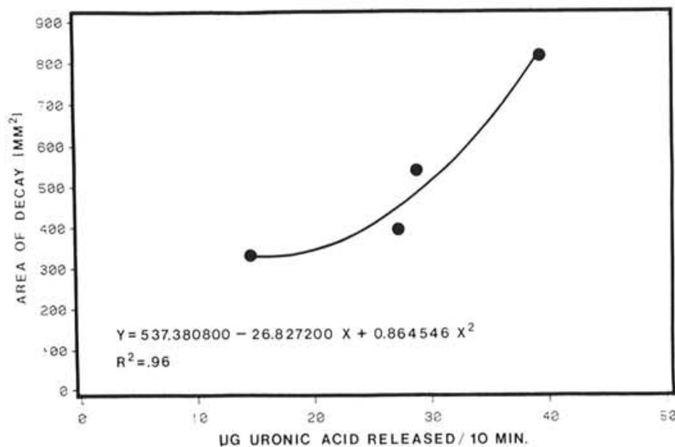

Fig. 3. Relationship between area of decay and amount of uronic acid released by polygalacturonase (produced by *Penicillium expansum*) from cell walls extracted from Golden Delicious apples pressure infiltrated (68.95 kPa) with $CaCl_2$ solutions. Data points indicate the concentration of $CaCl_2$ solutions (4, 2, 1, or 0%) from left to right, respectively.

that produced in vitro, possibly as a result of the extraction process (18). By using PG produced in fruit, there may be less possibility for changes from the native enzyme during the extraction procedure. The characteristics of this PG were similar in regard in molecular weight, pH, and buffer optima to PG produced by other *Penicillium* sp. (2,18).

The reduction in area of decay in apples caused by pressure infiltration of a 4% $CaCl_2$ solution compared with nontreatment was about 50–60% in this and a previous study (5), and the amount of increase in Ca content of the cell wall was similar as well (5). Because the amount of uronic acid released by the activity of PG was 60% less in the cell walls extracted from fruit treated with the 4% $CaCl_2$ solution, this amount of reduction in area of decay was most probably due to a reduction in the fungal PG activity.

The relationship between Ca ions and the cell wall has been shown to play a key role in disease resistance. Ca ions are bound to the pectins present in the cell wall (7). Few pectins, if any, are free of neutral sugars, notably rhamnose, and are composed of α-1-4-linked galacturonic acid residues in a chain with 2-linked rhamnose insertions in the chain (14). Rhamnose causes a marked kink in this chain. The resulting configuration of the polygalacturonic chain allows spaces for the binding between carboxyl groups of a series of cations. All binding sites may be filled because the binding of one ion causes chain alignment that facilitates binding of the next (9). The formation of cation cross bridges between pectic acids may make the cell wall less accessible to enzymes produced by fungal pathogens that cause decay (19).

The results of the study reported herein also support the conclusion that reduction in decay in apple caused by *P. expansum* is due, in part at least, to a decrease in maceration of cell walls by PG due to the improved structural integrity caused by an increase in Ca content. As the Ca content of the cell wall increases, enzyme activity decreases.

In conclusion, by increasing the amount of Ca in apple fruit, the level of defense in the apple to enzymatic tissue maceration is increased and decay and resulting storage loss is reduced.

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