MOLECULAR CLONING OF CHITINASE 33 (CHIT33) GENE FROM TRICHODERMA ATROVIRIDE

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ABSTRACT

In this study Trichoderma atroviride was selected as over producer of chitinase enzyme among 30 different isolates of Trichoderma sp. on the basis of chitinase specific activity. From this isolate the genomic and cDNA clones encoding chit33 have been isolated and sequenced. Comparison of genomic and cDNA sequences for defining gene structure indicates that this gene contains three short introns and also an open reading frame coding for a protein of 321 amino acids. The deduced amino acid sequence includes a 19 aa putative signal peptide. Homology between this sequence and other reported Trichoderma Chit33 proteins are discussed. The coding sequence of chit33 gene was cloned in pET26b(+) expression vector and expressed in E. coli.

Key-words: Trichoderma atroviride, chit33, chitinase activity, gene structure.

Chitin is a polymer of β-1,4 linked N-acetylglucosamine (GlcNAc) and a very abundant natural polymer. It is the main structural compound of cell wall of fungi, insect exoskeletons and shells of crustaceans (10). The fungal cell wall is a highly dynamic structure subject to constant change during cell expansion and division, and during spor germination, hyphal branching and septum formation in filamentous fungi. The cell wall degrading enzymes are glycosyl hydrolases that degrade chitin and glucan polymer, which comprise important structural elements in the cell walls of fungal organisms (9). Mycoparasitic Trichoderma species secrete chitinases and glucanases that attack cell wall polymer on other fungi and have been exploited in the development of biocontrol strategies (1). In this study, we describe the identification of T. atroviride as a high producer of chitinolytic enzymes and cloning and partial characterization of its endochitinase gene (chit33) along with the heterologous expression of this enzyme.

In the past two decades, extensive studies on chitinases have been done by a large number of laboratories. This high level of interest in chitinases is mostly due to the antifungal property of these enzymes. Most of these studies were on the characterization of the genes and cDNA and on examination of gene expression and its regulation. Trichoderma sp. exhibit considerable variability among strains with respect to their production of hydrolytic enzymes, biocontrol activity and host range (12). To determine the maximum level of enzyme production and hence use this period for mRNA isolation, Trichoderma species were grown in 200 ml of Czapeck-Dox medium containing the following per litter, 3 g NaNo3, 0.5 g MgSO4.7H2O, 0.5 g KCl, 0.01 g FeSO4.7H2O, 1 g KH2PO4 and supplemented with 10% glucose in 500 ml flask. The flask was inoculated with 2 ml conidial suspension (10⁶ conidia/ml) of 30 different isolates of Trichoderma and incubated for 96 hours at 25°C as stationary culture. Harvested mycelia were washed several times with 2% of MgCl2 and distilled water and transferred to Czapeck-Dox medium supplemented with 1.5% colloidal chitin. The secreted enzymes into the medium were used for enzyme activity measurement up to 5 days with one day intervals. Trichoderma atroviride was among the 30 isolates showing the high enzyme specific activity (0.97 U/ mg), on third day of incubation.

By screening thirty Trichoderma isolates we found an Iranian source strain identified as T. atroviride to be among the high producer of chitinase by using colloidal chitin as a substrate.
and inducer. Most of the chitinolytic enzyme systems reported in the literature are inducible (4,8,14). Monreal and Reese (8) suggested that the most probable inducers of chitinase in *Serratia marcescens* are soluble oligomers derived from chitin, but not the monomer (GlcNAC). Ulhoa and Peberdy (14) suggested that products of chitin degradation also regulate chitinase synthesis in *T. harzianum* 39.1. In agreement with these findings we found high chitinase activity only in cultures supplied with chitin.

It was found that chitinase activity increased with increasing colloidal chitin concentration up to 1.5%. Ulhoa and Peberdy (14) suggested that chitinase production was substrate concentration dependent, above 0.5% (w/v) chitin there was no further promotion of synthesis. Elad et al. (3) reported that chitinase secretion into the growth medium by *T. harzianum* was increasing up to concentrations of 1%.

For the purpose of amplification of *chit*33 gene from *T. atroviride*, we designed two specific primers against known *chit*33 sequences. The two tailed primers, mch33f and mch33r (Table 1), were designed based on sequence similarity of existing chitinase cDNA present in the database. To facilitate subsequent cloning of the PCR-derived fragments, *Xba*I restriction site (bolded) were added to the 5'-end of these primers (Table 1). Fungal chromosomal DNA was prepared as described by Sun et al., (13). PCR reactions contained 2.5 units of Fermentas *Pfu* DNA polymerase, 1X buffer, 200 μM of each deoxyribonucleotide triphosphate, 2 μM MgSO4, and 0.5 μM primers. Reaction conditions for PCR amplification were 94ºC for 90 sec, 55ºC for 30 sec, and 72ºC for 120 sec, for 34 cycles followed by a final extension of 5 min.

A specific band about 1.2 kb was amplified from *T. atroviride* chromosomal DNA (Fig. 1A) and confirmed by sequencing. The sequence was submitted to NCBI database under accession number EF439839. DNA sequence information confirmed that we have amplified the PCR fragment with high homology to the previously reported chitinase cDNA sequence of *T. harzianum* (6), *T. virens* (7), and *Hypocrea virens* (5). The new construct (pUCSM1) was confirmed by restriction pattern using *Xba*I (Fig. 1A).

![Figure 1](image)

**Figure 1.** A) line 1, PCR amplification of *chit*33 genomic DNA (approximately 1.2 Kb), line 2, digestion of recombinant plasmid containing *chit*33 gene, using *Xba*I, (two bands approximately 2.7 and 1.2 Kb), M= DNA size marker; B) line 1, PCR amplification of *chit*33 cDNA (approximately 1 Kb), line 2, digestion of PCR product using *SacI*, (two bands approximately 0.6 and 0.4 Kb), M= DNA size marker.

For RNA isolation, *T. atroviride* was grown in 250 ml shaking flasks containing 150 ml Czapek-Dox medium supplemented with 10% glucose at 28ºC and 200 rpm for 96 hours. Mycelia were collected after 96 hours by Whatman (No.1) filter paper and washed several times by MgCl2 (2%) and then inoculated into Czapek-Dox medium supplemented with 1.5% colloidal chitin (2). Cells were harvested after 42 hours of growth and frozen in liquid nitrogen. Frozen mycelium was ground and suspended in 5 volumes of guanidine isothiocyanate, 0.5% Na-lauryl sarcosinate, 25 mM sodium citrate pH 7.0 and 0.1 M β-mercaptoethanol (11). The messenger RNA was purified by mRNA isolation kit 1741985 (Roche).

cDNA synthesis using the poly(A)’ RNA was carried out by Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas). The reaction volume was 50 μl containing: 5 μg of poly(A)’ RNA, 20 pmol of oligo(dT)12, 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 MgCl2) 500 μM of each dNTP, and 200 units reverse transcriptase. The RNA was denatured at 70ºC, 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 42ºC for 1 h and then incubated at 70ºC for an additional 5 min. The cDNA from the reaction was kept at -70ºC and used for a PCR reaction with specific primers (mch33f and mch33r) containing *Xba*I site at their 5’ ends. DNA

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**Table 1.** Oligonucleotides (primers) used in this study.

| Name           | Oligonucleotides Sequence |
|----------------|---------------------------|
| MCH33f         | 5’-GGAGAATGCTTTGAGCTTGGCG-3’ |
| MCH33r         | 5’-CGTCTAGATCACCAATGGACTG-3’ |
| CH33PF         | 5’-GTTTCTAGATCTTGGACG-3’ |
| CH33Pr         | 5’-CGAATTCCTTTATGTA-3’ |
| M13F           | 5’-GGTATTGTGGTGGAGGCCGGG-3’ |
| M13R           | 5’-GCTACTAACATGCCAGT-3’ |
| T7 promoter    | 5’-TAATACGACTCACTATAG3’ |
| T7 terminator  | 5’-CGATCAATAACACGCAGTCGC-3’ |
amplification was carried out in a PCR reaction containing 2.5 units of Fermentas Pfu DNA polymerase. The cDNA fragment approximately 1 kb was obtained under optimized conditions. The PCR product was isolated and confirmed by restriction pattern analysis using SacI enzyme (Fig. 1B). This fragment ligated to pUC19 vector and designated as pUCSM2.

Comparison of the cDNA sequence with the genomic chit33 sequence demonstrated this gene is interrupted by three short introns, 73, 89, and 74 bp in length (Fig. 2). The consensus sequences, GT on the 5' end and AG on the 3' end for each intron of the chit33 gene are also observed. The coding region of chit33 codes for a polypeptide of 321 amino acids, the first 19 residues of which form a putative signal peptide. The calculated size of the predicted product is 34026 daltons. CLUSTAL W program used for multiple alignment of the deduced amino acid sequence obtained in this study with Chit33 enzymes from Hypocrea virens (AAL78811, AAL84693 and ABP96986), T. harzianum (CAA56315) and T. reesei (DA05860) (Fig. 3). Pairwise alignment shows very high homology between Chit33 polypeptide sequence in this study and Chit33 amino acid

Figure 2. Nucleotide and deduced amino acid sequences of T. atroviride chit33 gene. The introns sequences are presented with small characters. Signal peptide is indicated by underline.

| ATG | CCT | TCA | TTG | ACT | GCT | TCT | GCG | AGC | GCC | GCT | TCT | GCT | TTT | GCT | GGC |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| TGG | AAT | GTT | TAC | AGC | CAA | AAC | ATT | GCT | GTC | TAC | TGG | G | gtaaggccctttctttatgagc |
| W | V | N | V | N | S | K | Q | N | I | A | V | Y | W | G |
| ttttacaaatattatatataagggaaataatgc tatctcaacatgacatctgtatag | GA | CAA | AA |
| C | TGC | GCC | GGC | CAG |CAA | AGC | ACC | AAC | CAG | GTT | TCA | ACC | TGC | AGC | G | gtaagtttctctatatatgaaagaatggttgct |
| S | A | G | Q | S | T | Q | R | L | S | T | Y | C | S | D |
| gctttatttcccaacactgcaagtcttttagctggcacaaatatggaaagcatgtgtag |
| ttcgagcagtggccatagtag | AT | GCC | AAT | ATT | AAT | GTT | ATT | GAC | ATT | GCT | TTA | AAC | GGA | ATC | |
| ACT | CCT | CCC | ATG | ACC | AAC | TTT | GCC | AAT | GCT | GGT | GAC | CGA | TGC | ACT | CCC | TCC | GAC | AAC |
| T | P | P | M | T | N | F | N | A | G | D | R | C | T | P | F | S | D | N |
| CCT | TGG | CTC | CTG | AGC | TGC | CCT | GAA | ATT | GA | gtaagttttctctatatatgaaagaatggttgct |
| P | W | L | L | S | C | P | E | I | E |
| tgatgtgtatgcaaatatggactaatatattttatatttttaag | G | GCC | GAT | ATC | AAG | ACT | T |
| A | D | I | K | T | C |

| GC | CAG | GCT | AAC | GGC | AAG | ACC | ATC | CTC | TCT | TCT | TCT | GGT | GGT | GAC | TCT | TAC | ACC | CAA | GGT |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Q | A | N | G | K | T | I | L | L | S | L | G | G | D | S | Y | T | Q | G | G |
| GC | TGG | AGC | TCT | GCC | AGC | GCT | CAA | GCC | GCA | GCC | AGC | AAG | GTT | CGG | GGC | ATG | TCC | GAC | GGC |
| T | G | S | G | S | G | S | A | S | E | R | P | P | F | P | G | S | A | I | V | D | G | F | D |
| AT | TTC | GAC | TTT | GAG | ACC | ACC | AAC | ACC | CAT | GCT | GCT | TCC | GGC | CCC | ACC | CAG | CAT | AAC | AGC |
| T | F | D | F | E | A | T | T | N | L | A | A | F | G | A | Q | L | K | E | L |
| TC | TCC | AAC | GCT | GCC | GCC | GCC | AGG | AAG | TAC | TAC | TCC | TCT | GCT | CCT | CAG | TCG | TCC | CTC |
| S | N | A | A | G | G | K | K | Y | Y | F | S | A | A | P | Q | C | F | P | F |
| CA | GAC | GCC | GCT | GCT | GGT | GCG | CTG | ATC | AAC | GCC | GTC | CCC | ATG | GAC | TGG | ATC | CAG | ATT | CAG |
| D | A | A | V | G | A | I | N | A | V | P | M | D | W | I | Q | I | Q | F |
| TC | TAC | AAC | AAT | CCT | TGG | GCC | GTC | ATG | GCC | TAC | AGC | CCC | GGC | ACC | AGC | AGC | CAG | AAC | AAT | T|
| Y | N | N | P | C | G | V | S | Y | T | P | G | T | S | S | Q | N | N | Y |
| AC | AAC | TAC | CAG | ACC | TGG | GAT | ACC | TGG | GCC | AAG | AGC | AGC | CCC | AAC | ACC | GTC | AAG | CTT | C |
| N | Y | Q | T | D | T | W | A | T | K | T | S | P | N | P | N | V | K | L | L |
| TT | GTC | GGC | ATC | CCC | GCT | GGC | CCA | GTG | GTT | GGC | GGC | TAC | GTG | TCT | GGC | TCT | CAG | CTC | A |
| T | V | G | I | P | A | G | R | G | Y | V | S | G | S | Q | L | T |
| CT | TCA | GTC | TCC | CAG | TAC | TGG | AAG | GGG | TAC | AGC | AGC | ACC | TTT | GCC | GGT | GTC | ATG | TGG | G |
| S | V | F | Y | S | K | G | F | S | S | T | F | A | G | A | M | M | M | W | D |
| AT | ATG | TCC | CAG | TCT | CAG | TGC | ACC | ACT | GCC | TTT | GAG | GCC | CAG | GTT | GTC | AAT | GCT | TGG | AGG | T |
| M | S | Q | L | Y | Q | N | T | G | F | E | A | Q | V | V | N | A | L | R | * |

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sequence from *H. virens* (91.6-91.9%), *T. harzianum* (92.5%) and *T. virens* (83.2%).

The *chit33* cDNA coding region was expressed in *E. coli* BL21(DE3). The sequence containing the coding region of *T. atriviride* was amplified by PCR using two specific primers (CH33pf and CH33pr). The restriction site EcoRI was added in 5’ end of CH33pf forward primer to provide cloning of the cDNA fragment in frame with pelB leader sequence, when the vector is digested with NcoI enzyme. This cDNA was cloned in the pET26b(+) expression vector and designated as pETSM2. Cultures of *E. coli* BL21(DE3) carrying the pETSM2 was grown and induced with 0.2 mM IPTG. The expression of Chit33 by this vector was analyzed in liquid culture for 8 hours with 2 hours intervals by SDS-PAGE. *Escherichia coli* BL21(DE3) harboring pET26b(+) (empty vector) was used as negative control. The results indicated that this IPTG inducible polypeptide was expressed after 4 hours of induction with a molecular mass of about 35 kDa (Fig. 4), which corresponds to the deduced molecular weight of Chit33 and PelB leader peptide. The absence of this protein band afterwards may is due to the proteolytic action of the host cell. The antibody which is raised against this protein could be used for detection of expressed Chit33 in transgenic plants. 

**RESUMO**

Clonagem molecular do gene quitinase 33 (*chit33*) em *Trichoderma atroviride*

Neste estudo *Trichoderma atroviride* foi escolhido como superprodutor da enzima quitinase dentre 30 isolados de *Trichoderma* sp. com base na atividade específica de quitinase. Clones de cDNA e genômico codificando chit33 foram obtidos deste isolado e seqüenciados. A comparação das sequências genômica e de cDNA para definir a estrutura do gene indicou que este contém três pequenos introns e uma fase aberta de leitura codificando uma proteína de 321 aminoácidos. A sequência de aminoácidos deduzida inclui um possível peptídio sinal de 19 aminoácidos. Homologia entre esta sequência e outras proteinas Chit33 descritas de *Trichoderma* é discutida. A sequência codificadora do gene chit33 foi clonada no vetor de expressão pET26b(+) e expressa em *E. coli*.

**Palavras-chave:** Trichoderma atroviride, chit33, atividade de quitinase, estrutura gênica.

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