Plant Physiol. (1989) 90, 191–196
0032-0889/89/90/0191/06/$01.00/0

Received for publication June 8, 1988
and in revised form January 9, 1989

Pectic Enzymes in Pectolyase

Separation, Characterization, and Induction of Ethylene in Fruits

Elizabeth A. Baldwin and Russell Pressey*

U.S. Department of Agriculture-Agricultural Research Service, Richard B. Russell Agricultural Research Center, P. O. Box 5677, Athens, Georgia 30613

ABSTRACT

The pectic enzymes in Pectolyase were separated by ion exchange chromatography on Q-Sepharose. Three pectin lyases, two polygalacturonases, and a pectinmethylesterase were resolved. The enzymes were further purified on Mono Q and/or Mono S columns to remove traces of cellulase. The enzymes had molecular weights ranging from 25,000 to 36,000 daltons. They were optimally active between pH 4.0 and 6.2 and were not greatly affected by ions. The pectin lyases and polygalacturonases were endo-enzymes. They solubilized uronic acids from washed cell wall fragments, but the lyases were much more effective than the polygalacturonases. The mixture of enzymes constituting Pectolyase increased ethylene production 15- to 25-fold when introduced into tomato and orange fruits. The enzymes purified from Pectolyase all increased ethylene production in the fruits but the lyases were generally more effective than the hydrolases.

Pectolyase is a commercially available enzyme mixture prepared from culture filtrates of *Aspergillus japonicus* (8, 13). These enzymes in combination with cellulases are very effective in degrading cell walls and thereby liberating protoplasts. For this reason, Pectolyase is widely used to generate protoplasts in cell culture research. There have been several attempts to identify the cell wall degrading enzymes in Pectolyase to explain the effectiveness of this preparation. Ishii and coworkers (10, 11) separated an endo-PG[1] (EC 3.2.1.15) and an endo-PL (EC 4.2.2.3) from the culture filtrates. They also obtained evidence that the solutions contained a maceration stimulation factor which enhanced the action of both enzymes on cell walls (9). The combination of enzymes able to degrade pectate and pectin plus the enzyme activator appeared to be the basis for the potency of Pectolyase in liberating protoplasts.

Our interest in the pectic enzymes in Pectolyase stems from the observation that purified tomato PG induced ethylene production and accelerated ripening when infiltrated into green tomato fruit (4). We wanted to expand these studies to microbial pectic enzymes and selected Pectolyase as a source. Attempts to separate the PL and PG (10, 11) revealed that the Pectolyase contained several isoenzymes of each. This paper describes the separation and properties of the enzymes and their effectiveness in inducing ethylene production in tomato and orange fruit.

[1] Abbreviations: PG, polygalacturonase; PL, pectin lyase; PME, pectinmethylesterase.

MATERIALS AND METHODS

Substrates

Citrus pectin (Sigma Chemical Co.) was purified by precipitation from a 1% aqueous solution by the addition of two volumes of ethanol, followed by washing with ethanol and acetone. Polygalacturonic acid was prepared from pectate (Sigma Chemical Co.) by partial hydrolysis as described earlier (15). Cell walls were prepared from the fruits of apple, nectarine, cucumber, and tomato and the roots of carrot and radish by homogenizing 100 g of tissue with 200 mL of 0.5 M sodium phosphate (pH 7.0). The insoluble material was collected by centrifugation and washed with 200 mL of phosphate buffer and then two times with 200 mL of water. The cell walls were then washed with ethanol and acetone and dried under vacuum.

Assay for Pectin Lyase

Pectin lyase was assayed by measuring the increase in absorbance at 235 nm according to the method of Albersheim and Killias (1). The reaction mixture consisted of 0.2 mL of 0.1 M sodium acetate, 0.25 mL of 0.15 M NaCl, 0.5 mL of 1% pectin, and 0.05 mL of enzyme solution diluted as necessary. The acetate buffer and pectin were adjusted to the pH optimum of each enzyme. After incubation at 37°C for 15 min, the reaction mixture was diluted with 5 mL of water, and the absorbance at 235 nm was determined. A unit of pectin lyase is defined as that amount that catalyzes an increase in absorbance of 1.0 at 235 nm in 15 min at 37°C.

Assay for PG

PG was assayed by measuring the liberation of reducing groups from polygalacturonic acid (16). The reaction mixture contained 0.2 mL of 0.1 M sodium acetate, 0.25 mL 0.15 M NaCl, 0.5 mL of 1% polygalacturonic acid, and 0.05 mL of enzyme solution. The buffer and substrate were adjusted to the pH optimum for each enzyme. After 15 min at 37°C, the solution was analyzed for reducing groups by the arsenomolybdate method (14). A unit of polygalacturonase is defined

as that amount which liberates 1 μmol of reducing groups in 1 min at these conditions.

Assay for PME

PME was assayed by measuring the liberation of acid groups from pectin. The reaction mixture consisted of 10 mL of 1% pectin (pH 5.5), 15 mL of 0.15 M NaCl and 0.1 mL of enzyme solution dialyzed against 0.15 M NaCl to remove buffers. The release of acid groups was measured with an automatic titrator at pH 5.5 for 5 min at 25°C. A unit of pectinmethylesterase is defined as that amount which yields 1 μeq of acid at these conditions.

Assay for Cellulase

Cellulase was assayed by measuring the release of reducing groups from carboxymethylcellulose. The reaction mixture consisted of 0.2 mL of 0.1 M sodium acetate (pH 5.0), 0.2 mL 0.15 M NaCl, 0.5 mL of 1% carboxymethylcellulose (pH 5.0), and 0.1 mL of enzyme solution. Reducing groups in the solutions were determined after 16 h at 25°C.

Solubilization of Cell Walls

The enzymes were evaluated for their abilities to solubilize uronic acids from cell wall preparations. The reaction mixture consisted of 20 mg of cell walls, 2.5 mL of 0.15 M NaCl, 1.0 mL of 0.1 M sodium acetate adjusted to the pH optimum of each enzyme, and 0.05 mL of enzyme solution. After 30 min incubation at 37°C in a shaker water bath, the samples were centrifuged and filtered. The filtrates were analyzed for uronic acids by the hydroxydiphenyl method (6).

Chromatographic Methods

The enzymes were separated on a 2.5 × 24 cm column of Q-Sepharose (Pharmacia, Inc.) equilibrated with 0.01 M Bis-Tris propane (pH 7.1). Elution was conducted with 1 L of a linear gradient of 0 to 1.0 M NaCl in 0.02 M Bis-Tris propane (pH 7.1). Fractions of 5 ml were collected. The enzymes were then purified by chromatography on a Mono S and/or Mono Q columns in a FPLC system (Pharmacia, Inc.). Elutions were conducted with linear gradients of NaCl in appropriate buffers at a flow rate of 1 mL/min and 0.5 or 1.0 mL fractions were collected. Absorbance at 280 nm was routinely measured with UV monitors during chromatographic elution. Enzyme solutions were concentrated by ultrafiltration using PM-10 membranes (Amicon Corp). Protein in the concentrated solutions was determined by the Bradford method (7). The purified enzymes were examined by electrophoresis in a Protean II cell (Bio-Rad) on SDS-PAGE gels. Polypeptides were visualized by silver staining (5).

Treatment of Fruit and Determination of Ethylene

Tomato fruit of approximately equal size were harvested at the mature green stage and only those producing less than 4 nL/h of ethylene were selected. The tomatoes were surface sterilized with 0.2% NaOCl for 30 s and rinsed with water. They were then vacuum infiltrated with enzyme solution equivalent to 1% of the fruit weight by pipetting the solution onto the stem scar as described previously (4). The treated fruit were placed in glass jars which were periodically sealed for 1 h. One mL samples were withdrawn from the head volume and analyzed for ethylene by gas chromatography using an activated alumina column at 90°C. There were 4 fruit replications for each treatment.

Full sized green oranges (*Citrus sinensis* [L.] Obs) cv Hamlin were harvested from Huft groves in Macintosh, FL, with 4 cm stems which were immersed in water during transport to the laboratory. Enzyme solutions (20 μL) were injected at six points around the equator of each fruit. The oranges were placed individually in glass jars which were sealed for 1 h before withdrawing a sample for ethylene analysis.

RESULTS AND DISCUSSION

Separation and Purification of the Enzymes

Fifty mg of Pectolyase (Sigma Chemical Co.) was dissolved in 5 mL of 0.02 M Bis-Tris propane (pH 7.1), and applied immediately to the Q-Sepharose column. The fractions obtained by elution with the 0 to 1.0 M NaCl gradient were assayed for the pectic enzymes and cellulase. Three peaks of PL (PL-A, PL-B, and PL-C), two peaks of PG (PG-A and PG-B) and a single peak of PME were separated (Fig. 1). Very low levels of cellulase were widely distributed in the fractions with small peaks near fractions 12 and 28 (data not shown). The fractions corresponding to each pectic enzyme were pooled, adjusted to pH 5.0, and ultrafiltered to 2 mL. It was especially important to lower the pH of PG-A and PL-A as soon as possible because of their instabilities at alkaline conditions. Losses of these two enzymes were minimized by completing the Q-Sepharose chromatography, assay of the fractions and ultrafiltration of the solutions in about 6 h.

Pectolyase from three different batches has been analyzed for the pectic enzymes by chromatography on Q-Sepharose. The elution patterns were similar in terms of enzyme activities and absorbance at 280 nm, with significant differences only for PL-B and PME.

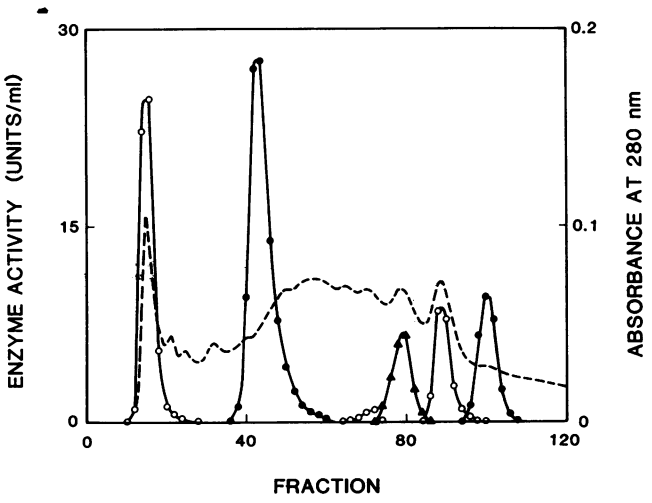

Figure 1. Chromatography of Pectolyase on Q-Sepharose. (– – –), Absorbance at 280 nm; (O——O), PL; (●——●), PG; (▲——▲), PME.

PL-A was further purified by chromatography on the cation exchanger Mono S equilibrated with 0.02 M sodium acetate (pH 5.0). One mL aliquots of enzyme solution were applied to the column and elution was conducted with 30 ml of a linear gradient of 0 to 0.25 M NaCl in 0.02 M acetate (pH 5.0) at a rate of 1 mL/min. The enzyme and protein elution profiles are shown in Figure 2A. Cellulase was found in fractions 4 to 6, well removed from the pectin lyase peak at fraction 9.

PG-A was purified by a two-step procedure. It was first chromatographed on the anion exchanger Mono Q equilibrated with 0.02 M sodium acetate (pH 5.5). Elution was conducted with 30 mL of a linear gradient of 0 to 0.4 M NaCl in 0.02 M acetate (pH 5.5) (Fig. 2B). The fractions containing PG were pooled, ultrafiltered to 2 mL, and dialyzed against 0.02 M NaCl. The enzyme was then chromatographed on the Mono S column as described for PL-A above. PG-A was eluted in fractions 12 and 13 (Fig. 2C), whereas cellulase was found in the first protein peak. On all three columns, Q-Sepharose, Mono Q, and Mono S, PG-A eluted as a sharp peak followed by a shoulder of enzyme activity. Rechromatography of the activity in the shoulder on Mono S showed that it was PG-A. An explanation for the skewing of PG-A during chromatography was not apparent. Recovery of this enzyme was somewhat lower by discarding the shoulders.

PL-B was purified by chromatography on the Mono Q column equilibrated with 0.02 M sodium acetate (pH 5.5). Elution was accomplished with 30 mL of a linear gradient of 0 to 0.8 M NaCl in 0.02 M acetate (pH 5.5). The results are presented in Figure 3A. Identical chromatographic conditions were used to purify PME (Fig. 3B), PL-C (Fig. 4A), and PG-B (Fig. 4B). Each enzyme was concentrated by ultrafiltration, dialyzed against 0.02 M NaCl, and stored at −20°C. A summary of the enzyme purifications is presented in Table I.

The enzymes were examined for purity by SDS-PAGE. PL-C and PG-B were the best preparations with one major band and one minor band in each. PL-A and PG-A contained a major band but several minor bands; PL-B and PME were the most heterogeneous with several bands in each.

Properties of the Enzymes

PME, PL-C, and PG-B were rather stable enzymes, with no loss of activity of either one in the Q-Sepharose fractions during refrigeration for 2 weeks. PL-B decreased slowly during the same period. In contrast, PL-A and PG-B were quite unstable at pH 7.1, with about 30% loss of each one after 24 h at 4°C. For this reason, the pH of both enzymes was lowered to 5.0 immediately after separation on the Q-Sepharose column. All of the enzymes were stable for at least several weeks when stored frozen as concentrated solutions. The stabilities of the enzymes were further evaluated by heating reaction mixtures of each before the addition of the substrate. All of the enzymes were stable to heating for 5 min at 40°C, and all were completely inactivated after 5 min at 60°C.

The three lyases were specific for the substrate pectin, with no reaction with polygalacturonic acid in terms of increase of

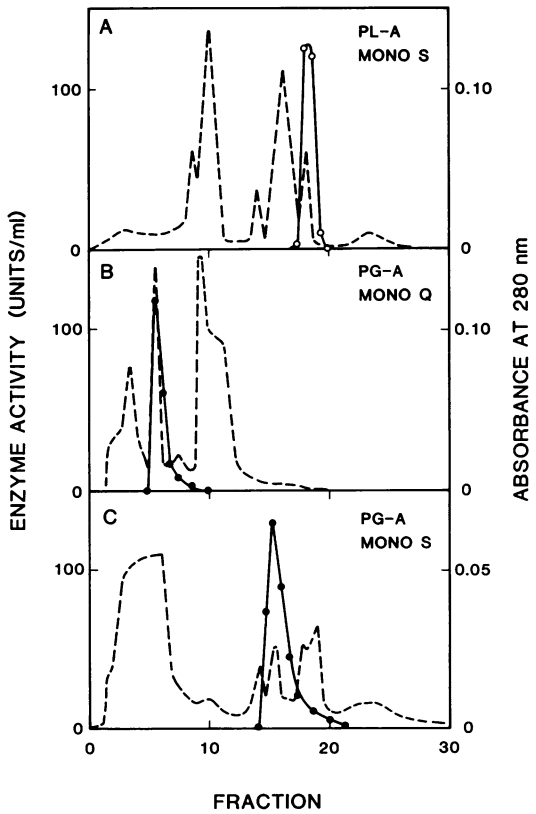

Figure 2. Chromatography of PL-A on Mono S (A), PG-A on Mono Q (B), and PG-A on Mono S (C). (- - -), Absorbance at 280 nm; (O——O), PL; (●——●), PG.

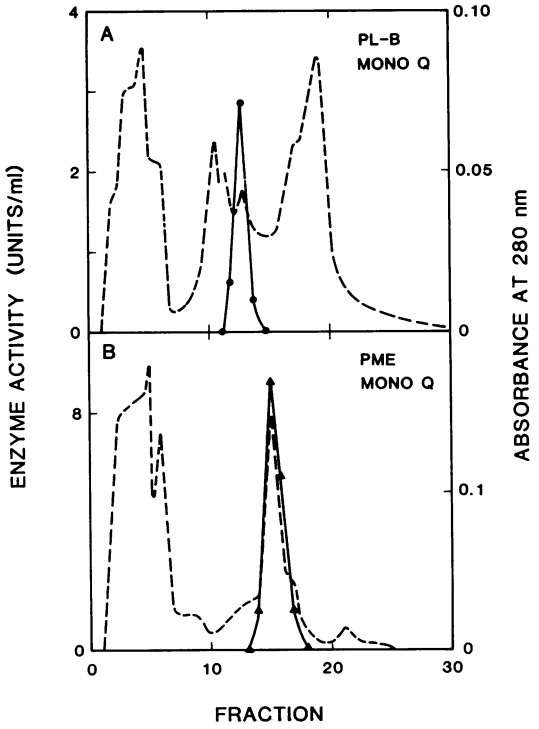

Figure 3. Chromatography of PL-B (A) and PME (B) on Mono Q. (- - -), Absorbance at 280 nm; (●——●), PL; (▲——▲), PME.

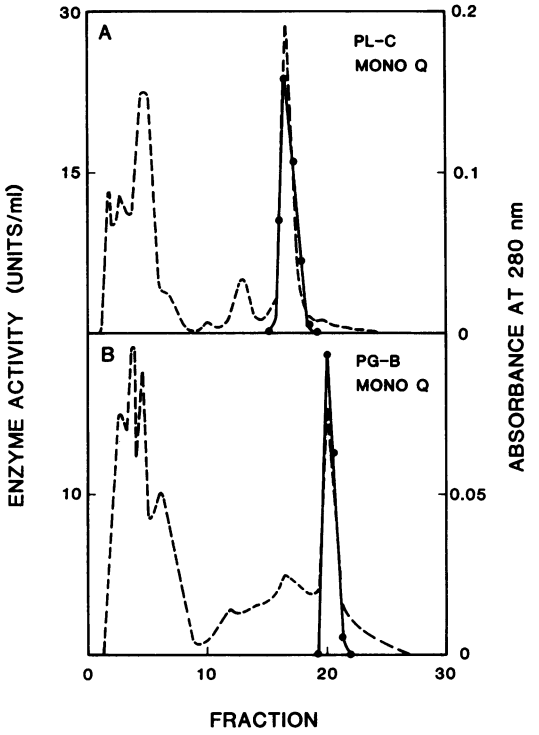

Figure 4. Chromatography of PL-C (A) and PG-B (B) on Mono Q. (●——●), PL; (●——●), PG; (- - -), absorbance at 280 nm.

Table I. *Summary of the Purification of Pectic Enzymes*

Enzyme	Step	Volume	Protein	Activity	Specific Activity
		mL	*mg*	*units*	*units/mg*
PL-A	Q-Sepharose	25	3.7	590	160
PL-A	Mono S	4	0.2	450	2250
PG-A	Q-Sepharose	45	4.2	680	162
PG-A	Mono Q	5	1.2	520	433
PG-A	Mono S	6	0.3	430	1430
PL-B	Q-Sepharose	20	2.4	21	8
PL-B	Mono Q	6	0.2	14	70
PME	Q-Sepharose	40	4.7	64	14
PME	Mono Q	6	0.8	48	60
PL-C	Q-Sepharose	30	3.8	223	59
PL-C	Mono Q	8	0.6	170	283
PG-B	Q-Sepharose	35	1.4	126	90
PG-B	Mono Q	5	0.3	95	316

absorption at 235 nm or of reducing groups. Therefore, these enzymes can be classified as pectin lyases which require an esterified substrate (17). The lyases were endoenzymes based on rapid reduction of he viscosity of pectin relative to slow increases in absorption at 235 nm. The PGs were specific for the deesterified substrates polygalacturonic and pectin acids, with very slow reactions with pectin. These enzymes did not increase the absorption at 235 nm of either pectate or pectin. Both PGs reduced the viscosity of pectate rapidly while releasing reducing groups slowly, indicating that they are endoenzymes.

The following values for the mol wt of the enzymes were obtained by gel filtration on Sephadex G-100: PL-A, 25,000; PL-B, 32,500; PL-C, 27,000; PG-A, 25,000; PG-B, 36,500; and PME, 26,500. The pH optima of the enzymes were determined to be as follows: PL-A, 6.2; PL-B, 5.9; PL-C, 4.9; PG-A, 4.7; PG-B, 4.0; and PME, 5.7. In general, the enzymes were not affected by buffers, salts, or chelating agents such as EDTA. The only exception was PL-B which was more active in citrate than in acetate buffers.

Ishii (8) found that PL and PG from *Aspergillus japonicus* differed in abilities to macerate tissues of different plant species. Susceptibility of the tissues to maceration depended on enzyme specificity rather than enzyme concentration. Our isoenzymes of PL and PG, therefore, were further characterized for the ability to solubilize pectin from washed cell walls (Table II). The levels of enzymes added to the reaction mixtures were selected to yield linear releases of uronic acids over the 30-min reaction period. The PLs were assayed at 1.5 units enzyme/reaction mixture and the PGS at 5.0 units. PL-A was

Table II. *Solubilization of Uronic Acids from Cell Walls by Pectic Enzymes from Pectolyase*

Cell walls (20 mg) were treated with each enzyme for 30 min at 37°C. Filtrates from the reaction mixtures were analyzed for uronic acid (6).

Cell Walls	PL-A	PL-B	PL-C	PG-A	PG-B
			mg uronic acid		
Carrot	2.0	0.9	0.2	1.7	0.7
Apple	3.9	2.5	1.8	1.0	0.8
Nectarine	2.8	3.5	2.6	1.4	0.6
Radish	2.7	2.5	0.2	3.5	0.6
Cucumber	2.7	3.7	1.9	2.4	1.2

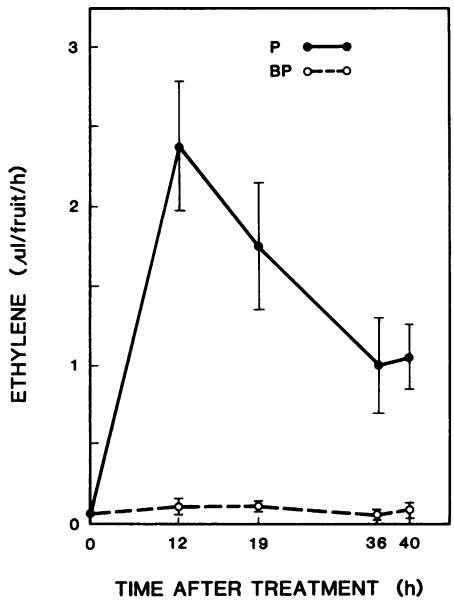

Figure 5. Effect of Pectolyase on ethylene production in green 'Jumbo' tomatoes. A 0.01% solution of Pectolyase in 0.1 M succinate (pH 5.0), was vacuum infiltrated into the fruit. The points are means of four replications ± SE. P, Pectolyase; BP, Pectolyase after heating 5 min at 100°C.

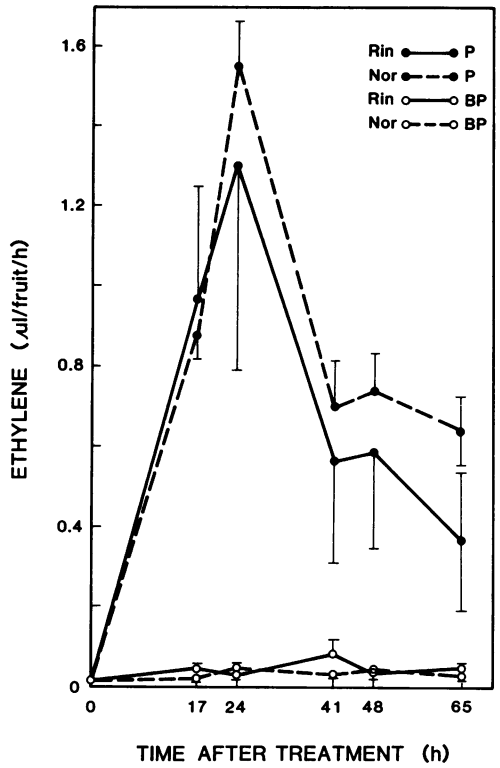

Figure 6. Effects of pectolyase on ethylene production in the nonripening mutant tomatoes *rin* and *nor*. Conditions were the same as described for Figure 5.

Table III. *Effects of Pectolyase Enzymes on Ethylene Production in Tomato Fruit*

Volume of enzyme solutions corresponding to 1% of tomato weight and containing 83 units/mL of PL or 122 units/mL of PG were vacuum infiltrated into cherry tomatoes. The fruit were periodically sealed in glass jars for ethylene determinations. Data are means of four replications ± SE.

Hours	PL-A	PL-C	PG-A	PG-B	Buffer
		nL ethylene/fruit/h			
1	8 ± 4	9 ± 2	1 ± 1	1 ± 1	0 ± 0
3	41 ± 5	44 ± 7	41 ± 8	37 ± 7	24 ± 11
6	31 ± 16	129 ± 12	92 ± 21	53 ± 20	8 ± 5
10	70 ± 32	48 ± 15	9 ± 9	1 ± 1	2 ± 1
19	76 ± 15	18 ± 7	3 ± 1	1 ± 1	2 ± 1
24	55 ± 7	11 ± 8	3 ± 1	2 ± 1	2 ± 1

the most effective PL for solubilizing uronic acids from the cell walls. It released high amounts of uronic acids from all six cell wall preparations, but optimally from apple cell walls. PL-B was also very effective except on carrot cell walls. PL-C was the least effective of the PLs, particularly on carrot and radish cell walls. Of the PGs, PG-A was the more effective enzyme. It released the most uronic acid from radish cell walls and the least from apple cell walls. In contrast, PG-B was quite unreactive with all of the cell wall preparations, and it released the least uronic acids of the five enzymes. The low reactivities of PL-C and PG-B with cell walls may be due to their highly acidic natures based on the strong binding to Q-Sepharose. Presumably these enzymes bind irreversibly to cell

Table IV. *Effects of Pectolyase Enzymes on Ethylene Production in Oranges*

Enzyme solutions containing 68 units/mL of PL or 103 units/mL of PG were injected at six locations around the equator of the fruit (20 μL/injection). The fruit were periodically sealed in glass jars for ethylene determinations. Data are means of four replications ± SE.

Hours	PL-A	PL-C	PG-A	PG-B	Buffer
		nL ethylene/fruit/h			
1	38 ± 32	40 ± 17	0 ± 0	1 ± 2	1 ± 2
2	138 ± 47	49 ± 14	3 ± 2	16 ± 8	3 ± 4
5	234 ± 14	62 ± 6	21 ± 8	26 ± 20	3 ± 2
8	365 ± 164	119 ± 13	88 ± 7	168 ± 36	27 ± 8
18	318 ± 117	91 ± 34	122 ± 20	125 ± 32	9 ± 10
24	65 ± 46	93 ± 29	74 ± 18	104 ± 31	5 ± 8
42	26 ± 24	218 ± 151	47 ± 6	84 ± 26	6 ± 16
66	32 ± 10	57 ± 21	41 ± 11	80 ± 14	9 ± 16

wall fragments at the low pHs corresponding to their pH optima (about 4–5).

It should be noted that the PGs were much less effective than the PLs in degrading cell walls. Based on calculations using the extinction coefficient for unsaturated uronic acids (12) which were confirmed by measuring reducing groups formation by the lyases, PGs cleave the polyuronide chain about 25 times faster than the lyases per unit of activity. But 3.3 times more PGs were needed for solubilization rates comparable to that for the lyases. The relative ineffectiveness of the PGs may be due to highly esterified pectin in cell walls which would not be susceptible to these enzymes. The PGs would react optimally in the presence of PME. In contrast, PME would reduce the reactivity of the lyases by deesterifying the pectin in the cell walls. Thus, for maximum effectiveness of the PLs, PME should be removed and this can be accomplished easily by chromatography on Q-Sepharose as we have described.

Effects of the Enzymes on Ethylene Production

Baldwin and Biggs (3) reported that Pectolyase induces ethylene production in citrus fruit. We found that the mixture of enzymes in Pectolyase is also effective in inducing ethylene formation in tomatoes. Infiltration of Pectolyase solution resulted in increased production of ethylene for not only normal tomato fruit (Fig. 5) but also for fruit from the nonripening mutants *rin* and *nor* (Fig. 6). The normal tomato fruit exhibited unusual color development in response to the Pectolyase treatment. Red color appeared first around the stem scar, which was the site of enzyme infiltration, rather than at the blossom end as in normal ripening. There was no visible lycopene formation in the mutant fruit, however. The two PGs and the major PLs, PL-A and PL-C, were then tested for induction of ethylene in tomatoes. PL-C produced the highest amount of ethylene after 6 h but PL-A was effective for a longer period (Table III). Of the PGs, PG-A was the more efficient enzyme and the effects by both enzymes were rather short. The responses for individual enzymes peaked earlier than for Pectolyase (Figs. 5 and 6) with the exception of PL-A.

Both PL-A and PL-C induced ethylene formation in orange

fruit after only an hour following treatment (Table IV). PL-A was the most effective enzyme in oranges, but the response to PL-C lasted longer. In contrast, the PGs exhibited a much longer lag time, although fairly high levels of ethylene were eventually produced by both enzymes. The difference in response time for the lyases and hydrolases may be due to highly esterified pectin in citrus which would be less susceptible to degradation by PGs (17). The enzyme-treated oranges developed areas of Chl breakdown around the injection sites which increased with time.

There have been other reports that cell wall degrading enzymes induce ethylene production in plants. Anderson *et al.* (2) found that Cellulysin, an enzyme mixture from *Trichoderma viride* increased ethylene production when added to tobacco leaf discs. Tong *et al.* (18) reported that Macerase, an enzyme mixture from *Rhizopus* induced ethylene in pear cell cultures, and that cell wall fragments released by the enzyme mixture were also effective. Purified tomato PG infiltrated into mature green tomato fruit induced ethylene formation and ripening (4). The results of the present study show that pectinases of fungal origin also induce ethylene in fruits and that PLs were more effective than PGs. The lyases may be more effective inducers of ethylene because they are more efficient than the hydrolases in solubilizing pectin from cell walls, as discussed above. It is also possible that the unsaturated pectin fragments released by pectin lyases are more effective than the simple oligouronides released by PGs.

LITERATURE CITED

1. **Albersheim P, Killia U** (1962) Studies relating to the purification and properties of pectin transeliminase. Arch Biochem Biophys **97:** 107–115
2. **Anderson JD, Mattoo AK, Lieberman M** (1982) Induction of ethylene biosynthesis in tobacco leaf discs by cell wall digesting enzymes. Biochem Biophys Res Commun **107:** 588–596
3. **Baldwin EA, Biggs RH** (1988) Cell-Wall lysing enzymes and products of cell-wall digestion elicit ethylene in citrus. Physiol Plant **73:** 58–64
4. **Baldwin EA, Pressey R** (1988) Tomato polygalacturonase elicits ethylene production in tomato fruit. J Am Soc Hortic Sci **113:** 92–95
5. **Blum DH, Beier H, Gross HJ** (1987) Improved silver staining for plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:** 93–99
6. **Blumenkrantz N, Asboe-Hansen G** (1973) New method for quantitative determination of uronic acids. Anal Biochem **54:** 484–489
7. **Bradford M** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
8. **Ishii S** (1976) Enzymatic maceration of plant tissues by endopectin lyase and endo-polygalacturonase from *Aspergillus japonicus*. Phytopathology **66:** 281–289
9. **Ishii S, Kiho K** (1976) Evidence for a factor that stimulates tissue maceration by pectolytic enzymes. Phytopathology **66:** 1077–1081
10. **Ishii S, Yokotsuka T** (1972) Purification and properties of endopolygalacturonase from *Aspergillus japonicus*. Agric Biol Chem **36**(11): 1885–1893
11. **Ishii S, Yokotsuka T** (1975) Purification and properties of pectin lyase from *Aspergillus japonicus*. Agric Biol Chem **39:** 313–321
12. **Macmillan J, Vaughn R** (1964) Purification and properties of a polygalacturonic acid-trans-eliminase produced by *Clostridium multifermentas*. Biochemistry **3:** 564–572
13. **Nagata T, Ishii S** (1979) A rapid method for isolation of mesophyll protoplasts. Can J Bot **57:** 1820–1823
14. **Nelson N** (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem **153:** 375–380
15. **Pressey R, Avants JK** (1971) Effect of substrate size on activity of tomato polygalacturonase. J Food Sci **36:** 486–489
16. **Pressey R, Avants JK** (1982) Solubilization of cell walls by tomato polygalacturonase: Effects of pectinesterases. J Food Sci **6:** 57–74
17. **Rexova-Benkova L, Markovic O** (1976) Pectic enzymes. Adv Carbohydr Chem Biochem **33:** 323–385
18. **Tong CB, Labavitch JM, Yang SF** (1986) The induction of ethylene production from pear cell culture by cell wall fragments. Plant Physiol **81:** 929–930