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Short Communication

Three Dimensional Structure Prediction of Recombinant Endochitinase from *Trichoderma virens* UKM-1

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ABSTRACT

Chitinases (EC 3.2.11.14) are capable of hydrolyzing chitins by splitting their ß-1,4-glucosidic bonds. They are present in a wide range of organisms including the fungus *Trichoderma virens* UKM-1. A gene encoding endochitinase from *Trichoderma virens* UKM-1 had successfully been cloned (with GenBank Accession number DQ 865246) and expressed in *Escherichia coli* BL21 (DE3). As a member of glycosyl hydrolases, chitinases are assumed to have a similar catalytic mechanism and structure as other enzymes such as lysozyme. A predicted three dimensional (3D) structure of endochitinase derived from *Trichoderma virens* UKM-1 was successfully constructed using the Swiss-Prot model server and analyzed by PyMOL software. The prediction of the structure was done by comparing *T. virens* UKM-1 endochitinase with seven published 3D structures of chitinases from the Swiss-Prot database. Recombinant endochitinase from *T. virens* UKM-1 was shown to have a TIM-barrel structure with eight parallel ß-sheets and eight α-helices laid down in the inner barrel together with three-stranded ß-sheets. These characteristics revealed the aspects of the catalytic centers of family 18 chitinases. An extensive study was done on the multiple sequence alignment of various class V, family 18 chitinases by using DNASIS Software. Two conserved consensus motif boxes SxGG (Box 1) and DxxDxDxE (Box 2) were found at the N-terminal amino acid sequence of endochitinase from *T. virens* UKM-1 which were involved in catalysis.

Keywords: Catalytic domain, recombinant endochitinase, three dimensional prediction structure, *Trichoderma virens*

ABSTRAK

Kitinase (EC 3.2.11.14) berupaya menghidrolisis kitin dengan memutuskan ikatan ß-1,4-glikosidik yang wujud pada sebilangan organisma termasuk kulat, *Trichoderma virens* UKM-1. Gen yang mengkodkan endokitinase telah berjaya dipencilkan dari *Trichoderma virens* UKM-1 dan dizahirkan di dalam *E. coli* BL21 (DE3) dengan nombor pendaftaran GenBank DQ 865246. Kitinase dikelaskan di bawah kumpulan glikosil hidrolase, oleh itu kitinase dianggap mempunyai mekanisma katalitik dan struktur tiga dimensi (3D) yang sama seperti sebahagian enzim, sebagai contohnya lisozim. Melalui

kajian ini, struktur tiga dimensi bagi endokitinase daripada *Trichoderma virens* UKM-1 dibentuk daripada jujukan asid amino hasil daripada analisis Pengkalan Swiss-Prot serta perisian PyMOL. Struktur tersebut dibentuk berdasarkan analisis perbezaan antara endokitinase dari *Trichoderma virens* UKM-1 dengan tujuh struktur kitinase lain dalam simpanan rangkaian data Swiss-Prot. Endokitinase rekombinan daripada *Trichoderma virens* UKM-1 menunjukkan struktur tong-TIM dengan lapan kepingan-ß selari serta lapan α-heliks berada di tong dalaman dengan tiga jalinan kepingan-ß di luar. Ciri-ciri katalitik seperti ini membuktikan ia tergolong dalam kumpulan 18 kitinase. Kajian lanjut telah dibuat melalui kaedah penjajaran berbilang jujukan ke atas beberapa enzim kitinase dari kelas V kumpulan 18, dengan menggunakan perisian DNASIS. Hasil dari kaedah tersebut didapati terdapat dua motif yang mempunyai jujukan amino asid yang sama, iaitu SxGG (Kotak 1) dan DxxDxDxE (Kotak 2) di terminal-N, yang berkait rapat dengan aktiviti katalitik enzim tersebut.

Kata kunci: Domain katalitik, endokitinase rekombinan, struktur ramalan tiga dimensi, *Trichoderma virens*

INTRODUCTION

Chitinases (EC 3.2.11.14), catalyze the hydrolysis of chitin, a homopolymer of ß-1,4-linked of N-acetylglucosamine (GlcNAc). These enzymes are widely distributed in nature and play an important role in the degradation of chitin which is present in different organisms, mainly arthropods and fungi. The classification system of chitinase is according to their amino acid sequence similarity. According to the system, chitinases are grouped into two families of glycosyl hydrolase, family 18 and family 19. Family 18 contains chitinases from bacteria, viruses, animals, fungi and some plants (class III and class V) while family 19 is from plant origin except some which are from *Streptomyces* or *Caenorhabditis elegans* (Zhang, 1999; Brurberg *et al*., 2000; Patil *et al*., 2000).

Enzymes exhibit their function when the proteins fold properly to form appropriate three dimensional (3D) structures. Thus, the information on the three dimensional structure is essential in order to study and understand the catalytic mechanism of the enzymes. Currently, X-ray crystallography and NMR spectroscopy are important strategies for obtaining three-dimensional structure of macromolecules. Among the chitinolytic enzymes other than avian lysozymes, crystal structure of 26 kDa chitinase from barley seeds, *Horduem vulgare*, which is classified into family 19 was first solved by Robertus and his colleagues at the University of Texas (Hahm *et al*., 1995). Moreover, the structures of 60 kDa chitinase A from *Serratia marcescence* and a 29 kDa chitinase from *Hevea brasiliensis* (hevamine) were solved and reported in similar publication. Both are from the family 18 chitinase enzymes (Zhang, 1999).

Previously, Alias *et al.* (2009) had successfully cloned the endochitinase gene from *Trichoderma virens* UKM-1 and expressed in *E. coli* BL21 (DE3). In this paper, we describe the prediction of the three dimensional structure of recombinant endochitinase from *Trichoderma virens* UKM-1 and compared it with other published structures from family 18, class V chitinases.

MATERIALS AND METHODS

Recombinant Endochitinase from *Trichoderma virens* UKM-1

The recombinant construct containing endochitinase cDNA, pET22b-ech1 was previously cloned into *E. coli* BL21 (DE3) and acted as the template for structure prediction (Alias *et al*., 2009). Both the DNA and its mRNA sequence were submitted to GenBank with Accession number DQ865247 and DQ 865246, respectively.

3D Structure Prediction of Endochitinase

The 3D structure of *Trichoderma virens* UKM-1 endochitinase was predicted using Swiss-Model Repository and was analyzed with PyMOL software. The predicted structure was done by comparing *T. virens* UKM-1 endochitinase with a few published 3D structures of chitinases from the Swiss-Prot database as listed in Table 1. The crystal structure of 1W9P from *Aspergillus fumigatus* was chosen as the template to predict the structure of endochitinase from *Trichoderma virens* UKM-1.

Table 1. List of class V chitinase structures from *Aspergillus fumigatus* available in the Swiss-Prot database.

PDB no.	Protein description
1W9P	Chitinase B1 complex with natural product cyclopentapeptide inhibitor
1WNO	Native chitinase structure
1W9V	Chitinase B1 complex with argifin
2A3E	Chitinase B1 complex with allosamidin
2A3B	Chitinase B1 complex with caffeine
1W9U	Chitinase B1 complex with argadin
2A3A	Chitinase B1 complex with theophylline

Multiple Sequence Alignment

Multiple sequence alignment of various class V, family 18 chitinases (Figure 6) was carried out using DNASIS software. The approach was to locate, identify and analyze the conserved region, catalytic and chitin binding domains present in the region.

RESULTS AND DISCUSSION

Structure Prediction of *T. virens* UKM-1 Endochitinase

Predicted 3D structure of *Trichoderma virens* UKM-1 endochitinase (Figure 1) with its surface electron density map (Figure 2) was constructed from the amino acid sequence sent to the Swiss-Model Repository and was analyzed with PyMOL software. The predicted 3D structure was done based on the *Aspergillus fumigatus* chitinase B1 (1W9P) as template and which belonged to the same family 18 and class V chitinase as endochitinase.

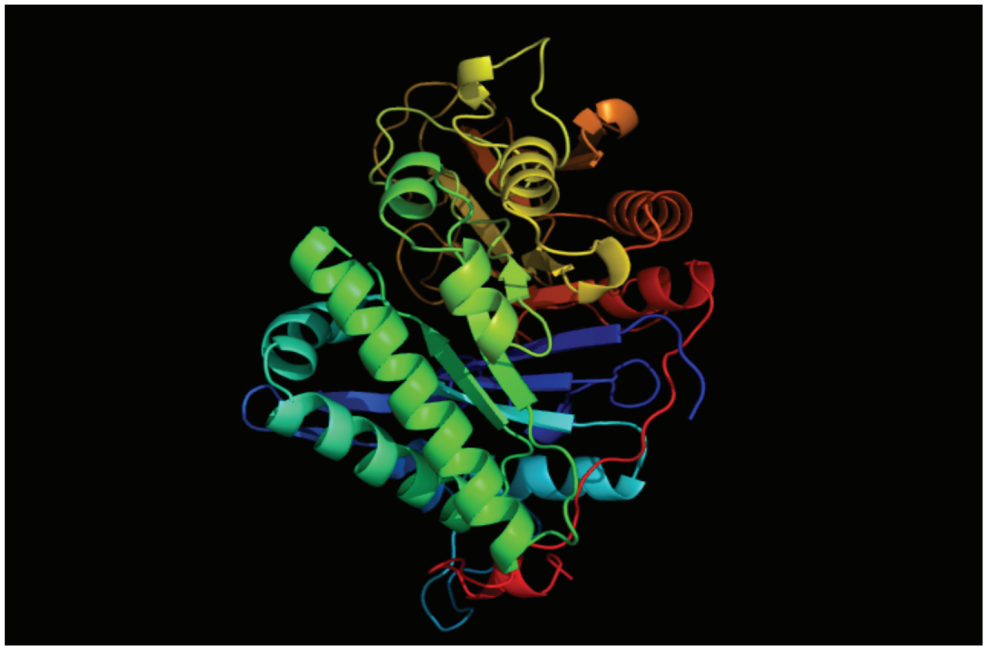

Fig. 1. Predicted 3D structure of recombinant endochitinase from *Trichoderma virens* UKM-1 by SWISS-PROT

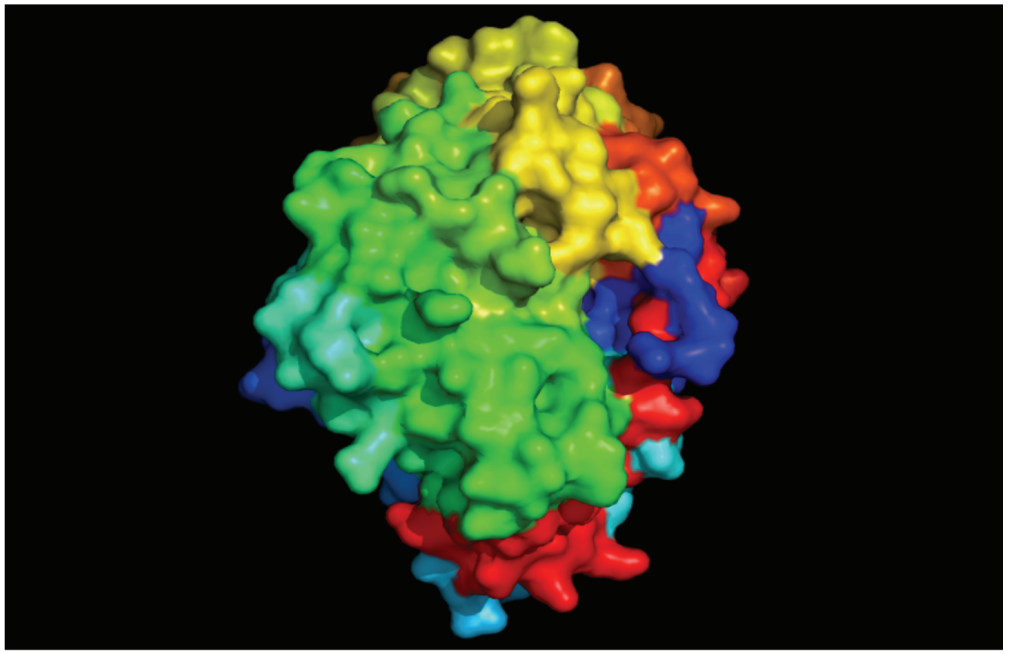

Fig. 2. Surface electron density map of the recombinant endochitinase from *Trichoderma virens* UKM-1

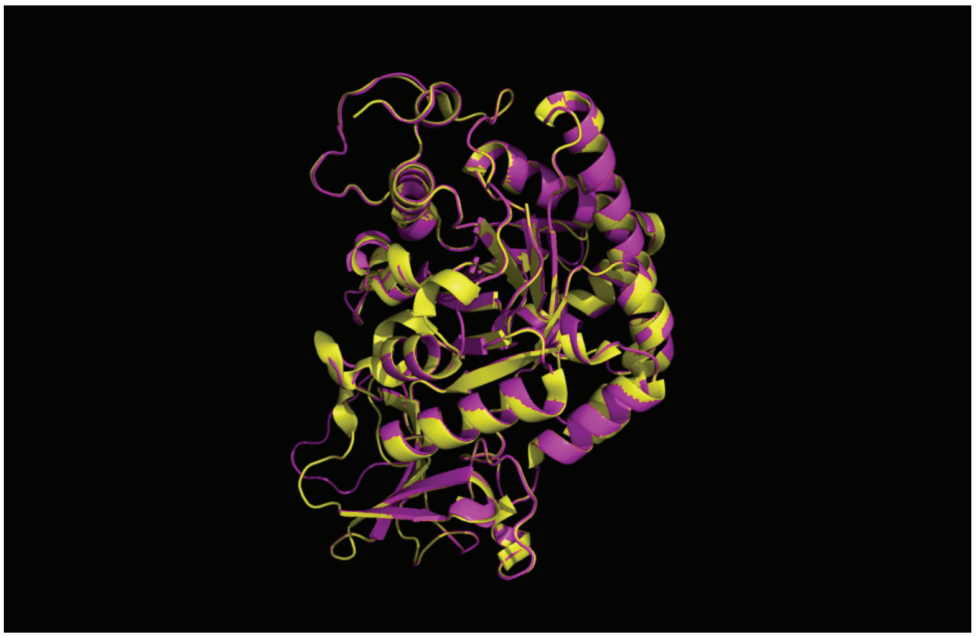

Fig. 3. Superimposed 1W9P structure (template) in yellow and predicted endochitinase structure from *Trichoderma virens* UKM-1 in purple

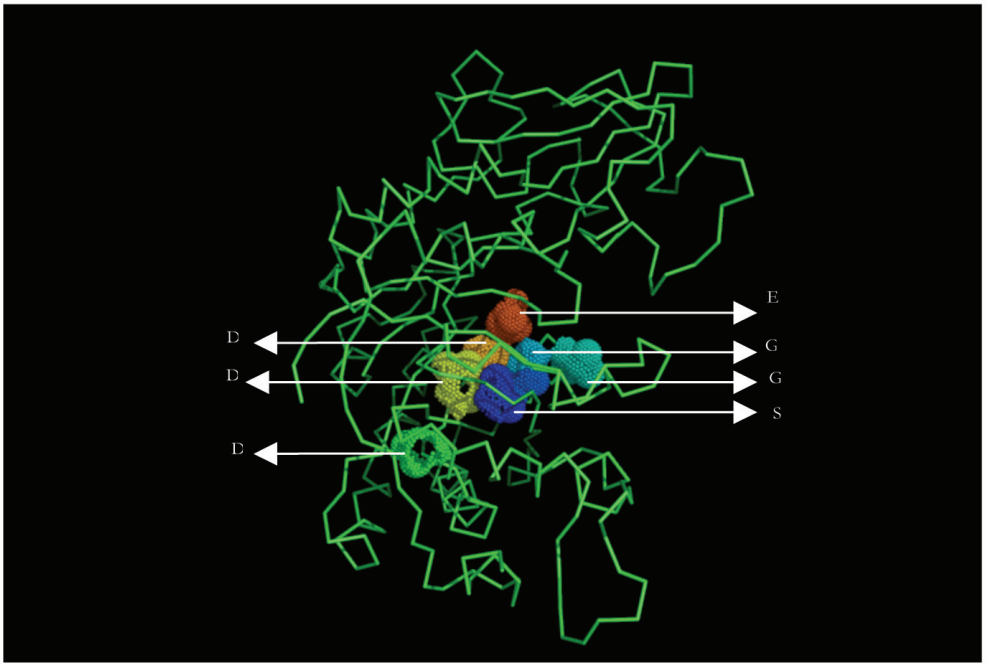

Fig. 4. Location of SxGG (Box 1) and DxxDxDxE (Box 2) motifs in the structure of recombinant endochitinase from *Trichoderma virens* UKM-1

Data retrieved from the Swiss-Model Repository showed 56% of the structure was identified to the template as shown in the superimposed figure (Figure 3). Besides that, amino acid alignments of both the template and endochitinase from *T. virens* UKM-1 also confirmed the result as shown in Figure 5. Furthermore, the structure was also compared with other published 3D structures of Class V chitinases from Swiss-Prot database in order to refine the final structure of the protein itself.

Crystal structures of one domain family 18 chitinase hevamine from *Hevea brasiliensis* (Van Scheltinga *et al.*, 1994) and the multi domain of *S. marcescens* ChiA were the landmark research in family 18 chitinases. The predicted structure of *T. virens* UKM-1 endochitinase has a TIM-barrel structure with eight parallel ß-sheets and eight α-helices laid down in the inner barrel together with three-stranded ß-sheets (Figure 1). This characteristic revealed the aspects of the catalytic centers of family 18 chitinases as stated by Zhang (1999), Fukamizo (2000), Brurberg *et al.* (2000) and Ikegami *et al.* (2000).

Since 1994, more structures of family 18 chitinases have been published such as chitinase from barley seeds, *Hordeum vulgare* (Hart *et al.*, 1995), structure of hevamine combined with chitinase and lysozyme from plant, *Hevea brasiliensis* (van Scheltinga *et al.*, 1996), and the catalytic domain of Chitinase A1 from *B. circulans* (Matsumoto *et al.*, 1999). Later, a complete structure of one-domain chitinase from fungus *Coccidioides immitis* (Hollis *et al.*, 2000) and the complete structure of the two-domain chitinases (ChiB and ChiA) from *S. marcescens* endochitinase were also reported (Van Aalten *et al.*, 2000).

Chitin Catalytic Domain

From the multiple sequence alignment of various classes V, family 18 chitinases, two conserved consensus motif boxes (Box 1 and Box 2) were found at the N-terminal of endochitinase from *T. virens* UKM-1 (Figure 4). The conserved region which comprised of serine (S) and glycine (G) as Box 1, are known to be hydrophilic and hydrophobic amino acids that are responsible for reacting with the surface of chitin molecules during a hydrolysis reaction. While in Box 2, glutamic acid (E) and aspartic acid (D) residues dominate the motif box which are both acidic and negatively charged amino acids. As reported by Watanabe *et al.* (1993), glutamic acid in this context acts as a general acid catalyst, which donates a proton to the glycosidic oxygen while aspartic acid stabilizes the transient carbonium ion intermediate electrostatically by lowering the energy barrier of the reaction. The mechanism was confirmed by chemical modification (Lin and Koshland, 1969) and site-directed mutagenesis of the proposed catalytic residues (Malcolm *et al.*, 1989).

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TARGET    36      ANGYANS VYFTNWGIYE RNFQPADLVA SDVTHVIYSF MNLQADG-TV
1W9P      39    a--ssgyrsv vyfvnwaiyg rnhnpqdlpv erlthvlyaf anvrpetgev

TARGET    82    VSGDTYADFE KHYADDSWND VGTNAYGCVK QLFKVKKANR GLKVLLSIGG
1W9P      87    ymtdswadie khypgdswsd tgnnvygcik qlyllkkqnr nlkvllsigg

TARGET    132   WTWSTNFPSA ASTDANRKNF ARTAITFMKD WGFDGIDVDW EYPADSTQAS
1W9P      137   wtyspnfapa astdagrknf aktavkllqd lgfdgldidw eypendqqan

TARGET    182   NMILLLKEVR SQLDAYAAQY APGYHFLLTI AAPAGKDNYS KLRLADLGQV
1W9P      187   dfvlllkevr taldsysaan aggqhflltv aspagpdkik vlhlkdmdqq

TARGET    232   LDYINLMAYD YAGSFSPLTG HDANLFANPS NPNATPFNTD SAVKDYIKGG
1W9P      237   ldfwnlmayd yagsfsslsg hqanvyndts nplstpfntq taldlyragg

TARGET    282   VPANKIVLGM PIYGRSFQNT AGIGQTYNGV GGGGGGSTGS WEAGIWDYKA
1W9P      287   vpankivlgm plygrsfant dgpgkpyngv ------gqgs wengvwdyka

TARGET    332   LPRAGATIKY DDVAKGYYSY NSNTKELISF DTPDMINTKV AYLKSLGLGG
1W9P      331   lpqagatehv lpdimasysy datnkflisy dnpqvanlks gyikslglgg

TARGET    382   SMFWEASADK KGTDSLIGTS HRALG---SL DSTQNLLSYP NSKYDNIRKG
1W9P      381   amwwdsssdk tgsdslittv vnalggtgvf eqsqneldyp vsqydnlrng

TARGET    429   LK
1W9P      431   mqt
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Fig. 5. Amino acid sequence alignment between endochitinase from *Trichoderma virens* UKM-1 (Target) with Chitinase B1 complex with natural product cyclopentapeptide inhibitor from *Aspergillus fumigatus* (1W9P) which served as the template for protein structure prediction.

The two motif boxes were also observed in chitinase-1 (CiX1) of the human fungal pathogen, *Coccidioides immitis* (Hollis *et al.*, 2000). These residues lie down in the TIM barrel and form the active site cleft on the carboxyl end of the ß barrel (Zhang, 1999; Hollis *et al.*, 2000). The clustering of both motif boxes and other conserved residues make the active site cleft easy to identify. The motifs in the 3D structure of *T. virens* UKM-1 endochitinase are shown in Figure 4. Analyses of the primary sequences of ChiA, ChiB and ChiC from *S. marcescens* indicated the existence of the same two motif boxes that comprised of SxGG (Box 1) and DxxDxDxE (Box 2) at the catalytic domain site (Zhang, 1999; Brurberg *et al.*, 2000). These motifs are identified as characteristics of family 18 glycosyl hydrolase which are involved in the catalytic reaction of endochitinases.

Active Site Residues

The conserved region, comprised of the catalytic domain, contains a glutamic acid residue (Glu172) located at the barrel of the *T. virens* UKM1 endochitinase. The active site residues of class V, family 18 chitinases were first reported by Watanabe *et al.* (1993) which indicated that glutamic acid residue plays an important role in the catalytic domain of chitinase A1 from *Bacillus circulans* WL-12. The same findings were also reported by Ueda *et al.* (1998), Zhang (1999), Hollis *et al.* (2000) and Kim *et al.* (2002). A summary of the active site region of some chitinase producers are shown in Table 2.

Table 2. Active site region of some chitinase producers (Gooday, 1994). Amino acid numbering is based on *Trichoderma virens* UKM-1 endochitinase gene sequence as stated in GenBank.

Sources	Active site region
Chitinase from fungus *Trichoderma virens* UKM-1 *Trichoderma harzianum* *Aphanocladium album*	164:FDGIDVDWEYP:174 FDGIDVDWEYP FDGIDIDWEYP
Chitinase A1 from prokaryotes *Bacillus circulans*	FDGVDLDWEYP
Chitinase from plant *Nicotiana tabacum* class V	FHGLDLDWEYP
Chitinase from insect *Manduca sexta*	FDGLDLDWEYP
Chitinase from nematode *Brugia malayi*	FDGFDLDWEYP
Chitinase from virus *Autographa californica*	FDGVDIDWEYP
Human chitinase from macrophage Chitotriosidase	FDGLDLDWEYP

Substitution of glutamic acid (Glu204) to glutamine at the active site in chitinase A1 from *B. circulans* WL-12 completely eliminated the enzyme activity. This residue is suggested to be involved as a proton donor in the catalysis of the enzyme (Fukamizo, 2000; Ikegami *et al*., 2000; Watanabe *et al.,* 1993). This glutamic acid corresponds to Glu172 in *T. virens* UKM-1 endochitinase. Point mutation on chitinase-1 (CiX1) from *Coccidioides immitis* indicated that substitution of Glu171 to Gln completely inactivated the enzyme, which was in agreement with mutagenic data of a bacterial chitinase A1 from *B. circulans* WL-12 (Hollis *et al*., 2000).

Moreover, site-directed mutagenesis of two putative catalytic residues of Chit42 from *T. harzianum* were carried out to both Glu172 and Asp170 sites (Boer *et al*., 2007). The mutations were made by changing the Glu172 to Gln172 and Asp170 to Asn170. The kinetic analysis of both mutants showed that the activity of the Glu172Gln172 mutant had dropped to 0.3% and the Asp170Asn170 mutant to 0.6% of the original activity, thus confirming their important roles in the catalysis (Boer *et al*., 2007).

The negatively charged amino acids Asp165, Asp168 and Asp170 (DxxDxD) which are common to class V chitinase were also found in Box 2 endochitinase from *T. virens* UKM-1 as well as in the other aligned chitinase sequences in Figure 6. This aspartic acid triad (DxxDxD) also exists in chitinase A1 of *B. circulans* WL-12. Substitution of aspartic acid residues, Asp200 and Asp202 in *B. circulans* WL-12 chitinase A1 was found to impair the enzymatic activity but did not completely eliminate the activity (Fukamizo, 2000).

Comparison of the three-dimensional model structure of *T. harzianum* Chit42 with the solved structure of *Coccidioides immitis* CiX1 and *Serratia marcescens* chitinase A, revealed several conserved amino acid residues interacting with the substrate which were Glu172, Asp170 as well as nearby residues Asp168, Asp241, Tyr44 and Tyr240 (Hollis *et al*., 2000; Boer e*t al*., 2007). The study by Boer *et al.* (2007) revealed that Glu172 of Chit 42 from *T. harzianum* was proposed to act as the general acid/base while Asp170 was postulated to stabilize the positively charged reaction intermediates where both of them were important residues in catalytic reaction. Both of these amino acids were also conserved in *T. virens* UKM-1 endochitinase as shown in Figure 6.

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UKM-1   :    118   KANRGLKVLLSIGGWTWSTNFPSAASTDANRKNFARTAITFMKDWGFDGI   :167
TV      :    118   KANRGLKVLLSIGGWTWSTNFPSAASTDANRKNFARTAITFMKDWGFDGI   :167
TA      :    118   KANRNLKVMLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGI   :167
TH      :    118   KANRGLKVLLSIGGWTWSTNFPSAASTDANRKNFARTAITFMKDWGFDGI   :167
TZ      :    118   KPTHLKFLSMVVDWPQLPSEN--AASTDATRKNFAKTAITFMKDWGFDGI   :165
TR      :    118   KANRNLKVMLSIGGWTWSTNFPSAASTDANRKNFAKTAITFMKDWGFDGI   :167

UKM-1   :    168   DVDWEYPADSTQASNMILLL   :187
TV      :    168   DVDWEYPADSTQASNMILLL   :187
TA      :    168   DVDWEYPADDTQATNMVLLL   :187
TH      :    168   DIDWEYPADSTQAANMVLLL   :187
TZ      :    166   DVDWEYPADDTQATNMVLLL   :185
TR      :    168   DVDWEYPADDTQATNMVLLL   :187
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Fig. 6. Alignment of putative active site regions in family 18 glycosyl hydrolases. The amino acid numbering is based on *Trichoderma virens* UKM-1 endochitinase gene sequence (DQ865247). Amino acids contained in Box 1 and Box 2 is as underlined. Important amino acids (DxxDxDxE) and (SxGG) in both boxes are bold. UKM-1: *Trichoderma virens* UKM-1 endochitinase; TV: *Trichoderma virens* class V chitinase (AF397020); TA: *T. atroviride* strain DAOM 165779 (AF188920); TH: *T. hamatum* chit-HAM (AB041754); TZ: *T. harzianum* endochitinase (AJ605116); TR: *T. viride* chitinase (AF208842).

Research done by Gustav *et al.* (2004) had shown that Asp142 in ChiB from *Serratia marcescens* is important for catalysis since the mutation to alanine abolished enzyme activity. However, substitution of Asp142 with Asn also decreased the activity but to a lesser extent. As previously reported, Asn (N) in the sequence N-X-S/T are usually N-glycosylated (Hayes *et al*., 1994). However, according to Mononen and Karjalainen (1984), glycosylation was blocked when the amino acid X was a proline residue. One such site was also found in the endochitinase gene of *T. virens* UKM-1 which is N-Y-S at amino acid positions 219-221.

CONCLUSION

Endochitinases from *T. virens* UKM-1 consisted of two conserved consensus motif boxes (Box 1 and Box 2) which were found at the N-terminal of the protein. The conserved region is comprised of the chitin hydrolysis domain which contains serine (S) and glycine (G) residues in Box 1, and glutamic acid (E) and aspartate (D) residues in Box 2. These motifs are typical characteristics of family 18 glycosyl hydrolase which is involved in the catalytic reaction of the enzyme. Catalytic domains of family 18 chitinases have a TIM-barrel structure with eight parallel ß-sheets laid down in the inner barrel together with eight α-helices and three-stranded ß-sheets which revealed the aspects of the catalytic centers. The conserved region which comprises the catalytic domain contained a glutamic acid residue (Glu172) located at the active site down in the barrel. The negatively charged amino acids Asp165, Asp168 and Asp170 (DxxDxD) which are common to class V chitinase were also found in Box 2 of endochitinase (*ech1*) from *T. virens* UKM-1 as well as in most of the other chitinase sequences.

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