The isolation and characterization of an endochitinase gene from a Malaysian isolate of *Trichoderma sp.*

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Abstract

Chitinases have been reported to be capable of hydrolyzing chitin by splitting their ß-1,4-glucosidic bonds. The chitinases are divided into the exo and endochitinases. The aim of this study was to isolate and characterize an endochitinase gene from a local isolate of *Trichoderma spp*. that was isolated from the Malaysian soil samples. In total, six highly antagonistic *Trichoderma* isolates were screened for chitinolytic activity via dual plate method and greenhouse studies. *Trichorderma* isolate T2 was identified as a target for isolation of an endochitinase gene due to its high chitinolytic enzyme activity by observing the degradation of chitin substrates. The genomic DNA of *Trichoderma* isolate T2 was extracted, amplified and sequenced. The putative endochitinase gene, *ChitT2* was then subjected to *in silico* analysis to obtain physico-chemical, evolutionary and structural information of this protein. The ChitT2 protein sequence had 352 amino acids and showed 99% homology to *Trichoderma harzianum* endochitinase Chit36Y. The maximum parsimony analysis showed that ChitT2 protein was clustered into Group V with other fungi. Ultimately, the *in silico* analysis of *ChitT2* implicated the involvement of this gene/protein in chitin catabolism.

Keywords: endochitinase, *Trichorderma*, conserved domains, glycosyl hydrolases.
Abbreviation: MEGA_Molecular Evolutionary Genetics Analysis; UPGMA_Unweighted Pair Group Method with Arithmetic Mean.

Introduction

Biological control of plant pathogens is an attractive proposition to decrease heavy dependence of modern agriculture on costly chemical fungicides, which not only causes environmental pollution but also leads to the development of resistant strains (Harjono and Widyastuti, 2001). Various fungi and bacteria have been shown to exhibit antagonistic activity which can be used to control pathogenic organisms. A number of biocontrol agents have been registered and are available as commercial products, including strains belonging to *Agrobacterium, Pseudomonas, Streptomyces* and *Bacillus*, and fungal genera such as *Gliocladium, Trichoderma, Ampelomyces, Candida* and *Coniothyrium* (Francesco et al., 2008).

One of the most popular candidates is the *Trichoderma* sp. which has been shown to be an efficient biocontrol agent with high reproductive capacity, survival rate, nutrient utilization and the ability to promote plant growth and defense mechanisms (Weindling, 1932; Elad et al., 1982; Grondona et al., 1997; Ziedan, 1998; Kharwar et al., 2010; Todorova and Kozhuharova, 2010; Andrei et al., 2012; Fahmi et al., 2012; Panahian et al., 2012). *Trichoderma* spp. are capable of recognizing and attacking phytopathogen of distinct phyla of Basidiomycete, Ascomycete and Oomycetes such as *Rhizoctonia solani, Botrytis cinerea, Fusarium graminearum, Phytophthora* spp*,* and *Pythium* spp. The mycoparasitic activity of *Trichoderma* spp. may be due to

antibiosis (Ghisalberti and Sivasithamparam, 1991), competition (Chet, 1987), production of cell wall-degrading enzymes (Chet, 1987; Schirmbock et al., 1994) or a combination of these antagonistic activities.

The *Trichoderma* spp. has been reported as efficient producers of chitinase. This ability has been used in provision of resistance against pathogenic organisms in plant defense. Chitinase (EC 3.2.1.1.14) is a hydrolase that is able to lyase chitin-chitosan to β-1, 4 linked N-acetyl glucoseamine (Kitamura and Kamei, 2003). Chitinases are basically classified into two families, 18 and 19, where the chitinases in family 18 are of bacterial, fungal, viral, animal and plant origin (class III and V), while family 19 includes chitinases of plant origin from classes I, II and IV(Saito et al., 1999). The Chitinases in Family 18 are classified into the categories of endochitinases, exochitinase and acetylhexosaminidases (Henrissat and Bairoch, 1996).

Very little is known about the regulation and expression of these chitinolytic genes. Chitinase expression in fungi is thought to respond to degradation products that serve as inducers and to easily metabolizable carbon sources that serve as repressors (Blaiseau et al., 1992; Sahai and Manocha, 1993; Smith and Grula, 1983; St. Leger et al., 1986). Most studies of the regulation of chitinase formation in *Trichoderma* spp. have identified chitinases only by

enzyme assays and have not addressed the possibility of differential regulation for the various isoenzymes.

Over the past decade, several Chitinases such as ech42, ech46, chit36, chit37, and ech30 have been isolated and characterized (Verena et al., 2005; Klemsdal et al., 2006). The small amount of data presently available indicates that *ech42*, *chit33*, and *nag1* are inducible by fungal cell walls and colloidal chitin (Carsolio et al., 1994; Garcia et al., 1994; Limon et al., 1995; Peterbauer et al., 1996) or by carbon starvation (Limon et al., 1995; Margolles-Clarck et al., 1996). These chitinases were either isolated via the genomic DNA, or the cDNA approach through the use of PCR amplification with specifically designed primers (Kim et al., 2002; Viterbo et al., 2002; Steyaert et al., 2004; Reithner et al., 2005; Verena et al., 2005; Ike et al., 2006; Klemsdal et al., 2006).

In this study we isolated an endochitinase gene (*ChitT2*) from a Malaysian isolate of *Trichoderma* and analyzed it *in silico*. The amino acid sequence of amplified products was analyzed for the presence of two conserved motifs that have been identified with endochitinases: the chitinase family active site ([LIVMFY] - [DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-X-E) and the chitin binding domain (XXXSXGG) (Renkema et al., 1998). In addition to the identification of the active domains, the protein was also subjected to physico-chemical, phylogenetic and structural analysis (Carolina et al., 1994; Nielsen et al., 1997; Emanuelsson et al., 2000; Susana, 2006; Ziyauddin, 2005; Lip et al., 2009).

This study was also conducted to examine the variation in the chitinolytic potential of various *Trichoderma* spp isolated from soil. The best chitinase producer was then used as a target for gene amplification of the endochitinase gene. This gene was then characterized *in silico* to compare and contrast our gene with other *Trichoderma* spp endochitinase.

Results and Discussion

Screening of Trichoderma spp. for chitinase activity

Twenty two (22) *Trichoderma* isolates were obtained from soil samples through serial dilution and spread plate technique. Out of the twenty two different *Trichoderma* isolates, six isolates (2, 7, 8, 9, 11 and 21) were shortlisted for chitinase activity studies based on their antagonistics activities against plant pathogens via the dual method and greenhouse studies (Hamdia and Kalaivani, 2013). These isolates were grown on chitin containing agar plates to screen the chitinase activity. The results of the chitin plate assays are presented in Table 1. The chitinase activity of all isolates was subjected to a statistical analysis to determine isolate(s) that produce significant activity.

A correlation was detected on the ability to utilize chitin and chitinase activity, where high colonization (high CFU) was indicative of high enzyme activity. Among the six isolates, *Trichoderma* isolate T2 and T21 showed high CFU ability and hence we correlated this to better chitinase enzyme activity (2×10^9 and 1×10^8 CFU/mL respectively). The growth ability of these isolates on chitin can be seen from the growth shown on these plates as opposed to the control. Chitinase production reached maximum within 48 hours of induction and was stable up to 96 hours. Therefore, culture filtrate taken after 48 hours of induction can be used for routine screening of *Trichoderma* isolates for chitinolytic activity. A statistical analysis was conducted on the data obtained from the chitin plates and media. The analysis showed that there is a significant difference ($p \leq 0.05$) in the growth of *Trichoderma* isolates T2, 4 days post incubation in colloidal chitin, compared to the other five isolates. The chitinase activity in crude supernatant was low in the dialyzed filtrate of all six *Trichoderma* isolates. However, some researchers reported contrary results (Susana, 2006), in which they observed difference in crude and pure enzyme activity that may be attributed to the differences of media enzyme assays (Bruce et al., 1995). *Trichoderma* isolate T2 (Fig. 1).

ChitT2 sequence analysis

The T7 and Sp6 primers were used to amplify the gene insert from the pGEM[R]-T Easy Vector, then the nucleotide sequence of *ChitT2* gene was obtained. The complete endochitinase gene was obtained by processing the sequences through the BioEdit program. The sequence was verified as an endochitinase gene through homologous analysis via blast analysis. Fig. 2A provides the *ChitT2* gene and protein sequence. The *ChitT2* gene sequence of 1151bp was fed into the ORF Finder, which revealed a single ORF (open reading frame) with 352 amino acids. The protein was then blasted against all protein sequences in the NCBI database and returned a 99% homology to *Trichoderma harzianum* endochitinase Chit36Y (chit36Y) (AF406791.1). Fig. 2B shows the location of peptidase cleavage site and conserved domains within the amino acid. No glycosylation site was detected in this protein.

Chitin catalytic domain

The amino acid sequence of the ChitT2 was blasted against thirteen amino acid sequences of endochitinase genes from *Trichoderma* species and one amino acid sequence of endochitinase from *Elaeis guineensis* (plant) found in NCBI. The Multialin analysis was conducted with default parameters i.e with gap opening penalty of 10.0 and gap extension penalty 1.0. Fig. 3 shows the multiple alignment results with several regions of homology as indicated by derived consensus sequence in red. Comparison of the ChitT2 amino acid sequence revealed that they shared a high degree of similarity among fungal endochitinases and the regions of SIGGW and FDGIDVDWE (the conserved domains are SxGG and DxxDxDxE) which were highly conserved among chitinases of the glycosyl hydrolase family 18. We observed minimal variation in the sequences within the SxGG and DxxDxDxE conserved domains of the amino acid sequences other than the variation observed in the chitin binding domain (SxGG) of *Elaeis guineensis* chitinase-like protein (Chit5-1) (JX312738.1) and the absence of the signature DxxDxDxE domain in the putative endochitinase ECH30 (ech30) of *Trichoderma atroviride* (AY258147.1) (Fig. 3).

The conserved region of serine [Ser (S) 129] and glycine [Gly (G): 131,132] in the Orange Box are known to be hydrophilic and hydrophobic amino acids that are responsible for reacting with the surface of chitin molecules during a hydrolysis reaction (Fig. 3). The Blue Box was dominated by the glutamic acid (E) and aspartic acid (D) residues that resulted in the protein being acidic and negatively charged (Fig. 3). The glutamic acid residue acts as an acidic catalyst that donates protons to the glycosidic oxygen while aspartic

Table 1. The colony forming unit of *Trichoderma* isolates

Trichoderma isolates	Colony forming unit (CFU) /ml
T2	2×10^9
T7	3×10^6
T8	4×10^6
T9	2×10^5
T11	1×10^5
T21	1×10^8

The CFU forming ability was indirectly used as an indicator of the ability of the isolate to degrade chitin and utilize it as substrate. Chitin catabolism would involve the production of chitinase enzyme.
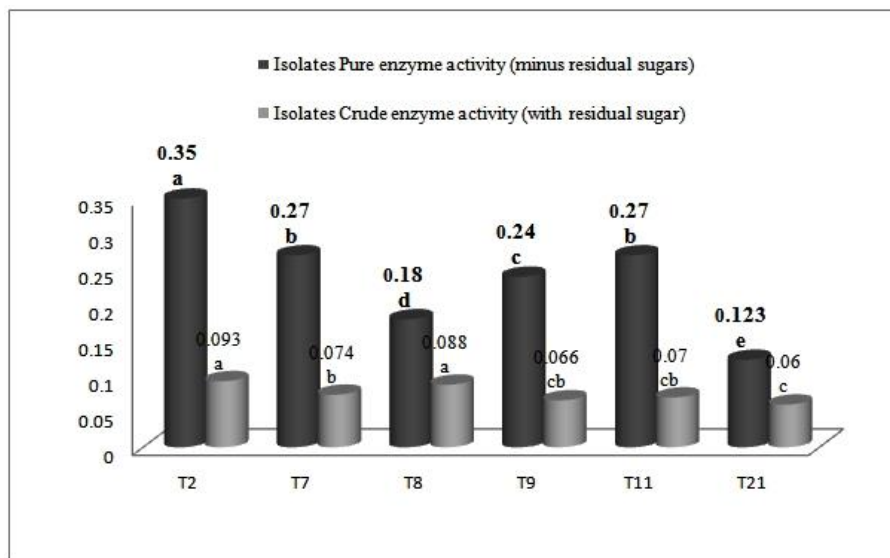

Fig 1. Crude and pure chitinase enzyme activity for six Trichoderma isolates.The chitinase activity was obtained in chitin media containing sugars (light grey) and in chitin media minus the sugars (dark grey).

acid stabilizes the transient carbonium ion intermediate electrostatically by lowering the energy barrier of the reaction (Watanabe et al., 1993). The glutamic residue; therefore, plays an important role in the catalytic domain of the endochitinase (Hollis et al., 2000; Kim et al., 2002). The glutamic acid residue in ChitT2 endochitinase corresponds with the location of the residue in other endochitinase (Fig. 3). Site-directed mutagenesis of the catalytic residues in Chit42 from *Trichorderma harzianum* resulted in reduced enzyme activity of the mutant strains. This indicates that the residues within the conserved domains play a critical role in the enzyme kinetics of the endochitinases (Boer et al., 2007).

In addition, the InterProScan analysis on ChitT2 identified the glycosyl hydrolases family 18, chitinase, and other catalytic and active domains (Fig. 4). Table 2 shows the location of each domain within the protein. The SignalP (Version 4.1) indicated the presence of a putative signal sequence ASA/QN in the protein (Supplementary Fig. 1). The presence of a signal peptide site predicts that the protein is secreted and; therefore, may either be an enzyme or toxin exuded by the fungi as part of their pathogenicity. The

TMHMM analysis also indicated that this is a secreted form of enzyme (Supplementary Fig. 2). Based on the sequence analyses, we predict that ChitT2 is synthesized as a preproenzyme, and the first 33 amino acids have characteristics of a signal peptide and the signal peptidase cleavage site is between A33 and Q34. After processing of the 33 N-terminal amino acids, the calculated molecular weight of ChitT2 is 37.151 kDa, with a theoretical pI of 4.59. The protein was deemed stable with a net negative charge through ProtParam analysis.

Phylogenetic analysis of the ChitT2 protein

The evolutionary history was inferred using the maximum parsimony method (Fig. 5). Fifty three (53) endochitinase proteins from plant, fungi, and bacteria were used in this phylogenetic study (Supplementary Fig. 3). Based on their primary structures, endochitinases have been categorized into 2 families, 18 and 19 glycosyl hydrolases (Henrissat and Bairoch, 1996). Family 18 chitinases have plants, bacteria, fungi (Classes III and V), mammals, and viruses as members

Table 2. The domains and the location of the domains in the ChitT2 protein.

Domain/Motif	Location (amino acid)
Glycoside hydrolase, family 18, catalytic domain (IPR001223)	226-315
Glycoside hydrolase, chitinase active site (IPR001579)	268-276
Chitinase	205-276
Signal Peptide	1-33

(A)

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ATGCTCCGGCCGCCatggcggccgcgggaattcgattcatgacacgccttcttgacgccagcttttctgctg    71
              M  A  A  A  G  I  R  F  M  T  R  L  L  D  A  S  F  L  L
tgcctgctatcgcatcgacgctatttggcaccgcctctgcacagaatgcgacatgcgcacttaagggaaagg   143
L  P  A  I  A  S  T  L  F  G  T  A  S  A  Q  N  A  T  C  A  L  K  G  K
ccggcaggcaaagtcttgatgggatattggggaaaattgggatggagcagccaacggtgttcaccctggattt   215
P  A  G  K  V  L  M  G  Y  W  E  N  W  D  G  A  A  N  G  V  H  P  G  F
Ggttggacaccaatcgaaaaccccatcattaaacagaatggctacaatgtgatcaacgccgccttccccgtt   287
G  W  T  P  I  E  N  P  I  I  K  Q  N  G  Y  N  V  I  N  A  A  F  P  V
attctgtcagatggcacagcgttatgggaaaacgacatggctcctgacactcaagtcgcaactccagctgaa   359
I  L  S  D  G  T  A  L  W  E  N  D  M  A  P  D  T  Q  V  A  T  P  A  E
Atgtgtgaggctaaagcagctggagccacaattctgctgtcaattggaggtgctactgctggcatagatctc   431
M  C  E  A  K  A  A  G  A  T  I  L  L  S  I  G  G  A  T  A  G  I  D  L
agctccagtgcagtcgctgacaagttcatcgccaccattgtaccaatcttgaagcagtacaattttgacggc   503
S  S  S  A  V  A  D  K  F  I  A  T  I  V  P  I  L  K  Q  Y  N  F  D  G
Gatattatagacattgagacggggttgaccaacagcggtaatatcaacacactctccacatcccagaccaac   575
I  D  I  D  I  E  T  G  L  T  N  S  G  N  I  N  T  L  S  T  S  Q  T  N
Ttgattcgcatcattgatggtgttcttgctcagatgccttccatcttcggcttgactatggcacctgagaca   647
L  I  R  I  I  D  G  V  L  A  Q  M  P  S  I  F  G  L  T  M  A  P  E  T
gcgtacgttacaggcggtagcatcacgtatggctctatttgggggagcgtacctacctatcatccagaaatat   719
A  Y  V  T  G  G  S  I  T  Y  G  S  I  W  G  A  Y  L  P  I  I  Q  K  Y
Gttcaaaacggccggctgtggtggctaaacatgcaatattacaacggcgacatgtacggttgctctggcgac   791
V  Q  N  G  R  L  W  W  L  N  M  Q  Y  Y  N  G  D  M  Y  G  C  S  G  D
Tcttacgcagctggcactgtccaaggattcatcgctcagactgactgcctaaatgcaggacttaccgtccaa   863
S  Y  A  A  G  T  V  Q  G  F  I  A  Q  T  D  C  L  N  A  G  L  T  V  Q
Ggcaccacaatcaaggttccgtacgacatgcaagtaccaggtctacctgcgcaatcaggagctggcggtggt   935
G  T  T  I  K  V  P  Y  D  M  Q  V  P  G  L  P  A  Q  S  G  A  G  G  G
Tatatgaatccaagcttggttggacaagcacgggatcactacaacggtgctctgaaaggcttgatgacgtgg  1007
Y  M  N  P  S  L  V  G  Q  A  R  D  H  Y  N  G  A  L  K  G  L  M  T  W
tcaatcaactgggatggagcgggtaactggacatttggcgacaattgcttactcgcattggttagAAAATA  1079
S  I  N  W  D  G  A  G  N  W  T  F  G  D  N  L  L  T  R  I  G  *
ATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTGATGTAAGTTTT  1151
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(B)

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MAAAGIRFMTRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDGAANGVHPGFGWT   70
PIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDTQVATPAEMCEAKAAGATILLSIGGATAGIDLS  140
SSAVADKFIATIVPILKQYNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSIFGLTMAPE  210
TAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYYNGDMYGCSGDSYAAGTVQGFIAQTDCLNAGL  280
TVQGTTIKVPYDMQVPGLPAQSGAGGGYMNPSLVGQARDHYNGALKGLMTWSINWDGAGNWTFGDNLLTR  350
IG                                                                     352
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Fig 2. The *ChitT2* gene. (A) The Nucleotide and Protein Sequence of *ChitT2*. (B) The Amino Acid Sequence of ChitT2. The sequences that are highlighted are the conserved domains. Peptide cleavage site indicated in green bold underlined (ASA/QN).
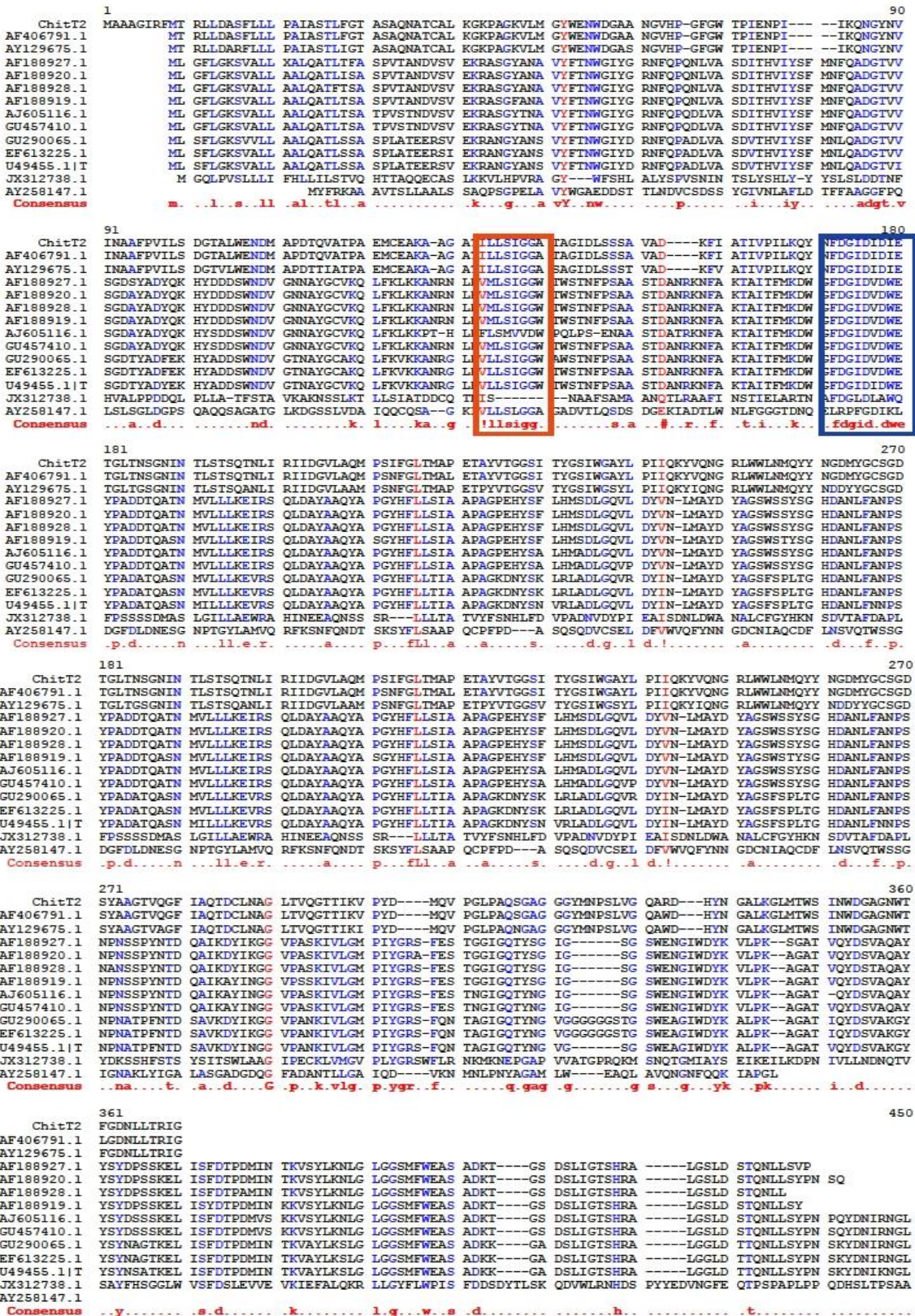
Fig 3. Multiple alignments of endochitinase genes. The boxed regions indicate the location of the two conserved domains in the gene. **Legend:** ChitT2, AF188927.1| *Trichoderma viride* GJS 90-20 42 kDa endochitinase gene, AF188920.1| *Trichoderma atroviride* DAOM 165779 42 kDa, AF188928.1| *Trichoderma viride* BBA 66069R 42 kDa, AF188919.1| *Trichoderma viride* ATCC 18652, AF406791.1| *Trichoderma harzianum* endochitinase Chit36Y (*chit36Y*), AY129675.1| *Trichoderma atroviride* endochitinase (*chit36P1*),GU290065.1| *Trichoderma saturnisporum* 42kDa endochitinase gene, AJ605116.1| *Trichoderma harzianum* (*ech42* gene), JX312738.1| *Elaeis guineensis* chitinase-like protein (*Chit5-1*), EF613225.1| *Trichoderma viride* endochitinase (*chit46*), U49455.1|THU49455 *Trichoderma harzianum* endochitinase (*chi1*), GU457410.1| *Trichoderma asperellum* endochitinase gene, AY258147.1| *Trichoderma atroviride* putative endochitinase ECH30 (*ech30*). The blue boxes indicate that the conserved domains for all sequences.

(Rifat et al., 2013). The chitinases of the two different families do not share amino acid sequence similarity, and have completely different 3-dimensional (3D) structures and molecular mechanisms. Therefore, they are likely to have evolved from different ancestors. Fig. 5 shows the separation of the 53 endochitinase samples into various classes (Hayes et al., 1994). Class I contains all plant endochitinases. This Class of proteins have cysteine-rich N-terminal chitin-binding domains (CBD) that are separated from the catalytic domain (Suarez et al., 2001).

Fig. 5 shows that Class I is divided into five sub-clades. These subclades were then further separated into Group 1a (for acidic plant chitinases) and Group 1b (for basic endochitinases). Class III chitinases are unique in structure and are part of the family 18 glycosyl-hydrolases and are divided into sub-clades; where the fungal and plant endochitinases are separated into Group IIIa for fungus and IIIb for plants. Class IV and V have single clades. The Maximum Parsimony tree presented here places ChitT2 in Group V together with other fungi. As stated earlier, all fungal endochitinases are either Group III or V. Maximum Likelihood and UPGMA analysis conducted on the same set of sequences, placed ChitT2 alone between Group III and V (Supplementary Fig. 4A and 4B). Since ChitT2 has family 18 motifs and has the same conservation of Glutamic acid and aspartic acid within these domains as other Group V endochitinase, we are biased towards the MP model that placed this protein in Group V endochitinases.

Protein structure of ChitT2

The 2D representation of the barrel and sheet structure of the ChitT2 protein shows the presence of 8 strands of parallel β sheets within the external α helices (Supplementary Fig. 5). Subsequently, a 3D modelling was conducted via I-TASSER which was based on top 10 PDB hits. The 2D (Supplementary Fig. 5) and 3D (Fig. 6) model showed the β sheets are located within the barrel structure of the protein. This is as previously reported by other researcher (Gooday, 1999).

The active glutamic acid (Glu169) residue and the negatively charged amino acids Asp162, Asp165 and Asp167 (DxxDxD) of class V, family 18 chitinases was found within the catalytic domain in the barrel of the ChitT2. The glutamic acid residues have been implicated in catalytic activity by other researchers (Ueda et al., 1998; Hollis et al., 2000; Kim et al., 2002). Comparison of the three-dimensional model of *T. harzianum* Chit42 with the solved structure of *Coccidioides immitis* CiX1 revealed several conserved amino acid residues such as Glu169, Asp165 as well as nearby residues Asp167, Asp241, Tyr44 and Tyr240 (Hollis et al., 2000; Boer et al., 2007). In addition to the highly conserved Glu169, Asp162 Asp165, Asp167, which have important roles in the enzyme function, the I-TASSER program also predicted active residues in Tyr248 and Tyr249. Both these residues are not highly conserved in all proteins analyzed except for in *Trichoderma harzianum* endochitinase Chit36Y (*chit36Y*), and *Trichoderma atroviride* endochitinase (*chit36P1*). This is expected as our Blast analysis of ChitT2 returned highest homology to *Chit36* gene.

Materials and Methods

Isolation of Trichoderma spp. from the soil and their maintenance

Trichoderma spp were isolated from soil samples collected from different parts of the National Forest Reserve in Merapoh Pahang, Malaysia. Soil suspensions were prepared by homogenizing 1 gram of soil sample into 10 mL sterilized distilled water. The homogenized soil suspension was subjected to serial dilutions followed by plating via spread plate technique on potato dextrose agar (PDA) plates. Post incubation, colonies that were possibly *Trichoderma* sp were identified for sub-culturing. Once pure cultures were obtained, they were microscopically validated as *Trichorderma spp.*

Preparation of substrates for chitinolytic assays

Preparation of colloidal chitin

Colloidal chitin was prepared according to the Atlas Handbook of Media for Environmental Microbiology 2nd Edition, 2005 with some modifications. Chitin (16.0g) was dissolved in cold concentrated HCl (160 mL) and further dissolved in 1L distilled/ deionized water at 5°C before filtering through Whatman #1 filter paper. The precipitated chitin was dialyzed against tap water for 12 hrs and the pH adjusted to 7.0 using KOH before topping up to 1L with distilled water.

Preparation of chitin plates

The Chitin plates were prepared as follow: Per 1L: 15.0 g Agar, 3.0 g colloidal chitin; 2.0 g $(NH_4)_2SO_4$, 1.1 gNa_2HPO_4, 0.7 gKH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 mg $FeSO_4$, 1.0mg $MnSO_4$) and dissolved in 1L of distilled water. The media was autoclaved at 121 °C for 15 minutes (Kamil et al., 2007).

Chitin plate assays

Spores of *Trichoderma* isolates were harvested in sterilized water to contain $1x10^8$ spore / mL and 100µL of spore suspension was plated on chitin plates via spread plate technique. After four days of post culturing, the Colony Forming Units (CFU) for each isolate was determined.

Chitinase enzyme activity assay

The isolates from the chitin agar were then sub-cultured into liquid chitin synthetic medium (SM) by inoculating plugs obtained from chitin plate cultures into 100 mL of liquid SM chitin in 250 mL conical flask. The liquid chitin SM was prepared according to Rodriguez-Kabana et al. (1983). Four (4) days post incubation in SM media, the mycelia was removed by filtration and culture filtrates were sterilized by passing them through 0.45 µm membrane filters. Filtrates were then dialyzed overnight (to remove residual sugars) in a continuous cold water flow at 10-12°C using 2.4 nm pore size dialysis tubing prior to assay for chitinase activity (Bruce et al., 1995; Susana, 2006).
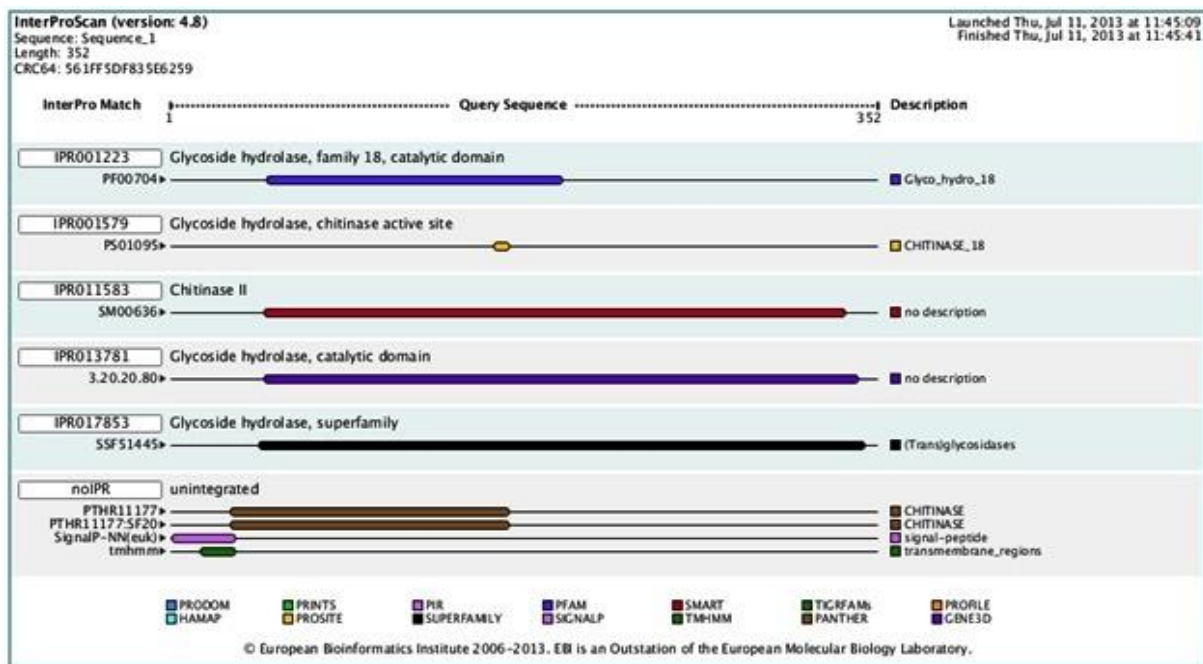
Fig 4. InterProScan analysis of the domains within the ChitT2 protein. Results indicate the presence of GH18 family proteins, chitinases, and other catalytic and active domains. The legends listed within the figure are the 14 protein and nucleotide databases which were used by InterProScan to identify domains within sequence.

For each dialyzed culture filtrate four tubes were set up. One tube from each filtrate set was boiled for 10 min to destroy enzyme activity before incubation at 37°C for 24 hrs. After incubation, all tubes were boiled for 10 min then 0.5 mL was removed from each tube for assay. To each of the four 0.5mL samples, 0.1mL of 0.8 M potassium tetraborate was added and the tubes were boiled again for 3 min before cooling.

The p-dimethylaminobenzaldehyde reagent (DMAB) was prepared according to Reissig et al. (1955). Three mL of DMAB reagent was added to the tubes which were then incubated at 36-38°C for 20 min, cooled, vortex, and absorbency read at 544 nm against water blank that has gone through the above assay procedure (Aminoff et al., 1952). A calibration curve was obtained to quantitate chitinase enzyme activity. Two sets of N-Acetylglucosamine concentrations were prepared: Group one contained concentrations 0, 5, 10, 15, 20 and 25 μg/mL in McIlvaine buffer while Group two contained: 0, 20, 40, 60, 80 and 100 μg/mL in McIlvaine buffer. The average of three replicate readings for each isolate was recorded.

Isolation of genomic DNA from fungus

Genomic DNA was isolated from *Trichoderma* isolate T2 via the Birnboim and Doly (1979) method. The concentration of DNA was estimated as described by Sambrook and Russel (2001).

Cloning of endochitinase gene

Specific primers were designed based on the reported full length gene from *Trichoderma asperellum* chitinase (*ChiB*) gene, (Accession DQ312296.1) from the NCBI database (Susana, 2006). The primers Chi-F-CATGACACGCCTT-

CTTGACG (20 mers) and Chi-R-ATTTCTAACCAA-TGCGAGTAAGC (23 mers) were used to amplify the endochitinase gene from our *Trichoderma* isolate T2. The thermal cycler conditions were: 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 54.7°C for 1 min and 72°C for 2 min, and a final extension at 72 °C for 10 min.

The purified PCR product of ~1.2kb (50ng/μl) was ligated into pGEM[R-] T Easy Vector System (3.0 kb and 50ng/μl) and transformed into *E. coli* DH5α competent cells by heat-shock treatment at 42°C for 1 min followed by immediate chilling for 2 min. Luria broth was added and the mix was incubated in a Thermomixer (Eppendorf, Germany) at 37°C at 600 rpm for 1.5-2 hours to allow bacteria to recover and express the antibiotic marker encoded by the plasmid. The culture was centrifuged and the pellet was dissolved in 100μl Luria broth and plated onto supplemented Luria Bertani agar plates [Ampicilin 100μg, 5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside(X-gal) 80 μg/mL and isopropyl B-D-thiogalacto-pyranoside (IPTG) 0.5mM], and incubated overnight at 37°C. The recombinant clones were identified by blue/white colony assay.

Sequencing and in silico analysis

The insert was sequenced using T7 and Sp6 primer pair. Homology search was conducted using BLAST search available at (http://www.ncbi.nlm.nih.gov/BLAST). *In silico* translation was determined using NCBI BLAST by selecting the CDS feature and pair wise alignment in BLAST option. Potential N-glycosylation sites were analyzed by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Molecular weight, theoretical pI and amino acid composition were analyzed by ProtParam tool (http://us.expasy.org/tools/protparam.html) (Gasteiger et al., 2005).
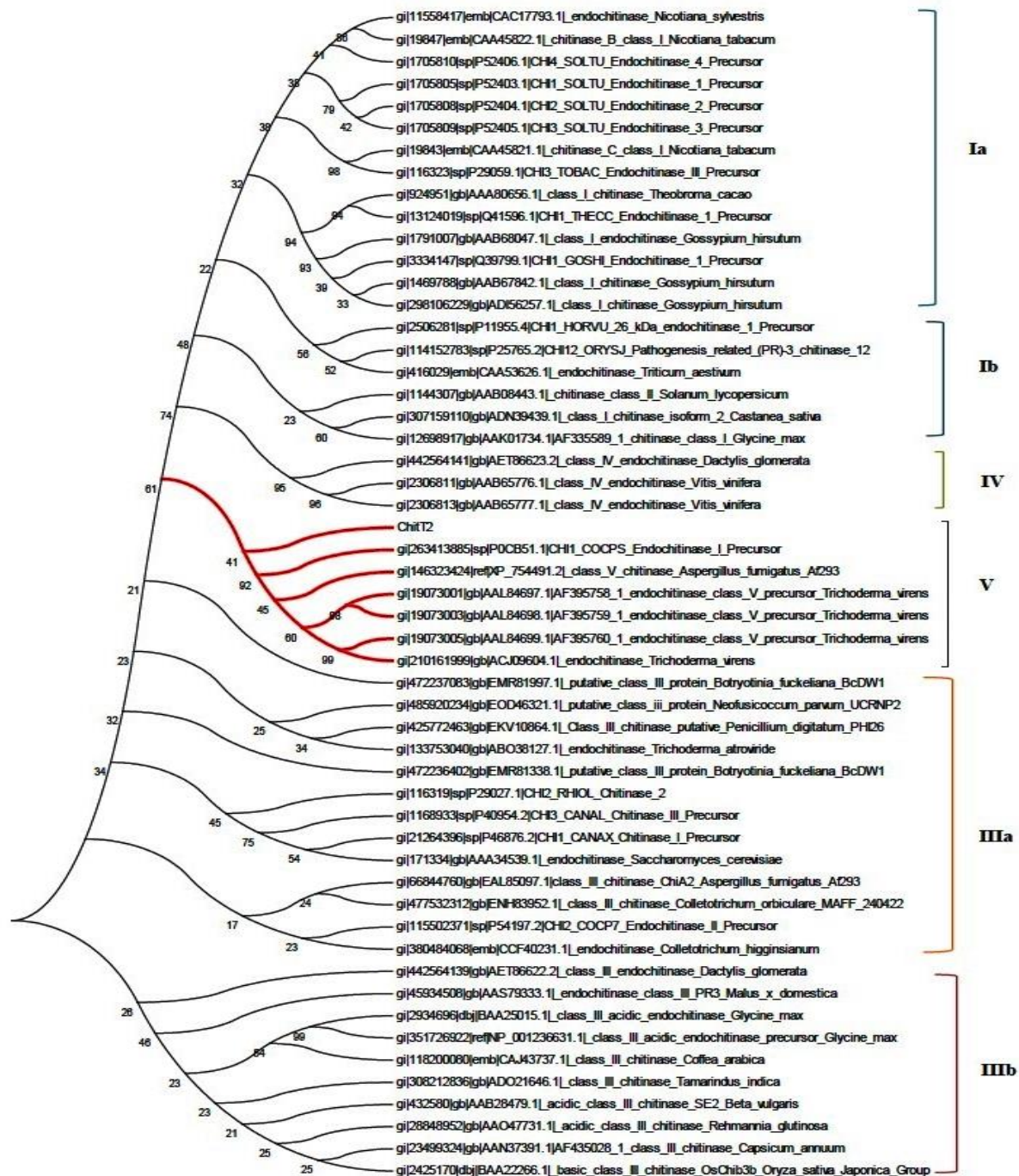
Fig. 5 Phylogenetics analysis of endochitinases from various organisms. The evolutionary history was inferred using Maximum Parsimony. The most parsimonious tree with length = 707 is shown in Fig. The evolutionary distances were computed using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) and are in the units of the number of amino acid substitutions per site. The analysis involved 53 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 61 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.10 (Tamura et al., 2012).

Putative signal peptide sequence was predicted using SignalP (Version 4.1) based on neural networks (NN) and Hidden Markov Models (HMM) trained on eukaryotes. InterProScan modular architectural analysis programs (http://www.ebi. ac.uk/Tools/pfa/iprscan/) was used to predict the domain architecture of the proteins identified as chitinase-like (Znobnov and Apweiler, 2001). Multiple alignment for homology search was performed using Multialin (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin. html).

To investigate the evolutionary relationship among the endochitinase proteins in the database and the putative endochitinase isolated in this study, a phylogenetic analysis was performed via MEGA 5.10 (http://www.megasoftware. net/) (Kumar et al., 2004). Location and number of helixes and sheets are shown in a 2D representation by Psipred (http://bioinf.cs.ucl.ac.uk/psipred/) (McGuffin et al., 2000) and a 3D model of the protein was built using I-TASSER http://zhanglab.ccmb.med.umich.edu/I-TASSER (Zhang, 2008; Roy et al., 2010; Roy et al., 2012).
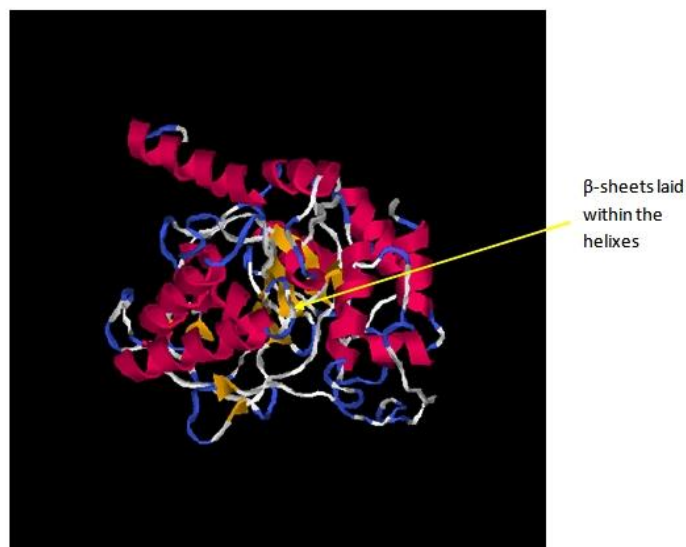
Fig. 6 The 3D Model of ChitT2. 3D structure of ChitT2 showing the 8 β-sheets laid in the middle surrounded by the helixes (as indicated by arrow). The 3D modeling of ChitT2 used the following PDB structures in prediction: 3n11A, 3n12A, 4axnA, 4ay1A, 3fxyA. The top 10 alignments (in order of their ranking) are from the following threading programs: 1: MUSTER, 2: SP3, 3: HHSEARCH, 4: SP3, 5: SP3, 6: SPARKS, 7: PROSPECT2, 8: PPA-I, 9: HHSEARCH I and 10: FFAS03. The model presented was selected based on the highest C-score of -0.79 (max 2). The C-score predicts the confidence score for estimating the quality of models by I-TASSER. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. In addition the Z-score was also taken into consideration where a normalized Z-score >1 means a good alignment.

Conclusion

Based on the overall *in silico* evaluation of ChitT2, we believe that this particular protein belongs to the family 18 hydrolases as it contains a number of conserved repeats of amino acids (Gluc, Asp and Tyr) known to be conserved in family 18 endochitinases. The presence of the signature 8 β sheet structures and the layout of the sheets within the helixes further substantiate the classification into family 18. ChitT2 has a multi domain structure which includes a catalytic domain, cysteine-rich chitin-binding domain and a serine/threonine-rich glycosylated domain. This multi domain structure is characteristic of chitinases in various animals and microorganisms (Tellam, 1996). Based on the domain analysis, structural and evolutionary classification, the ChitT2 is likely a Class V fungal endochitinase.

Acknowledgements

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