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Antifungal Activity of Purified Endochitinase Produced by Biocontrol Agent *Trichoderma reesei* Against *Ganoderma philippii*

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Abstract: Purified 32-kDa endochitinase from *Trichoderma reesei* isolate T13 was tested for its antifungal activity in bioassay against the hyphal growth of soil-borne pathogenic fungus *Ganoderma philippii*. The results indicated that the degree of inhibition was proportional to the level of enzyme concentration applied. Inhibition zone visually occurred at the outer line of colony of *G. philippii* at 80μg ml^{-1} concentration of enzyme and above. The enzyme caused necrotic lesion on mycelial cell wall and stimulated branching response of mycelial tip at the concentration of 60 to 200 μg ml^{-1}. At the concentration of 100 to 200μg ml^{-1}, endochitinase lyzed the hyphae of pathogenic fungus.

Key words: Antifungal activity, endochitinase, *Trichoderma reesei*, *Ganoderma philippii*.

Introduction

Ganoderma spp. are potential root rot pathogens causing serious damage of many types of tree plantations in Malaysia (Lee, 1996), India (Sharma and Florence, 1996), Australia (Old *et al.*, 1996) and Indonesia (Widyastuti *et al.*, 1998; Nair and Sumardi, 2000). Of the methods developed to control the disease, none gives successful result. Biological control of plant pathogens is an alternative approach to decrease the strong dependence on modern agriculture and chemical fungicides, which cause environmental pollution and development of resistant strains. Filamentous fungi *Trichoderma* spp. are the mycoparasites of plant pathogens and have been one of the most potent agents to be used for biocontrol of plant diseases (Papavizas, 1985). Although the mechanism of mycoparasitism is not fully understood, expression of extracellular cell-wall degrading enzymes is assumed to be involved in this process, including chitinolytic and glucanolytic enzymes. As reported for other chitinolytic systems, the endochitinase (EC 3.2.1.14) are the most effective for both antifungal and lytic activities in comparison with other types of chitinolytic enzymes (de la Cruz *et al.*, 1993; Lorito *et al.*, 1996a). Recently, 32-kDa endochitinolytic enzymes have been purified from *T. reesei* and are characterized (Harjono and Widyastuti, 2001; Harjono *et al.*, 2001). In this article we reported the *In vitro* antifungal activity of the pure endochitinase to inhibit the colony development and cause abnormal hyphal growth of *G. philippii*.

Materials and Methods

An experiment was conducted in the Laboratory of Microbiology, Inter University Center for Biotechnology, GMU and Laboratory of Forest Protection, Faculty of Forestry, GMU during September 1999 to November 2000.

Enzyme assay: Endochitinase activity was measured on the basis of reduction in turbidity of a suspension of colloidal chitin (Tronsmo and Harman, 1993), whereas colloidal chitin was prepared following the method of Vessey and Pegg (1973) from crab shell chitin (Sigma Chemical Co., St. Louis, MO). One enzyme unit was defined as the amount of enzyme required reducing the turbidity of a chitin suspension by 5% at 510 nm (Harman *et al.*, 1993; Tronsmo and Harman, 1993).

Production and purification of enzyme: Endochitinase was produced and purified using the method of Harman *et al.* (1993) with modification on culture condition by Harjono and Widyastuti (2001). *T. reesei* isolate T13 was grown for 7 days on a rotary shaker in Richard's modified medium, which contained 10 g of KNO_3, 5 g of KH_2PO_4, 2.5 g of $MgSO_4.7H_2O$, 2 mg of $FeCl_2$, 0.5% colloidal chitin, 1% polyvinylpyrolidene (Sigma), 150 ml of V8 juice (Campbell Soup Co. Camden, NJ), and 850 ml of H_2O at final pH 6.0. The biomass was removed by filtration, precipitated with ammonium sulfate, dialyzed against 50 mM potassium phosphate buffer (pH 6.7), and the enzyme was separated by gel filtration chromatography in a chromatography column packed with Sephacryl S-300 (Pharmacia LKB Biotechnology, Uppsala, Sweden), followed by chromatofocusing. Purity was confirmed using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Protein content in the enzyme was determined by the method of Hartee-Lowry (Hartree, 1972). At the final stage of the procedure, a single protein (32 kDa) with endochitinase activity was obtained (Harjono and Widyastuti, 2001).

Microorganisms: *T. reesei* isolate T13, which suppress *Ganoderma* spp. and other soil-borne pathogenic fungi (Widyastuti *et al.*, 1998, 1999, 2001, 2002; Widyastuti and Sumardi, 1998), was used to produce endochitinase enzyme. Antifungal activities of endochitinase enzyme were assayed against an isolate of *G. philippii* isolated from *Acacia mangium* (Widyastuti *et al.*, 1998). The isolates are the collection of Laboratory of Forest Protection, Faculty of Forestry, Gadjah Mada University. The fungi were maintained on a potato dextrose agar (PDA) slant.

Antifungal activity bioassay: Cylinder plate method (Johnson and Curl, 1972) was used to make wells on medium. Plug colony of *G. philippii* was grown on Petri dishes containing 1.5% potato dextrose agar (PDA). Three days after inoculation, when the colony diameter was 3-4 cm, wells were filled with 80 μl enzyme solution of the following concentrations: 0, 40, 50, 60, 70, 80, 90 100 and 200 μg protein ml^{-1}. The plates were observed after 12 h. Abnormal growth of hyphae and morphological responses such as branching, bursting, appearance of necrotic zones and lysis of the hyphal tips were noted.

Results and Discussion

Inhibition zone of 32-kDa endochitinase against colony development of *G. philippii* clearly appeared at concentration 90 to 200 μg ml^{-1} (Fig. 1a), whereas concentration of 80 μg ml^{-1} just showed obscure inhibition zone. No inhibition zone was observed at concentration 40 to 70 μg ml^{-1}.

Microscopic observation revealed that a number of hyphal morphological changes were induced by *T. reesei* endochitinase (Table 1). Branching and segmentation of hyphal tips occurred at 60 μg ml^{-1} of endochitinase application each with mean percentages of 0.84 and 1.67 (Table 1 and Fig. 1b,

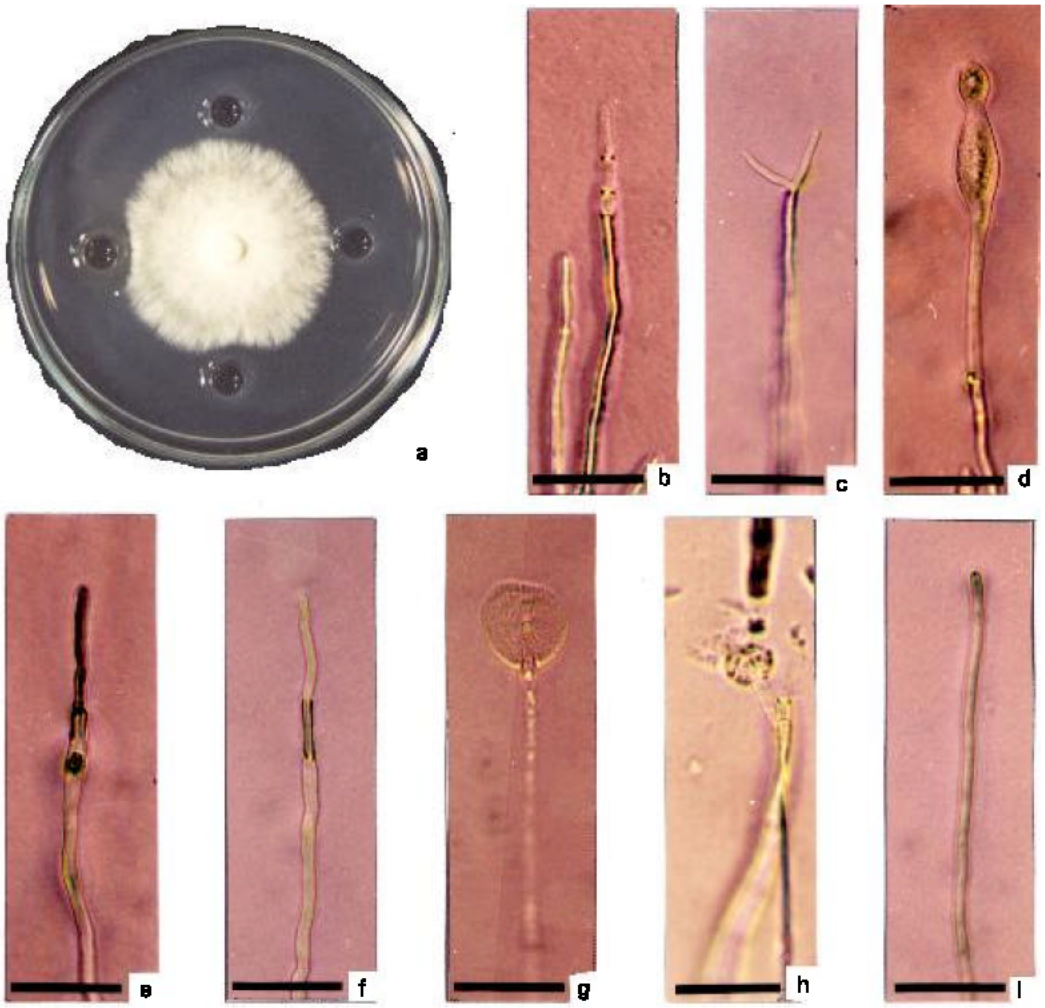

Fig. 1: Effects of the 32-kDa *Trichoderma reesei* endochitinase to hyphae of plant pathogenic fungus *Ganoderma philippii*. (a) Growth inhibition of *G. philippii* in response to various endochitinase concentrations. Wells contain 80 μl of the following enzyme solution in μg ml^{-1} (from top, in clockwise direction): 0, 50, 100 and 200. (b + c + d) Segmentation, branching and swelling of hyphal tip of *G. philippii* respectively, in presence of 60 μg ml^{-1} endochitinase and above. (e) Necrotic lesion at hyphal tip of *G. philippii* hyphae grown in the presence of endochitinase higher than 70 μg ml^{-1}. (f) The hyphae of *G. philippii* has been able to recover enzyme disturbance and continued its growth as normal hyphae at enzyme concentration of 80 μg ml^{-1}. (g) Lysis of hyphal tips of *G. philippii* grown in presence of endochitinase 90 to 200 μg ml^{-1}. (h) Lysis of mature hyphae of *G. philippii*. (I) The *G. philippii* hyphae in the absence of endochitinase. (Bars: 30μm).

Table 1: Microscopic observation of morphological anomalies of *Ganoderma philippii* hyphae caused by 32-kDa *Trichoderma reesei* endochitinase[1]

Endochitinase concentration (μg ml^{-1})	Appearance of abnormal hyphae[2]				
	Segmentation	Branching	Swollen	Necrotic	Lysis
0	-	-	-	-	-
40	-	-	-	-	-
50	-	-	-	-	-
60	1.67	0.84	1.67	-	-
70	1.67	2.50	0.84	-	-
80	4.17	2.50	2.50	5.00	-
90	6.67	5.00	2.50	8.33	1.67
100	10.83	5.83	7.50	11.67	9.17
200	7.50	8.33	6.67	15.83	14.17

[1] The data was obtained 12 hours after enzyme application.

[2] Percentage of 120 hyphae observed under microscope by two replications.

c). At this concentration, endochitinase also gave rise to swollen hyphal tips (Fig. 1d), whereas enzyme concentration of 70 μg ml^{-1} gave the same microscopic appearance.

Hyphal necrotic lesions were indicated at concentrations 80, 90, 100 and 200 μg ml^{-1} with mean percentages 5.00, 8.33, 11.67 and 15.83, respectively (Table 1). These endochitinase concentrations caused permanent hyphal necrosis of *G. philippii* (Fig. 1e). It was noticed that enzyme application at low concentration failed to suppress the growth of hyphae (Fig. 1f). It might be possible that in short time period of application, the enzyme also failed to inhibit the hyphae.

Hyphal lysis by enzyme was observed at the concentration of 90 μg ml^{-1} and the mean percentage increased drastically at the concentration of 100 μg ml^{-1} and 200 μg ml^{-1} (Table 1). Cell bursting and protoplast release, not only on hyphal tips (Fig. 1g) but also on mature hyphae (Fig. 1h) followed this process.

The results of this experiment are consistent with other reports (Schlumbaum *et al.*, 1986; Mauch, *et al.*, 1988; Lorito *et al.*, 1993) and was expected because *Ganoderma* contain chitin in their cell wall (Rajarathnam *et al.*, 1998). It has been

reported by Lorito *et al.* (1993) that since the antifungal activity of the purified chitinolytic enzymes was roughly proportional to the quantity of chitin in the cell wall of test fungi, the ability of these proteins to inhibit the fungal growth is probably a consequence of their chitinolytic activity, not of secondary toxic properties.

The purified endochitinase from *T. harzianum*, which play an antagonistic and a nutritional role, is a strong inhibitor of many important plant pathogens and able to lyse not only the "soft" structure at the hyphal tip but also the "hard" chitin wall of mature hyphae, conidia, chlamydospores and sclerotia (Benhamou and Chet, 1993; Lorito *et al.*, 1993; Rousseau *et al.*, 1996). Furthermore, the antifungal activity is synergistically enhanced when different *Trichoderma* cell wall degrading enzymes are used together (Lorito *et al.*, 1994) or in combination with other compounds (Collinge *et al.*, 1993; Sahai and Manocha, 1993). The level of inhibition for *Trichoderma* chitinases were usually higher than those described for plant, bacteria or other fungal chitinolytic enzymes assayed under same conditions (Lorito *et al.*, 1996b). Antifungal and morphological changes by *T. reesei* observed in this experiment represent some of the evidence to date supporting the involvement of the chitinolytic enzyme system in mycoparasitism.

It is concluded that 32-kDa endochitinase produced by *T. reesei* has anti-fungal activity to hyphae of *G. philippii In vitro* and the degree of inhibition was proportional to the level of endochitinase concentration.

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