Antifungal activity of heterologous expressed chitinase 42 (Chit42) from *Trichoderma atroviride* PTCC5220

Mohammad Javad Harighi[1,2], Mostafa Motallebi[1]*, Mohammad Reza Zamani[1]

[1]Department of Plant Biotechnology, National Institute for Genetic Engineering and Biotechnology (NIGEB), P.O. Box 14155-6343, Tehran, I. R. Iran [2]Department of Biology, Faculty of Science, Razi University, Kermanshah, I. R. Iran

Abstract

The cDNA from the mycoparasitic fungus Trichoderma atroviride PTCC5220 encoding a 42 kDa chitinase (Chit42) was isolated. The nucleotide sequence of the cDNA fragment as having a 1263 bp open reading frame that encodes a 421 amino acid polypeptide, and a high homology was found with other reported Chit42 belonging to the Trichoderma sp. The 22 amino acid N-terminal sequence is a putative signal peptide for the possible secretion of the protein. The protein has been expressed and secreted as a mature form in Escherichia coli BL21(DE3) using the pelB leader sequence. The E. coli strain expressed Chit42 in an active form and secreted the protein into the medium. This recombinant chitinase has been shown to have inhibitory activity on mycelial growth and also, lytic activity on the cell wall of Rhizoctonia solani (AG2-2), causal agent of root rot in sugar beet in vitro. Expressed chitinase was optimally active at pH 5 and at 40°C. It is thermally stable at 60°C for more than 120 min at pH 5.

Keywords: *Trichoderma atroviride; Chitinase 42; Antifungal; activity; Heterologous expression.*

INTRODUCTION

Since chitin is the major component of most fungal cell walls, a principle role has been attributed to chitinases in the control of a wide range of phytopathogens (Collinge *et al.*, 1993; Gokul *et al.*, 2000). Chitin is an insoluble linear β-1,4 linked polymer of N-acetyl glucosamine. It is one of the most abundant polysaccharides in nature (Gokul *et al.*, 2000).

*Correspondence to: **Mostafa Motalebi,** Ph.D.
Tel: + 98 2144580363, Fax: +98 2144580399
E-mail: motalebi@nrcgeb.ac.ir, motallebi38@yahoo.com

Chitinases are involved in the process of producing mono and oligosaccharides from chitin. Furthermore, chitinases are potential antifungal agents through their chitin degradation activity (Kunz *et al.,* 1992; Mathivanan *et al.,* 1998; Ordenlich *et al.,* 1988; Roberts and Selitrennikoff 1988; Park *et al.,* 2000). Chitinases are found in a wide range of organisms including bacteria, fungi, higher plants, insects, crustaceans and some vertebrates (Shih *et al.,* 2001). Chitinases have a variety of roles in different organisms. For example, bacteria produce these enzymes to assimilate chitin as a carbon and/or nitrogen source. In fungi, crustaceans, and insects, chitinases are involved in modifying the chitin components of the cell wall or the exoskeletons of these organisms. In plants, chitinases are generally expressed constitutively at low levels (Shih *et al.,* 2001).

The interest in the chitinase gene is not only based on their potential application as antifungal agents but also because chitinase genes of mycoparasitic fungi are excellent candidates for reinforcing plant defenses (Gokul *et al.,* 2000; Adams, 2004; Carsolio *et al.,* 1994, 1999).

In this study, we describe the cloning and partial characterization of an endochitinase gene of *T. atroviride* as a high producer of chitinolytic enzymes isolated from Iran, whose expression in *E. coli* is effective in inhibition of fungal mycelial growth and lysis of the phytopathogenic fungal cell wall.

MATERIALS AND METHODS

Microorganisms and growth conditions: *T. atroviride* PTCC5220 was obtained from the Persian Type

Culture Collection. It was identified at the NIGEB, Tehran, I.R. of Iran from amongst 30 *Trichoderma* isolates as a high producer of chitinolytic enzymes (Harighi *et al.,* 2006) and used for RNA isolation. The stock culture was stored on agar (1.5% w/v) slants of MY medium (2% malt extract, 0.2% yeast extract, 1% maltose, w/v).

Rhizoctonia solani (AG 2-2), causal agent of root rot in sugar beet, was kindly provided by Prof. Banihashemi, Mycology lab., Dept. of Plant Pathology, College of Agriculture, Shiraz University, Shiraz, I.R. of Iran. The fungus was propagated on potato dextrose agar (PDA) and subcultured as needed.

Escherichia coli DH5α (Cinagene, Iran) was used as a host and pUC18 (Pharmacia) was used as a vector for routine cloning. *E. coli* BL21 DE3 (Cinagene, Iran) and the pET26b(+) vector (Novagen) were used for heterologous expression of Chit42. *E. coli* was cultured in LB medium (1% trypton, 0.5% yeast extract, and 1% NaCl, w/v)

RNA Isolation and cDNA synthesis: For RNA isolation, *T. atroviride* was grown at 28°C, in 250 ml shaking flasks containing 150 ml Czapek-Dox medium supplemented with 10% glucose, with shaking at and 200 rpm for 96 h. Mycelia were collected after 96 h by the Whatman (No.1) filter paper and washed several times with $MgCl_2$ (2%) and then inoculated into Czapek-Dox medium supplemented with 1.5% colloidal chitin (De la Cruz *et al.,* 1999). Cells were harvested after 42 h of growth and frozen in liquid nitrogen. Frozen mycelium was ground and suspended in 5 volumes of 4 M guanidine isothiocyanate, 0.5% (w/v) Na-lauryl sarcosinate, 25 mM sodium citrate (pH 7.0), and 0.1 M β-mercaptoethanol (Sambrook and Russell, 2001). The messenger RNA was purified by the mRNA isolation kit 1741985 (Roche).

cDNA synthesis using the poly(A^+) RNA was carried out by Revert Aid™ First Strand cDNA Synthesis Kit (Fermantas). The reaction volume was 50 µl containing: 5 µg of poly(A^+) RNA, 1 mg of oligo$(dT)_{18}$, 20 units of RNase Block Ribonuclease Inhibitor, 1 X buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$), 500 µM of each dNTP, and 200 units of reverse transcriptase. The RNA was denatured at 70°C, cooled slowly at room temperature to allow the annealing of primers before it was added to the reaction mixture. The reaction mixture was incubated at 42°C for 1 h and then incubated at 70°C for an additional 5 min. The cDNA from the reaction was kept at -70°C and used for a PCR reaction with specific primers (CUM1, 5´-GCA GGT CGA CGG ATG AAA ATT CCA TCC A-3´ and CDM2, 5´-GGG AAT TCG TTG AGA CCG CTT CGG ATG-3´) containing *Sal*I and *Eco*RI sites at their 5´ ends, respectively. DNA amplification was carried out in a PCR reaction containing 2.5 units of Fermentas *Pfu* DNA polymerase, 1X buffer, 200 µM of each dNTPs, 2 µM $MgSO_4$ and 0.5 µM of each primers. Reaction conditions for PCR amplification were 94°C for 1min, 56°C for 30 sec, and 72°C for 1.5 min, for 30 cycles followed by a final extension of 15 min. The cDNA was confirmed by nested PCR using specific internal primers (Bf, 5´-ACC TCA TGG CCT ACG ACT AT-3´ and Br, 5´- ATT ATG CAG GGA GCG TTA TT-3´).

The resulting cDNA fragment was recovered and purified from a 1.2% (w/v) agarose gel using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH), then digested with *Sal*I/*Eco*RI and cloned into *Sal*I/*Eco*RI sites of pUC18 cloning vector (Sambrook and Russel, 2001).

General DNA procedures: Plasmid DNA preparation and electrophoresis of DNA fragments were performed by routine procedures (Sambrook and Russell, 2001). Enzymatic treatments of DNA molecules were carried out as recommended by the manufacturer (Fermentas, Germany). Bacteria were transformed by the $CaCl_2$ method (Sambrook and Russell, 2001).

Construction of expression vector: cDNA encoding the Chit42, without the native signal sequence, was amplified using *Pfu* polymerase (Fermentas) with specific primers (Chs5, 5´-GGA AGA CAA CAT GTC TCC TGT AAC TGC AAA CG-3´ and CDP4, 5´-GGG AAT TCC TAG TTG AGA CCG CTT CGG A-3´) which contain *Bpi*I and *Eco*RI sites respectively at their 5´ end, and cloned into pET26b(+) at the *Nco*I/*Eco*RI restriction sites.

Sequencing and computer analysis: Cloned DNA fragments (70-220 ng/µl) were sequenced by a commercial service (Seqlab, Gottingen, Germany) using the primer walking sequencing method. Computer analysis of the sequences was carried out and the deduced amino acid sequence from *chit42* cDNA gene was obtained by the BLASTX Network Service (NCBI) and alignment of this amino acid sequence was carried out by the BLOSUM62 Service using the clustal method.

Expression in *E. coli:* The cDNA sequence encoding the entire Chit42 without the putative signal sequence was cloned into the expression plasmid pET26b(+) and transformed into *E.coli* BL21(DE3). The construct was verified by sequence analysis of the inserted frag-

ment. The tansformed strain was grown at 37°C in LB medium supplemented with 50 µg/ml kanamycinm, to an OD_{550} of 0.5. Expression of protein was induced by addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and growth continued at 28°C for 24 h. The medium was centrifuged (4000 ×*g*, 10 min, 4°C), and 10 ml of the resulting supernatant was mixed with 10 ml of cold acetone and stored at -20°C for 24 h. The proteins were harvested by centrifugation (12000 ×*g*, 10 min, 4°C) and the pellet was resuspended in 50 µl of PBS (Phosphate Buffered Saline). Equal volume of protein loading buffer was mixed, boiled for 3 min and used for SDS-PAGE.

Optimum temperature and stability: The optimum temperature was determined by performing a standard assay within the temperature range of 10 to 100°C, at 10ºC intervals. The inactivation temperature was also determined by incubation of enzyme for 300 min at the temperature range of 30 to 80°C in 15 mM sodium acetate buffer, pH 5 and then measuring the remaining activity at 37°C by addition of colloidal chitin as the assay substrate. The inactivation temperature was defined as the temperature at which the activity was reduced by 50%, under the same conditions.

pH optimization: The optimum pH was determined after incubation with colloidal chitin in the buffer at various pH (1 to 10). The buffers used were: 10 mM HCl (pH 1), 15 mM phosphate buffer (pH 2), 15 mM citrate buffer (pH 3), 15 mM acetate buffer (pH 4-5), 15 mM MES (Morpholino Ethan Sulfunic acid) (pH 6), 15 mM phosphate buffer (pH 7), 15 mm Tris-HCl buffer(pH 8), and 15 mM CHES (Cyclo Hexylamino Ethan Sulfunic acid) (pH 9,10).

Enzyme assay: Chitinase activity was assayed with 200 µl each of colloidal chitin (5 mg/ml), and recombinant enzyme. The mixture was incubated for 60 min at 40ºC, and the reaction was stopped by adding 1 ml of NaCl (1%) and centrifuged at 6000×*g* for 5 min. The supernatant was boiled with 100 µl of potassium tetra borate buffer for 3 min. 3 ml of DMAB reagent [10 g of Di-methyl amino benzaldehyde in 100 ml of glycial acetic acid (12.5% v/v) and 10N chloridric acid (87.5% v/v)] was added to the reaction and incubated at 40ºC for 20 min, and the amount of N-acetylglucosamine (GLcNAc) produced in the supernatant was determined by the method described by Zeilinger *et al.* (1999) using GLcNAc as a standard. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 µmol GLcNAc in 60 min at 40ºC. Cytoplasmic and periplasmic fractions were obtained as described by Sashihara *et al.* (1984).

Antifungal activity: The antifungal activity of recombinant Chit42 was examined by using a modification of the bioassay by Broglie *et al.* (1991). Samples were added (40 µl per well) to 2 days old *R. solani* (AG 2-2) cultured on PDA and incubated for 72 h at 34ºC.

Light microscopy: To study the effect of expressed Chit42 on the cell wall of mycelium in *R. solani* (AG2-2), 20 µl of the culture filtrate from media inoculated with *E. coli* BL21 (DE3) harboring the cloned cDNA was added to 2 days grown *R. solani* on a slide covered with a thin layer of PDA. After 60 min of incubation at 40°C, the slide was examined for degradation of *R. solani* cell wall under a light microscope.

RESULTS

cDNA synthesis: In order to amplify chitinase 42 cDNA (*chit42)* from *T. atroviride*, a high producer of chitinase enzymes, the specific oligonucleotide primers (CUM1 and CDP4) were designed based on *chit42* genomic DNA sequence. The mRNA was purified from mycelia grown (after 42 h) in medium induced by colloidal chitin and the first-strand cDNA was obtained by reverse transcriptase enzyme. The cDNA copy of *chit42* was amplified from first-strand cDNA, using specific primers. A specific band of 1377 bp was amplified from *T. atroviride* mRNA and confirmed by restriction pattern using *Hind*III/ *Hae*II and *Sac*I enzymes (Fig. 1). This PCR product was ligated to the pUC18 vector and confirmed with nested PCR using Bf and Br internal primers (Fig. 2) and designated pMJH2. The cloned cDNA was sequenced and submitted to the NCBI data base under the accession number DQ022674. The cDNA contained an open reading frame encoding a protein of 421 amino acids (Fig. 3).

Multiple alignment of the deduced amino acid sequence with fungal Chit42 proteins was performed with the CLUSTAL W program. When *T. atroviride* Chit42 was compared with the previously reported Chit42 sequences of *T. aureoviride* (AAW31956), *T. viride* (AAG09447), *T. harzianum* (AAZ95175), *T. longibrachiatum* (ABD42921), and *T. koningii* (AAF19612), it was shown that most of the residues are conserved (Fig. 3). Pair wise alignment showed very high homology between the *T. atroviride* Chit42 and other fungal Chit42 proteins, *T. aureoviride* (100%), *T. viride* (98%), *T. harzianum* (98%), *T. longibrachiatum* (97%), and *T. koningii* (95%) (Fig. 4).

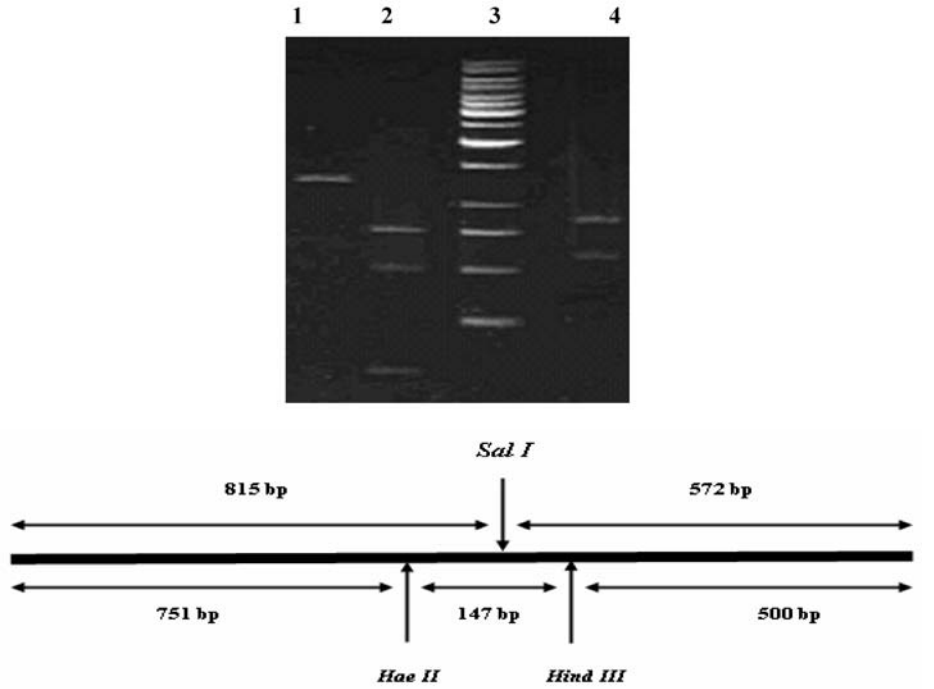


Figure 1. PCR amplification of the *chit42* gene (line 1, approximately 1.4 Kb), Confirmation of the PCR product by restriction pattern using *Hind*III/*Hae*I (line 2, approximately 0.75, 0.5, and 0.15 Kb), and *Sac*I (line 4, approximately 0.8, and 0.6 Kb), 1 KB ladder (M).

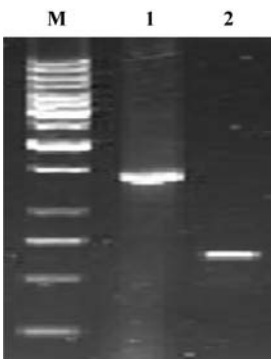


Figure 2. PCR confirmation of pMJH2 using specific primers CUM1/CDM2 (line 1, approximately 1.4 Kb) and Bf/Br (line 2, approximately 0.66 Kb), 1 KB ladder (M).

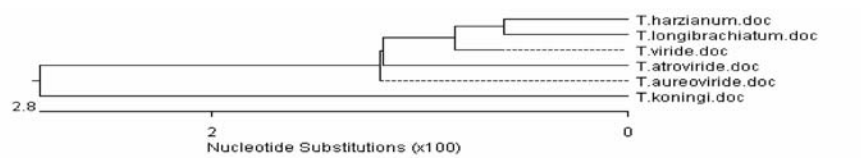
Expression in *E. coli*: To verify that the cloned *chit42* cDNA encodes an active enzyme, the *chit42* cDNA coding region was expressed in *E. coli* BL21(DE3). The sequence containing the coding region without the putative signal peptide of *T. atriviride* was amplified by PCR using two specific primers (Chs5 and CDP4). the *Bpi*I site was added at the 5´ end of the Chs5 forward primer to allow for cloning of the cDNA fragment in frame with *pel*B leader sequence, when the vector is digested with *Nco*I enzyme. This cDNA was cloned in the pET26b(+) expression vector and designated as pMJH3. Cultures of *E. coli* BL21(DE3) carrying the pMJH3 were grown and induced with IPTG. *E. coli* BL21(DE3) harboring pET26b(+) (empty vector) was used as a negative control. Chitinase activity was detected only in the protein extracts of induced bacteria carrying pMJH3 (Table 1). No activity was detected in the control cultures. These data confirm that the cloned *chit42* encodes active chitinase enzyme.

Characterization of the *chit42* gene product: To characterize the chitinase 42 expressed by recombinant *E. coli* BL21 (DE3) transformants, we used a direct activity staining technique that allows rapid, specific detection of the chitinase enzyme using a reaction mixture containing colloidal chitin (5 mg/ml) as a substrate. The predicted *chit42* gene product (Chit42 without the putative signal peptide) consists of 399 amino acids with an estimated molecular mass of 43881 daltons, which was identified by SDS-PAGE (Fig. 5).

To examine whether the expressed Chit42 is in the extracellular, cytoplasmic and/or periplasmic fractions, cell extracts prepared from *E. coli* BL21(DE3) containing plasmid pMJH3 were tested. The ratio of the enzyme activity of each fraction to total chitinase activity was analyzed in an exponential phase culture of *E. coli* BL21(DE3) harboring the plasmid pMJH3 (Table 1). More than 69% of total activity was found in the extracellular fraction. The Chit42 produced by

```
                    *       20          *       40          *       60          *       80
T.atroviri : ..................................................................................... :  81
T.aureovir : ..................................................................................... :  81
T.harzianu : ..............................T...................................................... :  81
T.koningi. : ........................AA........................................................... :  81
T.longibra : ..............................T...................................................... :  81
T.viride.d : ..................................................................................... :  81
             MLGFLGKSVALLAALQATLTSA3PVstNDVS6EKRASGY NAVYFTNWGIYGRNFQPQ1LVASDITHVIYSFMNFQADGTV

                    *       100         *       120         *       140         *       160
T.atroviri : ..................................................................................... : 162
T.aureovir : ..................................................................................... : 162
T.harzianu : ..................................................................................... : 162
T.koningi. : ..................................................................................... : 162
T.longibra : ..................................................................................... : 162
T.viride.d : ..................................................................................... : 162
             VSGDAYADYQKHYSDDSWNDVGNNAYGCVKQLFKLKKANRNLKVMLSIGGWTWSTNFPSAASTDANRK1FAKTAITFMKDW

                    *       180         *       200         *       220         *       240
T.atroviri : ..................................................................................... : 243
T.aureovir : ..................................................................................... : 243
T.harzianu : ..................................................................................... : 243
T.koningi. : ............S.....................................................F...S.............. : 243
T.longibra : ..................................................................E.........P...... : 243
T.viride.d : ..................................................................................... : 243
             GFDGID6DWEYPaDDTQATNM6LLL4EIRS2LDAYAAQYAPGYHFLLSIAAPAGPEHYSaLHMadLGQVLDYVNlMAYDYA

                    *       260         *       280         *       300         *       320
T.atroviri : ..................................................................S.............. : 324
T.aureovir : ..................................................................S.............. : 324
T.harzianu : ..................F.................................................................. : 324
T.koningi. : ....N.........................D..K.....................G......S.............. : 324
T.longibra : ..................................................................S.............. : 324
T.viride.d : ..................................................................................... : 324
             GSWSsYSGHDANLFANPSNPNsSPYNTDQAIKaYInGGVPASKIVLGMPIYGRSFESTngIGQTY GIGSGSWENGIWDYK

                    *       340         *       360         *       380         *       400
T.atroviri : ..................................................................................... : 405
T.aureovir : ..................................................................................... : 405
T.harzianu : ..................................................................................... : 405
T.koningi. : ..................................NT.....................................N.... : 405
T.longibra : ..................................................................................... : 405
T.viride.d : ..................................................................................... : 405
             VLPKAGATVQYDSVAQAYYSYDSSSKELISFDTPDM6skKVSYLKNLGLGGS6FWEASADKTGSDSLIGTSHRALGsLDST

                    *       420
T.atroviri : ..................--- : 421
T.aureovir : ..................--- : 421
T.harzianu : .............IRS... : 424
T.koningi. : ..................--- : 421
T.longibra : .............IRS... : 424
T.viride.d : .............IRS... : 424
             QNLLSYPNSQYDN
```

Figure 3. Sequence alignment with CLUSTAL W of the deduced amino acid sequences of *T.* atroviride PTCC5220 Chit42 with those of *T. aureoviride*, *T. harzianum*, *T. koningii, T. longibrachiatum*, and *T. viride*. Single-letter amino acid is used.



A

Percent Identity

	1	2	3	4	5	6	
1		100.0	98.1	95.7	97.4	98.3	1
2	0.0		98.1	95.7	97.4	98.3	2
3	1.9	1.9		93.8	98.8	99.3	3
4	4.4	4.4	6.5		93.1	94.1	4
5	2.7	2.7	1.2	7.2		98.6	5
6	1.7	1.7	0.7	6.2	1.4		6
	1	2	3	4	5	6	

Divergence

1 T.atroviride.doc
2 T.aureoviride.doc
3 T.harzianum.doc
4 T.koningi.doc
5 T.longibrachiatum.doc
6 T.viride.doc

B

Figure 4. Phylogenic tree and sequence distances of the Chit42 amino acid sequences comparing *T. atroviride* Chit42 with that of *T. aureoviride*, *T. harzianum*, *T. koningii, T. longibrachiatum*, and *T. viride* A) Phylogenic relationships B) Percentage of sequence similarty and divergence, generated from multiple alignments.

Table 1. Distribution of Chit42 activity in *E. coli* BL21 (DE3) harboring pMJH3 or pET26b(+).

	Chit42 activity (%)		
Plasmid	**Extracellular**	**Periplasmic**	**Cytoplasmic**
pMJH3	69	18.5	12.5
pET26b(+)	0.0	0.0	0.0

E. coli BL21 containing pMJH3 was also found as an intracellular enzyme. About 12.5 % of total chitinase activity was localized in the intracellular fraction and also about 18.5 % of the total enzyme remained in the periplasm, and residual activity of 69 % excreted into the medium (Table 1). No such activity was detected in the corresponding extracts prepared from cells containing pET26b(+) as a negative control.

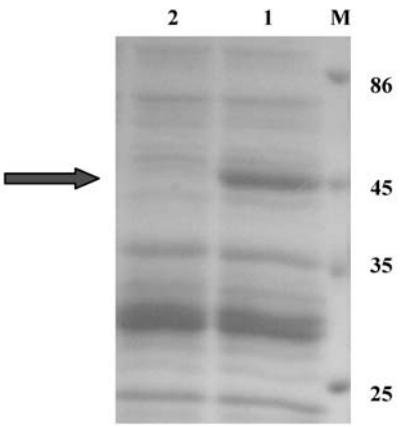


Figure 5. SDS-PAGE of Chit42 expressed in *E. coli* BL21(DE3) harboring the *chit42* gene (line 1), and *E. coli* BL21(DE3) harboring pET26b(+) as a control.

The maximal enzyme activity of the extracellular chitinase of Chit42, a product of the cloned *chit42* cDNA in *E. coli* BL21(DE3), was revealed at pH 5 (Fig. 6) and at 40ºC (Fig. 7). The stability of the expressed protein was examined by measuring the decrease in activity after incubation at a temperature range of 30 to 80ºC with 10ºC intervals. No significant decrease in enzyme activity was observed at 30 to 60ºC after incubation for 120 min and the activity of expressed Chit42 decreased by 50% after incubation for 180 min at 70°C and 120 min at 80ºC (Fig. 8).

Antifungal activity: The growth of *R. solani* was inhibited by addition of the culture filterate (extracellular enzyme) of *E. coli* BL21(DE3) (pMJH3) as compared to the culture filtrate from medium inoculated or not inoculated with *E. coli* BL21(DE3) harboring the pET26b(+)(empty vector) (Fig. 9). Also, the effect of expressed recombinant Chit42 from *E. coli* BL21(DE3) (pMJH3) on the cell wall of *R. solani* was tested by light microscopy. The results showed that the culture filtrate containing expressed Chit42 are able to lyse the *R. solani* mycelium cell wall (Fig. 10). No lysis activity was observed when the culture filtrate of *E. coli* BL21 (DE3) harboring pET26b(+) was used as a control.

DISCUSSION

Cultivated plants often suffer from various diseases caused by microbes including phytopathogenic fungi, resulting in reduced yield and quality of plant products. The direct mycoparasitic activity of *Trichoderma* species has been reported to be the major mechanism proposed to explain their antagonistic activity against phytopathogenic fungi, their lytic activity being mainly due to the chitinase and glucanase hydrolases (De la Cruz *et al.,* 1992). The substrate of chitinase is chitin, which is a common component of fungal cell walls (Broglie *et al.,* 1991). These enzymes are strong inhibitors of many important plant pathogens and the chitinases are able to lyse the “hard” chitin cell wall of

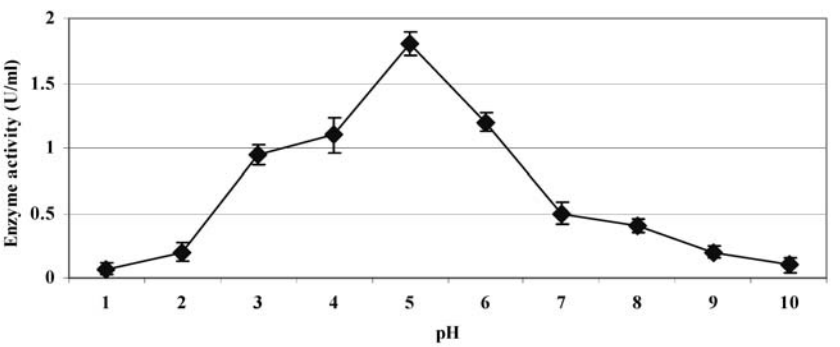


Figure 6. Effect of pH on enzymatic activity of the expressed Chit42 in *E. coli*.

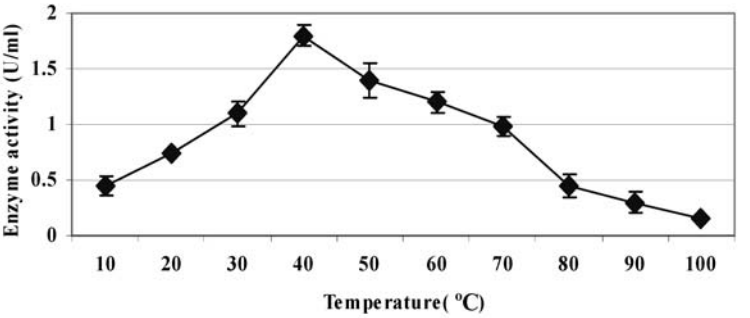


Figure 7. Effect of temperature on enzymatic activity of the expressed Chit42 in *E. coli*.

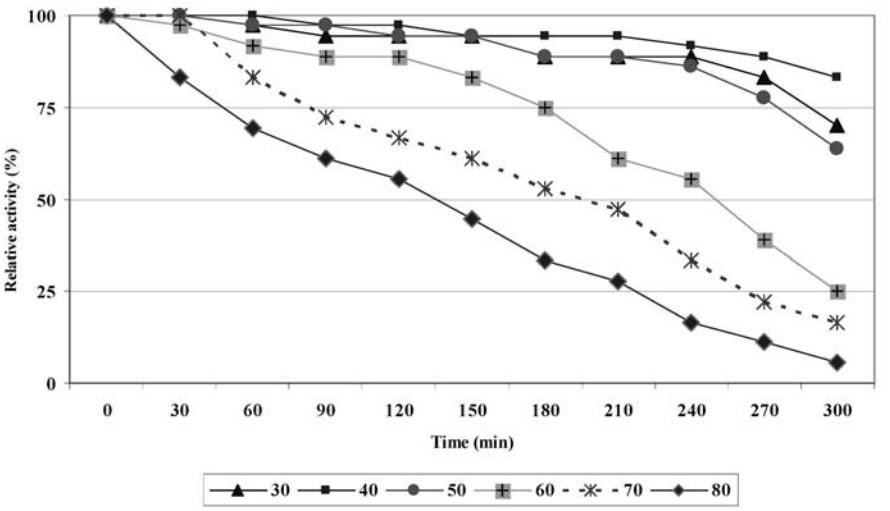


Figure 8. Effect of different temperatures on stability of the expressed Chit42. The remaining enzymatic activities after incubation at different temperatures were measured.

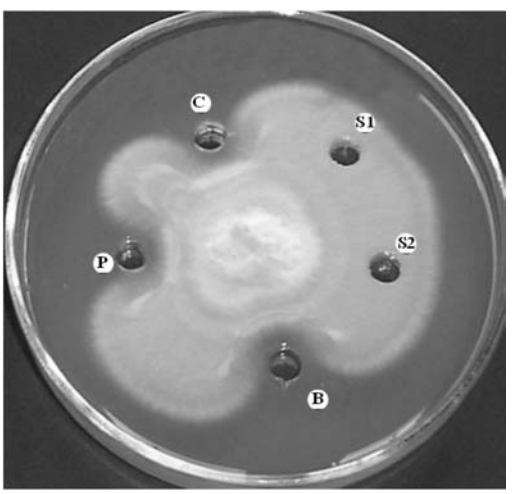


Figure 9. Inhibition of fungal growth by chitinase. B) expressed Chit42, C) crude enzyme obtained from culture medium of *T. atroviride,* P) purified Chit42 from culture medium of *T. atroviride,* S1) culture medium without inoculation (negative control), and S2) culture medium of *E. coli* BL21(DE3) harboring pET26b(+)(negative control).

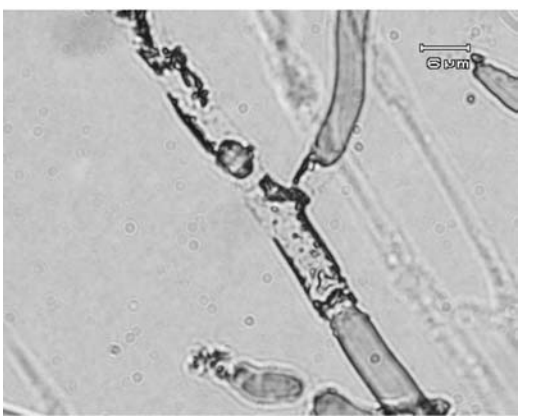


Figure 10. A light micrograph showing degradation of *R. solani* cell walls using expressed Chit42 in *E. coli*.

the mature hyphae, conidia, chlamydospores, and sclerotia (Lorito *et al.,* 1998). *Trichoderma* chitinases are substantially more antifungal than any other chitinases purified from any other sources when assayed under the same conditions. They are more active than corresponding plant enzymes, effective on a much wider range of pathogens, and are nontoxic to plants at high concentrations (Wang *et al.,* 2003; Lorito *et al.,*1998). The genome of mycoparasitic fungi such as *Trichoderma* sp. has evolved specifically to be capable of using other fungi but not plants as carbon sources and as such represent a potential source of powerful antifungal genes (Zhu *et al.,* 1994). In terms of antifungal activity, chitinase genes from *Trichoderma* sp. are clearly an improvement over corresponding plant chitinase genes. The *Trichoderma* sp. chitinase genes are capable of producing chitinolytic enzymes which reach the antifungal activity level of some chemical fungicides and extensive testing *in vitro* has shown that there are virtually no chitinous pathogens resistant to *Trichoderma* sp. chitinases and hence they have become excellent candidates for reinforcing plant defense hypersensitive reactions (Lorito *et al.*, 1993, 1998; De la Cruz *et al.,* 1992). Proof of this concept has been clearly demonstrated within the agricultural community by the use of the *Trichoderma* sp. 42 kD endochitinanse gene (Baek and Howell, 1999; Steyaert *et al.,* 2004).

In this study, a native Iranian isolate of *T. atroviride* capable of producing high levels of chitinolytic enzymes, was used for amplification of the cDNA copy of *chit42* gene. DNA sequence information confirmed that the cloned PCR fragment shows high homology to the previously reported *chit42* sequences of *T. aureoviride* (Yang and Song, 2004), *T. viride* (Zhang *et al.*, 1999), *T. harzianum* (Bhunchoth *et al.*, 2005), *T. longibrachiatum* (Bhunchoth *et al.*, 2006), and *T. koningii* (Cavingnac, 2000).

Comparison of the *chit42* cDNA sequence with the previously reported *chit42* genomic DNA from this microorganism (*T. atroviride*) demonstrated it that contains three small introns which have also been reported in the above cited *chit42* fungal genes. The consensus sequences, GT on the 5´ end and AG on the 3´ end for each intron of the *chit42* gene are also observed. The coding region of *chit42* codes for a polypeptide of 421 amino acids, the first 22 residues of which form a putative signal peptide by similarity (Draborg *et al.,* 1996). The calculated size of the predicted product is 45910 daltons. Thus, the mature secreted protein would have a calculated molecular mass of 43881 daltons corresponding to the only chitinase reported from *T. harzianum* with the ability to degrade *Botrytis cinerea* cell wall by itself (De la Cruz *et al.,* 1992). Computer analysis of the deduced amino acid sequence of the *T. atroviride* Chit42 protein indicated that this enzyme shows high homology to other *Trichoderma* sp. Chit42 (more than 95% identity). It is also related to bacterial chitainase and is homologous to a chitinase produced by the fungus *Aphanocladium album* (73% identity), another effective biocontrol agent (Blaiseau and Lafay, 1992).

To dissect and characterize the hydrolytic system of *T. atroviride*, and to evaluate the major role attributed to chitinolytic enzymes in the lytic activity of *T. atroviride* on phytopathogenic fungus (*R. solani* AG2-2) (Harighi *et al.,* 2006), the *chit42* cDNA of this fungus was cloned and expressed in *E. coli*. The PelB leader signal peptide in the pET system was used for secretion of Chit42. Comparison of secreted, periplasmic and cytoplasmic Chit42 showed high level of secretion (69%) of expressed Chit42. The expressed Chit42 effectively hydrolyses colloidal chitin at pH 5, and the half-life of the expressed protein at 60ºC (4 h) suggests that this cloned enzyme is a heat-stable chitinases. Also, the lytic activity of recombinant Chit42 was evaluated *in vitro* on the phytopathogenic fungus *R. solani*. Effective growth inhibition was observed when expressed Chit42 was added to *R. solani* on solid medium. The observed growth inhibitory effect may arise from the enzyme catalyzing hydrolysis of newly formed chitin and resultant disruption of the growing fungal mycelium (Broglie *et al.,* 1991).

Microscopic observation demonstrated the ability of expressed Chit42 to degrade the cell wall of *R. solani*. Similar to our findings, a number of other studies have implicated the chitinase enzyme to be responsible for the degradation of the cell wall of phytopathogenic fungi (Elad *et al.*, 1982; Ridout and Coley-Smith, 1988; Tweddell *et al.*, 1994).

In support of the role of chitinases in cell wall degradation, virtually no chitinous phytopathogenic fungi were found to be resistant to *Trichoderma* chitinases *in vitro* (Lorito *et al.*, 1993). A single *Trichoderma* chitinase expressed in tobacco and potato conferred tolerance or complete resistance to some phytopathogenic fungi (Lorito *et al.,* 1998) . The inhibitory effect of expressed Chit42 on the growth of *R. solani* and cell wall degradation of this fungus *in vitro* (of this study) indicated that this *chit42* gene could be used to develop transgenic plants with improved resistance to phytopathogenic fungi.

Acknowledgments

The authors would like to acknowledge National Institute for

Genetic Engineering and Biotechnology (project No. 248) for providing financial support.

References

Adams DJ (2004). Fungal cell wall chitinase and glucanases. *Microbiology* 50: 2029-2035.

Baek JM, Howell CR (1999). The role of an extracellular chitinase from *Trichoderma virens* GV29-8 in the biocontrol of *Rhizoctonia solani* . *Curr Genet.* 35: 41-50.

Bhunchoth A, Aeungkittikul J, Warin N, Romyanon K, Tienkhao A, Chamswang C, Inthanu W (2006). Endochitinase from *Trichoderma longibrachiatum.* Available under accession number of ABD42921 in Genebank.

Bhunchoth A, Warin N, Romyanon K, Tienkhao A, Intanoo W, Chamswarng C (2005). Endochitinase from *Trichoderma harzianum.* Available under accession number of AAZ95175 in Genebank.

Blaiseau PL, Lafay JF (1992). Primary structure of a chitinase-encoding gene (chi1) from the filamentous fungus *Aphanocladium album*. *Gene,* 120: 243-248.

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991). Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science*, 254: 1194-1197.

Carsolio C, Benhamou N, Haran S, Cortes C, Gutierrez A, Chet I, Herrera-Estrella A (1999). Role of the *Trichoderma harzianum*, endochitinase, *ech*42, in mycoparasitism. *Appl Environ Microbiol.* 65: 929-935.

Carsolio C, Gutierrez A, Jimenez B, Van Montagu M (1994). Characterization of *ech-42,* a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Pro Natl Acad Sci.* 91: 10903-10907.

Cavingnac Y (2000). 42 kDa endochitinase from *Trichoderma koningii.* Available under accession number of AAF19612 in Genebank.

Collinge DB, Kragh KM, Mikkelson JD, Nielson KK, Rasmussen U, Vad K (1993). Plant chitinases. A review, *Plant J*. 3: 31-40.

De la Cruz J, Liobell A (1999). Purification and properties of basic endo-β-1,6-glucanase (BGN16.1) from the antagonistic fungus *Trichoderma harzianum. Eur J Biochem.* 265: 145-151.

De la Cruz J, Hidalgo-Gallego A, Lora JM, Benitez T, Pintor-Toro JA, Liobell A (1992). Isolation and characterization of three chitinases from *Trichoderma harzianum* . *Eur J Biochem.* 206: 859-867.

Draborg H, Christgau S, Halkier T, Rasmussen G, Dalboge H, Kauppinen S (1996). Secretion of an enzymatically active *Trichoderma harzianum* endochitinase by *Saccharomyces cerevisiae. Curr Genet.* 29: 404-409.

Elad Y, Chet I, Henis Y (1982). Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Can J Microbiol. 28*: 719-725.

Gokul B, Lee JH, Song KB, Rhee SK, Kim CH, Panda T (2000). Characterization and applications of chitinases from *Trichoderma harzianum*- A review. *Bioprocess Eng*. 23: 691-694.

Harighi MJ, Motallebi M, Zamani MR (2006). Purification of chitinase 42 from *Trichoderma atroviride* PTCC5220. *Iranian J Biol.* 19: 203-214.

Kunz C, Ludwing A, Bertheau Y, Boller T (1992). Evaluation of the antifungal activity of the purified chitinase I from the filamentous fungus *Aphanocladium album. FEMS Microbiol Lett.* 90: 105-109.

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL, Di Pietro A (1993). Chitinolytic enzymes produced by *Trichoderma harzianum*; Antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* 83: 302-307.

Lorito M, Woo SL, Fernandez IG, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence C, Zoina A, Tuzun S, Scala F (1998). Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proc Natl Acad Sci USA*. 95: 7860-7865.

Mathivanan N, Kabilan V, Murugesan K (1998). Purification, characterization and antifungal activity of chitinase from *fusarium chlamydosporum* , a mycoparasite to groundnut rust, *Puccina arachdis*. *Can J Microbiol*. 44: 646-651.

Ordenlich A, Elad Y, Chat I (1988). The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathology* 78: 84-88.

Park SH, Lee JH, Lee HK (2000). Purification and characterization of chitinase from a marine bacterium, *Vibrio* sp. 98CJ11027. *J Microbiol*. 38: 224-229.

Ridout CJ, Coley-Smith JR, Lynch JM (1988). Fractionation of extracellular enzymes from a mycoparasitic strain of *Trichoderma harzianum*. *Enzyme Microb Technol*. 10: 180-187.

Roberts WK, Selitrennikoff CP (1988). Plant and bacterial chitinases differ in antifungal activity. *J Gen Microbiol*. 134: 169-176.

Sambrook J, Russell DW (2001). *Molecular Cloning.* Cold Spring Harbor: New York.

Sashihara N, Kudo T, Horikoshi K (1984). Molecular cloning and expression of cellulase genes of alkalophilic *Bacillus* sp. strain N-4 in *E. coli. J Bacteriol.* 158: 503-506.

Shih CT, Khan AA, Jia S, Wu J, Shih DS (2001). Purification, Characterization and molecular cloning of a chitinase from the seeds of *Benincasa hispida* . *Biosci Biotechnol Biochem.* 65: 501-509.

Steyaert JM, Stewart A, Jaspers MV, Carpenter M, Ridgway HJ (2004). Co-expression of two genes, a chitinase (*chit42*) and proteinase (*prb1*), implicated in mycoparasitism by *Trichoderma hamatum. Mycologia*, 96: 1245-1252.

Tweddell RJ, Jabaji-Hare SH, Charest PM (1994). Production of chitinases and β-1,3 glucanases by *Stachybotrys elegans,* a mycoparasitie of *Rhizoctonia solani. Appl Environ Microbiol.* 60: 489-495.

Wang Y, Kausch AP, Chandlee JM, Luo H, Ruemmele BA, Browning M, Jackson N, Goldsmith MR (2003). Co-transfer and expression of chitinase, glucanase, and *bar* genes in creeping bentgrass for conferring fungal disease resistance. *Plant Sci.* 165: 497-506.

Yang Q, Song J (2004). 42 kDa endochitinase from *Trichoderma aureoviride.* Available under accession number of AAW31950 in Genebank.

Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek CP (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genet Biol.* 26: 131-140.

Zhang HY, Ouyang F, Chen ZH (1999). Chitinase from *Trichoderma viride.* Available under accession number of AAG09447 in Genebank.

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ (1994). Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. *Biotechnology,* 12: 807-812.