Catabolite Repression of Polygalacturonase, Pectin Lyase, and Cellulase Synthesis in Penicillium expansum

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ABSTRACT

Production of pectin lyase (PL), polygalacturonase (PG), and carboxymethylcellulase (Cx), by *Penicillium expansum* in a 0.5% pectin-polypectate mineral salts medium, was repressed by 0.1 M arabinose, mannose, galactose, glucose, sucrose, raffinose, galacturonic acid, and glutamic acid. Raffinose and glutamic acid were equally repressive at 0.03 M and 0.1 M; affected PG, PL, and Cx equally; and were effective even when added after enzyme-production began. Repression reduced the maceration of apple tissue by culture filtrates.

Production of PG, PL, and Cx was high in dialyzed apple medium and was repressed by raffinose. In nondialyzed apple medium, production of enzymes was minimal, possibly repressed by sugars or other natural constituents of the apple tissue.

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Polygalacturonase (PG) and carboxymethylcellulase (Cx) production by certain fungi is repressed by glucose and galacturonic acid, both of which are catabolites of pectin and cellulose (2, 6, 8). Pectate lyase production by certain bacteria is also repressed by glucose (7, 14). *Penicillium expansum* Lk. ex Thom, the cause of blue mold rot in apples, produces pectin lyase (PL), in addition to PG and Cx, in artificial media and when attacking apple tissue (12). PL purified from apple tissue rotted by *P. expansum* caused maceration and death of plant tissue (12). Catabolite repression of PL production in fungi has not been reported and repressors, other than sugars and galacturonic acid, have not been tested for effects on PG, PL, and Cx.

Sugar repression of pectic and cellulolytic enzyme production by fungi has been related to disease resistance in plants (4). The present report relates the effects of repressors on enzyme production by *P. expansum* to rates of maceration of apple-tissue slices in fungus culture filtrates.

MATERIALS AND METHODS.—*P. expansum* was isolated from a 'Red Delicious' apple and maintained on potato-dextrose agar (PDA). The medium for enzyme repression studies was 0.5% Na polypectate and 0.5% citrus pectin (Nutritional Biochemicals Corp., Cleveland, Ohio) blended with a solution containing 0.05 M NH_4NO_3, 0.1 M KH_2PO_4, 0.01 M $MgSO_4 \cdot 7H_2O$, 70 μM $FeSO_4 \cdot 7H_2O$, 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. Media (25 ml/flask) were prepared in cotton-stoppered and aluminum foil-capped 125-ml Erlenmeyer flasks. A sugar or amino acid was added immediately and pH adjusted to 4.5 before autoclaving [20 min at 105×10^{-4} kg-force/mm^2 (15 psi)]; or, autoclaved concentrations of sugar or amino acid were added 24, 48, or 72 hr after inoculation. Sugars and amino acids generally were tested at 0.1 M, except when glutamic acid and raffinose were tested at 0.01, 0.03, and 0.1 M to determine the range for enzyme repression. Each flask was inoculated with 1.0 ml of a heavy spore suspension (5×10^6 spores/ml) prepared by washing spores from a 1- to 2-week-old culture on potato-dextrose agar PDA 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 per min. Flasks were removed at intervals and the contents vacuum-filtered on tared Whatman No. 1 filter paper in a Büchner funnel. Mycelium was weighed after drying for 48 hr at 80 C. The filtrate from each culture was readjusted to original volume with distilled water and used for enzyme determinations.

PG and Cx activities were determined as previously described (11) except that the Na 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 at 30 C.

PL activity was determined with a modification (1) of Neukom's thiobarbituric acid (TBA) test for breakdown products of pectin. 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 citric acid-sodium phosphate buffer at pH 6.5, and 0.5 ml 0.01 M $CaCl_2$. The blank run with each test contained enzyme inactiviated by heating in a boiling water bath for 15 min. After 4 hr in a water bath at 30 C, reaction mixtures were removed and 3 ml 0.04 M TBA and 2 ml 0.75 N HCl was added to each. Samples were then placed in a boiling water bath for 30 min, cooled, and read in a spectrophotometer at 547 nm. A unit of PL is the amount of enzyme required to produce a change in absorbance of 1.0 at 547 nm in 4 hr at 30 C in the TBA test described. Units were calculated per mg dry wt of growth.

Reviewers during publication reminded us that Neukom's procedure is not quantitative for the deoxyketouronic acids, and that the more sensitive and reliable assay for PL products is the procedure of Weissbach and Hurwitz as adapted by Preiss & Ashwell (9). Nevertheless, we believe that the large differences in PL activity that were observed (see results) offset the semiquantitative nature of the Neukom TBA assay.

Macerating activity was measured on apple-tissue disks 1-cm diam and 1-mm thick. Five discs were placed in 5 ml of enzyme solution (pH 6.5) and held in a water bath at 30 C. Activity was expressed as the reciprocal of the time (hr) required for disintegration of half of the disks.

Apple medium was prepared by blending 200 g sliced apple tissue for 2 min in 150 ml of 0.033 M sodium phosphate buffer at pH 6.8. Half of the medium was dialyzed against distilled water at 5 C for 24 hr. Mineral salts, as used in the pectin-polypectate medium, were added to both dialyzed and

TABLE 1. Growth and pectic enzyme production after 6 days at 25 C in cultures of *Penicillium expansum* amended with various sugars or amino acids

Compound tested (0.1 M)	Growth (mg/ml)	PG[a]	PL[a]
Check	3.52	153	1,539
Arabinose	8.48	44	94
Mannose	11.80	54	244
Galactose	10.32	19	0
Galacturonic acid	9.48	48	358
Glucose	10.24	66	120
Sucrose	11.20	91	188
Raffinose	11.68	14	0
Pyruvic acid	5.00	88	579
Glutamic acid	4.64	90	0
Methionine	2.41	119	792

[a] Polygalacturonase (PG) and pectin lyase (PL) activity expressed in units/mg dry wt $\times 10^5$. A unit of PG activity is the amount of enzyme required to reduce substrate flow rate by 50% in 1 min at 30 C. A unit of PL activity is the amount of enzyme required to produce a change in absorption of 1.0 at 547 nm in 4 hr at 30 C.

TABLE 2. Growth, pH, and enzyme production after 3 days at 25 C in cultures of *Penicillium expansum* amended with various concentrations of glutamic acid or raffinose

Concentration (M)	Growth (mg/ml)	pH	PG[a]	PL[a]	Cx[a]	Macerating enzyme[b]
Glutamic acid						
0	3.52	5.5	69	2,920	39	250
0.01	4.11	6.0	42	1,850	41	170
0.03	3.88	6.8	32	0	18	170
0.10	7.13	7.5	16	0	16	80
Raffinose						
0	3.78	5.6	30	635	10	40
0.01	6.44	5.4	15	155	4	20
0.03	7.16	4.8	6	56	3	20
0.10	10.13	4.4	2	0	2	20

[a] Polygalacturonase (PG), pectin lyase (PL), and carboxymethylcellulase (Cx) activity expressed in units/mg dry wt × 10^5.
[b] Macerating enzyme activity expressed as the reciprocal of the time in hr multiplied by 10^3 for 50% disintegration of apple tissue disks.

nondialyzed media. Raffinose (0.1 M) was added to half of the dialyzed and nondialyzed media. Media were sterilized, inoculated, and incubated as described above except that noninoculated controls were used to correct for weight of pulp when determining growth.

RESULTS.—*Survey of enzyme repressors.*—Repression of PG and PL activity was maximum 3 to 6 days after inoculation (data not shown). All sugars tested (arabinose, mannose, galactose, glucose, sucrose, and raffinose) and galacturonic acid increased growth and repressed production of PG and PL (Table 1). Glutamic acid repressed enzyme production more effectively than pyruvic acid and methionine. Raffinose and glutamic acid were selected for further study.

Effect of repressor concentration on enzyme production.—Increasing the concentration of glutamic acid in the medium to 0.1 M doubled growth, raised pH to 7.5, and repressed enzyme production (Table 2). As little as 0.03 M glutamic acid markedly repressed pectic and cellulolytic enzyme production. Repression was associated with reduction in macerating activity. Raffinose caused similar repression of enzyme production and increase in growth. Buffering capacity of the medium tended to keep the pH close to 4.5. As with glutamic acid, repression of enzyme production by raffinose caused some reduction in macerating activity.

TABLE 3. Growth and enzyme production after 72 hr at 25 C in cultures of *Penicillium expansum* amended with 0.1 M glutamic acid 24 and 48 hr after inoculation

Factor measured	Nonamended control	Time of addition	
		24 hr	48 hr
Growth[a]	3.05	3.94	4.02
PG[b]	39	9	16
PL[b]	1,040	0	0
Cx[b]	17	6	5
Macerating enzyme[c]	500	250	500

[a] Growth expressed as mg dry wt/ml medium.
[b] Polygalacturonase (PG), pectin lyase (PL), and carboxymethylcellulase (Cx) activity expressed in units/mg dry wt × 10^5.
[c] Macerating enzyme activity expressed as the reciprocal of the time in hr multiplied by 10^3 for 50% disintegration of apple tissue disks.

Repression of initiated enzyme production.—Addition of glutamic acid to a growing culture of *P. expansum* 24-48 hr after inoculation increased growth (Table 3). PG, PL, and Cx were repressed markedly, even when addition of repressor was delayed 48 hr after inoculation. Macerating enzyme activity was reduced slightly by addition of glutamic acid at 24 hr, but not at 48 hr.

Addition of raffinose up to 72 hr after inoculation more than tripled growth (Fig. 1A). Raffinose repressed PG production when added at 48 hr and 72 hr, but not at 24 hr (Fig. 1B). On the other hand, raffinose repressed PL production as effectively at 24 hr as at 48 hr or 72 hr (Fig. 1C). Repression of Cx production was similar to PL (Fig. 1D).

Effect of dialysis of apple medium on enzyme production.—Nonamended apple medium provided sufficient nutrients for growth of *P. expansum*, but production of PG, PL, and Cx was poor (Table 4). Raffinose at 0.1 M in the apple medium doubled growth, but had little effect on enzyme production. Dialysis of the apple medium reduced growth sharply, but stimulated enzyme production markedly. Raffinose increased growth in the dialyzed medium and either partially, or completely, repressed enzyme production.

DISCUSSION.—Our results demonstrate that not only sugars but an amino acid, glutamate, can repress production of pectic and cellulolytic enzymes by *P. expansum*. Possibly, other catabolites of polysaccharides and proteins also repress enzyme production, since repression is not limited to specific catabolites of the substrate attacked by a given enzyme.

Enzyme repression by raffinose could be caused by one or more hydrolysis products (glucose, galactose, and fructose) rather than by the parent compound. In our initial survey, glucose and galactose repressed enzyme production by *P. expansum*. Fructose may also be repressive.

Raffinose, rather than sucrose, a common sugar of apples, was used in order to compare unusual repressants not studied previously. The initial screening of sugars demonstrated that raffinose and sucrose both repressed enzyme activity and we chose to proceed with the trisaccharide. Since glutamic acid is a common amino acid in metabolism of plant tissue, we considered this choice to be very relative to possible host-parasite relations in apples.

Macerating activity with apple tissue slices tended to decrease with repression of pectic and cellulolytic enzymes (Tables 2 and 3). In addition, dialysis of apple medium increased pectic and cellulolytic enzyme production (Table 4). This effect probably was due to removal of sugars, since adding raffinose partially restored the enzyme-repressing activity of the apple medium. On the basis of present data, high sugar content in apples might be expected to correlate with resistance to *P. expansum*. However, Sitterly & Shay (10) found that infusion of apples on the tree with sugars permitted extensive tissue invasion by *Botryosphaeria ribis* Gross. & Dug. about 20 days earlier than in untreated fruit. They also found that infection occurred at or immediately before the climacteric rise. Mature apples are much more susceptible to *P. expansum* than immature apples when bruised and inoculated (13). Sugar content of apples increases steadily up to and even beyond the climacteric rise in respiration which usually occurs several days after normal harvest (5).

TABLE 4. Effect of dialysis of apple medium and 0.1 M raffinose on growth, pH, and enzyme production of *Penicillium expansum* in 3 days at 25 C

Test medium	Growth (dry wt) (mg/ml)	pH	PG[a]	PL[a]	Cx[a]
Nondialyzed	14.17	4.7	6	0	6
Nondialyzed plus raffinose	21.14	4.3	6	16	2
Dialyzed	3.10	4.9	216	107	1,070
Dialyzed plus raffinose	6.74	4.4	124	0	0

[a] Polygalacturonase (PG), pectin lyase (PL), and carboxymethylcellulase (Cx) activity expressed in units/mg dry wt × 10^5.

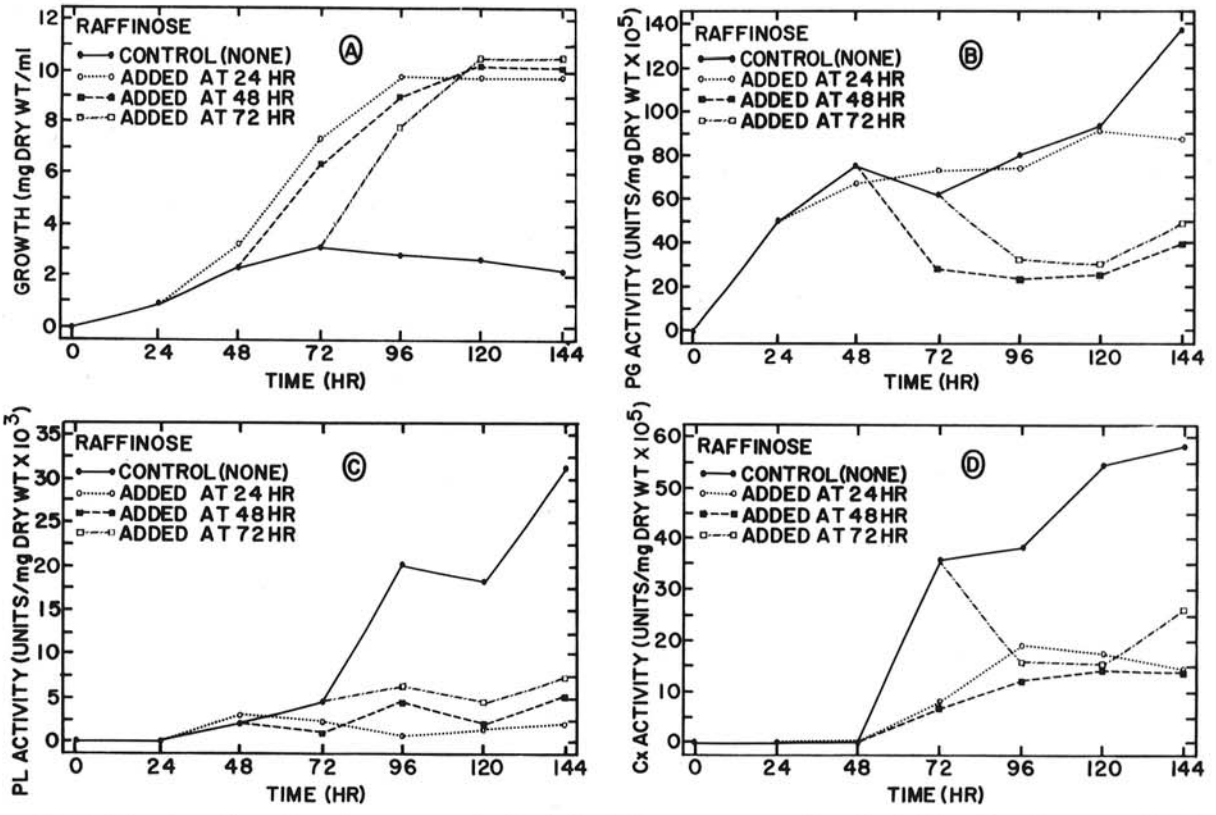

Fig. 1. Alteration of growth and enzyme production in *Penicillium expansum* cultured in 0.5% pectin-polypectate mineral salts medium supplemented at different timed intervals with 0.1 M raffinose: A) growth; B) polygalacturonase (PG) activity; C) pectin lyase (PL) activity; and D) carboxymethylcellulase (Cx) activity.

Thus, high sugar seems to be associated with high susceptibility to *P. expansum*. On the other hand, apples held in cold storage for 6 to 10 weeks were reported (3) to be more resistant to injury and decay, after vigorous washing, than similarly treated apples without storage. Additional work should be done to compare sugar and amino acid contents of apples and susceptibility to *P. expansum* at various times during storage.

LITERATURE CITED

1. AYERS, W. A., G. C. PAPAVIZAS, & A. F. DIEM. 1966. Polygalacturonate trans-eliminase and polygalacturonase production by Rhizoctonia solani. Phytopathology 56:1006-1011.
2. BIEHN, W. L., & A. E. DIMOND. 1971. Effect of pectin source and sugars on polygalacturonase production by Ceratocystis ulmi. Phytopathology 61:745-746.
3. ENGLISH, H., A. L. RYALL, & E. SMITH. 1946. Blue mold decay of Delicious apples in relation to handling practices. U.S. Dept. Agr. Cir. 751. 20 p.
4. HORTON, J. C., & N. T. KEEN. 1966. Sugar repression of endopolygalacturonase and cellulase synthesis during pathogenesis by Pyrenochaeta terrestris as a resistance mechanism in onion pink root. Phytopathology 56:908-916.
5. HULME, A. C. 1958. Some aspects of the biochemistry of apple and pear fruits. Adv. Food Res. 8:297-413.
6. KEEN, N. T., & J. T. HORTON. 1965. Induction and repression of endopolygalacturonase synthesis by Pyrenochaeta terrestris. Can. J. Microbiol. 12:443-453.
7. MORAN, F., & M. P. STARR. 1969. Metabolic regulation of polygalacturonic acid transeliminase in Erwinia. Eur. J. Biochem. 11:291-295.
8. PATIL, S. S., & A. E. DIMOND. 1968. Repression of polygalacturonase synthesis in Fusarium oxysporum f. sp. lycopersici by sugars and its effect on symptom reduction in infected tomato plants. Phytopathology 58:676-682.
9. PREISS, J., & G. ASHWELL. 1963. Polygalacturonic acid metabolism in bacteria. J. Biol. Chem. 238:1571-1576.
10. SITTERLY, W. R., & J. R. SHAY. 1960. Physiological factors affecting the onset of susceptibility of apple fruit to rotting by fungus pathogens. Phytopathology 50:91-93.
11. SPALDING, D. H. 1963. Production of pectinolytic and cellulolytic enzymes by Rhizopus stolonifer. Phytopathology 53:929-931.
12. SPALDING, D. H., & A. A. ABDUL-BAKI. 1973. In vitro and in vivo production of pectin lyase by Penicillium expansum. Phytopathology 63:231-235.
13. WRIGHT, T. R., & E. SMITH. 1954. Relation of bruising and other factors to blue mold decay of Delicious apples. U.S. Dept. Agric. Circ. 935. 15 p.
14. ZUCKER, M., L. HANKIN, & D. SANDS. 1972. Factors governing pectate lyase synthesis in soft rot and non-soft rot bacteria. Physiol. Plant Pathol. 2:59-67.