

Trichoderma asperellum Chi42 Genes Encode Chitinase

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(Received July 11, 2011. Accepted August 9, 2011)

Four *Trichoderma* strains (CH2, SH16, PQ34, and TN42) were isolated from soil samples collected from Quang Tri and Thua Thien Hue provinces in Vietnam. The strains exhibited high chitinolytic secretion. Strain PQ34 formed the largest zone of chitinase-mediated clearance (> 4 cm in diameter) in agar containing 1% (w/v) colloidal chitin. Analysis of the internal transcribed spacer regions of these strains indicated that they were *Trichoderma asperellum*. The molecular weights of the chitinases were approximately 42 kDa. Chitinase genes (*chi42*) of *T. asperellum* strains TN42, CH2, SH16, and PQ34 were 98–99% homologous to the *ech42* gene of *T. harzianum* CB-Pin-01 (accession No. DQ166036). The deduced amino acid sequences of both *T. asperellum* strains SH16 and TN42 shared 100% similarity.

KEYWORDS : *Chi42* gene, Internal transcribed spacer region, *Trichoderma asperellum*, 42 kDa chitinase

Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin, and is found in a wide variety of organisms including bacteria, fungi, invertebrates, plants, and animals [1]. Chitin, a (1,4)- β -linked homopolysaccharide composed of *N*-acetylglucosamine residues, is considered the second most abundant biopolymers in nature after cellulose. It is present in the cell walls of bacteria and fungi, mushrooms, exoskeleton of crustaceans and insects, the microfilarial sheath of parasitic nematodes, and the lining of the digestive tracts of many insects [2-4].

Chitinases are produced by a variety of organisms and may play important physiological and ecological roles. Numerous genes encoding chitinase have been cloned from a variety of organisms. Chitinases have been classified into two glycoside hydrolase families, 18 and 19, based on amino acid sequence similarity [5]. Family 18 chitinases cleave the β -1,4-glycosidic linkage of not only GlcNAc-GlcNAc but also GlcNAc-GlcN, whereas family 19 chitinases cleave GlcNAc-GlcNAc and GlcN-GlcNAc linkages [6, 7]. Therefore, it is generally accepted that chitinases cannot cleave the β -1,4-glycosidic linkage of GlcN-GlcN [4].

Trichoderma asperellum is a well-known mycoparasite and effective biocontrol agent of several plant pathogens. A complex system of more than six chitinolytic enzymes that degrades chitin (two are *N*-acetylhexosaminidases and others are endochitinases) has been described in *Trichoderma*

strains [8]. These mycoparasites utilize chitinolytic enzymes along with β -1,3-glucanases and proteases to degrade the cell walls of host pathogens and thus reduce the disease level [4]. However, recent studies on these enzymes have focused in *T. harzianum* [9, 10], *T. reesei* [4], *T. atroviride* [11-14], *T. virens* [15, 16], and *T. aureoviride* [17].

The present work deals with the isolation and identification of the chitinase producing *Trichoderma* strains and isolation of their chitinase genes.

Materials and Methods

Isolation and identification of *Trichoderma* strains.

Fifty-six soil samples were collected from the surface layer (15~20 cm) from eight different sites of agricultural fields in Thua Thien Hue and Quang Tri provinces, Vietnam. One gram of each sample was dissolved in 50 mL water by mixing for 10 min at room temperature. The supernatant was diluted 10^{-2} and 0.5 mL was spread on the surface of *Trichoderma* isolation medium [18], supplemented with propamocarb [19]. Cultures were incubated at 28°C until fungi colonies become visible. To induce chitinase activities, all selected isolates were inoculated on solid Czapeck-Dox without glucose, supplemented with 1% (w/v) colloidal chitin at 28°C for 36 hr [20]. Hydrolysis of colloidal chitin was detected by Lugol's solution [21].

The *Trichoderma* strains showing the highest chitinase activity was transferred onto potato dextrose broth medium

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[22] at 28°C for 2 days for biomass production. Total DNA from fungi biomass was isolated by the phenol-chloroform method [23]. PCR was performed by internal transcribed spacer (ITS)1 and ITS4 primers of fungi rDNA [24]. PCR components in a 25 µL volume included 2.5 µL *Taq* buffer, 1 unit *Taq* polymerase, 10 pmol each primer, 20 µM deoxynucleotides (dNTP), 1.5 mM MgCl₂, and 20 ng genomic DNA. PCR was carried out using the following program: 95°C for 5 min; followed by 32 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were sequenced by the method of fluorescent dideoxy-terminator on 3130 ABI system (Applied Biosystems, Foster City, CA, USA).

Substrate electrophoresis of chitinase. Crude chitinase from the broth after 4 days of culture of *Trichoderma* strains on liquid Czapeck-Dox medium without glucose was precipitated by ammonium sulphate (70% saturation) at 4°C for 2 hr and centrifuged at 15,000 rpm at 4°C for 10 min. The pellet was then resuspended in suitable buffer (100 mM sodium acetate, pH 5) and dialyzed overnight against 0.05 M acetate buffer (pH 5). Molecular weight of enzyme was determined by 12% SDS-PAGE including 0.1% (w/v) colloidal chitin following a standard procedure [25]. Electrophoresis was performed at 4°C for 3 hr and the gel was then incubated at 37°C for 2 hr with gentle shaking in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 to remove SDS [26]. The gel was stained in Lugol's solution [21] and the image was analyzed by Quality One software, ver. 4.1 (BioRad Laboratories, Richmond, CA, USA).

DNA isolation and PCR amplification of chitinase gene. Genomic DNA of *T. asperellum* was isolated by a phenol extraction method [23]. Then, genomic DNA was used as template in PCR amplification with the forward primer CHI-F (5'-ATG TTG GGC TTC CTC GGA-3') and the reverse primer CHI-R (5'-TTC GGG ATG GTT GTC ATA CTG-3'), which were specifically designed to detect the chitinase gene based on the nucleotide sequence of chitinase gene from *T. harzianum* CB-Pin-01 (accession No. DQ166036). The PCR mixture consisted of 1.25 unit *Taq* DNA polymerase (Fermentas, Maryland, CA, USA), 5 µL 10× *Taq* DNA polymerase buffer, 200 µM deoxynucleotides (dNTP mixture), 20 pmol of each primer, 1.5 mM MgCl₂, 40 ng genomic DNA, and distilled water to a final volume of 50 µL. After 5 min of genomic DNA denaturation at 95°C, 30 cycles of 1 min for denaturation at 95°C, 1 min for annealing at 55°C and 1 min for polymerization at 72°C were carried out in an iCycler thermocycler (BioRad Laboratories, Richmond, CA, USA). In the final cycle, the temperature of 72°C was held for an additional 5 min. PCR products were detected by

electrophoresis on 0.8% agarose gel and stained with ethidium bromide.

Cloning and sequencing of the chitinase gene. The PCR products were cut from the gel and purified by Wizard[®] SV Gel and PCR Clean-Up Kit (Promega, Madison, WI, USA). They were then ligated to a pGEM[®]-T easy vector (Invitrogen, Carlsbad, CA, USA). The ligation reaction consisted of 5 µL ligase buffer, 1 µL vector, 1 µL PCR product, 1 µL ligase, and 2 µL distilled water. The reaction mixture was incubated at 16°C overnight and then was transformed into *Escherichia coli* TOP10 cells by the heat-shock method. Positive colonies were cultured on 5 mL LB medium with 50 mg/mL ampicillin and recombinant plasmids were isolated using Quick Plasmid Miniprep Kit (Invitrogen). The presence of the insert was determined by PCR followed by 0.8% agarose gel electrophoresis. For determination of chitinase gene, PCR products were fluorescent dideoxy-terminator sequenced on 3130 ABI system (Applied Biosystem, Foster City, CA, USA).

Results and Discussion

Isolation and identification of *Trichoderma* strains.

Eighty-one *Trichoderma* strains were isolated from soil samples collected from Quang Tri and Thua Thien Hue provinces. Chitinase activity was detected in 41 strains. However, only four *Trichoderma* strains (CH2, SH16, PQ34, and TN42) exhibited high chitinolytic secretion in the assay of colloidal chitin hydrolytic activity on agar plates (Fig. 1). Among these four chitinase producing fungi isolates, strain PQ34 formed the largest zone of clearance, exceeding 4 cm in diameter.

The ITS regions of these strains were amplified by PCR (Fig. 2), then sequenced and used to search BLAST

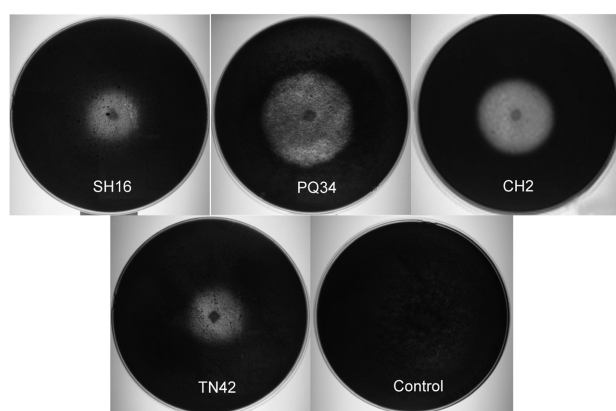


Fig. 1. Determination of action of chitinase from *Trichoderma asperellum* strains on assayed plate containing 0.1% (w/v) colloidal chitin. CH2, SH16, PQ34, and TN42, all four *Trichoderma* strains exhibited high chitinolytic secretion; Control, without *Trichoderma*.

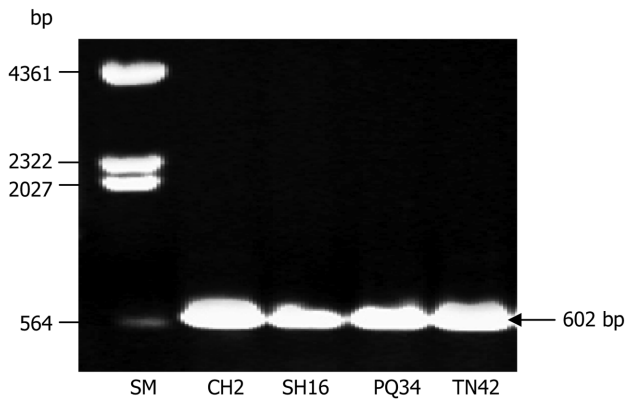


Fig. 2. Agarose gel electrophoresis (0.8% w/v) of PCR products from ITS region of *Trichoderma* strains CH2, SH16, PQ34, and TN42. SM, DNA size marker (λ DNA/*Hind* III).

in Genbank database for identification. They were deposited on NCBI (accession Nos. HM545079, HM545080, HM545081, and HM545083). Nucleotide comparison of ITS regions showed 100% identity of both these strains and *T. asperellum* strain ZJPH0810 (accession No. GU318216). Hence, these strains were classified as *T. asperellum*. Among them, *T. asperellum* strain SH16 has characterized chitinase properties [27].

Molecular weight determination of chitinase. The chitinase activity and molecular weight were determined by SDS-PAGE with colloidal chitin as substrate after ammonium precipitation at 4°C for 2 hr and dialysis overnight in acetate buffer. One chitinase band, confirming enzyme activity, clearly occurred in the dark-colored gel after staining in Lugol's solution. The molecular weights of enzyme were found to be approximately 42 kDa (Fig. 3).

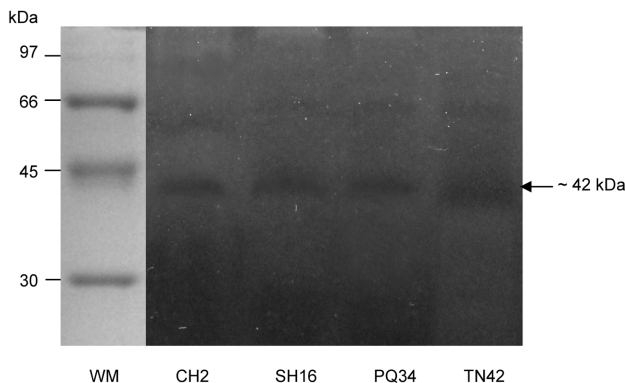


Fig. 3. SDS-PAGE (12%) with 0.1% (w/v) colloidal chitin as a substrate. WM, protein weight markers (97~14.4 kDa). CH2, SH16, PQ34 and TN42, chitinase enzyme of four *Trichoderma asperellum* strains. A clear band on the gel confirmed the chitinolytic activity of enzyme with an estimated molecular weight of 42 kDa.

Chitinase of 42 kDa is a common enzyme in a complex chitinase system of *Trichoderma* species. For example, 42 kDa chitinase has been demonstrated in *T. harzianum* [28-30], *T. atroviride* [31], *T. hamatum* [32], and *T. reesei* [33]. The present substrate SDS-PAGE in this work showed that 42 kDa chitinase in *T. asperellum* is dominant of complex chitinase system.

Chitinase gene of *T. asperellum*. As shown in Fig. 4, DNA fragments of approximately 1,450 bp were amplified from the genome of *T. asperellum* strains CH2, SH16, PQ34, and TN42, suggesting that a chitinase gene might be present in them. These results also showed that two CHI-F and CHI-R primers were specific for this chitinase gene. To verify the amplification of the chitinase gene from *T. asperellum* strains (called *chi42*), PCR products were cloned into pGEM[®]-T easy vector, and then their sequence was analyzed. Sequencing profiles indicated that all these sequences had the same 1,459 nucleotides. The *chi42* genes of *T. asperellum* strains TN42, CH2, SH16, and PQ34 (accession Nos. HM208346, HM191682, HM191683, and HM191684, respectively) exhibited 98~99% homology to the *ech42* gene of *T. harzianum* CB-Pin-01 (accession No. DQ166036).

Jinzhu (2005) suggested that the gene coding for a 42 kDa chitinase of *Trichoderma* species may be strongly conserved because of the high homology (more than 90%) [17]. Our sequences also showed high similarity (more than 98%).

Chi42 genes encode proteins of 424 amino acids; the molecular weight calculated from deduced amino acid sequence was approximately 42 kDa (Fig. 5). The deduced amino acid sequences of both *T. asperellum* SH16 and TN42 strains showed 100% similarity. Using the ScanProsite

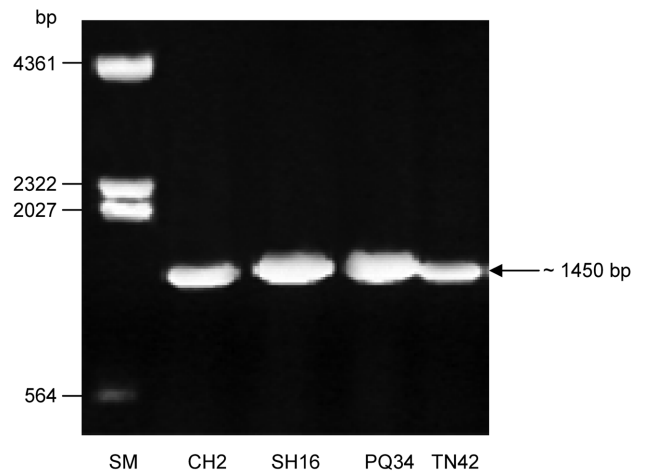


Fig. 4. Agarose gel electrophoresis (0.8% w/v) of PCR products of CHI-F and CHI-R primers from *Trichoderma* strains CH2, SH16, PQ34 and TN42. SM, DNA size marker (λ DNA/*Hind* III).

ECH42	MLGFLGKSVALLAALQATLTTSATPVSTNDVSVSEKRASGYTNAVYFTNWGIYGRNFQPPDL	60
CHI42-SH16	MLGFLGKSVALLAALQATLTTSATPVSTNDVSVSEKRASGYTNAVYFTNWGIYGRNFQPPDL	60
CHI42-TN42	MLGFLGKSVALLAALQATLTTSATPVSTNDVSVSEKRASGYTNAVYFTNWGIYGRNFQPPDL	60
CHI42-CH2	MLGFLGKSVALLAALQATLTSA S PVSTNDVSVSEKR S SGY A NAVYFTNWGIYGRNFQPPDL	60
CHI42-PQ34	MLGFLGKSVALLAALQATLTSA S PVSTNDVSVSEKRASGY A NAVYFTNWGIYGRNFQPPDL	60
ECH42	VASDITHVIYSFMNFQADGTVVSGDAYADYQKHYSDDSWNDVGNNAYGCVKQLFKLKKAN	120
CHI42-SH16	VASDITHVIYSFMNFQADGTVVSGDAYADYQKHYSDDSWNDVGNNAYGCVKQLFKLKKAN	120
CHI42-TN42	VASDITHVIYSFMNFQADGTVVSGDAYADYQKHYSDDSWNDVGNNAYGCVKQLFKLKKAN	120
CHI42-CH2	VASDITHVIYSFMNFQADGTVVSGDAYADYQKHYSDDSWNDVGNNAYGCVKQLFKLKKAN	120
CHI42-PQ34	VASDITHVIYSFMNFQADGTVVSGDAYADYQKHYSDDSWNDVGNNAYGCVKQLFKLKKAN	120
ECH42	RNLKVMLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGIDVDWEYPADDTQA	180
CHI42-SH16	RNLKVMLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGIDVDWEYPADDTQA	180
CHI42-TN42	RNLKVMLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGIDVDWEYPADDTQA	180
CHI42-CH2	RNLKVMLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGIDVDWEYPADDTQA	180
CHI42-PQ34	RNLKVMLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGIDVDWEYPADDTQA	180
ECH42	TNMVLLLKEIRSQDAYAAQYAPGYHFLLSIAAPAGPEHYSALHMADLGQVLDYVNLMA	240
CHI42-SH16	TNMVLLLKEIRSQDAYAAQYAPGYHFLLSIAAPAGPEHYSALHMADLGQVLDYVNLMA	240
CHI42-TN42	TNMVLLLKEIRSQDAYAAQYAPGYHFLLSIAAPAGPEHYSALHMADLGQVLDYVNLMA	240
CHI42-CH2	TNMVLLLKEIRSQDAYAAQYAPGYHFLLSIAAPAGPEHYSALHMADLGQVLDYVNLMA	240
CHI42-PQ34	TNMVLLLKEIRSQDAYAAQYAPGYHFLLSIAAPAGPEHYSALHMADLGQVLDYVNLMA	240
ECH42	DYAGSWSSYSGHDANLFANPSNPN S SPYNTDQAIKAYINGGVPASKIVLGMPIYGRSFES	300
CHI42-SH16	DYAGSWSSYSGHDANLFANPSNPN S SPYNTDQAIKAYINGGVPASKIVLGMPIYGRSFES	300
CHI42-TN42	DYAGSWSSYSGHDANLFANPSNPN S SPYNTDQAIKAYINGGVPASKIVLGMPIYGRSFES	300
CHI42-CH2	DYAGSWSSYSGHDANLFANPSNPN S SPYNTDQAIKAYINGGVPASKIVLGMPIYGRSFES	300
CHI42-PQ34	DYAGSWSSYSGHDANLFANPSNPN S SPYNTDQAIKAYINGGVPASKIVLGMPIYGRSFES	300
ECH42	TNGIGQTYNGIGSGSWENGIWDYKVLPKAGATVQYDSVAQAYYSYDSSSKELISFDT	360
CHI42-SH16	TNGIGQTYNGIGSGSWENGIWDYKVLPKAGATVQYDSVAQAYYSYDSSSKELISFDT	360
CHI42-TN42	TNGIGQTYNGIGSGSWENGIWDYKVLPKAGATVQYD S VAQAYYSYDSSSKELISFDT	360
CHI42-CH2	TNGIGQTY S GIGSGSWENGIWDYKVLPKAGATVQYDSVAQAYYSYDSSSKELISFDT	360
CHI42-PQ34	TNGIGQTY S GIGSGSWENGIW A YKVLPKAGATVQYDSVAQAYYSYDSSSKELISFDT	360
ECH42	VSKKVSYLKNLGLGGSWFWEASADKTGSDSLIGTSHRALGSLDSTQNLLSYPNQYDNIR	420
CHI42-SH16	VSKKVSYLKNLGLGGSWFWEASADKTGSDSLIGTSHRALGSLDSTQNLLSYPNQYDNIR	420
CHI42-TN42	VSKKVSYLKNLGLGGSWFWEASADKTGSDSLIGTSHRALGSLDSTQNLLSYPNQYDNIR	420
CHI42-CH2	VSKKVSYLKNLGLGGSWFWEASADKTGSDSLIGTSHRALGSLDSTQNLLSYPNQYDNIR	420
CHI42-PQ34	VSKKVSYLKNLGLGGSWFWEASADKTGSDSLIGTSHRALGSLDSTQNLLSYPNQYDNIR	420
ECH42	SGLN	424
CHI42-SH16	SGLN	424
CHI42-TN42	SGLN	424
CHI42-CH2	SGLN	424
CHI42-PQ34	SGLN	424

Fig. 5. Multiple alignment of deduced amino acid sequence of the chitinase gene from *Trichoderma harzianum* CB-Pin-01 (ECH42, GenBank DNA sequence, accession No. DQ166036) and four *T. asperellum* strains SH16, TN42, CH2, and PQ34 (CHI42-). Amino acid residues blocked in black differing from the corresponding one of ECH42.

tool (<http://expasy.org/tools/scanprosite>), we found an active site of chitinase family 18 in *chi42* genes of these *T. asperellum* strains.

Chitinases have been demonstrated to have several importance applications, especially in biological control and medicinal industry [34-36]. However, the large-scale production of chitinase using the wild type may be disadvantageous, due to the limitation of enzyme secretion and the difficulty of purification. Therefore, cloning and expression of chitinase genes in the optimal heterologous expression system is a potential means of overproduction of these enzymes. This study is the first report of the discovery of genetic material to express *chi42* gene in an appropriate host. This processing will be investigated.

Acknowledgements

This study was supported by Vietnam Ministry of Education and Training (2009~2011).

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