



# Isolation, partial characterization, and cloning of an extracellular chitinase from the entomopathogenic fungus *Verticillium lecanii*

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Genet. Mol. Res. 14 (1): 2275-2289 (2015)

Received March 24, 2014

Accepted September 23, 2014

Published March 27, 2015

DOI <http://dx.doi.org/10.4238/2015.March.27.13>

**ABSTRACT.** The entomopathogenic fungus *Verticillium lecanii* is a well-known biocontrol agent of fungal phytopathogens, as well as insect pests. A 42-kDa chitinase belonging to family 18 of the glycosyl hydrolases was isolated and partially characterized. Chitinase was purified using successive column chromatography on phenyl-sepharose, DEAE-sepharose, and CM-sepharose. The enzyme showed the highest activity at 40°C and pH 4.6. Enzyme activity was strongly activated in the presence of Mg<sup>2+</sup>. The purified enzyme showed inhibitory activity of spore germination against several plant pathogens, particularly *Fusarium moniliforme*. The genomic DNA and cDNA sequences were resolved by polymerase chain reaction amplification and sequencing. Protein modeling and comparative investigation of different chitinase amino acids showed that chitinases are conserved in parasitic fungi.

**Key words:** Acidic extracellular chitinase; Inhibitory effect; Protein modeling; *Verticillium lecanii*

## INTRODUCTION

Biological control agents are regarded as alternatives to conventional synthetic chemicals, which are known to have negative effects on the environment and non-target organisms, including animals and humans (Harman et al., 1993). However, the major commercial biopesticides have been limited by their lack of efficacy. Many efforts have been made to isolate high-performance pesticidal strains or novel compounds derived from such organisms for use in biological control (Prasanna et al., 2013). The versatile fungus *Verticillium lecanii* is one of the most promising natural parasites, as it shows tremendous potential as a pest biological control agent against numerous insects, including Coleoptera, Orthoptera, Homoptera, and Lepidoptera (Xu et al., 2011), as well as numerous phytopathogenic fungi, including *Sphaerotheca macularis*, *Puccinia coronata*, *Penicillium digitatum*, *Pythium ultimum*, and *Fusarium moniliforme* (Benhamou and Brodeur, 2000, 2001; Miller et al., 2004; Yu et al., 2012). In several phytopathogenic fungal species, *V. lecanii* may induce host defense reactions without showing the pathogenicity phenotype (Benhamou and Brodeur, 2000). Chitin is one of the highest  $\beta$ -1,4-*N*-acetyl-D-glucosamine-containing units in nature after cellulose. This molecule provides architectural reinforcement of biological structures, such as insect exoskeletons, crustacean shells, higher fungal cell walls, and other biological matrices involved in protection and self-defense (Hammami et al., 2013). Chitin is also a well-known pathogen-associated molecular pattern that elicits plant immunity. Identified chitin receptors include OsCEBiP in rice and AtCERK1 in the model experimental plant *Arabidopsis*, both containing an extracellular lysine motif domain that is widely distributed for *N*-acetyl-D-glucosamine recognition, which is required for chitin-triggered immunity (Liu et al., 2012). In addition, this polysaccharide is a main target molecule of biological agents that may secrete chitinolytic enzymes, such as several bacteria, actinomycetes, fungi, higher plants, and animals (Li, 2006). Chitinases in fungi play important physiological and ecological roles in defense mechanisms in response to pathogens and abiotic stress, and are also involved in nutrition and pathogenesis. Fungal pathogenesis is a complex process that includes adherence, penetration, and digestion and requires the production of hydrolytic enzymes that penetrate the chitinous cell wall and cuticle, an extremely thick barrier preventing the entry of most fungal pathogens and insects (Li et al., 2004). Chitinases (EC 3.2.1.1.4) are major extracellular-degrading enzymes responsible for the random hydrolysis of internal links of the chitin chain to small-molecule nutrients, which are required during fungus penetration into the insect cuticle or fungal cell wall (Rocha-Pino et al., 2011). In addition, chitinases in plants are important enzymes of protective approaches targeting fungal pathogens, since chitinases in plants can degrade chitin in the fungi cell wall. Chitinases are widely employed in laboratory and field experiments, such as for the biological control of fungal pathogens, oligosaccharide and *N*-acetyl-D-glucosamine preparations, and protoplast preparation for filamentous fungi. Therefore, chitinases have recently gained attention worldwide for their potential practical uses (Sahai and Manocha, 1993).

Based on the similarity of amino acid sequences, chitinases from various organisms have been grouped into Family 18 and Family 19 of the glycosyl hydrolases, and all fungal chitinases identified to date belong to Family 18 (Henrissat and Bairoch, 1993). In recent years, studies of fungal chitinases have demonstrated progress in gene isolation, functional characterization, and research on chitinase as alternative biocontrol compounds. Purification and characterization of chitinases from mycoparasitic fungi have also been reported, such as from *Aphanocladium album*, *Nomuraea rileyi*, *Gliocladium virens*, and *Fusarium*

*chlamydosporum* (Li, 2006). A chitinase gene, *Pcchi44*, from *Pochonia chlamydosporia* was characterized as a virulence factor in infection against nematodes (Mi et al., 2010), while the *Lecanicillium psalliotae* chitinase gene *Lpch1* was found to play a role in the biocontrol of the root-knot nematode *Meloidogyne incognita* (Gan et al., 2007). Chitinases in the *Trichoderma* genus have been investigated intensively and extensively for their widespread application in field biocontrol; for instance, chitinases in *Trichoderma harzianum*, *Trichoderma virens*, *Trichoderma atroviride*, and *Trichoderma asperellum* have been isolated and biochemically characterized (Ihrmark et al., 2010). Recently, a purified chitinase from *Stenotrophomonas maltophilia*, a bacterium that is antagonistic toward fungal phytopathogens, was shown to inhibit the growth of fungal phytopathogens belonging to the genera *Fusarium*, *Rhizoctonia*, and *Alternaria* (Jankiewicz et al., 2012). A purified chiIO8 from *Bacillus cereus* exhibited activity against *Botrytis cinerea* in an *in vivo* assay (Hammami et al., 2013). A genetic transformation and expression system for temporal and spatial regulation of gene expression has been established in *V. lecanii* to facilitate the study of gene function *in vivo*. Development of *V. lecanii* as a biopesticide requires additional functional characterization of candidate genes via genetic manipulation and biochemical approaches, so that the desirable traits can be combined to produce superior strains (Hasan et al., 2011). In this study, a chitinase from *V. lecanii* was isolated and biochemically characterized and *in vitro* activities toward several phytopathogenic fungi were tested. Subsequently, a chitinase gene, *VICHI*, was cloned and analyzed to improve the desirable traits of *V. lecanii* through genetic engineering.

## MATERIAL AND METHODS

### Organisms and culture conditions

*Verticillium lecanii* strain CA-12 employed in this study was originally isolated from a dead whitefly *Trialeurodes vaporariorum* in Changchun urban, Jilin Province, China (43°88'N, 125°35'E) (Yu et al., 2012). A single-spore isolate of *V. lecanii* CA-12 was stored and cultures were grown on freshly prepared potato dextrose agar (PDA) plates at 25°C. *Escherichia coli* DH5 $\alpha$  was used for DNA manipulation. The plasmid pMD18-T was purchased from Takara Co. (Shiga, Japan). For enzyme inducible production, the fungus was grown in 500-mL flasks containing 250 mL synthetic medium (SMCS) with chitosan and sucrose as carbon sources. SMCS was composed of (mg/L): KH<sub>2</sub>PO<sub>4</sub>, 680; K<sub>2</sub>HPO<sub>4</sub>, 870; KCl, 200; NH<sub>4</sub>NO<sub>3</sub>, 1000; CaCl<sub>2</sub>, 200; MgSO<sub>4</sub>·7H<sub>2</sub>O, 200; FeSO<sub>4</sub>, 2; ZnSO<sub>4</sub>, 2; MnSO<sub>4</sub>, 2; as well as 5 g chitosan and 5 g sucrose; the pH of the medium was adjusted to 6.0 (Li et al., 2004).

### Assay of enzymatic activity

Chitinolytic activity was determined using a colorimetric assay (Boller et al., 1983) with colloidal chitin as a substrate. The procedures were generally adapted from Li et al. (2004). One unit chitinase activity was defined as the amount of enzyme that catalyzed the release of 1  $\mu$ M  $\beta$ -1,4-*N*-acetyl-D-glucosamine/min at 40°C.

### Enzyme production and purification

For the production of chitinase, a fresh aliquot of 100  $\mu$ L strain *V. lecanii* CA-12

conidial suspension ( $1 \times 10^7$  conidia/mL) was inoculated into SMCS medium while shaking (200 rpm) at 25°C. Cultivation of *V. lecanii* CA-12 for monitoring the chitinase production was carried out in SMCS medium. An enzyme assay to monitor the production of chitinase was carried out using the crude enzyme as the source of chitinase and swollen chitin as the substrate. The optimum time for maximum production of chitinase was monitored every 12 h. Large-scale production of chitinase was carried out by mass fermentation, and the mycelia of the cultures after incubation were filtered and centrifuged at 10,000 g for 30 min at 4°C. The resulting supernatant was used as the crude enzyme solution. All stages of purification were carried out at 4°C. The following buffers were used: buffer A, 50 mM HAc-NaAc, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 6.0); buffer B, 50 mM Tris-HCl, 1 mM EDTA, and 1 mM PMSF (pH 8.0).

### Fractional ammonium sulfate precipitation

Salting out of the enzyme preparation was carried out in the first fractionation stage to 35% ammonium sulfate saturation and in the second to 90% saturation. After standing incubation for 12 h, the resulting precipitate was collected by centrifugation at 12,000 g for 30 min, dissolved in buffer A, and dialyzed overnight against 3 changes of 50 mM phosphate buffer, pH 6.0. Insoluble material was removed by centrifugation at 10,000 g for 10 min.

### Hydrophobic chromatography on phenyl-sepharose

Solid ammonium sulfate was added to the dialyzed sample to 50% saturation, and then immediately loaded onto a phenyl-sepharose column (1 x 20 cm) equilibrated with buffer A including 50% ammonium sulfate. After the column was washed with 5 column volumes of buffer A, the proteins were eluted with a 100-mL ammonium sulfate gradient solution (50-0% saturation in buffer A at a flow rate of 1 mL/min). Fractions with chitinase activity were collected and dialyzed overnight against buffer A.

### Ion-exchange chromatography on DEAE-sepharose and CM-sepharose

The dialyzed sample was passed over a DEAE-sepharose column (1 x 20 cm) that had been pre-equilibrated with buffer A. First, the column was washed with 5 column volumes of buffer A, and then the enzymes were fractionated with a 240-mL linear gradient of 0-0.3 M NaCl in buffer A at a flow rate of 1 mL/min. Fractions with chitinase activity were pooled and dialyzed overnight against buffer B. The dialyzed sample was applied to a CM-sepharose column (1.6 x 20 cm) that had been equilibrated with buffer B. After the column was washed with 5 column volumes of buffer B, chitinase was eluted with a 180-mL linear gradient of NaCl (0-0.3 M in buffer B) at a rate of 0.5 mL/min. Fractions with chitinase activity were pooled and concentrated to 2 mL using polyethylene glycol 6000 (PEG6000) according to the manufacturer instruction.

### Determination of protein content and chitinase molecular weight

Protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard. The molecular weight of chitinase was determined

by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 10% (w/v) isolation gel and 5% (w/v) concentration gel according to the method of Laemmli (Laemmli, 1970). Gels were stained with Coomassie blue for protein visualization.

### Characterization of chitinase

To determine the optimal temperature of enzyme chitinolytic activity, purified chitinase was incubated with colloidal chitin and examined for HAC-NaAc (pH 6.0) for 1 h at various temperatures (20°-80°C). In addition, the thermal stability of the enzyme was measured in terms of residual activity after incubation of purified chitinase at various temperatures ranging from 40°-70°C in 50 mM Tris-HCl, pH 6.0, for 30 min. The pH activity profile of chitinase was also determined by measuring enzyme activity at 37°C for 1 h under standard conditions over a pH range of 3.0-8.0, 100 mM CH<sub>3</sub>COOH-CH<sub>3</sub>COONa (pH 3-6.5), and 100 mM Tris-HCl (pH 7.0-8.0). The effects of metal ions (50 mM of NaCl, KCl, CaCl<sub>2</sub>, MgCl<sub>2</sub>, and CuSO<sub>4</sub>) on enzyme activity were investigated by pre-incubating the enzyme with colloidal chitin and 0.05 M HAC-NaAc, pH 6.0, as well as different metal ions for 1 h at 40°C. All experiments were performed in triplicate.

### Antifungal activity of purified enzyme *in vitro*

Antifungal activity of the purified enzymes *in vitro* was determined using the method described by Yu et al. (2012) with slight modifications. Several agronomically important phytopathogens, including *Penicillium italicum*, *F. moniliforme*, *Magnaporthe oryzae*, and *Setosphaeria turcica* were selected and propagated on a PDA Petri dish at 28°C for 8 days. After sporulation, spores were collected, washed 3 times with 0.05 M Tris-HCl buffer, (pH 8.0), and resuspended in 0.1% (m/v) glucose (approximately 1 x 10<sup>8</sup> spores/mL). Spores were utilized directly to examine the effects of chitinase on spore germination. Briefly, 5 µL conidial suspensions and 5 µL PD solution were mixed with 50 µL purified chitinase at different concentrations in the wells of sterile depression slides, and incubated at 28°C for 24 h. The control slides contained buffer B in place of enzyme solution. The results were recorded by light microscopy (Olympus BHS313, Tokyo, Japan), and the percentage of spore germination (100 randomly selected conidia) was determined and compared with that of the control.

### *VICHI* gene cloning and sequence analysis

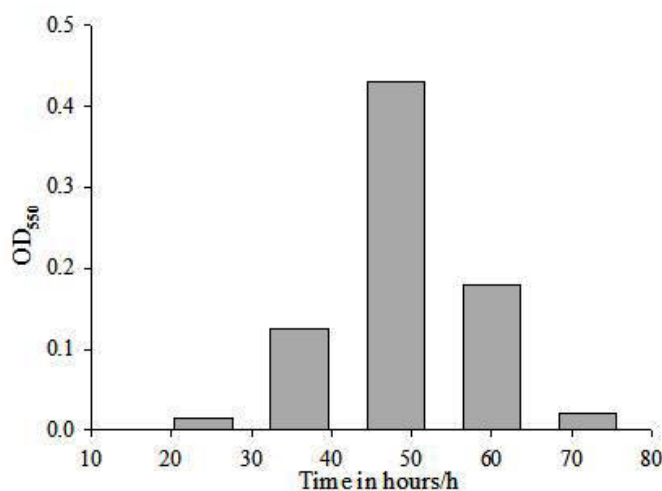
*Verticillium lecanii* strain CA-12 was used for cloning of *VICHI* (*V. lecanii* chitinase) in this study. Mycelia of *V. lecanii* were harvested and ground to fine powders in liquid nitrogen with a mortar and pestle. Genomic DNA (gDNA) was extracted using a cetyltrimethylammonium bromide (CTAB) method, while total RNA was prepared using TRIzol Reagent following the manufacturer instructions (Ambion, Austin, TX, USA). cDNA was synthesized using the Prime-Script™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara). The degenerate primers were: forward: 5'-TCCCCCGGGATGTTGAGYCTACTCAAAAARTCG ATG-3', and reverse: 5'-GCTCTAGACTATTTTCAT GCCATTCTTGATGTTG-3', and were designed according to the conserved region of the aligned amino acid and nucleotide sequences deduced from chitinase gene sequences in *Cordyceps confragosa* (GenBank Accession No. ABD77095), *Trichoderma longibrachiatum* (GenBank Accession No. ACZ63268), *Hypocrea*

*rufa* (GenBank Accession No. AAF19618), *Laganum attenuatum* (GenBank Accession No. AGS15320), *Trichoderma hamatum* (GenBank Accession No. AAC60385), and *L. psalliotae* (GenBank Accession No. ABQ57240). A conventional polymerase chain reaction (PCR) amplification with high-fidelity *Pfu* DNA polymerase (Fermentas, Vilnius, Lithuania) was performed in a Bio-Rad S1000™ Thermal Cycler (Hercules, CA, USA). The PCR product was recovered and ligated into the pMD18-T vector (Takara), which was then transformed into competent cells of *E. coli* DH5 $\alpha$ . Positive *E. coli* clones were characterized by PCR, and 3 positive clones were individually sequenced by Invitrogen (Carlsbad, CA, USA). The sequence was subjected to a homology search in the NCBI database using the BLASTx program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). For phylogenetic analysis, amino acid sequences of chitinase were selected from GenBank and aligned using the ClustalX software (version 1.83). A phylogenetic tree was constructed using the MEGA software (version 4.0.2), and the neighbor-joining method using 1000 bootstrap replicates was used to ascertain the reliability of a given branch pattern in the neighbor-joining tree. In addition, the conserved domain of chitinase in parasitic fungi was aligned using the ClustalX software and analyzed using the BoxShade server ([http://ch.embnet.org/software/BOX\\_form.html](http://ch.embnet.org/software/BOX_form.html)). The 3-dimensional (3D) structure of VICHI was predicted by protein modeling with the SWISS-MODEL server (<http://swissmodel.expasy.org/>), using a chitinase template of *Bionectria ochroleuca* chitinase (GenBank Accession No. EU000575.1; PDB code 3G6M).

## RESULTS

### Kinetics of enzyme production by *V. lecanii*

The production of extracellular chitinase from *V. lecanii* grown in liquid SMCS culture supplemented with chitosan for inducible production was determined at different growth phases. Extracellular chitinase activity was detected after 24 h in the culture supernatants and levels of chitinase activity increased after 48 h of incubation, after which expression decreased (Figure 1). Thus, 48 h was selected as the fermentation time course for large-scale production of chitinase.



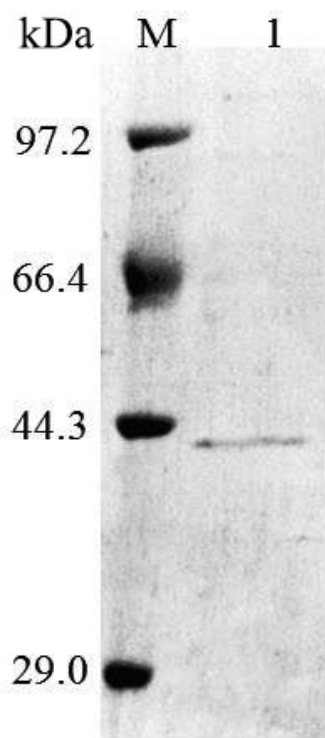
**Figure 1.** Chitinase production at different incubation periods (hours) in *Verticillium lecanii* CA12.

### Purification of chitinase from *V. lecanii*

Fractions of the chitinase peak on the DEAE-sepharose fast flow column were collected and dialyzed overnight at 4°C against 50 mM Tris-HCl containing 1 mM EDTA and 1 mM PMSF, pH 8.0; EDTA and PMSF were included to inhibit protease activity. The enzyme was then applied to a CM-sepharose ion-exchange column for chromatography. The chitinase activity peak fraction was collected and run on a sodium dodecyl sulfate-polyacrylamide gel. The chitinase was purified 18.76-fold with a final yield of 16.9% and specific activity of 29.26 U/mg. The purification procedures of extracellular chitinase secreted by *V. lecanii* CA12 are summarized in Table 1. Enzyme purity and molecular mass were determined by gel electrophoresis, showing a single polypeptide chain of approximately 42.0 kDa (Figure 2).

**Table 1.** Purification of extracellular chitinase from *Verticillium lecanii* CA12.

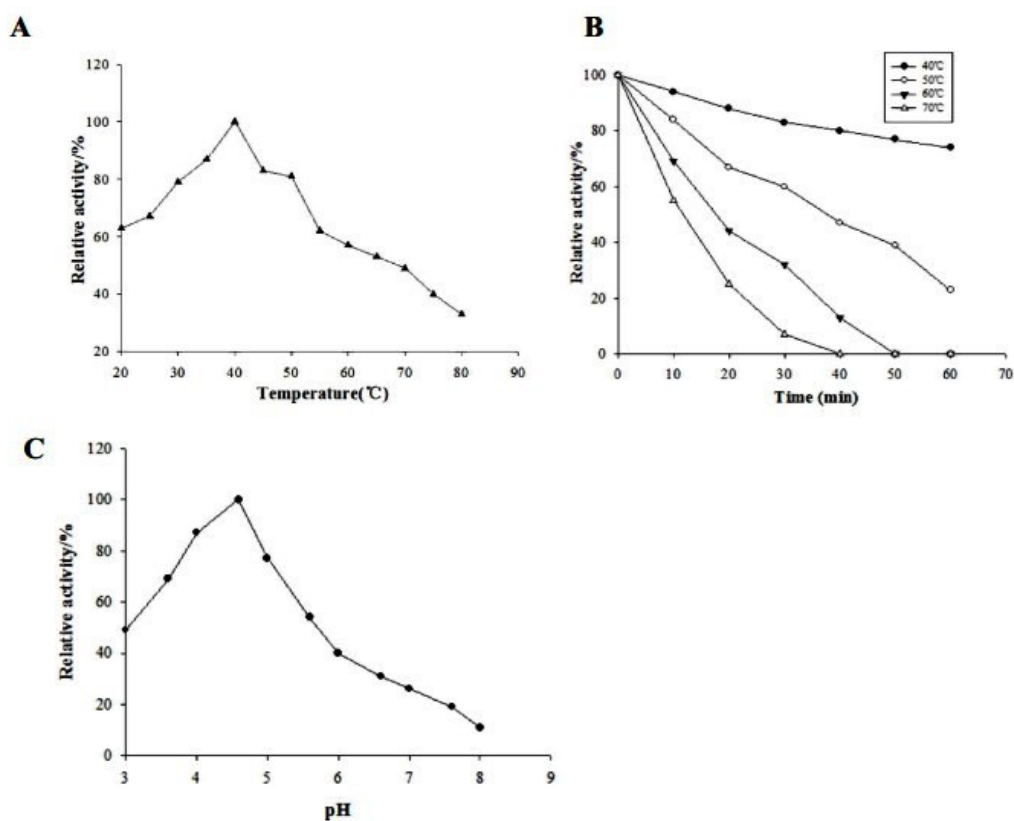
Purification step	Volume (mL)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification (fold)
Crude extract	900	837.43	1.56	1306.7	100	1.00
90% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	26	113.18	3.91	442.3	33.8	2.51
Phenyl-Sepharose	23	44.77	8.24	369.1	28.2	5.28
DEAE-Sepharose	18	17.07	16.8	286.7	21.9	10.76
CM-Sepharose	15	7.54	29.26	220.6	16.9	18.76



**Figure 2.** SDS-PAGE of purified chitinase from *Verticillium lecanii* strain CA12. Lane M = standard protein molecular weight marker; lane 1 = protein samples from CM-sepharose (0.8 µg protein was loaded onto the gel).

## Characterization of chitinase

To determine the optimum temperature of purified chitinase activity, enzyme reactions were performed over a range of temperatures from 20° to 80°C in HAc-NaAc, pH 6.0, for 1 h, using colloidal chitin as substrate. Optimum enzyme activity was observed at 40°C. When the reaction temperature reached higher than 50°C, there was a sharp decrease in activity, with only 33% of the maximum activity observed at 80°C (Figure 3A). Regarding the thermostability of purified chitinase, only 23% activity was retained after 60 min at 50°C, whereas complete inactivation was observed when the enzyme was incubated at 60°C for 50 min and 70°C for 40 min (Figure 3B). Experiments examining the influence of pH on enzyme activity were also carried out. Residual relative activity of more than 70% was found in the pH range of 4.0-5.0, with optimum activity at pH 4.6 (Figure 3C), indicating that the purified chitinase was acidic. Enzyme activity was measured in the presence of different metal ions by incubating the enzymes with colloidal chitin and 50 mM HAc-NaAc, pH 4.8, including different metal ions such as Ca<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup>, K<sup>+</sup>, and Na<sup>+</sup> for 1 h at 40°C. Activity without metal ions was estimated to be 100%. Chitinase activity was strongly activated by 50 mM Mg<sup>2+</sup>, and partially by Ca<sup>2+</sup> (Table 2), whereas chitinase activity was slightly affected by Cu<sup>2+</sup>, K<sup>+</sup>, and Na<sup>+</sup>.



**Figure 3.** Activity and stability of purified chitinase. **A.** Optimum reaction temperature of purified chitinase. **B.** Stability of purified chitinase at different temperatures. **C.** Activity of purified chitinase at different pH values.

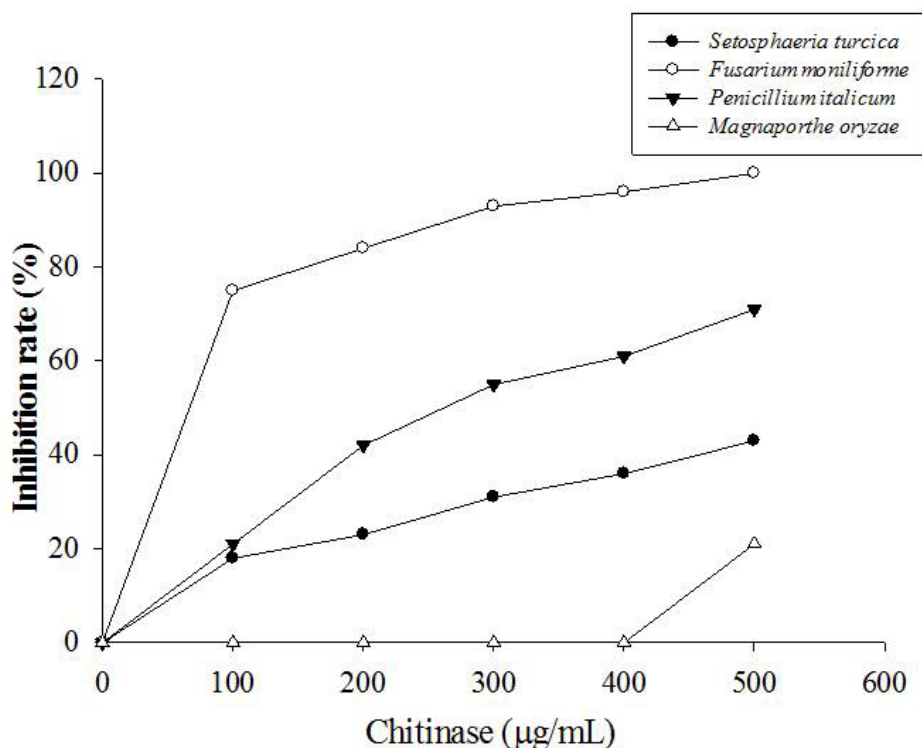


**Table 2.** Effect of various metal ions on the activity of chitinase VICHI.

Metal ions	Concentration (mM)	Relative activity (%)
Control	0	100
Na <sup>+</sup>	50	63
K <sup>+</sup>	50	55
Ca <sup>2+</sup>	50	108
Mg <sup>2+</sup>	50	208
Cu <sup>2+</sup>	50	78

### Effect of chitinase on phytopathogenic fungi

Antifungal activity was determined using isolated chitinase at different concentrations. Spore germination of the different phytopathogenic fungi tested was inhibited by different concentrations of chitinase. Partially purified chitinase exhibited prominent antifungal activity against the phytopathogenic fungus *F. moniliforme*, followed by *P. italicum*. This fungus was inhibited by more than 90% under the conditions used in this study at a concentration of 300 µg/mL (Figure 4).



**Figure 4.** *In vitro* antifungal activity (%) of purified chitinase (VICHI) from 0 to 500 µg/cm<sup>3</sup>. Approximately 1000 fungal spores were cultured in the agar plate with or without different amounts of chitinase at 25°C for 10 h. Inhibition of spore germination was examined under a light microscope.

## Cloning and sequence analysis of *VICHI* gene

Using *V. lecanii* total cDNA and gDNA as templates, the full-length cDNA open reading frame was generated via reverse transcription-PCR. The fragment was 1272 bp, indicating that *VICHI* is composed of 423 amino acids with a predicted molecular weight (MW) of 45.8 kDa; the pI of mature chitinase protein was predicted to be 5.67, which is consistent with the optimum pH for the enzyme purified in this study. In addition, the genomic DNA sequence of chitinase was also determined. The gene-coding sequence of the genomic DNA spanned 1432 bp and contained 3 introns of 57, 51, and 52 bp, respectively (Figure 5). Many extracellular chitinase molecules are translated with a signal sequence, followed by a cleavage into a mature protein. The signal P4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>) analysis, which is used to predict the presence of signal peptides in protein sequences and the location of potential cleavage sites, predicted that *VICHI* contained a putative signal peptide of 20 amino acids (MLSLKKSMAVAVALQAVTA) for excreted proteins, and the MW of the predicted mature chitinase matched the size of the purified enzyme in this study. The *VICHI* cDNA and gDNA sequences were deposited in GenBank (accession numbers are KF041476 for gDNA and KF041477 for cDNA). A GenBank database search showed that the nucleotide and amino acid sequence of *VICHI* had extensive similarities with members of the Family 18 chitinases, and the chitinase from *V. lecanii* obtained in this study can be assigned to the GH18 (glycosyl hydrolases, Family 18) type II chitinases. The sequences of its homolog in parasitic fungi were aligned using the ClustalX program. Chitinases in fungi were found to be conserved. *VICHI* contained the conserved substrate-binding and catalytic domains (SXGG and DXXDXDXE) of glycosyl hydrolase Family 18 (Yang et al., 2010), and a conserved catalytic triad center containing Asp169, Trp170, and Glu171, which are very important for chitin binding and catalytic activity (Figure 6). Chitinase *VICHI* showed overall sequence similarities ranging from 70 to 90% to 4 other chitinases (LfChi, ThChi, MaChi, and BoChi), and with 91% similarity to a basic chitinase chi2 from *V. lecanii* strain DAOM 175104 (Lu et al., 2005). However, the amino acid residues around the substrate-binding site and the catalytic center were very conserved (Figure 6). The 3-D structure of *VICHI* was modeled using the SWISS-MODEL server employing the crystal structure of chitinase from *B. ochroleuca* chitinase (GenBank No. EU000575.1; PDB code 3G6M) as a template, which is highly homologous to *V. lecanii* chitinase, and whose crystal structure was previously reported (Yang et al., 2010). The predicted 3-D structure revealed that 8 strands of parallel  $\beta$  barrels were surrounded by 8  $\alpha$ -helices to form the core domain, named the  $(\beta/\alpha)_8$  triosephosphate isomerase barrel, which is common in other Family 18 chitinases (Yang et al., 2010), and the N-terminal catalytic domain contained 3 putative active residues facing each other (Figure 7).

## DISCUSSION

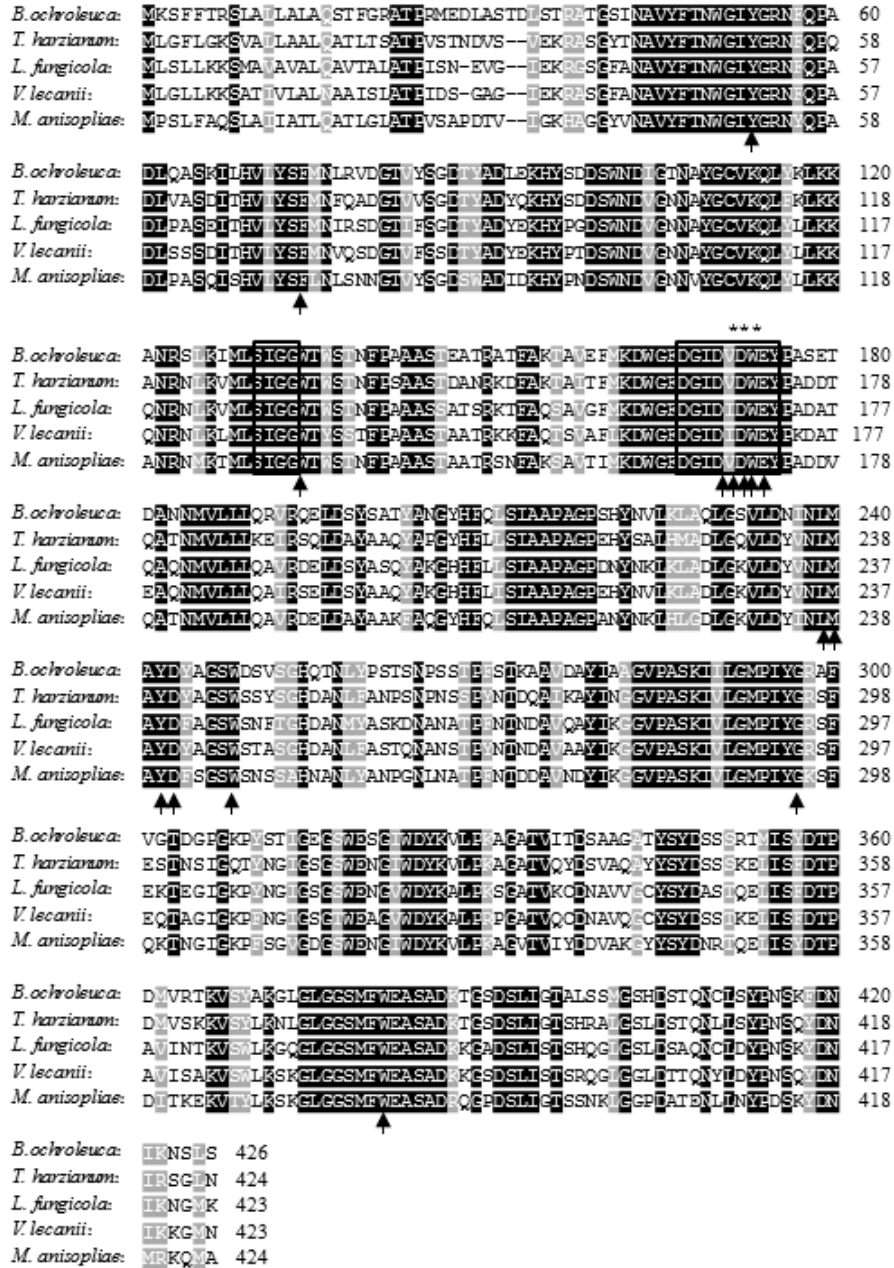
Biological control agents may effectively overcome most of the challenges related to extensive use of pesticides, and subsequently, they have gained worldwide attention in recent studies of sustainable crop products and protection. There are currently 2 main research fields in biological control, including searching for highly efficient strains and screening of compounds or proteins with activity toward desired targets. More recently, researchers have

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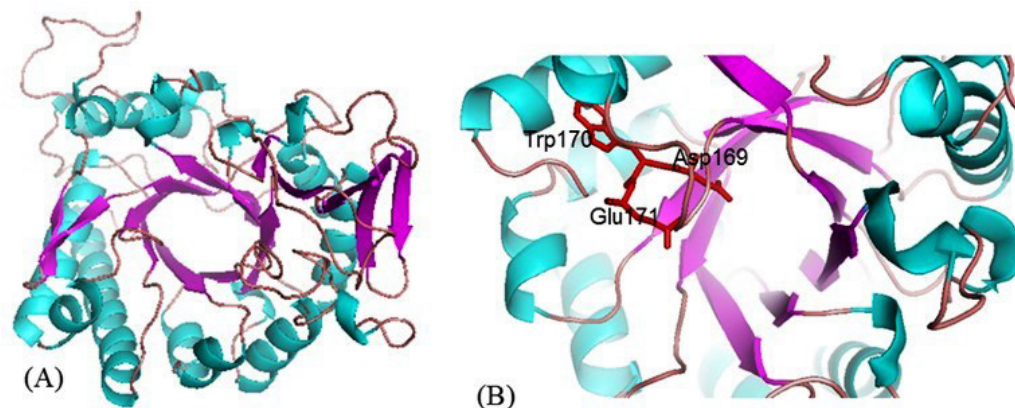
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1 M L S L L K K S M A Y A V A L Q A V T A
61 CTGGCTACGCCAATCTCCAACGAGGTTGGCATTGAGAAGCGCGGTAGTGGCTTCGCAAAT
21 L A T P I S N E V G I E K R G S G F A N
121 GCTGTTACTTCACCAACTGGTTTGTTCGACTTTTAATCTTCAGCTTCTCCACACTT
41 A V Y F T N W
181 GATTAACAAAGGAGCAGGGGCATCTACGGCCGTAACCTCCAGCCCAGACCTTCCAGCT
48 G I Y G R N F Q P A D L P A
241 TCGGAGATCACCCATGTGCTCTATTCTTTCATGAACATCCGCTCAGATGGCACCATGTAA
62 S E I T H V L Y S F M N I R S D G T I
301 GTCATCTTCATATAGGCTGAATTACGAAACTGATCAAGGCGAAAAGCTTTTCTGGCGAT
80 F S G D
361 ACCTATGCGGACTACGAGAAGCACTACCCTGGTGACTGTA AAGAAGCCCA CAATTCTTT
85 T Y A D Y E K H Y P G D
421 GGTATAAGTG AATCACTAACATGCTTTAGCCTGGAATGATGTGGGAAATAATGCTTACGG
97 S W N D V G N N A Y G
481 ATGCGTTAAGCAGCTTTATTGCTCAAGAAGCAAAACCGCAACTTGAAAGTTATGCTGTG
108 C V K Q L Y L L K K Q N R N L K V M L S
541 GATTGGTGGTGGACCTGGTCCACCAACTTCCCGCTGTGCCAGCTCTGCTACCAGCCG
128 I G G W T W S T N F P A A A S S A T S R
601 AAAGACTTTCGCTCAATCTGCCGTTGGTTTCATGAAGGATTGGGGATTTCGATGGTATCGA
138 K T F A S A V G F M K D W G F D G I D Q
661 CATCGATTGGGAGTACCCTGCGGATGCTACGCAGGCTCAGAACATGGTCCTCTGTGCTCA
158 I D W E Y P A D A T Q A Q N M V L L L Q
721 GGCCGTCGCGACGAGCTCGACTCTTATGCTCGCAGTATGCCAAAGGCCACCATTTCCT
178 A V R D E L D S Y A S Q Y A K G H H F L
781 GCTTTCTATTGCCGCCCCGCTGGACCTGACAACTACAACAAGTGAAGCTCGCCGATCT
198 L S I A A P A G P D N Y N K L K L A D L
841 TGGAAAGGTTCTTGACTACGTTAACCTGATGGCCTACGACTTGGCCGGCTCGTGGAGTAA
218 G K V L D Y V N L M A Y D F A G S W S N
901 CTTACCCGGCCACGACGCCAACATGTACGCCAGCAAGGACAATGCTAATGCAACTCCTTT
238 F T G H D A N M Y A S K D N A N A T P F
961 TAATACAAATGATGCTGTTACGGGTACATCAAAGGAGGTGTCCCTGCCAGCAAAATTTGT
258 N T N D A V Q A Y I K G G V P A S K I V
1021 CCTGGGTATGCCCATCTACGGTGGTCTTTCGAGAAGACTGAGGGAATTGGCAAGCCATA
278 L G M P I Y G R S F E K T E G I G K P Y
1081 CAACCGTATCGGCTCTGGCAGCTGGGAGAACGGTGTATGGGATTACAAGCTCTTCCCAA
298 N G I G S G S W E N G V W D Y K A L P K
1141 GTCTGGGGCCACCGTCAAGTGCACAACGCTGTTGTGGGTGCTATAGCTACGATGCGAG
318 S G A T V K C D N A V V G C Y S Y D A S
1201 CACTCAGGAGCTCATTCTTTCGACACTCCAGCTGTTATCAACACCAAGGTTAGCTGGCT
338 T Q E L I S F D T P A V I N T K V S W L
1261 TAAGGGCCAGGGACTCGGTGGAAGCATGTTTTGGGAGGCTCCGCTGATAAGAAGGGCGC
358 K G Q G L G G S M F W E A S A D K K G A
1321 CGATTCCCTCATCAGCACAAAGCCACCAAGGCTTGGTAGCCTGGACAGCGCTCAGAACTG
378 D S L I S T S H Q G L G S L D S A Q N C
1381 CCTTGACTACCCCAACTCAAAGTATGACAACATCAAGAATGGCATGAAATAG
398 L D Y P N S K Y D N I K N G M K *

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**Figure 5.** Nucleotide and deduced amino acid sequence of chitinase gene *VICHI* (GenBank accession No. KF041476). The nucleotide and/or amino acid sequence is numbered on the left. The upper lines show the nucleotide sequence with 3 introns (single-line underlined). The putative signal peptide is double-underlined. The stop codon is indicated with an asterisk (\*).



**Figure 6.** Multiple sequence alignment of VICHI homologs. The proteins shown are chitinase from *Verticillium lecanii* (KF041477.1), *Lecanicillium fungicola* (AAP45631.1), *Metarhizium anisopliae* (AAY32603.1), and *Trichoderma harzianum* (ABD42923.1), as well as chitinase from *Bionectria ochroleuca* (EU000575.1). A black background indicates conserved residues; a brown background indicates residues identified to be more than 80% conserved. The conserved substrate-binding and catalytic domains (SXGG and DXXDXDXE) are marked with a black frame, and the key residues (Asp, Trp, Glu) important for chitin binding are marked with asterisks (\*). The key residues in the active site are indicated with short black arrows.



**Figure 7.** Overall hypothetical 3-dimensional structures of VICHI. **A.** The structure was generated based on homology modeling using chitinase from *Bionectria ochroleuca* chitinase GenBank (GenBank No. EU000575.1; PDB code 3G6M) as a template. Helices, strands, and coil are colored cyan, purple, and violet, respectively. **B.** Putative active sites in the catalytic domain of VICHI.

exploited molecular manipulation to generate genetically modified microorganisms combined with different bio-control traits. Wu et al. (2013) generated a transgenic *Streptomyces lydicus* by transforming an exogenous resistance gene, a *chit42* gene from *T. harzianum*, which not only enhanced antibiotic production but also highly improved the activity of pathogen cell wall degradation, particularly against *B. cinerea*. Li et al. (2012) constructed a transgenic *Trichoderma koningi* containing a *Metarhizium anisopliae chit42* gene, which significantly increased its activity towards the Asian corn borer. Xia et al. (2009) expressed a subtilisin-like protease, CDEP, from *Beauveria bassiana* that was co-fused with the Cry1Ac protein in *Bacillus thuringiensis* and showed that it could improve insecticidal activity toward *Helicoverpa armigera* larvae. *V. lecanii* is a candidate organism used as a pest biological control agent against insects and phytopathogens (Benhamou and Brodeur, 2001; Yu et al., 2012). Isolating and characterizing candidate proteins and genes in *V. lecanii*, then testing their activity *in vivo* and *in vitro* is important, as the transformation system of *V. lecanii* has been established (Hasan et al., 2011).

In this study, we isolated an acidic extracellular chitinase from the entomopathogenic fungus *V. lecanii* using different columns for chromatography. The enzyme exhibited the highest activity at 40°C and pH 4.6, and was significantly activated by  $Mg^{2+}$ , while Lu et al. (2005) isolated a chitinase, whose optimum enzymatic activity was observed at 22°C and pH 7.5. There are at least 2 chitinases in *V. lecanii*, an acidic and a basic chitinase. In plants, there are 2 classes of chitinases, basic or acidic chitinases, targeting different parts of the cell, and are differentially regulated. Acidic chitinases are secreted into the extracellular region, whereas basic chitinases generally accumulate intracellularly and are induced by stress-related environments (Lu et al., 2005). Our results indicate that the acidic chitinases are highly expressed during *in vitro* culture of *V. lecanii*. *In vitro* antifungal assay experiments of VICHI revealed spore germination inhibitory activity against several devastating fungal phytopathogens that extensively attack plants, particularly *F. moniliforme*, *P. italicum*, *S. turcica*, and *M. oryza*. Future experiments should be conducted to determine the efficacy of VICHI against other fungi

as well. Next, we cloned the correlative gene *VICHI* from this organism using degenerate primers. Sequence alignment showed that the chitinase examined in this study belonged to the GH18 superfamily and is conserved in parasitic fungi according to BLASTp results. In general, GH18 family chitinases bind their substrates in an extended recognition site, and *VICHI* in this study contained the conserved substrate-binding and catalytic domains (SXGG and DXXDXDXE), as well as a conserved catalytic center containing 3 vital amino acid residues, Asp169, Trp170, and Glu171 (Yang et al., 2010). Several structures of fungal chitinase have been determined, such as chitinase from *Aspergillus fumigatus* (AfCHI) (PDB code: 1W9P) (Rao et al., 2005), *Coccidioides immitis* (cmChi) (PDB code: 1D2K) (Hollis et al., 2000), *Yersinia entomophaga* (YeCHI) (PDB code: 4A5Q) (Busby et al., 2012), and *B. ochroleuca* (also known as *Clonostachys rosea*) (BoChi1) (PDB code: 3G6M), and these structure were used to predict chitin binding, while biochemical assays were used to characterize chitinase in fungi. In this study, we utilized *B. ochroleuca* (BoChi1) (PDB code: 3G6M) as a template to generate a 3-D protein model of *VICHI* using the SWISS-MODEL server. Protein modeling and structural comparison of different chitinases validated the conserved active sites (Figures 6 and 7).

In summary, we isolated an acidic chitinase *VICHI* in *V. lecanii* during *in vitro* culture when chitosan was added as a substrate and tested the optimum pH and temperature for its activity. In addition, *VICHI* exhibited inhibitory activity toward several agronomically important phytopathogens, specifically *F. moniliforme*, and few previous studies showed that proteins in *V. lecanii* exhibited activity against phytopathogens (Yu et al., 2012). The cDNA sequence was obtained and analyzed, and the putative amino acids shared extensive similarities with other parasitic fungal chitinases, indicating that chitinase is well-conserved in parasitic fungi. However, the relationship between different amino acid residues in chitinase from different microorganisms and the parasitic specificity should be investigated for the generation of improved biocontrol strains. Our study provides insight for modifying recombinant biocontrol strains used in an acidic environment, as well as toward some plant pathogens.

## ACKNOWLEDGMENTS

Research supported by the Twelfth Five Year Plan Project of Science and Technology Support, China (#2014BAD14B02 to P.H., #2013BAC09B01 to Z.H., #2012BAD19B04 to P.H.), by the Research and Development of Industrial Technology Special at Jilin Provincial Development and Reform Commission (#2013C001 to P.H), and by the Ministry of Agriculture Key Project of GM Cultivation of New Varieties (#2013ZX08004004 to L.Q.), China.

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