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MOLECULAR CLONING OF CHITINASE 33 (*CHIT*33) GENE FROM *TRICHODERMA ATROVIRIDE*

Matroudi S.; Zamani M.R.; Motallebi M.

National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, I.R. of Iran

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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 *chit*33 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 *chit*33 gene was cloned in pEt26b(+) expression vector and expressed in *E. coli.*

Key-words:*Trichoderma atroviride , chit*33, 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 spore 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 (*chit*33) 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 (10^6 conidia/ml) of 30 different isolates of *Trichoderma* and incubated for 96 hours at 25ºC 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

*Corresponding Author. Mailing address: Shahrak-e-pajoohesh, 17km Tehran-Karaj high way, National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, I.R. of Iran. P.O. Box 14965/161. Tel./Fax:+9821 44580363. E-mail: mrzamani97@yahoo.com, zamani@nigeb.ac.ir

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 deoxynucleotide triphosphate, 2 µM $MgSO_4$ 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*1 (Fig. 1A).

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

Name	Oligonucleotides Sequence
MCH33f	5'- GC**TCTAGA ATG** CCTTCATTGACTGCTCTTGCG-3'
MCH33r	5'- CG**TCTAGA TTA**CCTCAAAGCATTGACAACC-3'
CH33Pf	5'- G**GGTCTC**GC **ATG** CCTTCATTGACTGCTCTTGCG-3'
CH33Pr	5'- C**GAATTC TTA**CCTCAAAGCATTGACAACC-3'
M13F	5'- GCTAGTTATTGCTCAGCGG -3'
M13R	5'- GTAAAACGACGGCCAGT -3'
T7 promoter	5'- TAATACGACTCACTATAG-3'
T7 terminator	5'- CGATCAATAACGAGTCGC-3'

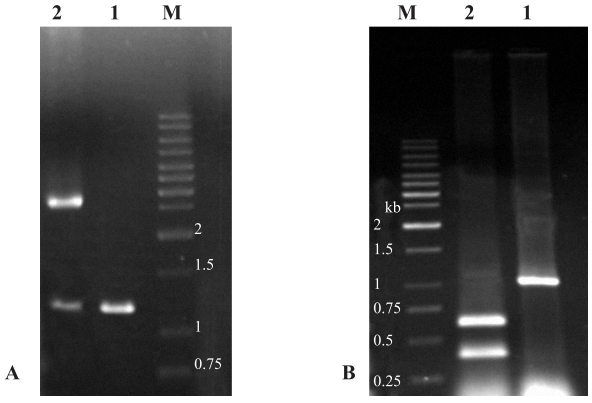

Figure 1. A) line 1, PCR amplification of *chit*33 genomic DNA (approximately 1.2 Kb), line 2, digestion of recombinant plasmid containing *chit*33 gene, using *Xba*I, (two bands approximately 2.7 and 1.2 Kb), M= DNA size marker; B) line 1, PCR amplification of *chit*33 cDNA (approximately 1 Kb), line 2, digestion of PCR product using *Sac*I , (two bands approximately o.6 and 0.4 Kb), M= DNA size marker

For RNA isolation, *T. atroviride* was grown in 250 ml shacking 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 $MgCl_2$ (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 ß-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 (Fermantas). The reaction volume was 50 µl containing: 5 µg of poly(A^+) RNA, 20 pmol 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 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

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 *Sac*I enzyme (Fig. 1B). This fragment ligated to pUC19 vector and designated as pUCSM2.

Comparison of the cDNA sequence with the genomic *chit*33 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 *chit*33 gene are also observed. The coding region of *chit*33 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* (DAA05860) (Fig. 3). Pairwise alignment shows very high homology between Chit33 polypeptide sequence in this study and Chit33 amino acid

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ATG CCT TCA TTG ACT GCT CTT GCG AGC GCG CTC GCT CTT GTT CCT TCC GTC TTT GCT GGC    60
M   P   S   L   T   A   L   A   S   A   L   A   L   V   P   S   V   F   A   G
TGG AAT GTT AAC TCG AAG CAA AAC ATT GCT GTG TAC TGG G gtaaggcccttcttatgagc        120
W   N   V   N   S   K   Q   N   I   A   V   Y   W   G
ttttacacaatttatataagggaaaatagctatctaacatgacatctgtatag GA CAA AA                  180
                                                        Q   N
C TCG GCC GGC CAG CAA AGC ACG CAA CAG CGT CTT TCA ACC TAC TGC AGC G gtacgtttct    240
  S   A   G   Q   Q   S   T   Q   Q   R   L   S   T   Y   C   S   D
gctttatttcccaacactgcaagctttgatcggccaaaatgagaaaaagagcatgttgaa                     300

tctgacagctgccatgtag AT GCC AAT ATT AAT GTT ATT GAC ATT GCT TTA AAC GGA ATC        360
                        A   N   I   N   V   I   D   I   A   L   N   G   I
ACT CCT CCC ATG ACC AAC TTT GCC AAT GCT GGT GAC CGA TGC ACT CCC TTC TCC GAC AAC   420
T   P   P   M   T   N   F   A   N   A   G   D   R   C   T   P   F   S   D   N
CCT TGG CTC CTG AGC TGC CCT GAA ATT GA gtaagtttctctatatgaagaaatgggtgtc           480
P   W   L   L   S   C   P   E   I   E
tgatgtgtatgcaaatatggactaactttaattcattttaaag G GCG GAT ATC AAG ACT T              540
                                              A   D   I   K   T   C
GC CAG GCT AAC GGC AAG ACC ATC CTC CTT TCT CTT GGT GGT GAC TCT TAC ACC CAA GGT G  600
   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 GCT CAA GCC GCA GCC AAC CAG GTC TGG GCC ATG TTC GGT C  660
   W   S   S   A   S   A   A   Q   A   A   A   N   Q   V   W   A   M   F   G   P
CC GTT CAG TCC GGC AGC TCT GCC GAG CGT CCG TTT GGC AGT GCA ATC GTG GAC GGC TTT G  720
   V   Q   S   G   S   S   A   E   R   P   F   G   S   A   I   V   D   G   F   D
AT TTC GAC TTT GAG GCC ACG ACC AAC AAC CTC GCT GCT TTC GGC GCC CAG CTC AAG AGC C  780
   F   D   F   E   A   T   T   N   N   L   A   A   F   G   A   Q   L   K   S   L
TC TCC AAC GCT GCC GGC GGC AAG AAG TAC TAC TTC TCT GCT GCT CCT CAG TGC TTC TTC C  840
   S   N   A   A   G   G   K   K   Y   Y   F   S   A   A   P   Q   C   F   F   P
CA GAC GCC GCT GTC GGT GCG CTG ATC AAC GCC GTC CCC ATG GAC TGG ATC CAG ATT CAG T  900
   D   A   A   V   G   A   L   I   N   A   V   P   M   D   W   I   Q   I   Q   F
TC TAC AAC AAT CCT TGC GGC GTC AGT GGC TAC ACG CCC GGC ACC AGC AGC CAG AAC AAC T  960
   Y   N   N   P   C   G   V   S   G   Y   T   P   G   T   S   S   Q   N   N   Y
AC AAC TAC CAG ACC TGG GAT ACC TGG GCC AAG ACG AGC CCC AAC CCC AAC GTC AAG CTT C  1020
   N   Y   Q   T   W   D   T   W   A   K   T   S   P   N   P   N   V   K   L   L
TT GTC GGC ATT CCC GCT GGC CCA GGT GCT GGT CGC GGC TAC GTC TCT GGC TCT CAG CTC A  1080
   V   G   I   P   A   G   P   G   A   G   R   G   Y   V   S   G   S   Q   L   T
CT TCA GTC TTC CAG TAC TCG AAG GGG TTC AGC AGC ACC TTT GCC GGT GCC ATG ATG TGG G  1140
   S   V   F   Q   Y   S   K   G   F   S   S   T   F   A   G   A   M   M   W   D
AT ATG TCC CAG CTT TAC CAG AAC ACT GGC TTT GAG GCC CAG GTT GTC AAT GCT TTG AGG T  1200
   M   S   Q   L   Y   Q   N   T   G   F   E   A   Q   V   V   N   A   L   R   *
AA                                                                               1202
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Figure 2. Nucleotide and deduced amino acid sequences of *T. atroviride chit*33 gene. The introns sequences are presented with small characters. Signal peptide is indicated by underline.

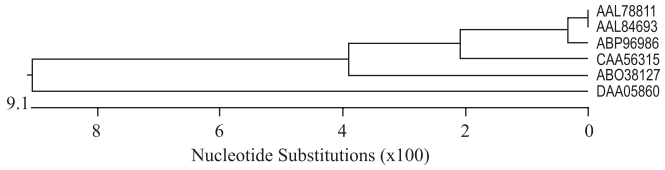

Figure 3. Phylogenetic tree of the Chit33 amino acid sequences comparing *T. atroviride* Chit33 (ABO38127) with those of *Hypocrea virens* (AAL78811, AAL84693 AND ABP96986), *T. harzianum* (CAA56315) and *T. reesei* (DAA05860) generated from multiple alignment.

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

The *chit*33 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 *Eco*31I was added in 5' end of CH33pf forward primer to provide 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 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.

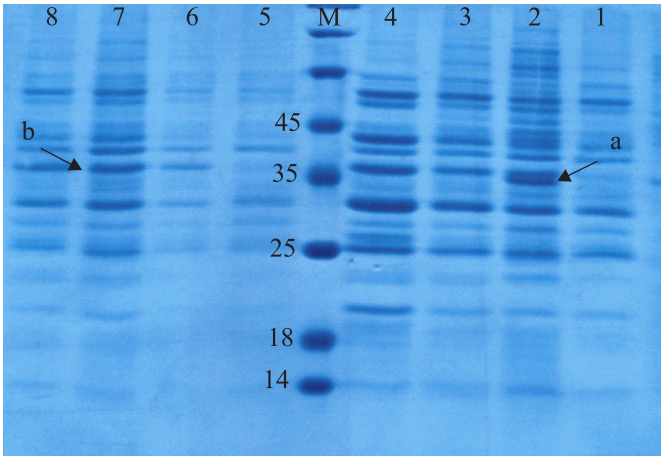

Figure 4. SDS – PAGE and molecular weight determination of Chit33 extracted from *E. coli* harboring *chit*33 gene. The gel was stained with Coomassie blue. Line 1, 2, 3, an 4- *E. coli* BL21 (DE3)- harboring pETSM2 induced with 0.2 mM IPTG for 2, 4, 6, and 8 hours, respectively. Line 5, 6, 7, an 8- *E. coli* BL21 (DE3)- harboring pET26b(+) induced with 0.2 mM IPTG for 8, 6, 4, and 2 hours, respectively. M- Protein molecular weight marker (kDa), arrows (a and b)- presence (a) or absence (b) of expressed protein after 4 hours induction with IPTG.

RESUMO

Clonagem molecular do gene quitinase 33 (*chit* 33) 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 *chit*33 foram obtidos deste isolado e seqüenciados. A comparação das seqüências 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 seqüência de aminoácidos deduzida inclui um possível peptídio sinal de 19 aminoácidos. Homologia entre esta seqüência e outras proteínas Chit33 descritas de *Trichoderma* é discutida. A seqüência codificadora do gene *chit*33 foi clonada no vetor de expressão pET26b(+) e expressa em *E. coli.*

Palavras-chave: Trichoderma atroviride, *chit*33, atividade de quitinase, estrutura gênica.

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