Cloning and characterisation of chitinase from *Trichoderma atroviride*

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**ABSTRACT:** Chitinases are an effective tool for the complete degradation of mycelia or conidial walls of phytopathogenic fungi. A gene encoding chitinase was cloned from *Trichoderma atroviride* to test its activity against fungal pathogens. Genomic DNA was isolated from the *Trichoderma atroviride* isolates, OTPT2 and OTPT3. Chitinase genes of 1kb were isolated using chitinase specific primers. The genes were cloned into pTZ57R/T vector and transformed into *E.coli* DH5α. The genes were sequenced and found to have high homology with the other reported *Trichoderma* chitinase sequences (GenBank AY028421, AY129675). The genes were cloned into pET27b protein expression vector and transformed into BL21 cells. The predicted protein contains 340 amino acids. Chitinase protein was expressed and isolated.

**Keywords:** Chitinase, *Trichoderma atroviride*, cloning

**INTRODUCTION**

Plant diseases caused by fungal pathogens are a major production constraint worldwide and destroys 125 million tonnes of the five major crops (rice, wheat, maize, potatoes and soybeans) each year. More than 600 million people could be fed each year by managing fungal diseases in the world’s five most important crops. The fungal diseases are controlled by different methods, which include crop rotation, host resistance and fungicides (Haggag., 2008). The limitations of the conventional practices like crop rotation and cultural practices are protection only against certain group of pathogens and failure of resistant varieties due to emergence of new virulent fungal races (Cornelissen and Melchers, 1993). The environmental pollution, non –specificity, residues in food chain, harmful effect on beneficial microbes and development of fungicidal resistance are associated problems with fungicidal applications (Getsinger, 1998; Rommens and Kishore 2000). One such alternative is the identification of biological agents in combination with molecular biology for the control of plant disease (Heydari and Pessarakli, 2010). Microorganisms with inhibitory activity against pathogens are a potential source of genes to confer disease resistance in plants. *Trichoderma* species alone or in combination with other *Trichoderma* species can be used in the biological control of several plant diseases (Gomathinayagam et al., 2010; Susanto et al., 2012).

Chitin is a homopolymer of 1,4-ã-linked N-acetyl-ã-D-glucosamine (GlcNAc) residues, which is the main component of fungal cell wall (Cohen-Kupiec et al. 1999) and exoskeleton of insects (Ruiz-Sanchez et al., 2005). Degradation of chitin can inhibit the growth of the fungi and insects, which could be an effective management strategy. Chitinases are potential antifungal agents through their chitin degradation activity (Patel et al., 2007; Kucuk and Kivanc, 2004; Gohel et al., 2006; El-Katatny et al., 2000) and also have been used in combination with the insecticidal Cry proteins of *Bacillus thuringiensis* to enhance their activity against important crop pests such as *Eldana saccharina* (Downin et al., 2000) and *Spodoptera littoralis* (Regev et al., 1996). The antifungal activity of chitinases cause rapid lysis of fungal hyphal tips and germinating spores. These enzymes are an effective tool for the complete degradation of mycelia or conidial walls of phytopathogenic fungi (Suryanto et al., 2010). 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; Anitha and Rabeeth, 2010).

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 been isolated from the *Trichoderma* species (Ganiger et al. 2009; Mora and Earle., 2001; Matroudi et al. 2008; Hoell et al. 2005). *Trichoderma* species are strongly antagonistic to other phytopathogenic fungi. They produce hydrolytic enzymes which are believed to play an important role in the parasitism of
phytopathogenic fungi (Gomathinayagam et al., 2010). They are more active than corresponding plant enzymes, effective on a much wider range of pathogens, and are nontoxic to plants at high concentrations (Harighi et al., 2006; Lorito et al., 1998). *Trichoderma* sp. has evolved specifically to be capable of using other fungi as carbon sources but not plants and as such represent a potential source of powerful antifungal genes (Susanto et al., 2005). In our studies, we have identified potential biocontrol agent against *Alternaria solani*, *A. brassicae* and *A. brassicicola*. Hence the present study is contemplated to isolate and characterize chitinase from the potential biocontrol agent *Trichoderma atroviride* OTPT2 and OTPT3.

MATERIALS AND METHODS

Fungal strain

*Trichoderma atroviride* isolates OTPT2 and OTPT3 were isolated from tomato phylloplane following leaf wash method (Gould et al., 1996) using Trichoderma selective medium (TSM) (Elad et al., 1981). Ten gram of leaf sample was taken in 90ml sterile water and mixed well in a rotary shaker at 140 rpm for 30min. Then, 0.1ml of sample was spread on the plate having selective media (yenjit et al., 2004). These plates were incubated at 26°C±2°C for 5-7 days. After isolation, colonies were sub cultured and *T. atroviride* isolates were maintained on PDA slants at 4°C.

Chitinase isolation and cloning

DNA was extracted from seven day old fungal mycelia grown in potato dextrose broth according to the procedure described by Raeder and Broda. (1985) and slightly modified by Chowdappa et al., (2003). The chitinase gene was amplified from the DNA using primer pair CHITF (5’-CATGACACGCCTTCTTGACG-3’) and CHITR (5’-ATTTCTAACCAATGCGAGTAAGC-3’) (Severgnini., 2006). PCR mixture consisted of approximately 100ng of template DNA, 5 il 10x PCR buffer, 40 il sterile distilled water, 1 il 2.0 mM dNTPs, 1il each of 50 pM primers CHITF and CHITR and 0.25 il Taq polymerase (5U/il). Thermal cycling conditions were initial denaturation at 95°C for 3 min, followed by 40 cycles consisting of denaturation at 95°C for 30s, annealing at 57°C for 45s and extension at 72°C for 1 min, followed by a final extension of 72°C for 10 min. Negative controls (no template DNA) were used in every experiment to test for the presence of contamination in reagents. PCR products were analysed by electrophoresis in 2% (w/v), agarose gel in 1x Tris Borate-EDTA buffer and stained with ethidium bromide (5µg/ ml) and visualized the gels by Alpha imager EP (Alpha Innotech Corporation, USA). The resulting PCR product was ligated into pTZ57R/T vector and transformed into *E.coli* DH5α. The plasmid was purified from *E.coli* and the cloned DNA fragment was sequenced to confirm that it has homology identical to the previously reported chitinase sequence available at Genbank (www.ncbi.nlm.nih.gov).

Protein expression

The chitinase gene was digested from the pTZ57R/T vector using the restriction enzymes EcoRI and BamHI, then ligated into pET27b vector between the sites EcoRI and BamHI. The ligation mixture was transformed into *Escherichia coli* BL21 cells. *E. coli* BL21 without vector, *E. coli* BL21 harboring pET27b (empty vector), *E. coli* BL21 harboring pET27b (with chitinase) were grown both at induced and uninduced conditions of IPTG. Cultures of *E. coli* BL21 carrying the pET27b vector with chitinase gene was incubated in LB medium with 30µg ml⁻¹kanamycin in a rotary shaker at 37°C at 210rpm, to a cell density of 0.6 at 600nm. IPTG was added to a final concentration of 1 mM, and the cells were further incubated for 4 h at 37°C followed by centrifugation (6000 rpm, 8 min at 4°C). The cell pellet was resuspended in Na-tris-EDTA buffer pH 8.0, and the cells were lysed by sonication at 20% amplitude with 10 s pulses of 5 times (with 10 s delay between pulses) on ice, with an Ultrasonic Processor. The crude protein was isolated from the cells by centrifuging at 10,000rpm for 30 min.

![Fig. 1. PCR amplification of chitinase gene with specific primers from gDNA and cloned vectors.](image)

Lane 1 and 6- 500bp ladder; Lane2- *T.atroviridae* OTPT2 gDNA; Lane3- OTPT2 chitinase cloned in pET27b vector; Lane4- *T.atroviridae* OTPT3 gDNA; Lane5- OTPT3 chitinase cloned in pET27b vector.
Fig. 2. Schematic representation of chitinase gene sequence (lower case) and their corresponding amino acid sequence (upper case).

The sequence was interrupted by stop codons at 3 places (indicated as *).

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RESULTS AND DISCUSSION

When amplified the genomic DNA of *T. atroviride* isolates OTPT2 and OTPT3 with the primers CHITF and CHITR, the amplified PCR products contained a single band of 1Kb in size (Fig.1). The gene sequence represented its size to 1022bp and had 100% homology with *T. harzianum* endochitinase gene (GenBank AY028421) and 99% with *T. atroviride* endochitinase gene (GenBank AY129675) available in NCBI. The gene sequence was deposited in the GenBank (www.ncbi.nlm.nih.gov) with the accession No HM180397. The gene was expressed only under induced condition (Fig.3) with 1mM IPTG. Under uninduced condition and in negative controls (Fig.3) the protein was not expressed. The protein was 30KDa and the expression was strong at 1mM IPTG concentration.

Several groups of chitinases, Chi18-1 to Chi18-18, have been reported from *Trichoderma* species (Siedl., 2005). However, only three subgroups of Chi 18 such as Chi18-2 (44.5KDa), Chi18-3 (38.7KDa), Chi 18-4 (44.2KDa) were isolated from *T. atroviride* (Siedl., 2005).

Several chitinases are known to contribute to the bio-control properties of *T. atroviride* (Limon et al., 1999; Woo et al., 1999; Viterbo et al., 2001). In this study, the 30 kDa endo chitinase from *T. atroviride* was isolated, characterised and its potential application against fungal pathogens like *Alternaria solani, A. brassicae* and *A. brassicicola* are currently in progress.

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Protein electrophoresis

The crude protein was electrophoresed on SDS-PAGE using stacking gel (2.5%) and separating gel (12%). The crude protein was denatured in a boiling water bath for 5min, mixed with loading buffer in the ratio of 1:1 and loaded in the wells of the gel. Electrophoresis buffer was Tris-glycine buffer (pH 8.3). Electrophoresis was performed at 80 V. The protein patterns were visualized by staining the gels for 7h with Coomassie brilliant blue G in water: methanol: perchloric acid (15:1:4) mixture and destained with mixture of water: methanol: acetic acid (7:2: 1).
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