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SHORT COMMUNICATION

EXTRACELLULAR "GALACTANASE" ACTIVITY FROM *PHYTOPHTHORA INFESTANS* (MONT.) DE BARY

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Abstract—Culture filtrates of *Phytophthora infestans* contain an enzyme which catalyses the liberation of galactose from potato pectin. Evidence is presented that the activity is specific for 1-4 linkages between galactose residues.

ALTHOUGH *Phytophthora infestans* has been shown to produce extracellular pectin methyl esterase in liquid culture[1,2] the attempted demonstration of pectolytic activity has given equivocal results. Using filtrates from cultures grown on a supplemented potato extract medium, Grossman[1] was able to show that these caused a fall in the viscosity of unspecified pectic and pectinic acid substrates. This reaction was not accompanied by a measurable increase in reducing power or by the appearance of monomeric galacturonic acid. Furthermore Clarke[2] could not demonstrate polygalacturonase activity of any kind in filtrates from cultures on a wide range of media.

Clarke and Grossman both used traditional pectin substrates in which poly-uronide predominated. Since we found that galactose is the major component of hydrolysates of potato pectin (Table 1) it was decided to look for hydrolytic activity against potato pectin and white lupin pectin,[3] which is of similar monosaccharide composition.

TABLE 1. MONOSACCHARIDE COMPONENTS OF PECTINS

Pectin source	Monosaccharide (%)*			
	Gal UA	Gal	Ara	Xyl
Potato	13·5	51·6	5·0	—
Lupin	10·0	48·8	16·2	3·8

* Results are expressed as percentage of anhydro sugar by weight of pectin. Gal UA = Galacturonic acid, Gal = Galactose, Ara = Arabinose, Xyl = Xylose.

Initially the fungus was cultured on a range of liquid media with and without added pectin. Samples of filtrates were incubated with potato pectin at pH 4·0, 6·0 and 8·0 for 24 hr.

[1] F. GROSSMAN, *Naturwissenschaften* **50**, 721 (1963).
[2] D. D. CLARKE, *Nature* **211**, 649 (1966).
[3] E. L. HIRST, J. K. N. JONES and W. O. WALDER, *J. Chem. Soc.* 1225 (1947).

Activity was estimated as an increase in reducing sugar over the period of incubation. Some activity at pH 4·0 was detected from cultures on media containing either potato or lupin pectin. Enzyme preparations from culture filtrates, concentrated by ammonium sulphate precipitation could be assayed over a much shorter period than the original filtrate.

Investigation of the pH-activity relationship with potato pectin substrate revealed that the optimum for a 30-min incubation period was pH 3·5. After longer incubation there was an apparent shift in the pH optimum to pH values between 4 and 5, presumably due to progressive inactivation of the enzyme at low pH values. All activity was destroyed by boiling.

Paper chromatography of the concentrated products of a 24-hr incubation of enzyme and potato pectin at pH 4·5 revealed galactose as the only monosaccharide present. None was evident in an incubation with the boiled enzyme.

Lupin pectin contains a linear polymer of 1-4 linked β-D galactopyranose units,[3] whilst larch galactan consists of a framework of 1-3 linked β-D galactopyranose with short side-chains of the same units in a β1-6 linkage.[4] The enzyme preparation degraded lupin pectin

TABLE 2. ACTIVITY AGAINST VARIOUS SUBSTRATES OF AN ENZYME PREPARATION FROM CULTURE FILTRATES OF *Phytophthora infestans*

Substrate	μ-Moles galactose liberated		
	Unboiled enzyme		Boiled enzyme
	30 min	60 min	60 min
Potato pectin	1·75	3·43	0·07
Lupin pectin	1·57	3·16	0 07
Larch galactan	0·13	0·05	0·17
PNPG (pH 4·0)	0·06	0·10	
PNPG (pH 7·0)*	0·09	0·13	

PNPG = para nitrophenyl β-D-galactopyranoside.
* 0·2 mg Yeast β-Galactosidase (Koch-Light Laboratories Ltd) assayed for comparison gave 1·75 and 2·65 μMoles galactose at 30 and 60 min.

at a rate similar to that for potato pectin, but it showed no activity against larch galactan (Table 2). These observations suggest that the activity is specific for β1-4 linkages and that the galactan of potato pectin contains a predominance of such linkages.

β-Galactosidase preparations will catalyse the selective release of galactose from the polysaccharide moieties of glycoproteins.[5] However the preparation from *Phytophthora* gives poor rates in the standard assay for β-galactosidase and this activity may be independent of that against pectin. (Table 2).

Evidence for "galactanase" activity has been obtained in apples infected by *Botryosphaeria ribis*[6] and such activity has been held to account for symptoms of plant disease.[6,7] The *Phytophthora* enzyme may be involved in the attack or penetration of potato tissue by the fungus and investigation of the nature of the enzyme and its biological significance is being continued.

[4] G. O. ASPINALL, E. L. HIRST and E. RAMSTAD, *J. Chem. Soc.* 593 (1958).
[5] R. G. SPIRO, *J. Biol. Chem.* **237**, 646 (1962).
[6] J. M. MCCLENDON, G. F. SOMERS and J. W. HEUBERGER, *Phytopathology* **50**, 258 (1960).
[7] J. G. HANCOCK, *Phytopathology* **57**, 203 (1967).

EXPERIMENTAL

Potato pectin. Peeled and chopped King Edward potatoes (2 kg) were disintegrated with ethanol (1 ml/g) in a blender, filtered on cheese cloth and washed with distilled water (25 l.). The filtrates, containing the bulk of the starch grains present in the original material were discarded. The residue on the cheese cloth was suspended in a distilled water (2 l.) and boiled for 10 min to gelatinize remaining starch grains. The suspension was brought to pH 7·0 (NaOH), NaCl (4 g) was added and the volume made up to 5 l. with water. 20 ml of freshly prepared human saliva were added, followed by a little toluene, and the digestion allowed to proceed for 24 hr at 20°. The whole was then filtered under suction and the filtrate discarded. The cell wall material which formed the residue was suspended in water (1 l.), refluxed for 12 hr and filtered under suction. Four volumes of ethanol were added to the filtrate and the resultant precipitate of potato pectin collected by centrifugation, washed twice with ethanol, twice with ether and dried *in vacuo*. (Yield of potato pectin = 9·6 g.)

Lupin pectin. This was prepared from seeds of *Lupinus albus* (Dunn's Farm Seeds Ltd.) by the method of Hirst *et al.* (1947).[3]

Analysis of pectins. Galacturonic acid was determined by the carbazole method.[8] Samples were hydrolysed[9] and the hydrolysates neutralized with NN-bis-n-octylmethylamine.[10] The neutral sugars present in hydrolysates were separated by paper chromatography using ethyl acetate-pyridine-water (8:2:1, by volume) and estimated by the method of Wilson (1959).[11]

Source and culture of fungus. A race 4 isolate of *Phytophthora infestans*, obtained from infected leaves of Duke of York potato, was used throughout. Mycelial discs were inoculated into flat bottles of "Birds Eye" french bean liquid,[12] incorporating 2 g/l of potato or lupin pectin. After 7 days growth at 22° the cultures were filtered on a coarse-porosity sintered glass funnel.

Enzyme preparation. Pooled culture filtrates were brought to 80 per cent saturation with ammonium sulphate at 10°. The resultant precipitate was collected by centrifugation, resuspended in a minimal volume of 5m M buffer (pH 7·0) and dialysed for 4 hr against the same buffer at 4°. This preparation was stable in storage at −20° for a period of weeks.

Enzyme assays. 1 ml of enzyme preparation was incubated with 1 ml of potato pectin solution (5 mg/ml) and 1 ml citrate phosphate buffer at 25°. pH 4·0 was chosen for routine assay and at this pH the buffer was 0·012 M with respect to citric acid. Reducing sugar in the mixtures was estimated in terms of galactose by Somogyi's[13] modification of Nelson's[14] method.

In the experiment with lupin pectin and larch galactan (Koch Light Laboratories Ltd.) 1 ml of a solution of the substrate (5 mg/ml) was added in place of the potato pectin in the above procedure.

p-Nitrophenyl β-D-galactopyranoside (Koch Light Laboratories Ltd) was used as a substrate for 1 ml of the enzyme preparation in a standard assay for β-Galactosidase.[15]

Identification of products. The products of the enzyme reaction were subjected to chromatography on Whatman No. 1 paper with galacturonic acid, galactose and arabinose standards, using as solvent ethyl acetate, acetic acid, pyridine, water, (5:1:5:3 by volume) in a tank equilibrated with ethyl acetate, pyridine, water (40:11:6 by volume).[16] The monosaccharides were visualized and estimated by Wilson's method.

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[8] R. M. McCready and E. A. McComb, *Anal. Chem.* **24**, 1986 (1952).
[9] J. F. Saeman, W. E. Moore, R. L. Mitchell and M. A. Millet, *Tappi* **37**, 336 (1954).
[10] R. W. Stoddart, A. J. Barrett and D. H. Northcote, *Biochem J.* **102**, 194 (1967).
[11] C. M. Wilson, *Anal. Chem.* **31**, 1199 (1959).
[12] D. J. Austin and D. D. Clarke, *Nature* **210**, 1165 (1966).
[13] M. Somogyi, *J. Biol. Chem.* **195**, 19 (1952).
[14] N. Nelson, *J. Biol. Chem.* **153**, 375 (1944).
[15] S. A. Kuby and H. A. Lardy, *J. Am. Chem. Soc.* **75**, 890 (1953).
[16] F. G. Fischer and H. Dörfel, *Hoppe-Seylers Z.* **301**, 224 (1955).