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PRODUCTION OF HEMICELLULASES BY *PENICILLIUM DIGITATUM*

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Abstract—*Penicillium digitatum* grew sparsely on media containing hemicelluloses, but filtrates from these cultures had high macerating activity. They also contained xylanase and released only galactose, arabinose and xylose from a potato cell-wall preparation and from orange protopectin. The fungus produced α-L-arabinofuranosidase on media containing glucose or pectin N.F. as the carbon sources. Evidence is presented which shows that this enzyme is not responsible for maceration.

INTRODUCTION

Penicillium digitatum Sacc. causes a serious post-harvest soft-rot disease of citrus fruit known as "green mould". Infection of fruit is associated with separation of individual cells, disorganization of the tissue and breakdown of cell walls. Maceration of host tissues by fungi has been attributed mainly to the activity of pectic enzymes, especially endo-pectic glycosidases and lyases. Cellulases are usually considered unimportant at least in the early stages of infection. As well as pectic substances and cellulose, plant cell-walls contain hemicelluloses and protein. The growth of fungal pathogens on hemicelluloses and the significance of hemicellulases in pathogenesis have been little studied though there is now some evidence that they may play a role in maceration.[1-3] Byrde and Fielding[4] suggested that α-L-arabinofuranosidase is one of the enzymes responsible for maceration caused by filtrates from cultures of *Sclerotinia fructigena*. Since the completion of this work it has been found that this is not the case and Byrde and Fielding[5] correlated maceration with pectin methyl *trans*-eliminase. Below is described similar work with *P. digitatum*.

RESULTS

Table 1 shows that growth was sparse in the three media containing hemicellulose as the sole carbon source. Growth on glucose under the same conditions would be about 200 mg. Nevertheless, each filtrate had considerable macerating activity.

The filtrate from cultures on the medium containing jute hemicellulose was also added to the following substrates: spruce glucomannan, spruce xylan + mannan, larch galactan,

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[1] J. H. MCCLENDON, *Am. J. Bot.* **51**, 628 (1964).
[2] J. G. HANCOCK, *Phytopatholgy* **57**, 203 (1967).
[3] M. KNEE, Ph.D. Thesis, University of Hull (1968).
[4] R. J. W. BYRDE and A. H. FIELDING, *Nature* **205**, 390 (1965).
[5] R. J. W. BYRDE and A. H. FIELDING, *J. gen. Microbiol.* **52**, 287 (1968).

esparto grass hemicellulose, jute hemicellulose and a potato cell-wall preparation in reaction mixtures containing 50 mg hemicellulose or 20 mg potato cell-wall, 1·0 ml culture fluid, 1·0 ml 0·1 M citrate buffer (pH 5·5). The mixtures were incubated for 21 hr in test tubes with a drop of toluene. Supernatant solutions from mixtures with insoluble substrates were obtained by centrifuging. 25-μl samples of the reaction mixtures were analysed by ascending chromatography. A 20 per cent breakdown of the substrates in this volume to monosaccharide would be expected to give spots of the same intensity as the markers; concentrations 10 times less than the marker spots were detectable. None of the hemicelluloses was degraded by the jute culture filtrates under these conditions, but potato cell-wall gave a strong galactose spot though neither galacturonic acid nor any of its oligomers were detected.

TABLE 1. GROWTH OF *Penicillium digitatum* IN MEDIA CONTAINING HEMICELLULOSES AND MACERATING ACTIVITY OF FILTRATES

Hemicellulose	pH of medium (final)	Mean dry wt. of mycelium (mg)	Macerating activity
Jute	6·8	22·7 (± 4·0)	6·9
Larch	6·9	19·3 (± 2·6)	6·3
Spruce	6·6	18·8 (± 7·7)	5·1

TABLE 2. CHROMATOGRAPHIC ANALYSIS OF REACTION MIXTURES CONTAINING JUTE HEMICELLULOSE CULTURE FILTRATE

Spots	Pectin	NaPP	Substrate protopectin	Potato cell-wall
MonoGA*	++	++	—	—
Glucose	—	—	—	—
Galactose	++	++	+++	+++
Arabinose	—	+	++	++
Xylose	—	+	++	—
R_{GA}† 1·64–1·85	—	+	++	tr

Key: + = intensity of spot, tr = trace spot, — = no spot, * monogalacturonic acid, † $R_{galacturonic\ acid}$ (in BAW solvent).

50 mg orange protopectin,[6] 10 mg potato cell-wall, or 1 ml 1 per cent pectin N.F. or sodium polypectate (NaPP) solutions were also incubated with 2 ml jute culture filtrate and 2 ml 0·1 M citrate buffer (pH 4·5), for 24 hr at 25°. The products were then examined by descending chromatography with the results shown in Table 2. No spots appeared in controls with autoclaved filtrate. Only galacturonic acid and galactose were detected in mixtures containing pectin. Sodium polypectate mixtures contained galacturonic acid and galactose, and to a lesser extent arabinose, xylose and an unknown product with an R_{GA} of 1·64–1·85. Galacturonic acid was not detected in mixtures containing protopectin or potato cell-wall, but large amounts of galactose and somewhat less arabinose were found in both. Xylose was present in protopectin but not potato cell-wall mixtures.

[6] Z. I. KERTESZ, *The Pectic Substances*, Interscience Publishers Inc., New York (1951).

Xylose was readily detected in filtrates from cultures of *P. digitatum* on a medium containing 1% esparto grass hemicellulose for 15 days at 25°, but was not present in uninoculated media; this suggests that *P. digitatum* produces a xylanase in culture.

Arabinosidase activity was detected in filtrates from cultures of the fungus grown on media containing glucose or pectin. The activity at pH 5·0 of filtrates from cultures of different ages grown on the glucose medium is shown in Table 3. Activity increased with age of culture from the youngest to the oldest (17 days) that were tested. The results given in Table 4 for cultures grown on the glucose medium show that the enzyme was active over a wide pH range with an ill-defined optimum between pH 3·0–5·0.

TABLE 3. α-L-ARABINOFURANOSIDASE ACTIVITY IN CULTURES OF DIFFERENT AGES

	Age of culture (days)					
	3	6	9	11	13	17
Hydrolysis (%)	6·3	6·6	7·0	7·5	11·9	13·7
Rate of hydrolysis (μmoles/hr/ml enzyme)	1·4	1·5	1·6	1·7	2·6	3·0

TABLE 4. EFFECT OF pH ON ARABINOSIDASE ACTIVITY

pH	Hydrolysis (%)	Rate of hydrolysis (μmoles/hr/ml enzyme)
3·0	13·4	3·0
3·5	14·2	3·1
4·0	12·4	2·7
4·5	13·1	2·9
5·0	14·2	3·1
5·5	9·8	1·1
6·0	10·4	2·3
6·5	7·0	1·5
7·0	6·9	1·5

To determine the relation between the "macerating factor" and α-L-arabinofuranosidase, a 10-day-old filtrate from a culture grown on the medium containing pectin was fractionated by gel filtration on Sephadex G 75. Water was used as the liquid phase in the column and was also the eluting solvent; 40 ml filtrate were applied to the column (44 cm × 3 cm) and 10-ml fractions were collected and assayed for arabinosidase and macerating activity (Fig. 1). The single peak of α-L-arabinofuranosidase activity was well separated from the single peak of macerating activity.

DISCUSSION

Filtrates from cultures grown on a medium containing jute hemicellulose as the carbon source gave reasonable yields of galactose, arabinose and xylose from orange protopectin and potato cell-wall. These sugars would not be expected as major products of the breakdown

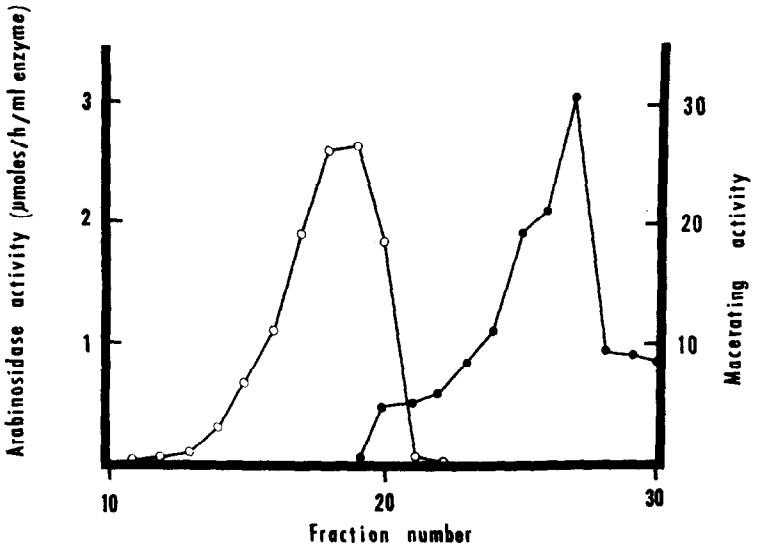

FIG. 1. SEPARATION OF α-L-ARABINOFURANOSIDASE AND MACERATING ACTIVITY FROM A 10-day-old CULTURE FILTRATE OF *Penicillium digitatum* BY SEPHADEX (G75) GEL FILTRATION.

α-L-Arabinofuranosidase O——O
Macerating activity ●——●

of pectic substances as classically conceived. Barrett and Northcote,[7] however, have indicated that the pectic substance of apple is in part composed of a large heteropolysaccharide in which there is a close association of arabans and galactans with polyuronides. The breakdown of such a pectic substance might well yield these sugars. Galacturonic acid or polymers of galacturonic acid were not obtained from protopectin or potato cell-wall, although the acid was found in mixtures containing pectin and sodium polypectate. The jute culture filtrate (and those from other hemicelluloses) had relatively high macerating activity so that the possibility that hemicellulases, especially galactanase, play a role in tissue maceration must now be considered feasible. Galactanase has been implicated in the attack or penetration of potato tuber tissue by *Phytophthora infestans*[8] and is reported to have some effect on the structural cohesion of potato discs.[3]

α-L-Arabinofuranosidase was produced constitutively by *P. digitatum* in a medium containing glucose as the carbon source and also in a medium containing pectin N.F. The extracellular "macerating factor" was not α-L-arabinofuranosidase. Bush and Codner[9] consider pectin methyl *trans*-eliminase to be responsible for maceration, however Cole and Wood[10] question this since macerating activity was detected in culture filtrates and diseased tissue extracts in the absence of pectin methyl *trans*-eliminase.

EXPERIMENTAL

A monospore isolate of *Penicillium digitatum* was obtained from a Navel orange with a typical "green-mould" lesion. Culture media for enzyme production:

[7] A. J. BARRETT and D. H. NORTHCOTE, *Biochem. J.* **94**, 617 (1965).
[8] M. KNEE and J. FRIEND, *Phytochem.* **7**, 1289 (1968).
[9] D. A. BUSH and R. C. CODNER, *Phytochem.* **7**, 863 (1968).
[10] A. L. J. COLE and R. K. S. WOOD, *Ann. Bot. Lond.* N.S., in press.

A. 4·6 g casamino acids, 1 g KH_2PO_4, 0·5 g $MgSO_4.7H_2O$, 2·5 g hemicellulose, 10 ml trace element solution and water to 1 l. Three hemicelluloses were used: jute; larch (arabino–galactan); and spruce (mannan + xylan).

B. 10 g KNO_3, 5 g KH_2PO_4, 2·5 g $MgSO_4.7H_2O$, 1·0 g Difco yeast extract, 20 g glucose or 1 g pectin N.F. and water to 1 l.

50 ml of media A in 150 ml Erlenmeyer or 50 ml of media B in 10 oz medical flats were autoclaved at 15 psi for 20 min and inoculated with 1 ml containing *c.* 1×10^7 conidia. Media in medical flats were incubated in stationary culture at 25° and in flasks incubated on a horizontal shaker at 25° for 6 days. Cultures were filtered through muslin or Whatman No. 541 filter paper and the mycelia that were obtained were transferred to weighed aluminium foil cups which were dried at 70°, cooled over $CaCl_2$ and weighed.

Culture filtrates were centrifuged at 26,000 *g* and the cell-free supernatant solutions were dialysed against distilled water for 18 hr at 5°. The final solutions were assayed for hemicellulases by analysing reaction mixtures by descending paper chromatography on Whatman No. 1 paper, irrigated for 66 hr with butanol–acetic acid–water (BAW), dried and developed with $AgNO_3$,[11] or by descending chromatography on Whatman No. 1 paper irrigated for 18 hr with *iso*-propanol–water, dried and developed with aniline diphenylamine.[11] Autoclaved enzyme was used in the controls. The R_{GA} (*GA = galacturonic acid*) values and characteristic colour of standard galacturonic acid and sugar solutions were compared with those of products in the reaction mixture.

α-L-Arabinofuranosidase activity was estimated by measuring colorimetrically the phenol liberated from phenyl α-L-arabinoside in the way devised by Byrde and Fielding. Reaction mixtures contained 0·5 mg phenyl α-L-arabinofuranoside/1 ml water, 0·5 ml 0·1 M citrate buffer, 0·2 ml enzyme preparation, and 0·3 ml water. After incubation at 25° for 30 min, 1 ml sat. Na_2CO_3 solution was added, thoroughly mixed and followed by 5 ml water, 0·4 ml Folin Ciocalteu's reagent and water to 10 ml. The colour that developed after 1 hr at room temperature was measured with a Gallenkamp colorimeter and an Ilford 621 red filter (670 mμ). Substrate and enzyme blanks served as controls; freshly prepared aqueous phenol solutions were used to obtain standard absorption curves.

Macerating activity of culture filtrates was measured with disks, 0·35 mm thick, 10 mm diam., cut from the medulla of a potato tuber that had been injected with water under vacuum. Test solutions contained 1 ml of 0·1 M citrate buffer (pH 5·5) and 1 ml of enzyme preparation with autoclaved enzyme solutions as controls. Maceration time was taken as the mean time (min) for the potato disks to lose coherence when tested by gently pulling between forceps. Activity is expressed as 100/mean maceration time.

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[11] I. SMITH, in *Chromatographic and Electrophoretic Techniques* (edited by I. SMITH), Vol. I, p. 246, Heinemann, London (1960).