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CELL WALL DEGRADING ENZYMES PRODUCED BY THE PHYTOPATHOGENIC FUNGUS PHYMATOTRICHUM OMNIVORUM

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CELL WALL DEGRADING ENZYMES PRODUCED BY THE PHYTOPATHOGENIC FUNGUS *PHYMATOTRICHUM OMNIVORUM*

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Abstract.—The extracellular plant cell wall degrading enzymes of *Phymatotrichum omnivorum* and the effect of different carbon sources on the production of these enzymes were investigated. Cellobiohydrolase, endoglucanase and xylanase activities were detected in fluids collected from cultures containing sodium carboxymethyl cellulose (CMC) or xylan as carbon sources and enzyme inducers. The highest activities of cellobiohydrolase and xylanase were measured in fluids collected from cultures containing xylan. Results of this study indicate that *P. omnivorum* constitutively produces small amounts of endoglucanase.

Direct penetration of susceptible hosts by the infective hyphae of phytopathogenic fungi is facilitated by the production of cutinases (Agrios 1988), followed by softening or disintegration of host tissues by plant cell wall degrading enzymes produced by the pathogen (Kenaga 1974; Agrios 1988). Production of these enzymes is induced in many plant pathogenic fungi when the these organisms are grown on media containing various sugar polymers (Cooper & Wood 1973; Pegg 1981; Ortega 1990).

Phymatotrichum omnivorum attacks many field crop plants of economic importance. It causes root rot of alfalfa, cotton, peanuts, soybeans, sugarbeets (Nyvall 1989) and sweetpotates (Cook 1978). This pathogen also causes root rots of ornamental plants such as abelia, acacia, cedar, california poppy, privet, lobelia and many others (Pirone 1978).

The primary objectives of this study were to determine the components of extracellular plant cell wall degrading enzymes of *P. omnivorum* and to determine the effects of the carbon source on the production of these enzymes by *P. omnivorum.*

Materials and Methods

Organism and culture conditions.—Stock cultures of *P. omnivorum* were maintained on PDA slants (Difco, B13). The fungus was previously grown in 250 ml flasks with 125 ml of a medium containing: 0.02% $MgSO_4.7H_2O$, 0.01% $Ca(NO_3)_2.4H_2O$, 0.1% Peptone, 0.2% yeast extract, 2.0% glucose in sodium citrate buffer at pH 5.0. After four days growth at 26°C, five ml of mycelium inoculum was washed

twice in distilled water and then transferred to the cellulolytic growth medium. The medium for the production of cellulases contained: 0.25% NH_4NO_3, 0.10% K_2PO_4, 0.05% $MgSO_4$, 0.05% $Ca(NO_3)_2.4H_2O$, 0.72 ppm $Fe(NO_3)_3.9H_2O$, 0.44 ppm $ZnSO_4.7H_2O$, 2.0 ppm $MnSO_4.4H_2O$, 0.40 ppm $ZnCl_2$, and 0.8% carbohydrate. The carbohydrates used as carbon sources and enzyme inducers were: sodium carboxymethyl cellulose (CMC, type 7HF, Aqualon Company), microcrystalline cellulose and xylan (Sigma Chemical Company). Control cultures had glucose as the sole carbon source. The pH of the growing medium was adjusted to 5.0 with 0.1N KOH. Incubation of the cultures was carried out for eight days in covered 250 ml flasks on an orbital shaker at 80 rpm and 26°C.

Enzyme preparation and assays.—Culture fluids were collected after eight days of growth by centrifugation (4800 rpm, 20 minutes, 10°C). The supernatant was subsequently used for the determination of extracellular enzyme activity. For simplification, the collected supernatant is hereafter referred to as the enzyme. All tests were replicated four times.

Cellobiohydrolase (1,4-B-D-glucan cellobiohydrolase, EC 3.2.1.91).— Cellobiohydrolase activity was measured by combining one ml of enzyme with 25 mg of microcrystalline cellulose in one ml of 0.05 M sodium citrate buffer (pH 5.0) and incubating the reaction mixture for two hours at 40°C. The tubes were stirred several times during incubation. After centrifugation, the concentration of reducing sugar in the supernatant was determined using the dinitrosalicylic acid reagent (Miller 1959).

Endoglucanase (CM-cellulase, carboxymethyl cellulase, EC 3.2.1.4).— Endoglucanase activity was measured by combining one ml of enzyme with 10 mg of carboxymethyl cellulose in two ml of 0.05 M sodium citrate buffer (pH 5.0). The reaction mixture was incubated at 40°C for two hours. The concentration of reducing sugar in the reaction mixture was determined using the dinitrosalicylic acid reagent (Miller 1959).

Xylanase (EC 3.2.1.32).—Xylanase activity was measured by combining 10 mg of xylan in one ml of 0.05 M sodium citrate buffer, pH 5.0, with one ml of enzyme. The reaction mixture was incubated at 40°C for two hours. After centrifugation, the concentration of reducing sugars in the supernatant fluid were determined using the dinitrosalicylic acid reagent (Miller 1959).

Table 1. Effect of different carbon sources on the specificactivities[1] of extracellular cell wall degrading enzymes produced by *Phymatotrichum omnivorum*.

Carbon source	Enzymes			
	Cellobio-hydrolase	Endoglucanse	Xylanase	Extracellular protein[2]
Microcrystalline cellulose	0.0	0.0	0.0	0.22±0.50
CMC	8.48±0.13	15.36±0.53	22.72±0.38	0.64±1.08
Xylan	48.67±2.36*	26.57±0.20	265.91±5.17*	1.49±0.66*
Glucose	0.0	73.06±5.68*	0.0	0.28±0.63

[1] μM of glucose or its reducing sugar equivalent/min/ml/mg of protein. Means ± SD of four replications.
[2] mg/ml.
* Using one-way ANOVA and Duncan's MRT, significantly different from other values in the same group.

Protein determination.—Extracellular protein in the crude supernatants was determined with the BCA reagent (Pierce Chemical Company) using bovine serum albumin as a standard.

Data analysis.—The results were expressed as units of specific enzyme activity and represent means plus or minus the standard deviation of four replications. One unit of specific activity (Usp) was calculated as the amount of enzyme that liberated one micromole (μM) of glucose, xylose or their reducing sugar equivalents per minute per ml of enzyme per milligram of extracellular protein under the conditions of the assay. Statistical analyses of experimental data were made with one-way analysis of variance (ANOVA) and Duncan's multiple range test (MRT).

Results

Cellobiohydrolase.—Production of cellobiohydrolase was induced in cultures that contained CMC and xylan. No cellobiohydrolase activity was detected in fluids collected from cultures with microcrystalline cellulose or glucose as the sole carbon source (Table 1). Maximum cellobiohydrolase specific activity (48.67 Usp, Table 1) was measured in fluids collected from cultures containing xylan. This activity was significantly higher ($P = 0.05$) than the cellobiohydrolase activities evaluated in fluids from cultures containing microcystalline cellulose, CMC or glucose.

Endoglucanase.—Production of endoglucanase by *P. omnivorum* was detected in liquid cultures of the fungus containing CMC, xylan and glucose (Table 1). Maximum endoglucanase activity (73.06 Usp) was measured in culture fluids of the fungus grown in media containing glucose. This activity was significantly higher ($P = 0.05$) than the cellobiohydrolase activities evaluated in fluids from cultures containing microcystalline cellulose, CMC or xylan (Table 1). Microcrystalline cellulose did not induce the production of endoglucanase in the cultures of *P. omnivorum* under the conditions of this study.

Xylanase.—Xylanase activities of *P. omnivorum* were detected in the fluids collected from cultures containing CMC and xylan. Maximum activity of this enzyme (265.91 Usp) was measured in fluids collected from cultures that had xylan as the sole carbon source (Table 1). This activity was significantly higher ($P = 0.05$) than the xylanase activities evaluated in fluids from cultures containing CMC, microcrystalline cellulose, or glucose; the latter two carbon sources did not induce the production of xylanase.

Discussion

Maximum production of cellobiohydrolase by *P. omnivorum* was measured in fluids from cultures containing xylan as enzyme inducer. Smaller amounts of this enzyme were measured in the fluids of cultures containing CMC. Similar results were obtained in other studies of the phytopathogenic fungi *Fusarium oxysporum* f. sp. *lycopersici* (cf. Ortega 1990) and *Exserohilum rostratum* (cf. Ortega 1993a). Although microcrystalline cellulose did not induce the production of cellobiohydrolase in cultures of *P. omnivorum*, previous reports indicate that this carbohydrate can induce cellobiohydrolase production in the plant pathogens *F. oxysporum* f. sp. *lycopersici* (cf. Ortega 1990), *Alternaria brassicae* (cf. Ortega 1992), *E. rostratum* (cf. Ortega 1993a), and *Curvularia senegalensis* (cf. Ortega 1993b). Apparently, cellobiohydrolase was not constitutively produced by *P. omnivorum* under the conditions of this study. This was suggested by the absence of cellobiohydrolase activity in fluids collected from cultures containing glucose as the sole carbon source. However, other plant pathogens, such as *A. brassicae* (cf. Ortega 1992) and *E. rostratum* (cf. Ortega 1993) seem capable of producing consitutively small amounts of this enzyme.

Endoglucanase production was induced in cultures containing CMC or xylan as carbon sources. Similar results were obtained in other

studies of the phytopathogenic fungi *F. oxysporum* f. sp. *lycopersici* (cf. Ortega 1990), *A. brassicae* (cf. Ortega 1992) and *E. rostratum* (cf. Ortega 1993a). Endoglucanase was constitutively produced by *P. omnivorum* as indicated by the enzyme activity measured in fluids from cultures with glucose as the sole carbon source. The endoglucanase activity produced constitutively was significantly higher than the enzyme activity that was induced by CMC or xylan. This enzyme is also produced in a constitutive manner by the plant pathogenic fungi *F. oxysporum* f. sp. *lycopersici* (cf. Ortega 1990), *A. brassicae* (cf. Ortega 1992) and *E. rostratum* (cf. Ortega 1993a).

Maximum xylanase activity by *P. omnivorum* was detected in fluids from cultures with xylan as the carbon source and enzyme inducer. Similar results were obtained in studies of the phytopathogenic fungi *F. oxysporum* f. sp. *lycopersici* (cf. Ortega 1990), *A. brassicae* (cf. Ortega 1992) and *E. rostratum* (cf. Ortega 1993a). *P. omnivorum* did not produce xylanase constitutively. Other reports indicate that the phytopathogenic fungi *Rhizoctonia solani* (cf. Robson et al. 1989) and *E. rostratum* (cf. Ortega 1993a) do produce small amounts of xylanase in a constitutive manner.

Production of the cell wall degrading enzymes cellobiohydrolase, endoglucanase and xylanase was induced in liquid cultures of *P. omnivorum* when CMC or xylan were used as sole carbon sources. Xylanase activities were significantly higher than the activities of the enzymes cellobiohydrolase or endoglucanase. The most effective xylanase inducer was xylan.

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