In Vitro and In Vivo Production of Pectin Lyase by Penicillium expansum

D. H. Spalding and A. A. Abdul-Baki

Research Plant Pathologist and Research Plant Physiologist, Market Quality, ARS, USDA, 13601 Old Cutler Road, Miami, Florida 33158, and Plant Industry Station, Beltsville, Maryland 20705, respectively.
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ABSTRACT

Pectin lyase was produced by *Penicillium expansum* in artificial media and in apple tissue. Pectin lyase production was greatest in a medium containing a pectin-polypectate mixture, less in media containing malic acid or citric acid, and absent in media containing glucose. Crude pectin lyase from apples rotted by *P. expansum* was purified by ammonium sulfate fractionation and column chromatography on Sephadex G-100 and diethylaminoethyl cellulose. The purified pectin lyase was free of pectin esterase, polygalacturonase, carboxymethylcellulase, phosphatidase, and proteinase activity, and caused maceration and death of potato tissue. Pectin lyase activity was maximum at pH 6.5 with 0.4% citrus pectin as substrate. Calcium, magnesium, and manganese ions stimulated pectin lyase activity, and ethylenediaminetetraacetic acid inhibited enzymatic activity in vitro. Pectin lyase retained 60% of its original activity, carboxymethylcellulase retained over 80%, and polygalacturonase retained less than 5% after 10 min at 50 C.

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Additional key words: blue mold rot, pectin *trans*-eliminase, apples, enzyme purification, cellulase.

Previous investigators of pectic enzyme production by *Penicillium expansum* Lk. ex Thom, the cause of blue mold rot of apples, have reported either no pectin lyase (PL) production in culture media (8) or have restricted their studies to the polygalacturonase (PG) without testing for PL (17). The report by Bush & Codner (6) that *Penicillium digitatum* produces PL prompted us to examine whether *P. expansum* pro uces PL in culture and in apples and to determine some of its properties.

MATERIALS AND METHODS.—*Growth of P. expansum in artificial media.*—The fungus was grown in a medium containing 0.1 M glucose, 0.1 M malic acid, 0.1 M malic acid plus 0.1 M glucose, 0.1 M citric acid, 0.1 M citric acid plus 0.1 M glucose, or 0.5% pectin plus 0.5% sodium polypectate (Nutritional Biochemicals Corp., Cleveland, Ohio) as a carbon source(s). The mineral salts in the medium were 0.05 M NH_4NO_3, 0.1 M KH_2PO_4, 0.01 M $MgSO_4 \cdot 7H_2O$, 0.3 μM $CuSO_4 \cdot 5H_2O$, 0.8 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM $(NH_4)_2MoO_4$, 9 μM $MnSO_4 \cdot 4H_2O$, and 50 μM H_3BO_3. The pH was adjusted to 4.5, and the medium dispensed in 125-ml Erlenmeyer flasks with 25 ml/flask. The flasks were stoppered with cotton, capped with aluminum foil, and autoclaved for 20 min at 15 psi. Each flask was inoculated with 1 ml of a heavy suspension of spores of *P. expansum* (5×10^6 spores/ml). We prepared the suspension by washing spores from a 1- to 2-week-old culture on potato-dextrose agar into sterile 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate). Cultures were incubated in a water bath at 25 C with constant shaking at 110 2.5-cm strokes/min. Flasks were removed at intervals and vacuum-filtered through tared Whatman No. 1 filter paper in a Büchner funnel. Fungal growth was measured as dry weight after 48 hr at 80 C. The filtrate from each culture was readjusted to original volume with distilled water and used to determine PL.

Extraction and purification of PL from apples.—Red Delicious apples were punctured in about 15 places and inoculated with *P. expansum* as previously described (16). Rotted apples were removed from 21-C storage 1 week after inoculation and were held overnight at 4 C. Dry polyvinylpyrrolidone (PVP) (Polyclar AT, General Aniline and Film Corp.), purified by the method of Loomis & Battaile (10), was soaked overnight in 0.1 M citrate-phosphate buffer, sodium form, pH 6.5. PVP (0.15 g/g of apple) was used to retard inactivation of enzymes by phenolics present in crude homogenates. Rotted apple pulp (1.5 kg) was scooped into the buffer-PVP mixture and ground in a mortar. The crude homogenate was passed through two layers of cheesecloth and clarified by centrifugation at 13,000 *g* for 15 min.

The 55 to 80% saturated $(NH_4)_2SO_4$ fraction of clarified crude homogenate was applied to a 2.5×45 cm column of Sephadex G-100 (Pharmacia Fine Chemicals) pre-equilibrated with 0.1 M citrate-phosphate buffer, sodium form, pH 6.5. The same buffer was used to elute the column. About 60 fractions of 4 ml each were collected and assayed for PG and PL activity. All extraction and purification steps were carried out in a cold room between 0 and 4 C.

The fractions from the Sephadex G-100 column containing most of the PL activity were combined, and 40 units of PL added to a 2.5×45 cm column of diethylaminoethyl cellulose (hydroxyl form) (DEAE cellulose) buffered with 0.1 M citrate-phosphate buffer, sodium form, pH 6.5. The column was eluted with 95 ml of buffer followed by an NaCl gradient in buffer until 1.0 M NaCl was reached. About 100 fractions of 5 ml each were collected and assayed for PL, PG, and carboxymethylcellulase (Cx) activity.

Sephadex G-100 fractions were collected of the 55 to 80% saturated $(NH_4)_2SO_4$ fraction of clarified crude homogenate of the pulp of nonrotted Red Delicious apples. The fractions were assayed for pectin esterase (PE), PG, PL, and Cx activity.

Enzyme assay.—PL activity was determined by the

spectrophotometric method of Albersheim & Killias (1). Citrus pectin (Nutritional Biochemicals Corp.) solutions were clarified by centrifuging at 40,000 *g* for 1 hr at O C (6). The standard 2-ml reaction mixture contained 0.4% citrus pectin in 0.05 M Tris [tris (hydroxymethyl) amino methane] - CH_3 COOH buffer or 0.05 M sodium citrate buffer, pH 6.5; 25 mM divalent cation; and 0.2 ml enzyme solution. The reaction was started by addition of PL solution, and changes in absorbance at 235 nm were recorded at 1-min intervals at 25 C. One unit of PL activity was the amount of enzyme which caused an increase in absorbance of 0.01 at 235 nm in 1 min in a 1-cm lightpath at 25 C (6).

PL activity was also determined with a modification (3) of Neukom's thiobarbituric acid (TBA) test for breakdown products of pectin. The TBA test was used in in vitro studies with culture filtrates where conservation of sample was not a factor. The reaction mixture contained 2.0 ml enzyme preparation adjusted to pH 6.5, 2.5 ml 0.8% citrus pectin (clarified at 40,000 *g* for 1 hr) in 0.1 M citrate-phosphate buffer, sodium form, pH 6.5, and 0.5 ml 0.01 M $CaCl_2$. The alkaline solution of $ZnSO_4$ used by Ayers et al. (3) was not added to the reaction mixture after 4 hr at 30 C. A unit of PL is defined as the amount of enzyme required to produce a change in absorbance of 1.0 at 547 nm in 4 hr at 30 C.

PG and Cx activities were determined as previously described (14), except that the sodium polypectate and carboxymethylcellulose substrates were clarified by centrifugation at 40,000 *g* for 1 hr. One unit of activity is the amount of enzyme required to reduce substrate flow rate by 50% in 1 min.

PE activity was measured in a 5-ml reaction mixture of 0.5 ml of enzyme solution in 1.2% citrus pectin and 0.15 M NaCl adjusted to an initial pH of 7.0 with 0.1 N KOH. Two drops of toluol were added for prevention of bacterial growth. Reaction mixtures were incubated at 25 C for 17 hr, then titrated to pH 7.0 with 0.01 N KOH.

Phosphatidase activity was determined at pH 4.5, 6.5, and 8.5 using refined soybean lecithin (Nutritional Biochemicals Corp.) in the "cup plate" method (12).

We assayed protease activity at pH 3.0, 5.0, and 7.0 using the casein digestion method of Kunitz as followed by Wang & Hesseltine (19).

Protein was determined by the method of Lowry et al. (11).

Cellular death of cells of potato discs was determined and rated by the neutral red vital stain method of Tribe (18), except that tests were made at pH 6.5. Maceration and rating of potato discs after treatment were established as described by Mount et al. (13). Discs showing no maceration or cellular death received a score of 5, but those showing complete loss of coherence or death of all cells received a score of 0. Intermediate stages of maceration or cellular death were rated accordingly.

Heat-of-inactivation.—Six ml of filtrate at pH 5.7 from 9-day cultures grown on the pectin-polypectate medium were placed in a test tube in a water bath and maintained at the desired temperature for 10 min. The tube was then cooled rapidly in cold water, and the contents were used to determine PL, PG, Cx, and macerating activities.

RESULTS.—*In vitro production of PL.*—Production of PL by *P. expansum* was suppressed, but growth was stimulated, in media containing glucose (Table 1). PL activity in malic or citric acid cultures was not detected until 9 days after inoculation. The highest PL production was in the pectin-polypectate medium. A time-course study of growth and PL production on the pectin-polypectate medium (Fig. 1) showed that growth was maximum in 3 days, but PL production per unit growth continued to increase linearly; pH was 4.5 initially, 5.4 in 3 days, 5.9 in 6 days, and 5.7 in 9 days.

In vivo production of PL.—Crude homogenate of Red Delicious apples rotted by *P. expansum* contained PE (10.3 mg carboxyl groups released/hr/mg protein), PG (0.2 units/mg protein), PL (1.4 units/mg protein), and Cx (0.2 units/mg protein). The fraction purified 54-fold by $(NH_4)_2SO_4$ and Sephadex G-100 procedures (Fig. 2) was free of PE, phosphatidase, and proteinase activity, but still contained PG (6.2 units/mg protein), and Cx (22.9 units/mg protein) in addition to PL (75.0 units/mg protein). Fractions purified from nonrotted apple tissue contained no PE, PG, PL, and Cx activity.

Fractions of PL free of PG and Cx activity were obtained by passage of fractions from the Sephadex G-100 column through a DEAE cellulose column (Fig. 3). The most active PL fraction (73.7 units/mg protein) macerated potato tissue in 6 hr at 30 C and pH 6.5. Maceration and cellular death of potato discs in combined fractions of purified PL at 30 C and pH 6.5 proceeded at about the same rate (Fig. 4).

Specificity of PL to pH and substrate.—Activity of PL in the purified fraction was maximum at about pH 6.5 and at about 0.4% citrus pectin concentration (Fig. 5). PL activity decreased at concentrations above 0.4% pectin. No PL activity was detected when sodium polypectate was used as a substrate in place of citrus pectin.

TABLE 1. Growth and pectin lyase production by *Penicillium expansum* on various carbon sources after 9 days at 25 C

Carbon source	Final pH[a]	Growth (dry wt) mg/ml	Pectin lyase[b]
0.1 M Glucose	4.6	5.8	0
0.1 M Malic acid	6.4	2.7	560
0.1 M Citric acid	6.4	4.2	290
0.1 M Glucose + 0.1 M malic acid	6.4	8.4	0
0.1 M Glucose + 0.1 M citric acid	6.5	10.0	0
0.5% Pectin + 0.5% Na polypectate	5.7	2.3	2,770

[a] Initial pH was 4.5.
[b] Activity in units/mg dry wt of growth $\times 10^5$.

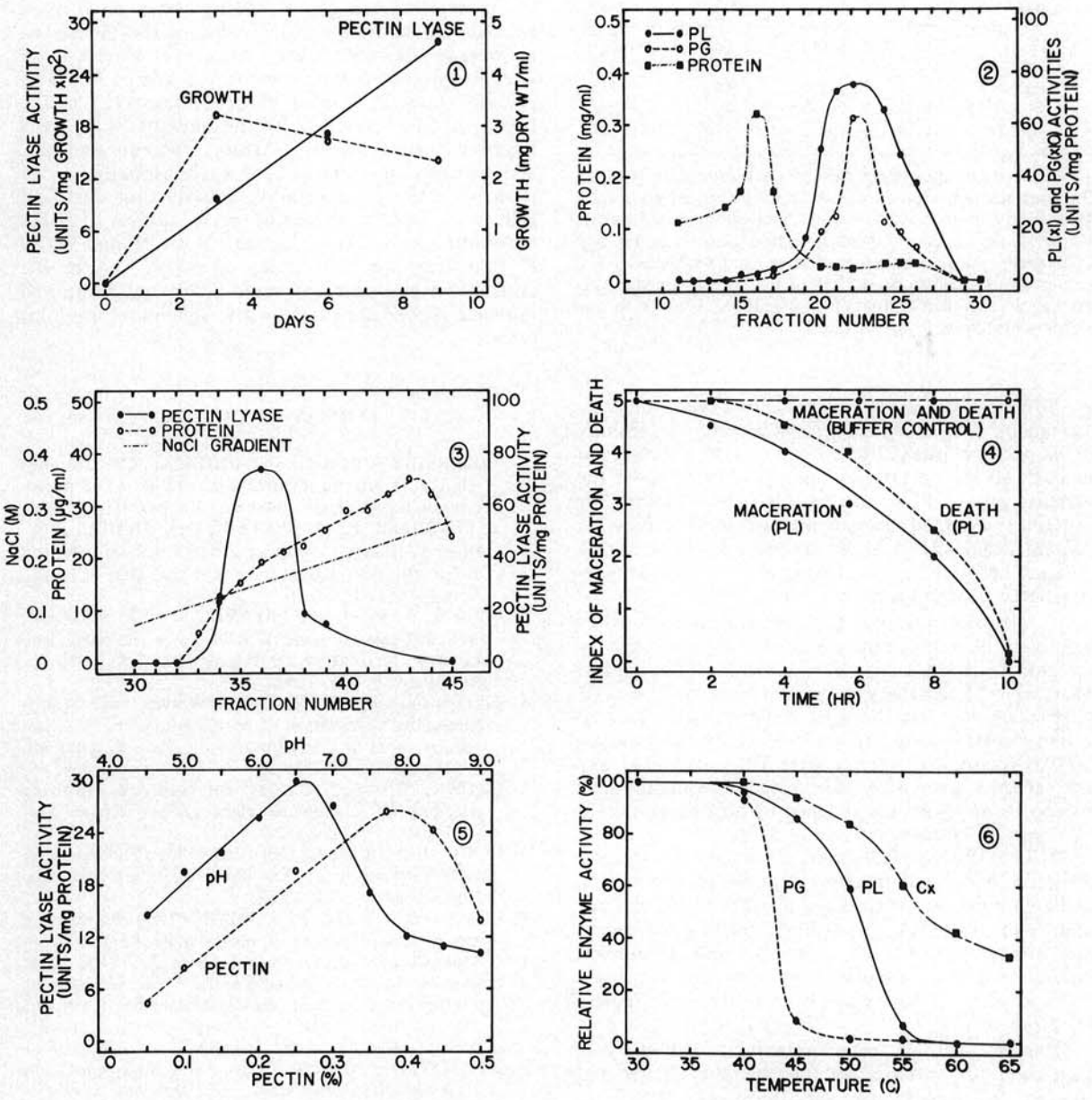

Fig. 1-6. **1)** Growth and pectin lyase (PL) production by *Penicillium expansum* in a pectin-polypectate medium during 9 days at 25 C. **2)** PL and polygalacturonase (PG) activity in fractions of *P. expansum*-rotted apple tissue after Sephadex G-100 column chromatography of the 55 to 80% saturated $(NH_4)_2SO_4$ fraction. **3)** Purification of PL of *P. expansum*-rotted apple tissue by column chromatography on diethylaminoethyl cellulose of partially purified PL from a Sephadex G-100 column. **4)** Maceration and cellular death of potato tissue discs in a reaction mixture containing 30 ml of purified PL (16.0 units), 2 ml of 0.8 M citrate-phosphate buffer (pH 6.5), and 24 potato discs (8 mm diam × 0.4 mm thick). **5)** Influence of pH and concentration of citrus pectin substrate on PL activity of 35 to 80% saturated $(NH_4)_2SO_4$-insoluble fraction of crude extract of *P. expansum*-rotted apple tissue. The 2-ml reaction mixture for the pH study contained 0.45% citrus pectin in 0.05 M Tris [tris(hydroxymethyl)amino methane] buffer adjusted to the desired pH with acetic acid, and 0.2 ml of enzyme solution, and was incubated at 29 C. The reaction mixture for the substrate concentration study contained the appropriate citrus pectin concentration in 0.05 M Tris-acetate buffer, pH 6.5, 0.2 ml of enzyme solution, and was incubated at 27 C. **6)** Heat-of-inactivation of PL, PG, and carboxymethylcellulase (Cx) in filtrate (pH 5.7) of 9-day cultures of *P. expansum* grown in a pectin-polypectate medium.

TABLE 2. Increase in activity of pectin lyase by addition of divalent cations

Final concentration (mM)	Pectin lyase activity[a]		
	Ca^{++}	Mg^{++}	Mn^{++}
0	160	160	160
1	170	195	235
10	277	288	349
25	381	352	400
50	406	374	349

[a] Activity in units/mg protein was determined at 24 C in a 2-ml reaction mixture containing 0.4% citrus pectin in 0.05 M Tris[tris(hydroxymethyl)amino methane]-acetate buffer, 0.1 ml of demineralized water or cation solution, and 0.2 ml of partially purified enzyme solution from Sephadex G-100 column. Substitution of 0.1 ml of 0.5 M ethylenediaminetetraacetic acid for cation solution reduced activity to one unit/mg protein.

Influence of divalent cations and ethylenediaminetetraacetic acid on PL activity.—Calcium, magnesium, or manganese ions (25 to 50 mM), as the chloride salt in 0.05 M Tris-acetate buffer, pH 6.5, increased the reaction rate of PL about 2.5-fold from 160 units/mg protein (no added salt) to as high as 406 units/mg protein (Table 2). Activity of PL was reduced to 1 unit/mg protein with 0.025 M ethylenediaminetetraacetic acid in the reaction mixture.

Heat-of-inactivation.—The temperature required to inactivate PL in 10 min was intermediate to that for Cx and PG. The Cx enzyme was the most resistant to heat, with 35% activity remaining after 10 min at 65 C (Fig. 6). PG was the least resistant, with 96% of activity destroyed in 10 min at 50 C. PL still retained 60% of its original activity after 10 min at 50 C, and Cx retained over 80%. The rate of maceration of potato tissue slices was reduced by 50% by 10 min at 50 C and by 100% by 10 min at 55 C.

DISCUSSION.—PL was detected in several artificial media and in apple fruit tissue. Production of PL in vitro was not related directly to the amount of growth. Growth was least in the pectin-polypectate medium in which PL production per unit growth was highest. Glucose stimulated growth, but repressed PL-production. Glucose has been reported to repress PG-production (9).

The PL purified from apple tissue rotted by *P. expansum* is specific for methylated pectin and thereby resembles the specificity of PL of *P. digitatum* (6), *Aspergillus fonsecaeus* (7), and the commercial preparation studied by Albersheim et al. (2). This specificity of PL for methylated pectin allowed the use of sodium polypectate to assay PG.

PL from rotted apple tissue has a higher pH optimum (pH 6.5) than that (pH 5.2) reported with two other fungal preparations (2, 7) and a considerably lower optimum than that (pH 8.0 and above) reported for pectate lyases (3, 13). The decrease in PL activity noted at pectin concentrations above 0.4% resembles the marked inhibition at 1.0% pectin of the PL preparation used by Albersheim & Killias (1). The stimulation of PL activity by divalent cations is analogous to increase in PL of other fungi in the presence of Ca^{++} (4, 7).

Brown (5) suggested that a protease, a lipase, or the macerating enzyme of the pathogen acted upon an essential structural constituent of the protoplast membrane of host tissue to cause death. Later workers presented evidence for the role of PG (15), pectate lyases (13), and phosphatidase (12) in the death of plant tissues. In the present study, the fraction causing death of tissue cells contained PL activity but no PG, PE, Cx, phosphatidase, or protease activity. Macerating activity and death of cells were closely associated as in studies of other host-pathogen systems (13, 15). Both PL and PG of *P. expansum* probably play a major role in the destruction and death of apple tissue, but additional evidence is needed to clarify their individual and joint roles.

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