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Purification and Characterization of â-1,3-Glucanase from the Antagonistic Fungus *Trichoderma reesei*

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***Trichoderma* enzymes that inhibit fungal cell walls have been suggested to play an important role in mycoparasitic action against fungal root rot pathogen *Ganoderma philippii*. This experiment was aimed to purify and characterize the â-1,3-glucanase of *T. reesei*. Extracellular â-1,3-glucanase was produced by growing mycoparasite *T. reesei* isolate T_{13} in colloidal chitin and sucrose as carbon sources. The enzyme was then purified to its homogeneity by precipitation with ammonium sulfate, followed by gel filtration chromatography and chromatofocusing. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 12% was used to confirm the purity of enzyme at each stage of preparation and to characterize purified protein. The results showed that *T. reesei* produced at least three extracellular â-1,3-glucanases. Estimation of molecular weight based on SDS-PAGE 12% have three isoform of â-1,3-glucanase were 90 kDa for â-1,3-glucanase-I, 75 kDa for â-1,3-glucanase-II, and 64 kDa for â-1,3-glucanase-III. Their optimum pH and temperature were 5 and 50 °C, respectively.**

Key words: â-1,3-glucanase, *Trichoderma reesei*, *Ganoderma philippii*

INTRODUCTION

Biological control by antagonistic organisms is a potential nonchemical tool for crop protection against phytopathogenic fungi (Papavizas 1985). Several strains of the genus *Trichoderma* have been described as antagonistic fungi to control a wide range of phytopathogenic fungi. The antifungal activity of *Trichoderma* involves in production of antibiotics, competition for key nutrients, and production of fungal cell wall-degrading enzymes (Hjeljord & Tronsmo 1998). Mycoparasitism by cell wall-degrading enzymes have been proposed as the major mechanism of *Trichoderma* antagonistic activity against fungal plant pathogens (Chet *et al*. 1998).

Trichoderma mycoparasitism is a complex process involving several successive steps. Initially, the mycoparasite grows directly towards its host and often coils arround it or attaches to it by forming hook-like structures and apressoria. Following these interactions, *Trichoderma* spp. sometimes penetrate the host miselium, apparently by partially degrading its cell walls. Finally, it is assumed that *Trichoderma* spp. utilize intracellular contents of the host (De La Cruz *et al*. 1995a).

A number of *Trichoderma* isolates produce hydrolytic enzymes such as chitinases, â-1,3-glucanases, â-1,6-glucanases, and proteases when grown on laminarin (â-1,3-glucan), chitin, or fungal cell walls as the carbon source. This observation and the fact that chitin and â-1,3 glucan are the main structural components of fungal cell walls, except those from members of the class Oomycetes, which contain â-1,3 glucan and cellulose, suggests that chitinase and â-1,3-glucanases produced by some *Trichoderma* spp. are the key enzymes in the lysis of cell walls during their mycoparasitic action against phytopathogenic fungi (De La Cruz *et al*. 1995b). In fungi, â-1,3-glucanases seem to have different functions. First, a physiological role in morphogenetic-morpholytic processes during fungal development and differentiation has been indicated. Second, â-1,3-glucanases have been related to the mobilization of â-1,3-glucans under conditions of carbon and energy source exhaustion, functioning as autolytic enzymes. â-1,3-glucanases are also involve in fungal pathogen-plant interactions, degrading callose (â-D-1,3-glucan) in the host's vascular tissues during pathogen attack. Finally, a nutrition role in saphrophytes and mycoparasites has been suggested (De La Cruz *et al*. 1995a). Therefore, purified enzymes are required in order to determine their specific roles in biological control.

In this study, we report production of â-1,3-glucanases by *T. reesei* isolate T_{13} grown in liquid culture and the purification and characterization of those enzymes.

MATERIALS AND METHODS

The experiments was conducted in the Laboratory of Microbiology, Inter University Center for Biotechnology, Gadjah Mada University and Laboratory of Forest Protection, Faculty Forestry, Gadjah Mada University, Yogyakarta.

Microorganisms. *Trichoderma reesei* isolate T_{13}, which suppress *Ganoderma* spp. and other soil-borne pathogenic fungi (Widyastuti *et al*. 1998, 1999, 2001; Widyastuti & Sumardi 1998), was used to produce â-1,3-glucanase enzyme. Antifungal activities of â-1,3-glucanase enzyme were assayed against *G. philippii* isolated from *Acacia mangium*

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(Widyastuti *et al*. 1998). The isolates are collection of Laboratory of Forest Protection, Faculty of Forestry, Gadjah Mada University.

â-1-3-Glucanase Activity Assays. â-1,3-glucanase activity was determined by measuring the amount of reducing sugar released from laminarin (Sigma Chemical Co., St. Louis, MO.) with modification on volume of substrat. The standard assay mixture (volume, 100 µl) contained 50 µl of protein concentrate, 5 mg of laminarin per ml, and 50 of µl 20 mM sodium acetate buffer (pH 5.0). Each reaction mixture was incubated for 1 h at 50 °C, and the production of reducing sugars was determined by the procedure described by Somogyi-Nelson method (as described in Vazquez-Garciduenas *et al*. 1998; Sudarmadji *et al*. 1984), using glucose as standard. One unit of â-1,3-glucanase activity was defined as the amount of enzyme that catalyzed the release 1 µmol of glucose equivalent per min.

â-1-3-Glucanase Induction. The mycelia of *T. reesei* were obtained by inoculating potato dextrose broth (PDB) with 5.10^7 conidia/ml and were incubated for 5 days on a rotary shaker. The mycelia were collected by filtration through filter paper and transferred to SMCS with modification on culture condition, a synthetic medium with colloidal chitin and sucrose as carbon source, which composed of 680 mg KH_2PO_4, 870 mg of K_2HPO_4, 200 mg of KCl, 1 g of NH_4NO_3, 200 mg of $CaCl_2$, 200 mg of $MgSO_4 \cdot 7H_2O$, 2 mg of $FeSO_4$, 2 mg of $ZnSO_4$, 2 mg of $MnSO_4$, 42 g of moist purified colloidal chitin, and 1 g of sucrose in 1 l of distilled water adjusted to pH 6.0 (Lorito *et al*. 1994). The supernatant were obtained by filtering the media through Whatman no. 4 paper. The supernatant was then precipitated with ammonium sulfate (80% saturation) at 4 °C. The precipitate was recovered by centrifugation (10,000 rpm for 30 min at 4 °C), resuspended in 20 mM Tris-HCl (pH 8.2), dialyzed against 20 mM Tris-HCl (pH 8.2), kept at 4 °C, and used as sources of â-1,3-glucanase (crude enzyme).

Gel Filtration. The crude enzyme was applied to a gel filtration column (1.4 by 60 cm) containing Sephacryl S-300 HR (Pharmacia LKB Biotechnology, Uppsala, Swedden). Elution was performed with 20 mM Tris-HCl buffer, pH 8.2 at flow rate of 3.6 ml/h. The most active fractions of 1.5 ml were collected and analyzed for laminarin activity as described previously, and protein was determined by A_{280}.

Chromatofocusing. Chromatofocusing was performed by using a column (1.4 by 20 cm) packed with PBE 94 (Pharmacia LKB). The column was eluted at a flow rate of 3.6 ml/h with Polybuffer 96. Active fractions were collected and analyzed for laminarin and protein content. The â-1,3-glucanase-positive fractions were used as a purified preparation of â-1,3-glucanase. Purity enzyme was confirmed using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) 12%.

SDS-PAGE of Protein. Electrophoresis under denaturing conditions was performed with 4% acrylamide in the stacking gel and 12% acrylamide in separating gel. Gel were stained with Coomassie R-250 brilliant blue. Molecular mass of protein standards (Broad range, Biorad) were used for molecular mass determination.

Optimum pH and Stability. The effect of pH on â-1,3-glucanase activity was measured by performing the standard assay within the pH range of 3.0 to 6.0 by using citric buffer system and by using phosphate buffer system in the pH range 7.0 to 8.0. Reaction mixture: 50 µl of enzyme solution and 50 µl various buffer, mix with 5 mg/ml of laminarin. The Reaction mixture were incubated at 50 °C for 1 h, followed by measuring reducing sugar content using method as described above.

Optimum Temperature and Stability. The optimum temperature was determined by performing the standard assay within the temperature range of 30-70 °C. Reaction mixture: 50 µl of enzyme solution and 50 µl of 20 mM sodium acetate buffer (pH 5.0), mix with 50 mg/ml of laminarin. The reaction mixtures were incubated at various temperatures for 1 h. Then, the reducing sugar content was using method as described above.

RESULTS

Enzyme Production. Enzyme production was attemted under two-step culture conditions. In first step, PDB medium was use to grow the mycelia; in the second step, â-1,3-glucanase was produced with colloidal chitin and sucrose as the carbon sources. The time course of â-1,3-glucanase production related to mycelia growth and increased continuously with time (Figure 1). The maximum activity of â-1,3-glucanase occured at 5 days of incubation (Figure 1).

Enzyme Purification. The purification scheme resulted in 25.37-fold purification of â-1,3-glucanase relative to the crude protein and substantial increase in the specific activity (from 47.35 to 1201.6 U/mg) (Table 1). Approximately 24.6% of the â-1,3-glucanase activity in the crude enzyme was recovered.

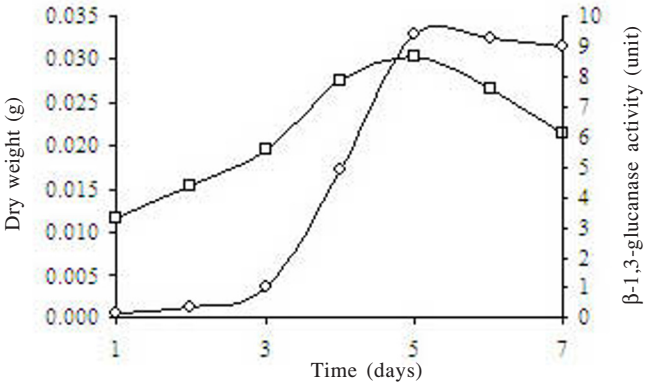

Figure 1. The activity and dry weight of extracellular of â-1,3-glucanase produced by *T. reesei* isolate T_{13} grown in colloidal chitin as the carbon source. (○) â-1,3-glucanase activity, (□) dry weight.

Table 1. Summary of purification of â-1,3-glucanase from *T. reesei* isolate T_{13}

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
80% ammonium sulfate	7.50	8.69	308.88	47.35	100.00	1.00
Sephacryl S-300 HR elution	3.00	0.81	141.37	581.25	45.77	12.27
Chromatofocusing elution	1.50	0.53	76.24	1201.60	24.60	25.37

Purification of *T. reesei* â-1,3-glucanase with activity against laminarin was performed from culture filtrates obtained after 5 days of growth in SMCS containing chitin. Hereafter, all purification steps were carried out at 4 °C.

Gel Filtration Chromatography. Showed two peaks containing enzyme activity (Figure 2). The activity of the first peak was tentatively called Peak-I (P-I) and the second peak was called Peak-II (P-II).

Preparative Polyacrylamide Gel Electrophoresis. After concentration of the P-I and P-II fractions eluted from gel filtration column, the active fraction were subjected to polyacrylamide gel electrophoresis (SDS-PAGE 12%) (Figure 3). Under SDS-PAGE, several protein band were detected in the P-I and P-II fraction.

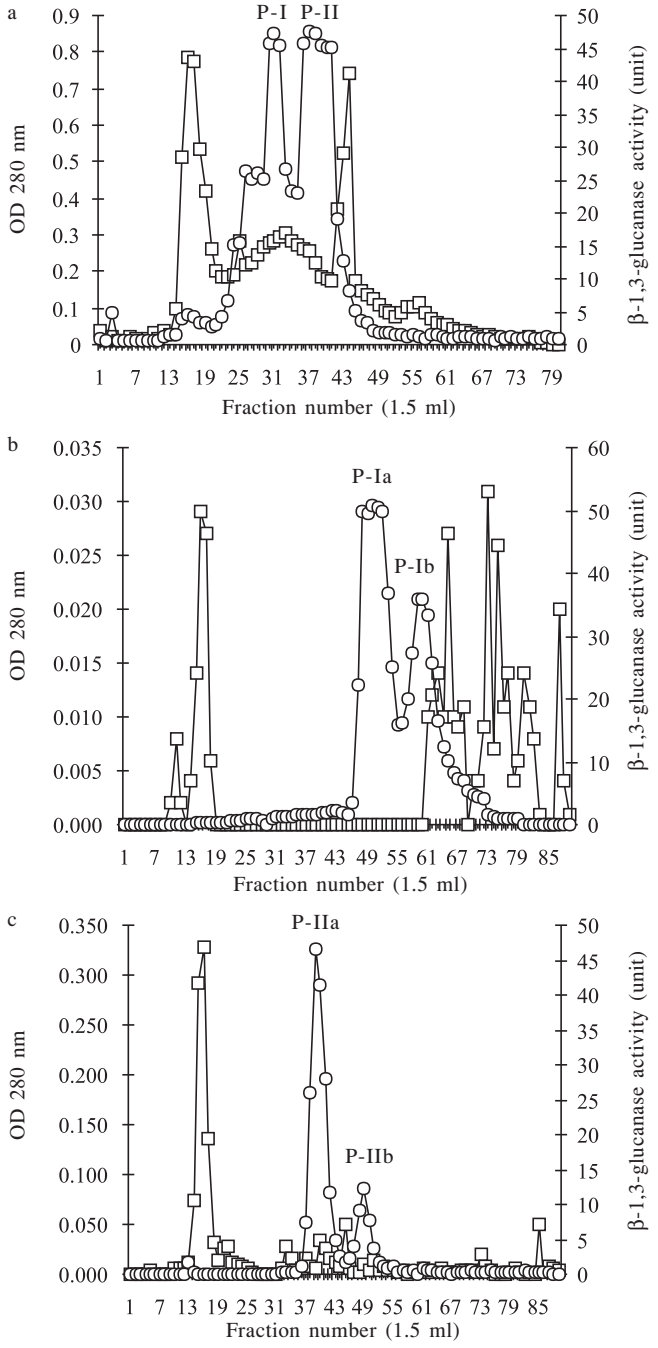

Figure 2. Profiles of chromatographic purification of â-1,3-glucanases from *T. reesei* isolate T_{13} by (a) gel filtration chromatography followed by, (b) chromatofocusing of P-I fractions, (c) chromatofocusing of P-II fractions. (○) â-1,3-glucanase activity, (□) Absorbance 280 nm.

Chromatofocusing. The fractions containing enzyme activities of interest were pooled (Figure 2a) and applied to a chromatofocusing column (1.4 by 20 cm) packed with PBE 94, pH 4-7. The purification of P-I fraction by chromatofocusing showed the presence of two peaks â-1,3-glucanase activity (Figure 2b; P-Ia, P-Ib). Subsequently, the purification of P-II fraction by chromatofocusing showed the presence of two peaks â-1,3-glucanase activity, as well (Figure 2c; P-IIa, P-IIb).

The Second Preparative Polyacrylamide Gel Electrophoresis. The purity of final enzyme praparations (â-1,3-glucanase-I, â-1,3-glucanase-II, â-1,3-glucanase-III) was examined by polyacrylamide gel electrophoresis. Estimation of molecular weight based on SDS-PAGE 12% have three isoform of â-1,3-glucanase were 90 kDa for â-1,3-glucanase-I, 75 kDa for â-1,3-glucanase-II, and 64 kDa for â-1,3-glucanase-III (Figure 4).

Optimum Temperature of the Enzyme Reaction. The optimum temperature of the enzyme reaction was determined by varying the incubation temperature at pH 5.0 (Figure 5a). The temperature-enzyme activity profiles of enzymes â-1,3-glucanase-I, â-1,3-glucanase-II, and â-1,3-glucanase-III were similar. The three enzymes were most active at 50 °C.

Effect of pH on â-1,3-Glucanase Activity. The â-1,3-glucanase activities of the enzyme preparations were measured at various pHs with laminarin as the substrat (Figure 5b). The reaction pHs were adjusted to 3.0 to 8.0 with a variety of buffers. The pH-enzyme activity profiles of enzymes â-1,3-glucanase-I, â-1,3-glucanase-II, and â-1,3-glucanase-III were similar. The pH optimum for these enzyme reaction were 5.

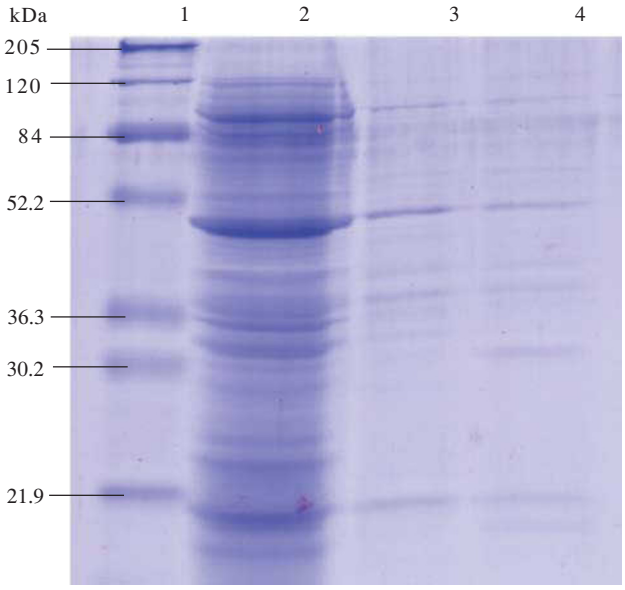

Figure 3. SDS-PAGE of the purified of â-1,3-glucanase. A polyacrylamide concentration of 12% was used, and gel were coomassie blue stained. Lane 1. Molecular mass protein standard (Broad range, Biorad); line 2. crude enzymes â-1,3-glucanase; line 3. P-I (30-32 fractions); line 4. P-II (36-39 fractions).

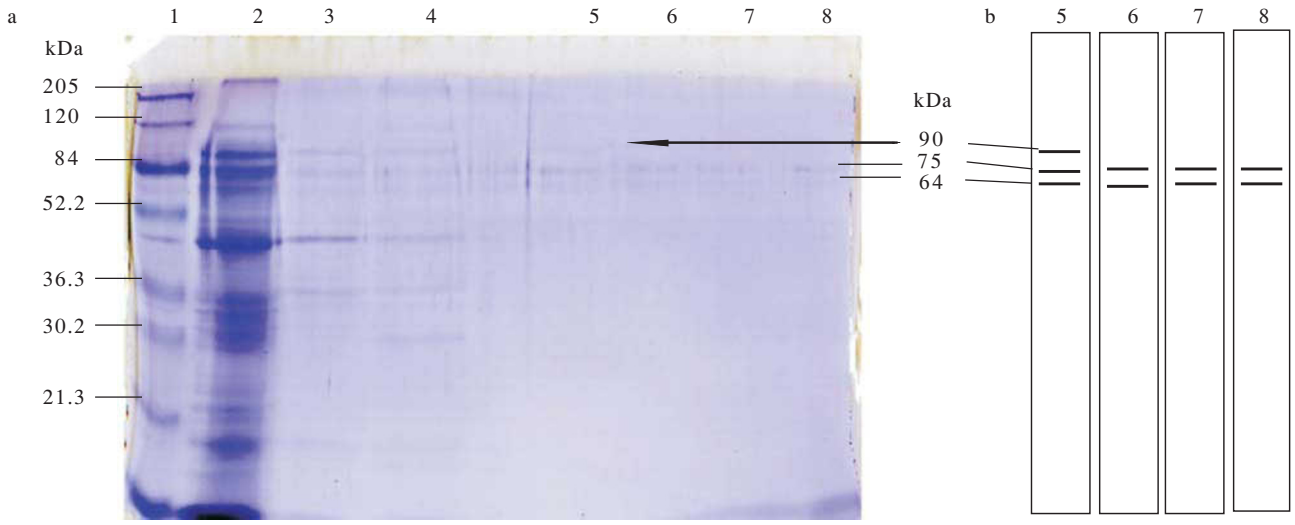

Figure 4. a. SDS-PAGE analysis after the final step â-1,3-glucanase purification. Lane 1. Marker molecular mass protein standard; lane 2. crude protein; lane 3. P-I; lane 4. P-II; lane 5. P-Ia (fraction 50); lane 6. P-Ib (fraction 59); lane 7. P-IIa (fraction 39); lane 8. P-IIb (fraction 49). (b) Line draw of lane 5-8.

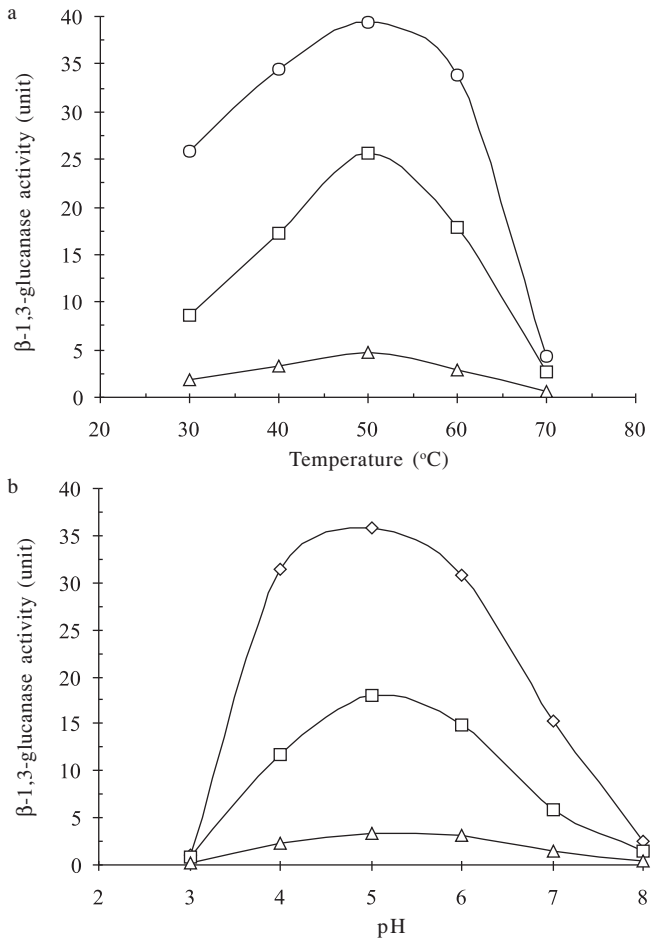

Figure 5. Effect of temperature and pH on â-1,3-glucanase activity. â-1,3-glucanase activity was measured at various temperatures (degrees Celcius) (a) and pH (b) under standard enzyme assay conditions. (○) â-1,3-glucanase I, (□) â-1,3-glucanase II, (△) â-1,3-glucanase-III.

DISCUSSION

Glucans and chitin are the major constituents of fungal cell walls, and the important roles of glucanolytic and chitinolytic enzymes in the degradationof fungal cell walls during mycoparasitism by *Trichoderma* have previously suggested (Lorito *et al*. 1994). The mycoparasitic action of *Trichoderma* has been proposed as the major mechanism for their antagonistic activity against phytopathogenic fungi. Their lytic activity was mainly due to the activity of chitinase and â-1,3-glucanase enzymes (De La Cruz 1995a). Previous research reported that 32-kDa extracellular chitinase of *T. reesei* T_{13} isolate was produced under inducing conditions (Harjono & Widyastuti 2001). This study examined the production of â-1,3-glucanase by *T. reesei* T_{13} isolate grown in liquid culture. Because of the complexity of the fungal cell walls, those hydrolytic enzymes were contributed to degradation of phytopathogenic fungal cell walls. This present study examined the production of â-1,3-glucanase in liquid culture by an effective biocontrol strain of *T. reesei*.

In this study, *T. reesei* isolate T_{13} produced three extracellular â-1,3-glucanases with activity against laminarin when it was grown in synthetic medium containing colloidal chitin and sucrose as carbon sources. Activity those three extracellular â-1,3-glucanases has been characterization. Multiple â-1,3-glucanases were reported to be present in the well-studied mycoparasite *Trichoderma harzianum* (Lorito *et al*. 1994; Noronha & Ulhoa 1996; Vazquez-Garciduenas *et al* 1998), and *Stachybotrys elegans* (Archambault *et al*. 1998).

In nature, many organism are known to produce simultaneously two or more enzymes that hydrolyze particular insoluble polysaccaharide. Multiple â-1,3-glucanase systems are known in many organisms. The multiple systems may contain not only genetically different isozymes but also enzymatically active small products resulting from one of the

enzymes by some proteolytic processing (Aono *et al*. 1995). Archambault *et al*. (1998) suggested that in most cases multiple â-1,3-glucanase rather than single enzyme have been found because â-1,3-glucanase have the multiple roles in apical growth, morphogenesis, and conidiogenesis. In this study, the three enzymes of â-1,3-glucanases from *T. reesei* was purified by gel filtration and chromatofocusing. The molecular weight of the three isoform of â-1,3-glucanases were 90 kDa for â-1,3-glucanase-I, 75 kDa for â-1,3-glucanase-II, and 64 kDa for â-1,3-glucanase-III, respectively. It was similar to those of the endo-1,3-â-D-glucanases from *Stachybotrys elegans*, 94 and 75 kDa (Archambault *et al*. 1998), the endo-â-1,3-glucanase from *T. harzianum*, 78 kDa (De la Cuz *et al*. 1995b), the endo-(1,3)-â-D-glucanase from *T. longibrachiatum*, 70 kDa (Tangarone *et al*. 1989), and the exo-â-1,3-glucanase from *T. viride*, 61 $\pm$ 1 kDa (Kulminskaya *et al*. 2001).

The optimal pH for enzyme activity (pH 5.0) was similar to those of the endo-1,3-â-D-glucanases from *Stachybotrys elegans*, pH 5.0 (Archambault *et al*. 1998), the â-1,3-glucanase from *Neurospora crassa*, pH 4.5 (Hiura *et al*. 1986), the endo-(1,3)-â-D-glucanase from *T. longibrachiatum*, pH 4.8 (Tangarone *et al*. 1989), and the exo-â-1,3-glucanase from *T. viride*, pH 4.5 (Kulminskaya *et al*. 2001). The optimal temperature for enzyme activity (50 °C) was similar to those of the endo-1,3-â-D-glucanases from *Stachybotrys elegans*, 40 °C (Archambault *et al*. 1998), the â-1,3-glucanase from *Neurospora crassa*, 45 °C (Hiura *et al*. 1986), the endo-(1,3)-â-D-glucanase from *T. longibrachiatum*, 55 °C (Tangarone *et al*. 1989), and the exo-â-1,3-glucanase from *T. viride*, 55 °C (Kulminskaya *et al*. 2001). Finally, to gain insight into enzyme multiplicity, it is important to obtain more information of the physiological role, biochemical characterization on each â-1,3-glucanase species secreted. Work is in progress to define further the role of â-1,3-glucanases in process mycoparasitism. And then, isolation and expression of the gene â-1,3-glucanase in transgenic plants might therefore improve resistance to fungal pathogens.

In conclusions, this study suggested that *T. reesei* isolate T_{13} produced at least three extracellular â-1,3-glucanases when it was grown on colloidal chitin and sucrose as carbon sources. Estimation of molecular weight of the three isoform of â-1,3-glucanase were 90 kDa for â-1,3-glucanase-I, 75 kDa for â-1,3-glucanase-II, and 64 kDa for â-1,3-glucanase-III. Their optimum pH and temperature were 5 and 50 °C, respectively.

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