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Full Length Research Paper

Purification and characterization of chitinase from *Gliocladium catenulatum* strain HL-1-1

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Chitinase was isolated and purified from *Gliocladium catenulatum* through ammonium sulfate sedimentation, sephadex G-25 gel filtration, deionization, ultra filtration condensation, negative ion exchange separation, and non-denaturing gel electrophoresis. A 51 kDa chitinase was purified from the fungus through sodium dodecyl sulfate polyacrylamide gel electrophoresis. The optimum temperature and pH for chitinase activity were 60°C and pH 6.0, respectively. The enzyme solution was stable from 20 to 40°C and pH 4 to 5. Co^{2+} and Ca^{2+} were conducive to the enzyme activity, whereas the Fe^{3+}, Cu^{2+}, and Ag^{+} evidently inhibited the enzyme activity. Fe^{2+}, Na^{+}, K^{+}, Zn^{2+}, and Mn^{2+} showed less inhibitory effects on the enzyme activity. The K_m of the chitinase was 2.832 mg ml^{-1}. The chitanase were found to inhibit the hyphal growth, conidial germination and sclerotial germination of various plant pathogenic fungi.

Key words: *Gliocladium catenulatum*, chitinases, purification, characteristics.

INTRODUCTION

The parasitism of fungus is one of the important mechanisms against fungal diseases for crops. This method has gained increasing attention from researchers because of its high potential applications. Bacterial parasites, as an important factor in biological control, inhibit the growth of pathogenic bacteria to prevent plant diseases through parasitizing fungus, antagonism, and host cell destruction via enzyme degradation. *Gliocladium* spp., a parasite of plant pathogenic fungi widespread in soil, inhabit the hyphae and sclerotia of many plant pathogenic fungi and act efficiently in biological control, as in the biopesticides SoilGard and Primastop, which contain *Gliocladium roseum* and *Gliocladium catenulatum* as the main active ingredients, respectively (McQuilken and Mohammadi, 1997; Paulitz and Belanger, 2001; Sharma and Singh, 1990; Wu, 1991).

The strong parasitic ability of the *G. catenulatum* strain (HL-1-1) was developed using a large screen in the laboratory. The parasitic sclerotium could not germinate, and more than 90% of the sclerotia decayed. This strain could inhibit damping-off in soybean caused by *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, which cause nightshade vegetables disease and rice sheath blight, by more than 80%. Moreover, it could also inhibit a variety of plant pathogenic fungi (Bao et al., 2004; Ma et al., 2004a). This bacterial strain is highly adaptable, easy to culture (Ma et al., 2004b), and it produces protease, chitinase, glucanase, and cellulase (Ma et al., 2007a, b).

Numerous histologic, morphologic, and biochemical studies on the mechanism of the parasitic activity of *Gliocladium* on *Sclerotinia* have been reported. *Gliocladium* causes the deformation of *Sclerotinia* hyphae, distortion or destruction of their sclerotia, and digestion of their cytoplasm (Zhang et al., 2004); chitinase (Ma et al., 2007a), β-1,3 glucanase (Li et al., 2007), and several other cell wall–degrading enzymes (CDEs) are involved in this process. However, Laing and Deacon (1991) did not agree on the direct action of CDEs on the breakdown and penetration into the host cell wall;

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Table 1. Plant pathogenic fungi tested.

Number	Strains of pathogenic fungi	Source
1	*Fusarium oxysporum*	Institute of Plant Protection, Chinese Academy of Agricultural Sciences
2	*Sclerotinia sclerotiorum*	Institute of Plant Protection, Chinese Academy of Agricultural Sciences
3	*Phytophthora capsici*	Institute of Plant Protection, Chinese Academy of Agricultural Sciences
4	*Fusarium nivale*	Institute of Microbiology, Chinese Academy of Sciences
5	*Coniella diplodiella*	Institute of Microbiology, Chinese Academy of Sciences
6	*Fusarium graminicola*	Institute of Microbiology, Chinese Academy of Sciences
7	*Cytospora mali*	Institute of Microbiology, Chinese Academy of Sciences
8	*Botrytis cinerea*	Institute of Microbiology, Chinese Academy of Sciences
9	*Curvularia lunata*	Institute of Microbiology, Chinese Academy of Sciences
10	*Alternaria solani*	Institute of Microbiology, Chinese Academy of Sciences
11	*Alternaria alternata*	Institute of Microbiology, Chinese Academy of Sciences
12	*Rhizoctonia solani*	Institute of Plant Protection, Chinese Academy of Agricultural Sciences

otherwise, both the enzymes and mechanical pressure may play important roles in this process (Whipps and Gerlagh, 1992).

Gao et al. (2009) constructed a subtractive cDNA library of *G. catenulatum* that parasitizes the sclerotia of *S. sclerotiorum* using suppression subtractive hybridization. Up to 60 sequences were obtained, some of which encode proteins similar to peroxidase, ribosomal protein L11, cytochrome P450, and heat shock proteins, which expressed under certain stresses. The genes might be part of the effect of the interaction between *Gliocladium* and *S. sclerotiorum* (Gao et al., 2009). To understand much better the nature and roles of chitinase in the parasitic process of *G. catenulatum,* the present study, the chitinase of *G. catenulatum* HL-1-1 strain was purified and its characteristics and antibacterial activity was studied.

MATERIALS AND METHODS

The tested strains and medium

The tested strains were *Fusarium oxysporum, S. sclerotiorum, Phytophthora capsici*, *Fusarium nivale*, *Coniella diplodiella*, *Fusarium graminicola*, *Cytospora mali, Botrytis cinerea*, *Curvularia lunata*, *Alternaria solani*, *Alternaria alternate*, and *R. solani*. The detailed information on the strains is shown in Table 1. The fungal medium used contained 10 g of Sclerotia powder, 3 g of NaCl, 3 g of K_2HPO_4, 3 g of $MgSO_4$, 1000 ml of double distilled water, and its pH was 4.5 (Ma et al., 2007a).

Culture conditions for enzyme production and crude enzyme preparation

The 15 lawns (6 mm diameter) cut from the edge of colony of *G. catenulatum* strain HL-1-1 were inoculated into 120 ml of potato dextrose medium, with the culture shaken at 30°C for 24 h. A 4 ml solution was pipette into 120 ml of fungal medium, and then cultured with agitation at 30°C for 6 h. The solution was centrifuged at 6,000 × g at 4 °C for 20 min; the resulting supernatant liquid was the crude enzyme solution.

Determination of enzyme activity

Up to 0.4 ml of 1% colloidal chitin and 0.4 ml of crude enzyme solution were added to 0.1 M of phosphate buffer at pH 5.8. The mixture was kept at 37°C for 2 h, and centrifuged at 10,000 × g for 10 min. Up to 0.4 ml of the supernatant liquid was mixed with 40 µl of 3% snailase solution, and maintained in a 37°C water bath for 30 min. Then, 0.2 ml of saturated borax solution was added and the mixture was placed in a boiling bath for 7 min. When the solution was cooled, 2 ml of glacial acetic acid and 1 ml of 1% dimethylaminoborane solution were added, and then maintained in a 37°C water bath for 15 min. The solution was used to determine the enzyme activity according to Ma et al. (2007a).

Isolation and purification of enzyme

Ammonium sulfate fractionation and desalting chromatography

The salt concentration of the crude enzyme solution in the ice bath was 35, 45, 55, 65, 75 and 85% after ammonium sulfate was added. After 2 h in an ice bath, the solution was centrifuged at 7,000 × g at 30°C for 30 min. The sediment was dissolved in 0.05 mol/L Tris–HCl buffer at pH 8.0, and desalted via Sephadex G-25 gel filtration. The sample volume was 15 ml, and the flow rate was 6 ml/min, and the eluent was 0.05 mol/L Tris–HCl buffer at pH 8.0. The solution was collected based on the different absorption peaks, and chitinase activity was measured. The solutions collected were pooled for further purification.

Ultra filtration of the enzyme solution

The previous collected solution was ultrafiltered using 30 kDa MWCO ultrafiltration in YM-30 centrifugal ultrafiltration tubes, and the activity levels of the chitinase below and above the filter of the ultrafiltration tubes were measured. The solution that showed enzyme activity was collected.

Ion-exchange column chromatography

The solution collected after ultra filtration was purified by ion-exchange chromatography. An AKTA explorer chromatography system (Amersham Company) and an ion exchange column

(SOURCE™ 15Q 4.6/100PE) were employed. The volume was 1.7 ml and the diameter of the chromatographic packing was 15 µm. The sample volume was about 3 ml, and the flow rate was 2 ml/min. The sample was pre-equilibrated with 0.05 mM Tris–HCl buffer at pH 7.5 containing 1 M NaCl. The elution concentration was 16, 20, and 100% of 1 M of NaCl, respectively. The elution solution was collected according to the absorption of protein (280 nm), and desalted with dialysis. The activity and concentration of the chitinase were measured.

Non-denaturing polyacrylamide gel electrophoresis (PAGE)

The chitinase was further purified using non-denaturing PAGE, and the different bands in the gels were collected and checked.

Measurement of enzyme purity and molecular weight

The purity and molecular weight of the enzyme were measured via sodium dodecyl sulfate (SDS)-PAGE. The concentration of the separation gels was 12%, and it was dyed with Coomassie Brilliant Blue. A low molecular weight protein was used as the standard protein.

The studies of chitinases characterization

Determination of optimum temperature and pH

The reaction was carried out at temperatures ranging from 10 to 80°C and the chitinase activity at different temperatures were measured to find the optimum temperature. A similar method was applied at varying pH levels from 3.0 to 10.0 to determine the optimum pH.

Metal ions

The effects of metal ions on chitinase activity were determined by adding 10 metal ions (NaCl, $CaCl_2$, KCl, $FeCl_3$, $FeCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $ZnCl_2$, and AgCl) into the enzyme solutions (the final concentration of the metal ions was 5 mM).

Determination of Km

The K_m values for the chitinase were calculated by fitting the reaction rates into a Lineweaver-Burk plot at various chitin concentrations (0, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 8.0, 10.0 and 20.0 mg/ml).

Measurement of antifungal activity for chitinase

The inhibitory effect of the chitinase on the growth of fungal hyphae was examined using the Oxford cup method. The different fungi was inoculated into a potato dextrose agar (PDA) plate, and after 1 week of culturing, the lawn (6 mm diameter) was removed via aseptic hole punch and then re-inoculated into a new PDA plate. The Oxford cup was placed 1 cm from the edge of the colonies, and 200 µl of 10 µg/ml extract or pure enzyme solution was added. The inhibitory effects were observed for 12 h.

Fungal spores were added to the solution with 200 µl of 10 µg/ml extract or pure enzyme solution and the final concentration was 10^6/ml. About 0.2 ml of the spore suspension was painted onto the PDA plate, and the germination of spores was checked after 2 h of culturing at 28°C.

The sclerotia of *R. solani* and *S. sclerotiorum* were sterilized and kept in the enzyme solution at 28°C for 24 h, and the germination rates of the sclerotia were determined after 2 days. The number of sclerotia for each fungus was 50.

RESULTS

Purification of the chitinase

The extract was purified through fractional precipitation with ammonium sulfate, column chromatography, and non-denaturing PAGE, and was then checked using SDS-PAGE. The result indicates high chitinase activity, which showed as a single band, at a molecular weight of about 51 kb (Figure 1). Additional information is shown in Table 2.

Characterization of the chitinase

The chitinase activity increased with increasing temperature from 20 to 60°C, but decreased when the temperature reached 70°C. The highest activity was at 60°C, with a value of 118.04 U (Figure 2A). Thermal stability was investigated via incubation from 20 to 80°C for 20 min. The chitinase showed good thermal stability at 20, 30, and 40°C, whereas it significantly decreased at 50°C and the activity was almost completely lost at 80°C (Figure 2B). The optimal pH for chitinase activity was 6.0, with 99.56 U (Figure 3A), but the chitinase was most thermally stable at pH 5.0 (Figure 3B). Different metal ions showed different effects on the GcCHI1 chitinase activity (Figure 4). Co^{2+} and Ca^{2+} played positive roles on the chitinase activity, whereas Fe^{3+}, Cu^{2+}, Ag^{+}, K^{+}, Zn^{2+}, and Mn^{2+} significantly decreased the GcCHI1 chitinase activity, with the most significant inhibition found in the Fe^{3+} treatment. No significant differences were found between the Fe^{2+} and Na^{+} treatments. The K_m values for the chitinase, calculated with the Lineweaver-Burk plot, was 2.832 mg/ml.

The activity of the chitinase in antifungals

The crude and the pure chitinase solutions inhibited all the ten pathogenic fungi. The wide inhibition zones of the crude GCHI1 were observed in *S. scleotiorum* and *B. cinerea*, whereas the narrowest inhibition zone of the crude GCHI1 was observed in *Cladosporium capsici* (only 0.59 mm). The pure GCHI1 showed wide inhibition zones in *B. cinerea* and *R. solani*, whereas minimal effects were found in the *C. capsici* and *Ahernaria solani*. Overall, the crude GCHI1 was more efficient in the inhibition of the hyphae growth than the pure GCHI1 (Table 3). The germination rates of all pathogenic fungi were inhibited by the crude and the pure GcCHI1 (Figure 5). The sclerotial germination rates of *R. solani* and *S. sclerotiorum* were significantly reduced

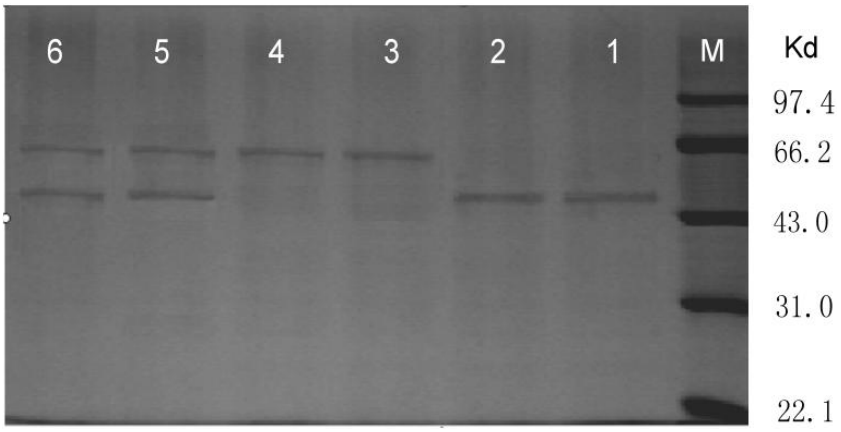

Figure 1. SDS-PAGE profile of protein samples collected via non-denaturing gel electrophoresis. M, protein maker; Lanes 1 and 2 inactive protein; Lanes 3 and 4 purified chitinase; Lanes 5 and 6 SOURCE™ 15Q 4.6/100 PE active components of column chromatography.

Table 2. Purification of chitinase from *G. catenulatum* HL-1-1.

Step	Total protein (mg)	Specific activity (U mg^{-1})	Total activity (U)	Yield (%)	Purification (fold)
Crude extract	190.4	1.21	230.84	100	1
85%$(NH_4)_2SO_4$	50.3	3.24	162.83	70.5	2.68
Source™ 15Q4.6/100PE	12.4	5.5	68.2	29.5	4.55
Native polyacrylamide gel electrophoresis	0.6	12.17	7.3	3.2	10.06

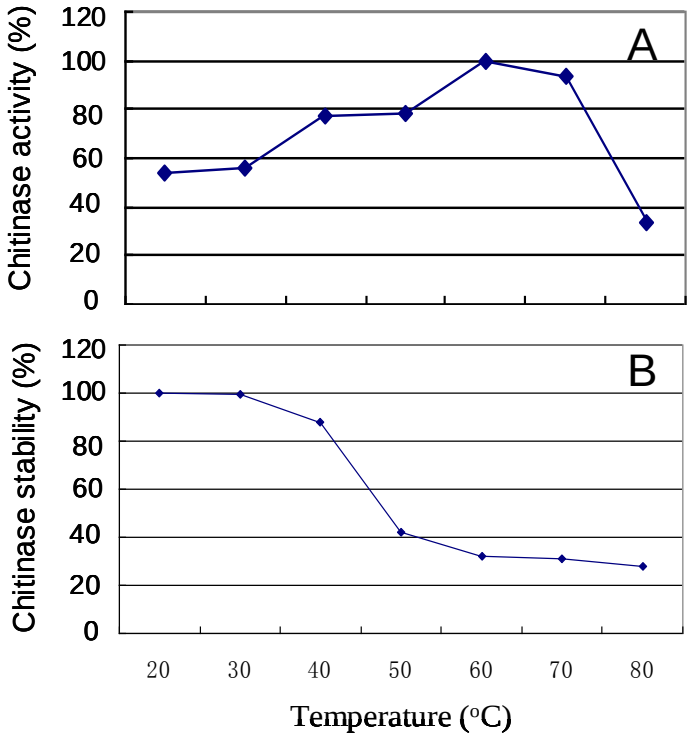

Figure 2. Effect of temperature on GcCHI1chitinase activity; A and stability; B.

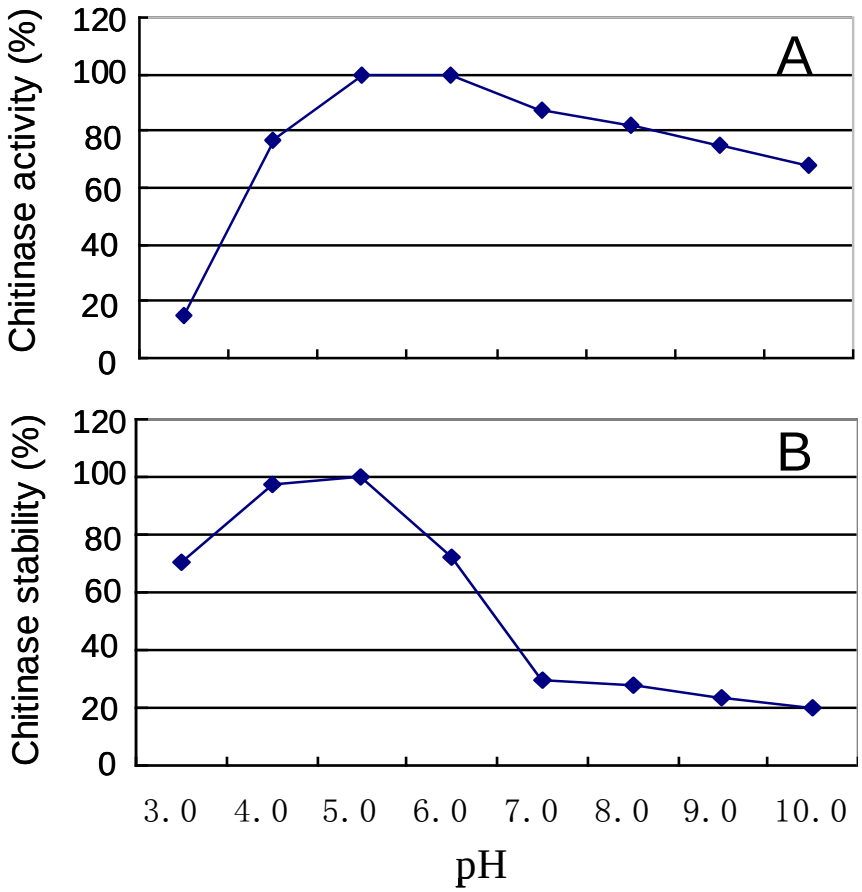

Figure 3. Effect of pH on GcCHI1chitinase activity; A and stability; B.

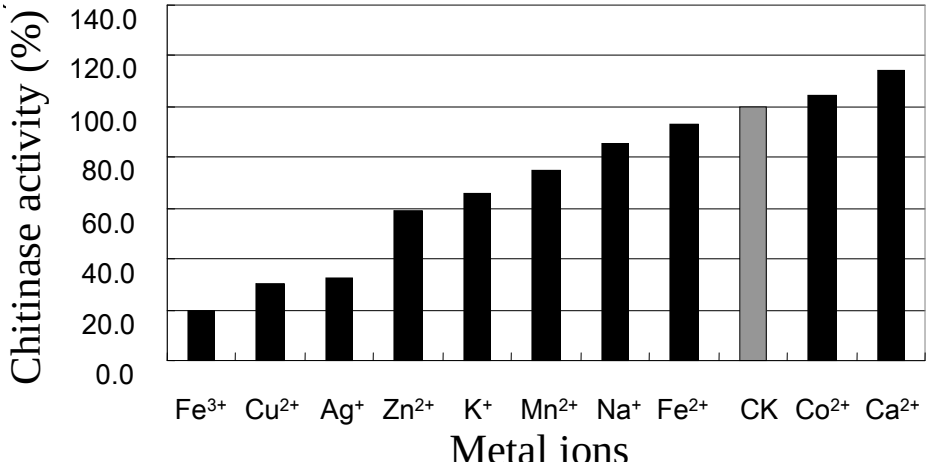

Figure 4. Effect of metal ions on GcCHI1 chitinase activity.

by the crude and the pure GcCHI1. The effects of chitinase on *R. solani* were more obvious than that on *S. sclerotiorum*. Furthermore, the pure chitinase showed more significant effects than the crude chitinase.

DISCUSSION

The chitinase, with a K_m value of 2.832 and a molecular weight of about 51 kDa, was purified from *G. catenulatum*

Table 3. Effect of GcCHI1 chitinase on the widths of *antifungals* bands in various pathogens.

Pathogenic bacteria	Crude solution	Pure solution
Fusarium oxysporum	2.47	1.26
Sclerotinia sclerotiorum	3.08	2.74
Phytophthora capsici	0.59	0.47
Coniella diplodiella	3.28	1.99
Fusarium graminicola	2.38	2.77
Botrytis cinerea	3.34	3.74
Curvularia lunata	2.18	1.28
Alternaria solani	1.58	0.45
Alternaria alternata	2.40	2.16
Rhizoctonia solani	2.41	3.60

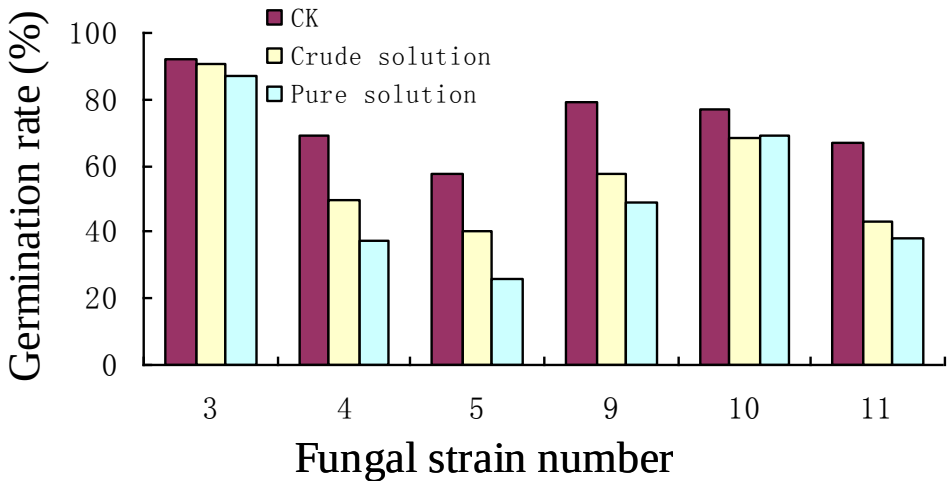

Figure 5. Effect of crude and pure chitinases on conidial germination rates of different fungal strains.

via ammonium sulfate precipitation, desalting, ultrafiltration, anion exchange chromatography, non-denaturing gel electrophoresis, and other methods. The optimum conditions for the chitinase activity were 60°C and pH 6.0. The enzyme solution was stable from 20 to 40°C, and pH 4 to 5. Two metal ions, Co^{2+} and Ca^{2+}, promoted chitinase activity, whereas Fe^{3+}, Cu^{2+}, and Ag^{+} significantly restrained the chitinase activity. The inhibitory effects of Fe^{2+}, Na^{+}, K^{+}, Zn^{2+}, and Mn^{2+} on the chitinase were less significant.

The present study demonstrates that the HL-1-1 strain produces large amounts of chitinase, which significantly inhibits the mycelial growth, as well as the spore and sclerotial germination of the tested pathogenic fungi. The inhibition of the oomycete fungal pathogen *P. capsici* was minimal. This result is consistent with that of *Trichoderma* chitinase (Lorito et al., 1993; Liu and Xu, 2003), which indicates that chitinase plays an important role in infecting the sclerotia of *Gliocladium* mold spores and restraining the growth of fungi. In the current experiment, the antibacterial effects of crude extract enzyme were more obvious than those of the pure enzyme in the inhibition of hyphae growth, probably resulting from the interaction between the chitinase and other antifungal microorganisms, and not solely from the chitinase. The co-inhibition of the chitinase and β-1,3-endoglucanase was higher than that of the single one (Van den Bulcke et al., 1989). This provides indirect evidence that the antibacterial activity of the crude extract enzyme were higher than that of the pure enzyme. The antibacterial effect of the crude extract enzyme was enhanced by the chitinase, other small molecule antibacterial substances, CDEs, and the related proteins.

The major components of mature fungal sclerotia are carbohydrates (about 75%), protein (10 to 25%), lipids (2 to 3%), and ash (3.5 to 5.0%). The carbohydrates include glucan, chitin, trehalose, mannose, and a small amount of reducing sugars. The main components of the fungal cell wall are glucan, chitin, proteins, and lipids, which are up to 61, 58, 13, and 9%, respectively. Chitinase is the limiting factor in the effective control of fungal diseases and pests (Shternshis et al., 2002). Chitinase degrades

chitin into chitin oligosaccharides and monosaccharides, thereby undermining the fungal cell wall and playing a role in the prevention and treatment of fungal diseases. Different strains of *Gliocladium* sp. reportedly have chitinase activity proportional to the ability of the corresponding nuclear parasite (Ma et al., 2007b). This indicates that chitinase plays a role in the bacterial parasitism of fungi, and is the key enzyme against fungal diseases.

Previous studies have shown that bacterial parasites produce a series of CDEs during host parasitism, such as glucanase, chitinase, cellulose, protease, and so on. The most important CDE is chitinase. Lorito et al. (1993) studied the antagonist activities of the chitinase in *Trichoderma harzianum* for nine plant pathogenic fungi, and the results demonstrate that the conidial germination rate of *B. cinerea* is significantly decreased by chitinase (1993). Moreover, stronger effects were found in the solution containing chitinase and five fungicides.

Parasitic fungi interact with their hosts, and normally produce several different chitinases, and infection of different parts and at different times generates different kinds of chitinases. Parasitic fungi produce three types of chitinases, namely, *N*-acetyl-β-glucosaminidase, endochitinase, and exochitinase, as well as a few enzyme succinic sugars. Recently, *N*-acetyl-β-glucosaminidase, endochitinase, and exochitinase have been isolated and purified from the different strains of *T. harzianum* (Lorito et al., 1993). The *T. harzianum* chitinases consists of six enzymes: two β-1,4-*N*-acetyl-glucosaminidases (CHIT102 and CHIT73) and four endochitinases (CHIT52, CHIT42, CHIT33, and CHIT31) (Lorito, 1998). Recently, *Trichoderma* expression of endochitinase gene ThEn42 has been cloned from parasitic fungus and successfully transferred into tobacco and other dicotyledonous plants, and has acquired a high level of constitutive expression (Schimbock et al., 1994). In the current study, a chitinase from *G. catenulatum* that significantly inhibits plant pathogenic fungi, which are different from other bacteria parasitic fungi that produce a variety of chitinases, was isolated. The results provide a foundation for learning the function of chitinases in bacterial parasitism. Further studies on the identification and clone of the chitinase need to be conducted in the future.

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