Short Communication

Purification and partial characterization of a chitinase from the mycoparasitic fungus *Trichothecium roseum*

Duo-Chuan Li,* Shi-Hun Zhang, Kei-Qi Liu, and Jing Lu

Department of Plant Pathology, Shandong Agricultural University, Taian, Shandong 271018, China

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Fungi that parasitize the mycelia of other fungi are termed mycoparasites. Mycoparasites of plant pathogenic fungi have received attention for their use as bio-control agents. Cell wall lysis by mycoparasites has been suggested to correlate with the biological control of plant pathogenic fungi. It has been shown that cell wall degrading enzymes (chitinase, β -glucanase and protease) may be involved in the cell wall lysis. Among the enzymes most commonly suggested to be involved in mycoparasitism are those that degrade chitin (Haran et al., 1996). A considerable amount of research has been carried out in recent years on the chitinolytic system of the mycoparasitic fungus *Trichoderma harzianum* (De La Cruz et al., 1992; Haran et al., 1995, 1996; Harman et al., 1993; Lorito et al., 1993; Ulhoa and Peberdy, 1991, 1992). *Trichothecium roseum* was demonstrated to be a mycoparasite, capable of parasitizing *Sclerotinia sclerotiorum* (Huang and Kokko, 1993) and *Pestalotiopsis funerea* (Urbach, 1992), but so far chitinase from this mycoparasite has not been isolated. In this article, we described the purification and characterization of a chitinase from a strain of *T. roseum*.

T. roseum strain 32 was originally isolated from *Ustilago nuda* (Basidiomycete) in China. Conidia of the

fungus were used to inoculate potato-dextrose agar (PDA) plates. For enzyme production, the fungus was grown in 300-ml flasks containing 100 ml synthetic medium (SMCS) with colloidal chitin and sucrose as carbon sources. SMCS contained 680 mg of 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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg of FeSO_4 , 2 mg of ZnSO_4 , 2 mg of MnSO_4 , 40 g of crab shell chitin (Sigma) and 5 g of sucrose in 1 L of distilled water, at a final pH 6 (Di Pietro et al., 1993). The flasks were inoculated with conidia collected from freshly grown PDA plates. The medium, with 10^6 conidia per milliliter, was incubated at 28°C on a rotary shaker at 200 rpm. At the intervals indicated, aliquots from the cultures were taken out, and chitinolytic activity was examined using a colorimetric assay (Boller et al., 1983). The reaction mixture contained 0.5 ml of colloidal chitin (suspended at a concentration of 5 mg/ml in 50 mM HAc-NaAc, pH 5.0, Vessey and Pegg, 1973), 250 μl enzyme sample, and 0.5 ml of 50 mM HAc-NaAc (pH 5.0). After incubation at 40°C for 3 h, it was centrifuged at $2,000 \times g$ for 5 min and the amount of GlcNAc released in the supernatant was determined by the method described by Reissig et al. (1955) using GlcNAc as a standard. One unit of chitinase activity was defined as the amount of enzyme that catalyzed the release of $1 \mu\text{mol GlcNAc} \cdot \text{min}^{-1}$ at 40°C. Protein concentrations were determined with the method of Lowry et al. (1951), with bovine serum albumin as the standard. The kinetics of *T. roseum* chitinase produc-

* Address reprint requests to: Dr. Duo-Chuan Li, Department of Plant Pathology, Shandong Agricultural University, Taian, Shandong 271018, China.

E-mail: lidc20@sdau.edu.cn

tion is illustrated in Fig. 1. When the fungus was grown in liquid SMCS culture supplemented with chitin as the carbon source, chitinase activity was detected after 72 h in the culture supernatants. Such cultures showed higher levels of chitinase activity after 7 days of incubation.

For enzyme purification, the mycelia of the cultures after 7 days of incubation were filtered. The filtrate was centrifuged at $10,000\times g$ for 30 min at 4°C . The resultant supernatant was used as crude enzyme solution. All procedures of the chitinase purification were carried out at 4°C . The following buffers were used: buffer A, 50 mM Tris-HCl (pH 8.0); buffer B, 50 mM HAc-NaAc (pH 3.6). (1) Fractional ammonium sulphate precipitation: solid ammonium sulphate was added to the culture filtrate to 90% saturation. After 12 h, the resultant precipitate was collected by centrifugation at $10,000\times g$ for 30 min, dissolved in buffer A and dialyzed overnight against three changes of the same buffer (buffer A). Insoluble material was removed by centrifugation at $10,000\times g$ for 10 min. (2) Ion-exchange chromatography on DEAE-Sephacryl: the dialyzed sample was put on a DEAE-Sephacryl column (1×20 cm) equilibrated with buffer A. After the column was washed with five column volumes of buffer A, a 240 ml linear gradient of NaCl (0–0.3 M in buffer A) was applied at a flow rate of 60 ml h^{-1} . The volume of single fractions was 3 ml. Fractions with chitinase activity were pooled. (3) Ion-exchange chromatography on CM-Sephacryl: the sample from the DEAE-Sephacryl column was dialyzed overnight against buffer B. Insoluble material was removed by centrifugation at $10,000\times g$ for 10 min. The dialyzed sample was applied to a CM-Sephacryl column (1×20 cm) previously equilibrated with buffer B. After the column was washed with five column volumes of buffer B, chitinase was eluted with a 180 ml linear gradient of NaCl (0–0.3 M in buffer B) with a flow rate of 30 ml h^{-1} . The volume of single fractions was 2 ml. Fractions with chitinase activity were pooled and concentrated to 2 ml using Centricon-30 Microconcentrators according to the manufacturer's instructions. (4) Gel filtration on Sephacryl S-100: 2 ml of the concentrated sample from the CM-Sephacryl column was applied to a Sephacryl S-100 gel filtration column (1.6×100 cm) previously equilibrated with buffer A. Chitinase was eluted with 100 ml buffer A with a flow rate of 24 ml h^{-1} . The volume of single fraction was 1 ml. Fractions with chitinase activity were pooled and con-

Fig. 1. Time course of chitinase production by *T. roseum*. Values are the mean $\pm$ SEM of two experiments with three replicates each.

Fig. 2. Detection of molecular weight of the purified chitinase from *T. roseum* by SDS-PAGE.

The gel was stained with Coomassie blue stain. Lane A contains molecular mass markers 94, 67, 43, 30 kDa. Lane B contains the purified chitinase.

centrated for determination of purity and characterization.

To determine purity of the chitinase, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was carried. The enzyme showed a single protein band when run in SDS-PAGE (Fig. 2). A summary of the purification result is given in Table 1. The chitinase was purified 45.8-fold with a yield of 6.1% to a specific activity of 64.1 U mg^{-1} of protein. Similar yields were observed for purified chitinases from other mycoparasitic fungi: *Trichoderma harzianum* (De La Cruz et al., 1992; Harman et al., 1993; Ulhoa and Peberdy, 1992), *Aphanocladium*

Table 1. Purification of chitinase from *Trichothecium roseum*.

Steps	Total volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Recovery (%)	Purification (fold)
Crude extract	980	533.62	765.0	1.4	100.0	1.0
90% (NH ₄) ₂ SO ₄	52	90.27	601.4	6.7	78.6	4.8
DEAE-Sepharose	36	4.06	190.7	47.0	24.9	33.6
CM-Sepharose	28	1.01	58.0	57.4	7.6	41.0
Sephacryl S-100	9	0.73	46.8	64.1	6.1	45.8

album (Kunz et al., 1992a), and *Gliocladium virens* (Di Pietro et al., 1993). This low value might be due to a synergistic action of the different isoforms for chitin degradation present in the crude supernatant and the loss of chitinase activity during purification. To detect chitinolytic activity after gel electrophoresis, the chitinase was prepared in Laemmli sample buffer (Laemmli, 1970) without 2- β mercaptoethanol and incubated for 10 min at room temperature prior to loading. The enzyme was run in SDS-PAGE. The chitinase was reactivated in the gels by removing SDS with the casein-EDTA procedure (McGrew and Green, 1990). Enzyme activity was detected on gels by using fluorescent substrates (Tronsmo and Harman, 1993). The chitinolytic enzyme appeared as fluorescent bands under UV light because of enzymatic hydrolysis of the fluorescent substance 4-methylumbelliferone from 4-methylumbelliferyl- β -D-*N,N'*-diacetylchitobioside (4-MU-[GlcNAc]₂, Sigma). The result shows that a single fluorescent activity band (chitinase activity band) corresponded to the position of the single protein band (data not shown). These results indicated that the chitinase had been purified to homogeneity by fractional ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sepharose, ion-exchange chromatography on CM-Sepharose and gel filtration chromatography on Sephacryl S-100.

The apparent molecular mass of the purified chitinase from *T. roseum* was approximately 39 kDa by SDS-PAGE (Fig. 2) and 38 kDa by gel filtration in a Superose 12 HR 10/13 column. The results showed that the chitinase consisted of a single polypeptide. Chitinases of low molecular mass have also been previously reported in other mycoparasites, such as *T. harzianum* (33, 37, 42 kDa, De La Cruz et al., 1992; 35, 38, 40 kDa, Harman et al., 1993), *G. virens* (41 kDa, Di Pietro et al., 1993) and *A. album* (39 kDa, Kunz et al., 1992b).

Fig. 3. Effect of pH on chitinase activity of *T. roseum*.

Values are the mean $\pm$ SEM of two experiments with three replicates each.

The influence of pH on chitinase activity was determined by incubating the enzyme with chitin for 3 h at 40°C in the presence of buffers of a wide pH range (pH 4–10). The buffers used were: 0.2 M CH₃COOH-CH₃COONa (pH 4–5); 0.2 M NaH₂PO₄-Na₂HPO₄ (pH 6–7); 0.2 M Tris-HCl (pH 8–10). The enzyme was active in the pH 4.0–8.0 range. The optimum pH of the purified chitinase from *T. roseum* was 5.0 (Fig. 3). The value is in agreement with those detected for the majority of the purified extracellular chitinases from other fungi and plants (Harman et al., 1993; Mauch et al., 1988a, b; Vessey and Pegg, 1973). Temperature optimum was determined by incubating the chitinase with chitin and 0.2 M CH₃COOH-CH₃COONa (pH 5) for 3 h at different temperatures. Activity was estimated as a percentage of the maximum. The optimum temperature for chitinase activity at pH 5.0 was 40°C, and the activity dropped sharply at temperatures above 40°C (Fig. 4), possibly due to heat inactivation of the enzyme. Similar temperature values were described for the purified chitinases from other mycoparasitic fungi, except for *A. album* (Kunz et al., 1992b).

A microscopic bioassay described by Lorito et al.

Fig. 4. Effect of temperature on chitinase activity of *T. roseum*.

Values are the mean $\pm$ SEM of two experiments with three replicates each.

Fig. 5. Effect of the purified chitinase from *T. roseum* on spore germination of different fungi.

■ *F. moniliforme*, ◆ *A. alternata*, ▲ *M. grisea*. Values are the mean $\pm$ SEM of two experiments with three replicates each.

(1993) was used to determine antifungal activity of the purified *T. roseum* chitinase. Briefly, 30 µl of a spore suspension of *Alternaria alternata*, *Fusarium moniliforme*, and *Magnaporthe grisea* was transferred into sterile Eppendorf tubes. Ten microliters of a 6× PDB (potato-dextrose broth) solution and 20 µl of 0.2 M HAc-NaAc buffer (pH 5.0) containing pure chitinase were added and mixed. The tubes were incubated at 25°C. The control tubes contained distilled water instead of enzyme solution. After 24 h, three 20-µl samples from each tube were transferred to a microscope slide and analyzed under a phase-contrast light microscope. Percent of spore germination (100 conidia randomly considered) was determined and compared with that of the control. Spore germination of the fungi tested was inhibited by the chitinase. At the concentration of 300 µg/ml of the chitinase, inhibition of spore germination of *A. alternata*, *F. moniliforme* and *M. grisea* was 73%, 56% and 81%, respectively (Fig. 5). Similar results were observed for purified chitinases from other mycoparasitic fungi *Trichoderma harzianum* (Lorito et al., 1993), *Gliocladium virens* (Di Pietro et al., 1993) and *Aphanocladium album* (Kunz et al., 1992a).

The effects of the purified chitinase on mycelial growth of fungi were assayed using *A. alternata*, *F. moniliforme* and *M. grisea*. *A. alternata*, *F. moniliforme* and *M. grisea* were cultured on PDA at 28°C. Mycelial agar discs (6 mm in diameter) obtained from the fungal cultures were placed in the center of agar plates and then incubated at 28°C until the colonies were at least 5 cm in diameter. At that time, sterile filter discs (4 mm in diameter), to which chitinase solutions equivalent to 8 µg protein were applied, were laid on the agar sur-

Fig. 6. Effect of the purified chitinase from *T. roseum* on mycelial growth of *F. moniliforme*.

Disc 1 without the purified chitinase of *T. roseum*, showing no inhibition zone; disc 2, 3, and 4 with the purified chitinase of *T. roseum*, showing distinct inhibition zones.

face about 1 cm from the mycelial colonies. The treated plates were then incubated at 28°C, until inhibition of mycelial growth was observed. After incubation for 3 days, distinct inhibition zones developed around the filter discs treated with the purified chitinase to *F. moniliforme* (Fig. 6). Similar results were observed for *A. alternata* and *M. grisea* (data not shown).

The role of the *T. roseum* wall-lysing enzymes in mycoparasitism has been suggested (Huang and

Kokko, 1993). The purified chitinase from *T. roseum* strongly inhibited the growth and the development of the chitin-containing fungi tested in vitro. Therefore, the chitinase may be useful for the production of transgenic microorganisms with superior biocontrol capabilities and of transgenic plants with high resistance to plant-pathogenic fungi. On the other hand, the chitinase may be involved in mycoparasitism between *T. roseum* and its hosts.

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