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INTRACELLULAR β-GLUCANASE ACTIVITY OF *PHYTOPHTHORA PALMIVORA*

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SUMMARY

The mycelium of *Phytophthora palmivora* contains enzymes capable of hydrolysing β-D-1,3-glucans by endwise removal of glucose units. This laminarinase (β-1,3-glucan glucohydrolase) activity is found intimately associated with particulate fractions (3000–100 000 × *g*); very little is secreted into the culture medium. Laminarin-oligosaccharides are also cleaved. The rate of hydrolysis was greatest with laminaribiose, reached a minimum with laminaritetraose and rose again with laminaripentaose and laminarin. These results suggest the existence of a β-1,3-glucosidase together with the β-1,3-glucanase in the subcellular particles but separation of these two activities was not achieved. Maximum laminarinase activity occurred at 40 °C and pH 5–6. The laminarinase does not require the addition of metal ions for activity and is inhibited by heavy metal ions particularly Hg^{2+}. Motile zoospores from *P. palmivora* contain both particulate and soluble β-1,3-glucanase activities. A cytoplasmic glucan isolated from the mycelium of *P. cinnamomi* was digested by enzymes from the mycelium or zoospores of *P. palmivora* but at a much slower rate than laminarin.

INTRODUCTION

It is well known that fungi often undergo morphological differentiation when placed under nutritionally deficient conditions[1,2]. The metabolic demands of the differentiating fungus for energy and structural compounds must therefore be satisfied, partly or entirely, by endogenous reserves. Our interest in defining the intracellular glucanase activity of *Phytophthora* spp. originated from the following observations: (1) β-1,3-glucans (with β-1,6 links at the branching points) represent a major reserve carbohydrate of *Phytophthora cinnamomi*[3]. (2) During C starvation of the mycelium of *P. cinnamomi* or during encystment of zoospores of *P. palmivora*[4] soluble cytoplasmic glucan(s) are degraded; concurrently, there is considerable synthesis of

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an insoluble cell wall glucan. This glucan is a major structural component of the cyst walls, sporangial walls, or hyphal walls formed by the differentiating fungus[5,9]. Clearly, the synthesis of cell wall glucan from cytoplasmic glucan must be an important part of the biochemical machinery of cell differentiation in *Phytophthora*.

In the present communication we describe the extraction and characterization of intracellular β-1,3-glucanase(s) from *Phytophthora palmivora*.

MATERIALS AND METHODS

Laminarin was obtained from the Institute of Seaweed Research, Inveresk, Midlothian, Scotland, or from K and K chemicals (Plainview, N.Y., U.S.A.) and was purified by alcohol precipitation from aqueous solution. Pustulan was prepared from *Umbilicaria pustulata*[6,7]. Laminarin oligosaccharides were obtained by H_2SO_4 hydrolysis of laminarin[8] and separated by column chromatography on Sephadex G-15. The cytoplasmic glucan from *P. cinnamomi*, which consists of β-D-1,3-linked glucose units with β-1,6-linked branches, has been prepared and characterized in our laboratory[3]. Other substances used were commercially available products.

Enzyme preparation

Phytophthora palmivora (papaya isolate) was kindly supplied by M. Aragaki, University of Hawaii. (This strain had been formerly referred to as *P. parasitica* but its nomenclature has been revised[4]). The fungus was grown as described previously in V-8 juice liquid medium[9]. Cultures were grown in stationary bottles at 26 °C for 4–18 days, and the mycelia were harvested by filtration. The mycelial mats (wet weight 2–5 g) were washed with 10 mM sodium acetate buffer, pH 5.7, and broken in a Braun MSK Mechanical Cell Homogenizer (Bronwill Scientific, Rochester, N.Y., U.S.A.) for 30–60 s. Samples were centrifuged at 3000 × *g* for 10 min. To the resulting milky supernatant, $(NH_4)_2SO_4$ was added to 45% saturation. The precipitate was removed by centrifugation at 20 000 × *g* for 20 min and resuspended in a minimal amount of acetate buffer.

To determine extracellular glucanase activity, the culture filtrate was concentrated and dialyzed against acetate buffer. To the non-dialyzable portion, $(NH_4)_2SO_4$ was added to 70% saturation. The resulting precipitate was removed by centrifugation at 20 000 × *g* and redissolved in a minimal amount of acetate buffer.

Zoospores of *P. palmivora* were prepared from cultures grown at 27 °C on V-8 juice agar plates for 4 days in the dark and 4–9 days in the light. To harvest the zoospores, plates were flooded with a buffer containing 1 mM KH_2PO_4, 0.1 mM $MgCl_2$ and 0.02 mM $CaCl_2$, pH 6. After 20–30 min the plates were decanted and the liquid filtered through Whatman filter paper No. 41; the filtrate was centrifuged at 3000 × *g* for 20 min.

The sedimented zoospores were resuspended in 5 ml of 200 mM Tris–HCl buffer, pH 7, rapidly frozen and thawed three times in a dry-ice–acetone bath followed by homogenization in a Sorvall omni-mixer for 30 s. The resulting suspension was centrifuged at 12 000 × *g* for 10 min; both sediment (resuspended in buffer) and supernatant were assayed for enzyme activity.

Enzyme assays and chemical determinations

Laminarinase activity was assayed at 40 °C; the standard reaction mixture contained 50 mM sodium acetate buffer, pH 5.7, and 2 mg of laminarin in a final volume of 1 ml. The reaction was initiated by the addition of 0.1 ml enzyme (approx. 0.3 mg protein) and 0.4-ml samples were removed for assay at 10 and 20 min. Assays were based upon the release of glucose (Glucostat "Special", Worthington Biochemical Corp., New Jersey, U.S.A.) or by the increase in reducing power according to the method of Somogyi and Nelson[10] using glucose as standard. When the former method was used, the reaction was stopped by heating the sample in a boiling water bath for 5 min, while with the latter, the sample was pipetted into 1 ml of alkaline copper reagent. In all cases initial reaction velocity was measured, and the amount of product formed was proportional to the enzyme concentration. One unit of enzyme activity is defined as that amount catalyzing the formation of 1 μmole of glucose per min under the experimental conditions employed.

β-1,3-Oligoglucan:orthophosphate glucosyltransferase activity (phosphorylase activity) was measured in both directions either by determining inorganic phosphate released[12] or by measuring the glucose 1-phosphate formed with the phosphoglucomutase–glucose-6-*P* dehydrogenase–$NADP^+$ system[13].

Protein was measured by the Folin–Lowry[11] method using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Specificity of the β-glucanase of P. palmivora mycelium

The glucanase present in the cell-free extract (45% $(NH_4)_2SO_4$ precipitate) of *P. palmivora* is a laminarinase (Table I). It attacks laminarin, a linear β-1,3-glucan and has no detectable effect on β-1,4-glucan nor β-1,6-glucan. The cytoplasmic glucan of *P. cinnamomi*[3], a β-1,3-glucan with side branches attached by β-1,6-links, was hydrolyzed but only at about 1/3 the rate of hydrolysis of laminarin. This crude preparation also displayed enzyme activity against β-1,3-linked glucose oligosaccharides (see below) and seemingly contained traces of other carbohydrases capable of hydrolysing cellobiose and gentiobiose (Table I).

TABLE I

SUBSTRATE SPECIFICITY OF THE INTRACELLULAR β-GLUCANASE OF *P. palmivora* MYCELIUM

The reaction mixtures contained 0.1 ml of redissolved 45% $(NH_4)_2SO_4$ precipitate (0.1 mg protein).

Substrate	*Total μmoles glucose released per min*	
	Reducing sugar	*Glucose oxidase*
Laminarin (β-1,3-glucan)	0.064	0.061
Pustulan (β-1,6-glucan)	0	0
Acid-swollen cellulose (β-1,4-glucan)	0	0
Cytoplasmic glucan from *P. cinnamomi* (β-1,3–β-1,6-glucan)	0.019	0.022
Cellobiose	—	0.002
β-Gentiobiose	—	0.007

TABLE II

DISTRIBUTION OF LAMINARINASE ACTIVITY IN THE CELL FREE EXTRACT AND CULTURE MEDIUM OF *P. palmivora* MYCELIUM

Reaction mixtures contained 0.1 ml of enzyme (approx. 0.3 mg protein) prepared from 8-day-old mycelium.

Fraction	*Total protein (mg)*	*Laminarinase*	
		Total units	*Specific* activity*
Cell free extract: 3000 × *g* pellet	35.4	7.59	0.214
Cell free extract: 3000 × *g* supernatant	75.3	13.11	0.174
Culture filtrate	21.3	0.13	0.007

* Specific activity measured in μmoles glucose/min per mg protein.

Subcellular distribution and attempted purification of laminarinase activity of P. palmivora

Both the 3000 × *g* pellet and accompanying supernatant, from the cell-free extract of mycelium of *P. palmivora*, contained significant amounts of laminarinase activity; very little activity was found in the concentrated culture medium fluid (Table II). Differential centrifugation of the 3000 × *g* supernatant showed almost equal levels of laminarinase in the 8000 × *g* and the 100 000 × *g* pellets; no activity was detected in the 100 000 × *g* supernatant. Since $(NH_4)_2SO_4$ (45% saturation) precipitated essentially all of the enzymatic activity from the 3000 × *g* supernatant, this procedure was used instead of centrifugation at 8000 × *g* or 100 000 × *g* for routine preparation of the enzyme. Also, the precipitation with $(NH_4)_2SO_4$ increased the specific activity of laminarinase from about 0.17 (in the 3000 × *g* supernatant) to 0.52 (in the precipitate).

Because the laminarinase activity appeared to be intimately associated with subcellular particles, various extraction procedures were tested in an effort to solubilize the enzyme. The methods used to dissolve the enzyme from the $(NH_4)_2SO_4$ precipitate included extraction with butanol or acetone; sonic oscillation in a 10 kHz Raytheon unit; homogenization in a Braun MSK cell homogenizer; lyophilization followed by extraction with 1 M sodium acetate buffer, pH 5.7; and autolysis. Following these treatments, 50–80% of the original laminarinase activity remained in the particulate fraction but no enzymatically active protein was solubilized. Sodium dodecyl sulfate (0.05% final concentration) could not be employed since it inhibited enzyme activity almost completely.

Laminaribiase activity in the crude laminarinase preparation of P. palmivora mycelium

In addition to hydrolyzing laminarin, the crude enzyme preparation was capable of splitting laminarin oligosaccharides (laminaribiose through laminaripentaose). Enzyme affinity for these substrates, as measured by Michaelis constants (K_m), was of the same order of magnitude for laminarin as for its oligomers (Table III). The rate of hydrolysis was greatest for laminaribiose, reached a minimum with laminaritetraose and rose again for laminaripentaose and laminarin. In accordance with the known behavior of glucosidases *vs* glucanases[14], our data suggest the presence of two enzymic activities: a laminaribiase with maximum catalytic action on the disaccharide and

TABLE III

KINETIC PARAMETERS OF HYDROLYSIS OF LAMINARIN AND LAMINARIDEXTRINS BY *P. palmivora* ENZYMES

Samples were assayed by the glucose oxidase methods. Amounts of enzyme were adjusted to measure initial reaction velocity. In all cases K_m and V values were calculated by extrapolation from Lineweaver–Burk double-reciprocal plots.

Substrate	$K_m \times 10^3$ *(M)*	*V (μmoles glucose/min per mg protein)*
Laminaribiose	6.1	2.6
Laminaritriose	5.0	2.1
Laminaritetraose	3.8	0.53
Laminaripentaose	4.9	0.73
Laminarin	(3.3)*	1.5

* For this calculation the molecular weight of laminarin was assumed to be 2448 (15 glucose units).

progressively less effect on longer oligosaccharides, and a glucanase whose greatest rate of action would be on the polysaccharide and minimal on short oligosaccharides. Although the trend in V data support the presence of both laminaribiase and laminarinase, no direct proof has been gathered for the existence of two separate enzymes. Decisive proof would require separation of the suspected enzymes, a task not yet completed.

Properties of the β-1,3-glucanase of P. palmivora mycelium

Maximum enzyme activity occurred at pH 5–6 in 50 mM sodium acetate buffer, or in potassium phosphate buffer (I 0.05) with either laminarin or laminaribiose as substrates (Fig. 1). Because of the limited amounts of substrates available, saturating amounts were not used for these experiments on pH effect. Thus, initial rather than maximal velocities are shown in Fig. 1. This explains why the laminarinase activity appears to be greater than laminaribiase. When maximal velocities are

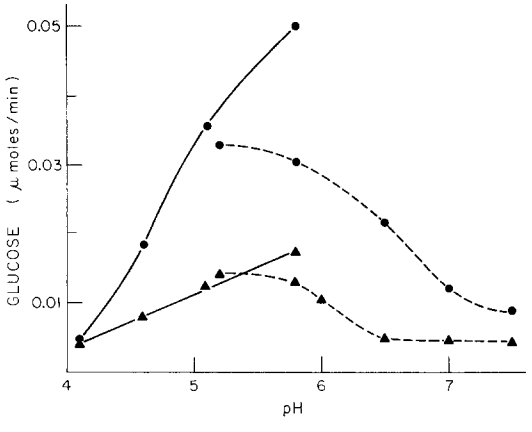

Fig. 1. Effect of pH on laminarinase (●) and laminaribiase (▲) activities of *P. palmivora*. pH effect was measured with either potassium phosphate buffer (I 0.05) (— — —) or 50 mM acetate buffer (———). In all cases, initial reaction velocity was measured. Laminarinase was assayed as described in Materials and Methods. For laminaribiase activity, the reaction mixture samples contained 0.56 mg of laminaribiose.

compared (Table III), the reverse is true. Optimal temperature for laminarin digestion was 40 °C. Almost all enzyme activity was destroyed at 60 °C. The enzyme was stable for 5 days at 5 °C, but after 2 weeks 50% of the activity was lost.

Of the various salts tested, at a final concentration of 1 mM, Ag^{+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+}, inhibited the reaction from 40–75%. Hg^{2+} was totally inhibitory. Mg^{2+}, Ca^{2+}, Ba^{2+} and Co^{2+} had no effect. The observation that 1 mM EDTA did not diminish the activity tends to corroborate the belief that metals are not required for activity.

The specific activity of laminarinase in the mycelium of *P. palmivora* was relatively constant over the cultivation period tested (7-, 12-, and 18-day cultures).

Mode of substrate degradation

The available evidence indicates that the laminarinase of *P. palmivora* mycelium is an exo-glucanase, *i.e.* it degrades the substrate by successive removal of terminal glucose units. Simultaneous assays of glucose (Glucostat) and reducing sugar indicated that the increase in reducing power was largely if not entirely due to the formation of free glucose. Paper chromatography of the products of laminarin hydrolysis revealed only one spot corresponding to glucose; there was no evidence for oligosaccharides of laminarin, thus suggesting the absence of appreciable levels of endo-β-1,3-glucanase activity.

β-Glucanase in zoospores of P. palmivora

The cell free extract from motile zoospores of *P. palmivora* contained laminarinase and laminaribiase activities in the 10 000 $\times$ *g* sediment and its supernatant. The supernatant also contained enzymic activity against β-1,4-glucan (acid-swollen cellulose) but not against β-1,6-glucan (pustulan). The cytoplasmic glucan of *P. cinnamomi* was slowly degraded. Glucosidase activity was also detected. Of the disaccharides tested limanaribiose was most rapidly degraded followed by gentiobiose and cellobiose.

Test of phosphorylase activity in P. palmivora

The possibility that glucose polymers were utilized *via* phosphorylative degradation was explored. Neither the mycelium nor the zoospores gave any evidence of such activity.

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REFERENCES

1 G. Klebs, *Die Bedingungen für Fortpflanzung bei einigen Algen und Pilzen*, Gustav Fischer, Jena, 1896.
2 L. E. Hawker, in G. C. Ainsworth and A. S. Sussman, *The Fungi, Vol. 2*, Academic Press, New York, 1966, p. 435.
3 L. P. T. M. Zevenhuizen and S. Bartnicki-Garcia, *J. Gen. Microbiol.*, 61 (1970) 183.
4 J. Tokunaga and S. Bartnicki-Garcia, *Arch. Mikrobiol.*, 79 (1971) 283.

5 J. Tokunaga and S. Bartnicki-Garcia, *Arch. Mikrobiol.*, 79 (1971) 293.
6 B. Lindberg and J. McPherson, *Acta Chem. Scand.*, 8 (1954) 985.
7 E. T. Reese, F. W. Parish and M. Mandels, *Can. J. Microbiol.*, 8 (1962) 327.
8 V. C. Barry and J. E. McCormick, in R. L. Whistler and M. L. Wolfrom, *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, p. 328.
9 S. Bartnicki-Garcia, *J. Gen. Microbiol.*, 42 (1966) 57.
10 J. E. Hodge and B. T. Hofreiter, in R. L. Whistler and M. L. Wolfrom, *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, p. 380.
11 O. H. Lowry, N. J. Rosebrough, A. L. Farr and J. R. Randall, *J. Biol. Chem.*, 193 (1951) 265.
12 H. H. Taussky and E. Shorr, *J. Biol. Chem.*, 202 (1953) 675.
13 H. G. Pontis and L. F. Leloir, in D. Glick, *Methods of Biochemical Analysis*, Vol. X, Interscience, New York, 1962, p. 116.
14 E. T. Reese, A. Maguire and F. W. Parrish, *Can. J. Biochem.*, 46 (1968) 25.