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Full Length Research Paper

Expression and characterization of the recombinant *Trichoderma virens* endochitinase Cht2

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An endochitinase, Cht2, from *Trichoderma virens* UKM1 was expressed in the methylotrophic yeast *Pichia pastoris,* and its biochemical properties were characterized. Both the cht2 gene and its cDNA have been cloned and sequenced, the endochitinase gene *cht2* encodes 321 amino acids from an open reading frame comprised of an 1169 bp nucleotide sequence separated by three introns. Cht2 is predicted to be an extracellular enzyme due to the presence of a signal peptide of 20 amino acids. Cht2 cDNA was cloned into the pPICZαC expression vector under the regulation of a methanol-inducing promoter and transformed into *P. pastoris* X33. Expression in *P. pastoris* showed that the recombinant Cht2 was secreted into the culture medium with a protein size of approximately 35 kDa when induced with 0.5% methanol. Biochemical characterization of the partially purified enzyme showed a specific enzyme activity of 1.34 U/mg towards colloidal chitin at a pH of 6.0 and at a temperature of 35°C. The enzyme showed optimal activity at this pH and temperature and also showed higher affinity toward colloidal chitin in comparison to glycol chitin. It is stable in the pH range of 5.0 - 7.0 and in the temperature range of 30 - 55°C, where it retained more than 70% of its residual activity.

Key words: Endochitinase, *Trichoderma virens*, recombinant, characterization.

INTRODUCTION

Chitin is one of the most abundant polysaccharides found in nature and is composed of linear chains of -1,4-N-acetyl glucosamine (GlcNAc). It is an important structural element in many organisms, such as insect exoskeletons, crustacean shells and fungal cell walls (Merzendorfer, 2006). Chitin and chitin derivatives, such as chitosan, glucosamine and N-acetylglucosamine, have commercial potential due to their high nitrogen content compared to other polysaccharides, such as cellulose (Kumar, 1999). They can be used in the paper production industry, textile finishes, photographic products, the treatment of water and agricultural waste, dietary supplements and pharmaceutical and biomedical applications (Prashanth and Tharanathan, 2007; Muzzarelli, 2008).

Chitinases are chitin-degrading enzymes that hydrolyze the -1,4-glycosidic bonds between the N-acetyl glucosamine residues of chitin and are widely distributed in nature. There are many chitinase-producing organisms including bacteria (Ningthoujam et al., 2009), insects (Merzendorfer and Zimoch, 2003), plants (Salami et al., 2008), fungi (Rattanakit et al., 2007) and vertebrates (Tunc et al., 2008). The roles of chitinases in these organisms are diverse. In bacteria, chitinases play roles in nutrition and parasitism (Sorbotten et al., 2005; Ningthoujam et al., 2009), whereas in fungi, they participate in morphogenesis, nutrition and parasitism (Duo-Chuan, 2006). Chitinases are considered a significant part of the defense system in plants and vertebrates (Salami et al., 2008; Tunc et al., 2008). In insects, chitinases aid in the degradation of old cuticle during the

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ecdysis process (Patil et al., 2000; Dahiya et al., 2006). Chitinases can be classified into two families, family 18 and 19, based on the amino acid sequence of their catalytic domains. While family 18 includes chitinases of viruses, fungi, bacteria, animals and some plants, family 19 includes all chitinases from plant origins and *Streptomyces griseus* (Patil et al., 2000; Dahiya et al., 2006).

Members of *Trichoderma* are some of the most studied fungi because of their potential in biological control, their ability to enhance the resistance of plants against pathogens and their ability to hydrolyze waste biomass (Brunner et al., 2005; Viswanathan et al., 2006; Alias et al., 2009). Chitinase secretion is one of the mechanisms used by *Trichoderma* to inhibit the growth of other fungi. *Trichoderma* chitinases belong to the glycosyl hydrolase family 18 and can be further grouped into class III and class V. Many chitinase genes from *Trichoderma* have been studied, including class III chitinases, such as *cht33* of *T. harzianum*, *cht33* of *T. atroviride* and *cht1* of *T. virens* and class V chitinase, such as *ech1*, *ech2* and *ech3* of *T. virens* (Kim et al., 2002; Markovich and Kononova, 2003; Duo-Chuan, 2006).

The use of *T. virens* UKM1 chitinases for the bioconversion of chitin waste to useful products has been explored and reported. Work by Abd-Aziz et al. (2008) showed that chitinases from *T. virens* UKM1 have a huge potential for the hydrolysis of chitin waste. Kim et al. (2002) demonstrate that *T. virens* strain 29 - 8 contains at least six chitinase-encoding genes in its genome, including the class III endochitinases *cht1* and *cht2* and the class V endochitinases *ech1*, *ech2* , *ech3* and *ech3b*. Some of these chitinases, such as Ech1, Ech3, Cht1 and Cht2, were predicted to contain a signal peptide, which indicates that they may be secreted from the fungus (Kim et al., 2002) . However, some, like Ech2, lack the signal peptide and may localize in the fungal cytoplasm and have different functions than the secreted chitinases. To elucidate the properties of *T. virens* endochitinases and to explore their potential in bioconversion of chitin waste, Alias et al. (2009) produced a recombinant Ech1, a class V endochitinase, from *T. virens* UKM1 and characterized its properties. To supplement this effort, in this study, a class III endochitinase from *T. virens* UKM1, Cht2, was chracterised. The objectives of this work are to produce the recombinant Cht2 endochitinase and elucidate its biochemical properties. Understanding the properties of this enzyme will enhance our knowledge of the chitin degradation system in this fungus.

MATERIALS AND METHODS

Fungal strain and plasmids

T. virens UKM1 was obtained from the fungal culture collection of the molecular mycology laboratory, School of Bioscience and Biotechnology, University Kebangsaan Malaysia. The plasmid pGEM-T Easy vector was purchased from Promega (USA). The expression vector pPICZ C and the expression host *Pichia pastoris* X33 were purchased from Invitrogen (USA).

Medium and culture condition

T. virens UKM1 cultures were maintained on potato dextrose agar (PDA) for 7 days at 30°C. For DNA isolation, the growing cultures were transferred into PDYE (Potato Dextrose Yeast Extract) broth and grown at 30°C in a shaking incubator at 180 rpm. After 3 days, the mycelia were harvested in sterile conditions, froze in liquid nitrogen and kept at − 80°C until further usage. For RNA extraction, *T. virens* was grown on PDA for 3 days at 30°C. Subsequently, spores were harvested and grown in 200 ml of Czapeck medium (3 g/l$NaNO_3$, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 0.01 g/l $FeSO_4$, 1 g/l KH_2PO_4) with 1.5% colloidal chitin as the sole carbon source. The culture was grown for 5 days at 30°C in a shaking incubator at 180 rpm. Then, the mycelia were harvested, frozen in liquid nitrogen, and kept at −80°C until further usage.

Cloning of *cht2* gene

Genomic DNA was extracted from the mycelia of *T. virens* UKM1 using the method described by Oh et al. (2009). Primers for cht2 amplification were designed using the *cht2* sequence from *T. virens* 29-8 (Kim et al., 2002). The forward primer used in this amplification was 5'-ATGCCTTCGTTGACTGCTCTCGC-3', and the reverse primer was 5'-TTATTTCAGAGCATTGACGACC- 3'. PCR amplifications were performed in total volume of 20 µl by mixing 500 ng of genomic DNA with 20 pmol of each primer, 4 mM of deoxynucleoside triphosphate, 2.5 mM of $MgCl_2$ and 0.5 U/µl of *Taq* DNA polymerase in 1 x PCR buffer. These reactions were subjected to initial denaturation for 5 min at 95°C, followed by 33 cycles of 2 min at 95°C, 1.5 min at 62.5°C, and 2 min at 72°C, with a final cycle of 20 min at 72°C for final extension. The products of PCR amplification were cloned into pGEM-T Easy vector (Promega, USA) and transformed into *Escherichia coli* DH5 . Plasmids were extracted using Wizard Plus SV Minipreps DNA purification Kit (Promega, USA), and the insert was sequenced using Big Dye^{TM} Terminator cycle sequencing ready reaction kit (PE Biosystems, USA), according to manufacturer instructions.

Cloning of *cht2* cDNA

RNA extraction was carried out on fungal mycelia using TRIzol reagent (Invitrogen, USA) as described by Oh et al. (2009). The primers of *cht2* gene amplification were used for cDNA synthesis. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplifications were carried out by using access RT-PCR kit (Promega, USA), according to manufacturer instructions. The same primer pair used for *cht2* amplification was used for its cDNA amplification.

These reactions were subjected to 45°C for 45 min to synthesize the cDNA, followed by 1 cycle of 2 min at 94°C and 35 cycles of 30 s at 94°C, 1 min at 62.5°C, and 2 min at 68°C, with a final extension of 7 min at 68°C. The products of RT-PCR amplification were cloned into pGEM-T Easy vector and transformed into *E. coli* DH5 . The cloned gene was sequenced and analyzed.

Nucleotide and amino acids sequence analysis

The software SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen et al., 2004) was used to predict the signal peptide of Cht2. ExPASY translate tools (http:// www.Expasy.ch; Gasteiger et al., 2003) were used to predict the amino acid sequence, the

molecular weight and the secondary structure of Cht2. BLAST analyses (NCBI; http://www.ncbi/BLAST; Altschul et al., 1997) were used to identify genes homologous to *cht2* sequences from *T. virens* and other species. ClustalW software (htpp://www.ch.embnet.org/software/ClustalW.html; Larkin et al., 2007) and BOXSHADE software (http:// www. ch. embnet. org/ software / BOX_form.html) were utilized to compare the Cht2 sequence with other chitinases available in public database.

Construction of expression vector

The cht2 cDNA was amplified without the sequence of the protein signal peptide and with addition of restriction sites before cloning into the pPICZ C expression vector. The restriction sites added to the primers were *Cla1* and *Xba1*, and the sequence of the forward primer was 5'-TCTGCCCTATCGATGTGGAATGC-3', while the sequence of the reverse primer was 5'-GTATCTAGATATTTCAGAGCATTG-3'. The resulting cDNA sequence was cloned in frame with the factor at the N-terminal and the six His tags at the C-terminal of pPICZ C to produce the expression cassette pPICZ C-Cht2. The plasmid was transformed into *E. coli* and plated on low- salt LB plates (0.5% NaCl, 0.5% yeast extract, 1% bacto- peptone) mixed with 50 µg/ml zeocin and incubated at 37°C overnight. Subsequently, the plasmid was extracted and linearized with *Pme*I before the expression cassette was transformed into *P. pastoris*.

***P. pastoris* transformation and selection**

A total of 10 µg of linearized pPICZ C-cht2 was transformed into *P. pastoris* X33 by electroporation. Transformants were selected on YPD with 100 µg/µl of zeocin. Zeocin^{+} transformants were repli-cated on minimal methanol (MM) plates, which contain 1.34% yeast nitrogen base (with ammonium sulphate and without amino acids), 0.5% methanol, 0.4 g/ml D-biotin, and 1% agar, to determine the Mut^{+} phenotype and on YPD containing different concentration of zeocin (500 - 2000 g/ml) to screen for the multicopy insertions. The confirmation of transformation was performed by PCR ampli-fication using primers AOXIF: 5'-GACTGGTTCCAATTGACAAGC-3' and AOXIR: 5'-GCAAATGGCATTCTGACATCC- 3'. The host and the transformed host with the vector (without cht2) were also amplified using AOX1F and AOX1R and used as controls. The template for the PCR reaction was a single colony picked and inoculated directly into the reaction. These reactions were subjected to an initial denaturation of 5 min at 95°C, followed by 30 cycles of 2 min at 95°C, 1.5 min at 67°C, and 2 min at 72°C, with a final cycle for 20 min at 72°C for the final extension.

Expression and purification of recombinant Cht2

The expression was carried out by following the *P. pastoris* expression manual (Invitrogen, USA) with some optimizations. Positive transformants were inoculated into 40 ml BMGYC medium (1.0% yeast extract, 2.0% peptone, 1.34% yeast nitrogen base without ammonium sulfate and without amino acids, 4 x 10^{-5}% biotin, 100 mM potassium phosphate, pH 6.0, 1.0% glycerol, and 1% casamino acid) and were grown at 30°C with shaking at 250 rpm.

The cells were harvested at OD_{600} of 18 - 20 by centrifugation and resuspended in 150 ml BMMY medium (1.0% yeast extract, 2.0% peptone, 1.34% yeast nitrogen base with ammonium sulfate and without amino acids, 4 x 10^{-5}% biotin, 100 mM potassium phosphate, pH 6.0, and 0.5% methanol) and were grown at 30°C with shaking at 250 rpm. Aliquots of the culture were removed after being induced with 0.5% methanol (v/v) for 24, 48, 72, 96 and 120 h. The concentration of the recombinant protein from the culture filtrate was performed by using Kvick Start Cassette (GE Healthcare, UK), which is a conventional cross-flow filtration system, followed by vivaspin with a molecular cut-off weight of 10 kDa (MWCO 10,000) (Millipore, Ireland). The protein concentration was measured by Bradford reagent (Bradford, 1976). Recombinant protein purification was achieved by using the immobilized metal ion affinity chromatography (IMAC) technique. This technique reflects the relationship between specific amino acids and particular metal ions. Amino acids, for example histidine, have the affinity to bind to some metal ions such as nickel. In this work, the recombinant endochitinase was cloned in-frame with the C-terminal of pPICZ C that includes the polyhistidine tag to facilitate the protein purification by the IMAC technique.

Molecular weight determination

The proteins were analyzed by SDS-PAGE electrophoresis (Laemmli, 1970) on a Thermo Mini Vertical Gel System (Major Science, Taiwan). The protein sample (30 - 40 µg) was mixed with an equal volume of 2X SDS sample buffer and was heated at 95ºC to denature the protein. The sample was loaded into the gel (12% resolving gel and 5% stacking gel) and run at constant voltage (170 V) for 1 h. Subsequently, the gel was stained with coomassie brilliant blue.

Assay of endochitinase activity

A standard curve representing different concentrations of N-acetyl glucosamine (NAG) was constructed using a range of NAG concentrations (1 - 5 µmol). The assay for NAG was carried out by a colorimetric method that was established by Miller (1959) using 3,5-dinitrosalicylic acid (DNS). Different concentrations of NAG were mixed with equal volumes of DNS solution, and samples were incubated in boiling water for 10 min. The absorbance was deter-mined using an ELISA reader at 540 nm. Recombinant Cht2 activity was measured using colloidal chitin as a substrate.

The reaction of the assay was prepared in 50 mM sodium phosphate buffer at pH 6, with 1% (w/v) colloidal chitin and 100 µg of recombinant Cht2. The reaction was incubated at 35°C for 30 min and stopped by incubation in boiling water for 5 min. Following that, the mixture was centrifuged at 12,000 rpm for 5 min. Sub-sequently, the supernatant was mixed with an equal volume of DNS solution to measure the reducing sugars that were produced from the enzymatic activity. Then, the mixture was incubated in boiling water for 10 min. The absorbance was read by an ELISA reader at 540 nm. One unit (U) of chitinase activity is defined as the hydrolytic rate of chitinase needed to release 1 µmol of N-acetyl-D-glucosamine in 1 min.

Effect of temperature and pH on enzyme activity

The optimal temperature of chitinase activity was determined by measuring product formation at pH 6.0 for a temperature range of 25 - 70°C. For the determination of temperature stability of the enzyme at pH 6.0, 100 µg of the recombinant Cht2 was pre-incubated at a temperature range of 25 - 70°C for 30 min and then assayed at 35°C with 1% (w/v) colloidal chitin as substrate.

To determine the optimum pH of the enzyme, 100 µg of the recombinant Cht2 was diluted in a range of solutions at pH values from 3 - 11 [citrate phosphate (pH 3.0 - 6.0), sodium phosphate (pH 6.0 - 8.0), Tris (pH 8.0 - 9.0), glycine- NaOH (pH 9.0 - 11.0) and assayed at that pH. The pH stability of recombinant Cht2 was determined by pre-incubation of the enzyme over a pH range of 3 - 11 for 30 min at 35°C and then assayed at pH 6.0 (the optimum

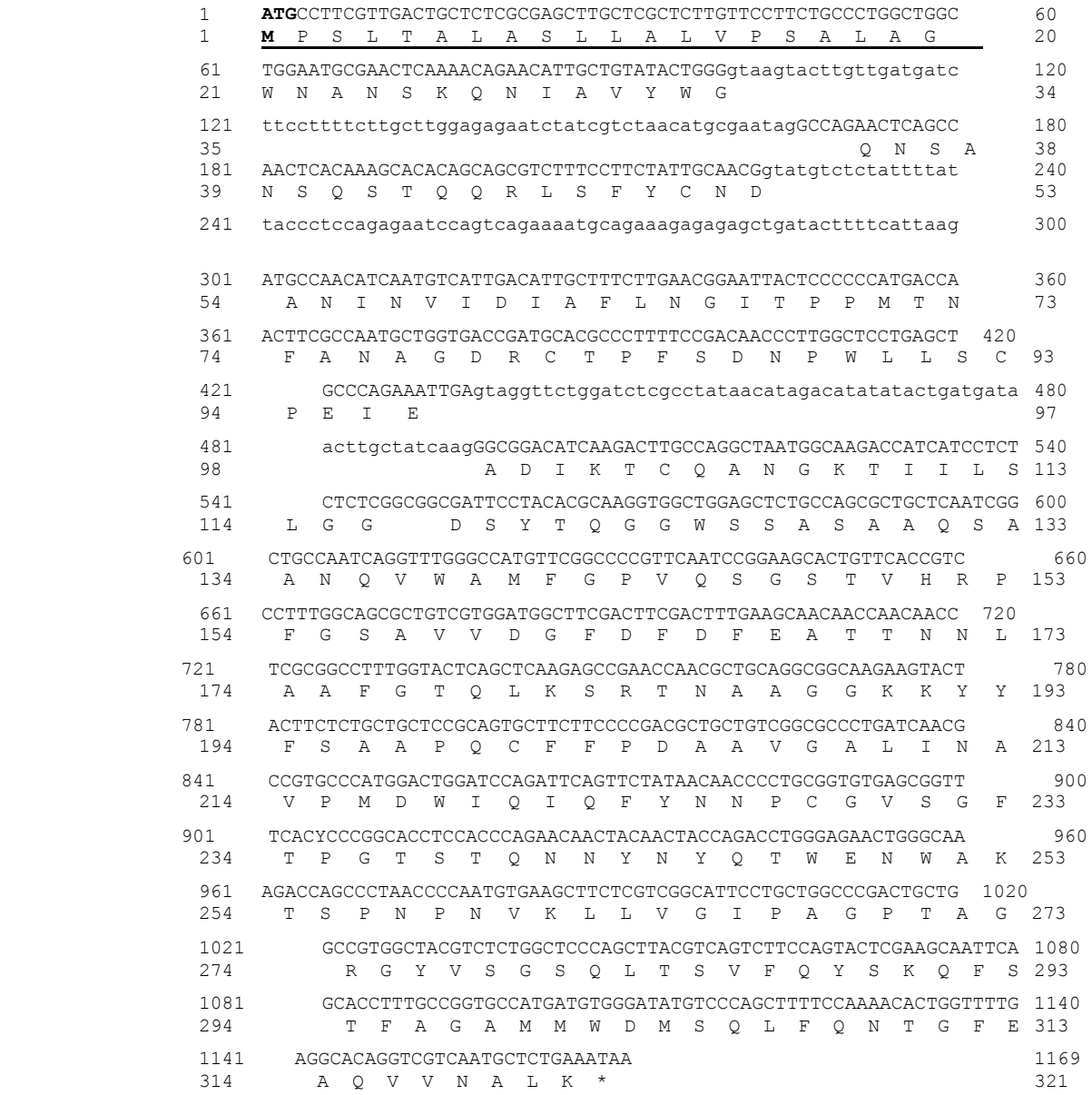

Figure 1. Nucleotide and deduced amino acid sequence (indicated below respective codons) of *T. virens* UKM1 *cht2* gene. The three introns are represented with lowercase letters. The start codon is in bold letters, and the stop codon is represented by an asterisk. The amino acid residues of the signal peptide are underlined.

pH) with 1% (w/v) colloidal chitin as substrate.

Substrate specificity

The affinity of recombinant Cht2 with a concentration of 100 µg was tested towards two different substrates, colloidal chitin and glycol chitin. The assays were carried out by using different concentrations of these substrates at 35°C and pH 6.0. Colloidal chitin concentrations were (0.05, 0.1, 0.5, 1, 2)% and glycol chitin concentrations were (0.125, 0.25, 0.5, 1, 1.5)%. The results were interpreted by the Michaelis-Menten constant (K_M), and the maximum velocity of substrate hydrolysis (Vmax) reflects the affinity of the enzyme to the substrate.

RESULTS AND DISCUSSION

Analysis of nucleotide and deduced amino acid sequence of Cht2

The gene and cDNA sequence of *T. virens* cht2 were amplified, cloned and sequenced. The alignment of the cht2 gene and its corresponding cDNA showed that the size of full-length *T. virens* UKM1 cht2 gene is 1169 bp and that the cht2 cDNA is 966 bp (Figure 1). The gene contains 3 introns of 66, 77 and 60 bp. The 966 bp of cht2 cDNA encodes for 321 amino acids. This sequence

was submitted to Genbank with the accession number GQ303455. Sequence alignment analysis between *cht2* of *T. virens* UKM1 and *T. virens* 29-8 (Kim et al., 2002) showed the presence of four nucleotide differences. However, these differences were silent mutations and did not change the amino acid sequence relative to one another. These differences in the nucleotide sequence could be due to strain variation between *T. virens* UKM1 and *T. virens* 29-8. Based on sequence analysis, Cht2 was predicted to have a signal peptide at the N-terminal end, which consists of 20 amino acids. The presence of a signal peptide suggests that this protein may be secreted into the culture medium.

Through sequence analysis, Cht2 can be grouped in the glycoside hydrolase family 18. Chitinolytic enzymes of family 18 are subdivided into two subclasses: class III that includes fungal and plant chitinases and class V that includes fungal and bacterial chitinases. The chitinases of class V are considered processive enzymes due to the special substrate binding site in these enzymes (Seidl, 2008) . This binding site is a deep, tunnel-shaped groove (Horn et al., 2006). The enzymes of this class associate with the substrate, keep the polymeric substrate inside the binding groove, and then release short-chained, 2-8-mer products. In contrast, chitinases of class III are non-processive enzymes and have a shallow, open substrate binding groove. At the end of the degradation process, the enzymes of this class dissociate from the substrate and release the product from the binding site. The released product can be long-chained, 6-30-mer products or multimeric products (Sorbotten et al., 2005; Horn et al., 2006).

Two potential functional domains that are commonly identified in chitinases of family 18 are the catalytic domain and the chitin binding domain (ChBD). The presence of the class III catalytic domain was detected in *T. virens* UKM1 (Figure 2). However, no conserved chitin binding domain was detected on Cht2. The catalytic domain is important for the protein to hydrolyze the substrate. Generally, in fungal class III chitinases, this domain has a conserved sequence, DXXDXDXE, which includes the glutamic acid residue that is essential for catalysis (Lu et al., 2002). Mutation of this glutamic acid in insect chitinases resulted in a loss of enzyme activity (Lu et al., 2002). Other studies suggested that the aspartic acid in this domain also plays an important role in the hydrolysis process. This role was observed when aspartic acid was mutated to asparagine and resulted in a decrease in catalytic activity (Vaaje-Kolstad et al., 2004). The sequence alignment of *T. virens* UKM1 Cht2 was performed with other *Trichoderma* class III chitinases including *T. virens* 29-8 Cht2, *T. harzianum* Chit33, *T. virens* Chi33, *T. atroviride* Chit33, *T. reesei* Chi18-12, *T. virens* Cht3 and *T. virens* Cht1. The results showed that Cht2 of *T. virens* UKM1 has 100% similarity with *T. virens* 29-8 Cht2, 95% with *T. harzianum* Chit33, 91% with *T. virens* Chi33, 92% with *T. atroviride* Chit33, 82% with *T. reesei* Chi18 - 12 and 60% with *T. virens* Cht3. This multiple alignment revealed the presence of a catalytic domain with a conserved active site of glutamic acid and aspartic acid in these chitinases (Figure 2).

Expression of recombinant endochitinase Cht2

The transformation of the expression cassette resulted in positive clones that were able to grow on both YPD supplemented with 2 mg/ml zeocin and MM media. The verification of positive clones was carried out by PCR, and the results confirmed the integration of the expression cassette into the *P. pastoris* genome (data not shown). Positive transformants of *P. pastoris* were inoculated into BMGYC medium, and the cells were harvested and resuspended into BMMY medium. The supernatant was collected every 24 h for 120 h, and all were run on SDS-PAGE along with two controls, which were proteins from the untransformed host and proteins from the host transformed with an expression vector without the cht2 cDNA. The SDS-PAGE profile showed the presence of -35 kDa recombinant Cht2. This result was confirmed by western blotting (Figure 3).

Toonkool et al. (2006) studied the expression of -galactosidase from *Dalbergia cochinchinensis* in *P. pastoris*; the expression was optimized by using different media including BMMY (Buffered complex medium containing methanol), BMMH (Buffered minimal medium containing methanol and histidine) and MMH (Minimal medium containing methanol and histidine). The results showed that the highest activity was observed in BMMY media. Similar results were obtained for Cht2 expression; Cht2 was only expressed in BMMY medium, and no recombinant protein expression was produced from other induction media. This indicates the importance of media selection for heterologous protein production in *P. pastoris*.

Enzymatic properties of partially purified recombinant endochitinase

The purification of Cht2 with IMAC technique was not successful. The problem was that the native structure of the protein hide the His tag and blocked its binding to the nickel resin. This assumption was proven when the protein was successfully purified after it was denatured (data not shown). Due to this problem, the purification was achieved partially with a conventional cross-flow filtration system followed by further concentration using a vivaspin with a molecular cut-off weight of 10 kDa.

The activity of Cht2 was examined at different temperatures (25 - 70°C). The results showed that Cht2 was active under a broad range of temperatures from 25 to 70°C with an optimum temperature at 35°C (Figure 4). The enzyme was stable at a temperature range from 30

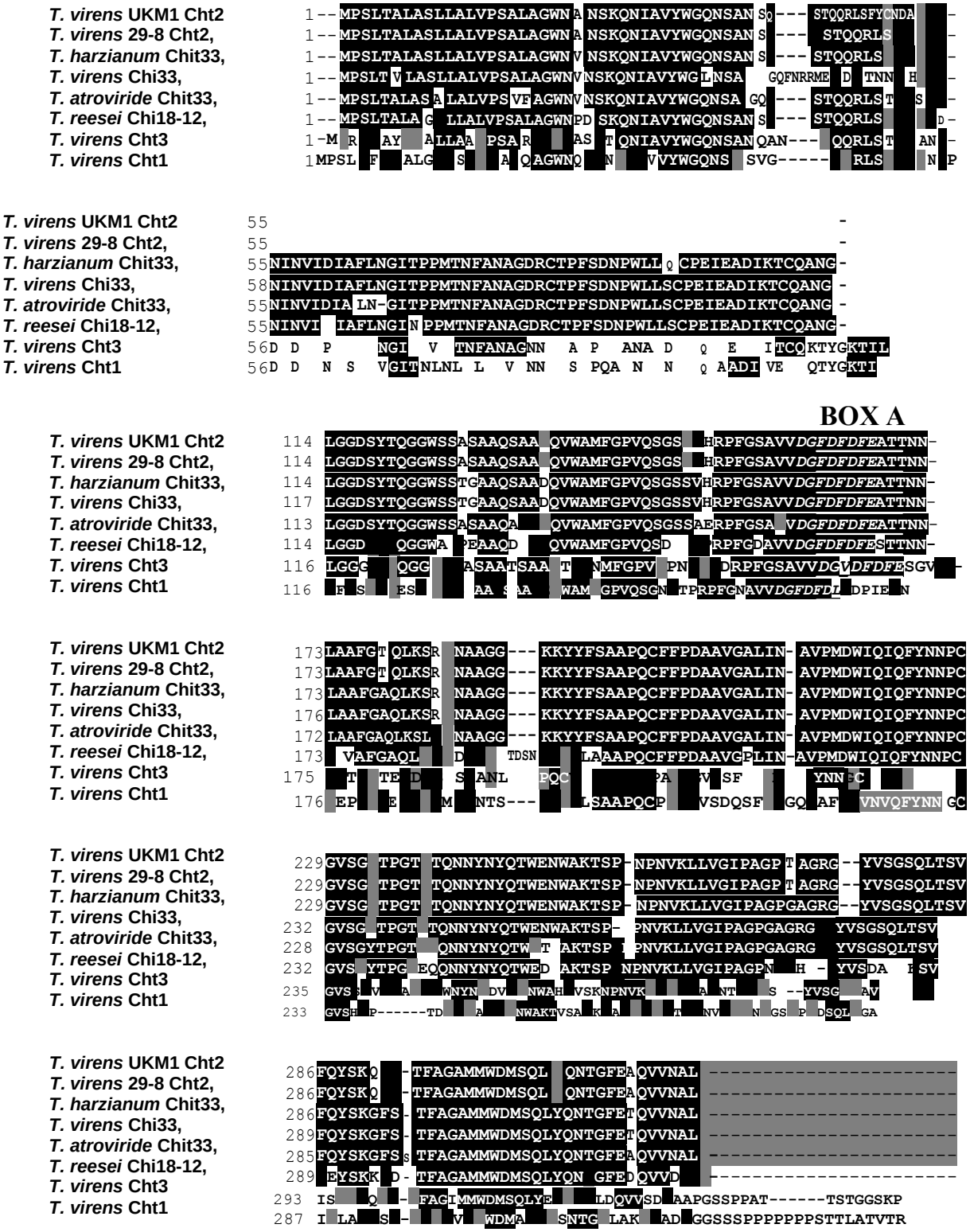

Figure 2. Multiple sequence alignment of *T. virens* UKM1 Cht2 and several fungal class III chitinases. The sequences of these chitinases were obtained from the GenBank with the following accession numbers: *T. virens* 29-8 Cht2 (Accession number: AF395754), *T. harzianum* Chit33 (Accession number: X80006), *T. virens* Chi33 (Accession number: FJ358733), *T. atroviride* Chit33 (Accession number: EF439839), *T. reesei* chi18-12 (Accession number: BK006085), *T. virens* Cht3 (Accession number: FJ237528), and *T. virens* Cht1 (Accession number: AF395753). The active site (BOX A) is in italics and underlined. The black shaded boxes represent identical amino acid residues, whereas the grey and white shaded boxes represent different amino acid residues. Gaps introduced to maximise the alignments are indicated by a horizontal dash.

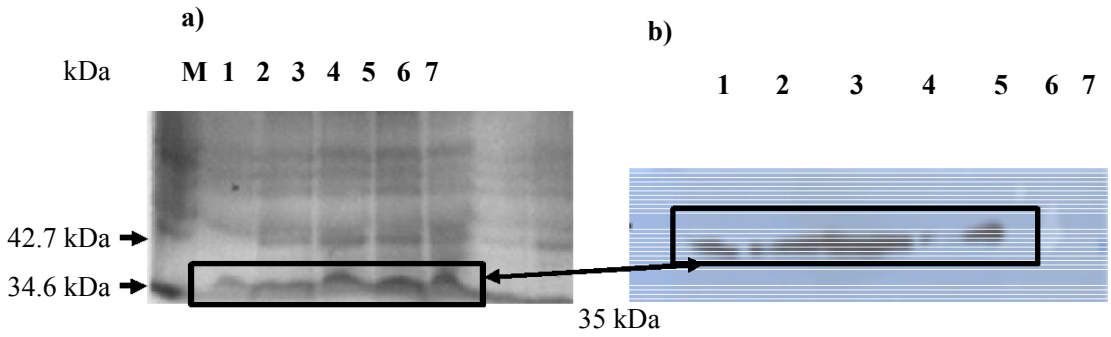

Figure 3. SDS-PAGE and western blot profiles of *T. virens* UKM1 recombinant Cht2. (a) SDS-PAGE profiles of Cht2; M: Unstained protein marker (Fermentas, Germany). Lane 1: Proteins collected after 24 h of expression; Lane 2: Proteins collected after 48 h of expression; Lane 3: Proteins collected after 72 h of expression; Lane 4: Proteins collected after 96 hours of expression; Lane 5: Proteins collected after 120 h of expression. Lane 6: Untransformed host protein collected after 72 h of expression; Lane 7: Proteins of host transformed with pPICZ C vector (without *cht2* cDNA) collected after 72 h of expression. (b) Western blot of profiles of (a).

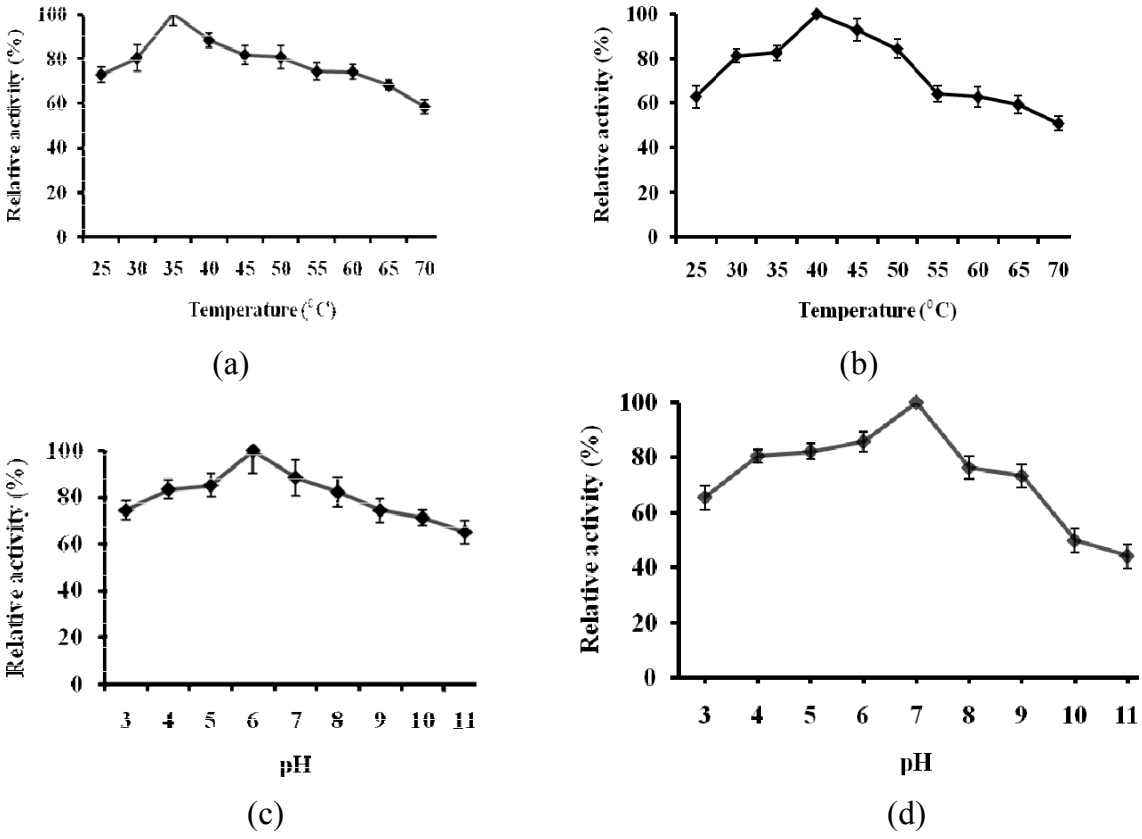

Figure 4. Temperature and pH profiles of Cht2 enzyme activity.(a) Effect of temperature on enzyme activity, (b) temperature stability of the enzyme, (c) effect of pH on enzyme activity, (d) pH stability of the enzyme.

to 55°C. It showed low stability at higher temperatures, and it retained approximately 50% of its activity at 70°C. The enzyme activity was also determined at different pH (pH 3 - 11) at 35ºC. The highest enzyme activity was observed at pH 6.0 with sodium phosphate buffer when it was incubated at 35°C for 30 min with 1% of colloidal chitin (w/v) as substrate (Figure 4). The enzyme was stable from pH 5.0 to 7.0, and its activity decreased

Table 1. Comparison of characteristics of *T. virens* UKM1Cht2 with other recombinant fungal chitinases.

Reference	Substrate	Optimum temperature	Optimum pH	Activity	Protein (size kDa)	Expression host	Chitinase
This study	colloidal chitin	35	6	1.34 U/mg (partially purified)	33	*P. pastoris*	*T. virens* UKM1 Cht2
Gan et al., 2007	colloidal chitin	37.6	7	12.3 mU/ml (purified)	45	*P. pastoris*	*L. psulliotae* Lpchi1
Viterbo et al., 2002	PNP $(GlcNAc)_2$	40-53	4.5	2.6 U/mg (purified) 1.2 U/mg (partially purified)	36	*P. pastoris*	*T. harzianum* Chit36
Fan et al., 2007	colloidal chitin and 4-MU-$(GlcNAc)_3$	55	5 - 6	3.9 U/mg (purified)	36	*P. pastoris*	*B. bassiana* Bbchit1
Caihong et al., 2007	colloidal chitin	40	5	0.75 U/ml (partially purified)	37	*S. cerevisiae*	*T. harzianum* Chit37
Ike et al., 2006	chitosan	55	5	18.1 U/mg (purified) 1.3 U/mg (partially purified)	46	*E. coli*	*T. ressei* Chi46
Lu et al., 2005	chitin	22	7.5	22 mU/ml (not purified)	46	*P. pastoris*	*Verticillium lecanii* Chi2
Alias et al. 2009	colloidal chitin	50	6	not determined	42	*E. coli*	*T. virens* UKM1 Ech1

gradually at lower and higher pH.

Chitinases, such as Chi1 of *Lecanicillium psulliotae* (Gan et al., 2007), Chit37 of *T. harzianum* (Caihong et al., 2007) and a chitinase of *T. harzianum* strain 39.1 (Ulhoa and Peberdy, 1991) have exhibited optimum temperature in the range of 37-40°C. However, several other chitinases, such as Bbchit of *Beauveria bassiana* (Fan et al., 2007), Chi46 of *T. ressei* (Ike et al., 2006) and Ech1 of *T. virens* UKM-1 (Alias et al., 2009), have shown optimum activity at higher temperatures between 50 - 55ºC. The comparison of optimum pH showed that some chitinases exhibited optimum activity at pH 6.0, such as *B. bassania* Bbchit1 and *T. virens* Ech1. *T. virens* Cht2 is stable from pH 3.0 to 7.0 and optimum at pH 6.0, which is in agreement with the pH stability of *T. virens* Ech1.

The activity of partially purified *T. virens* Cht2, which is 1.34 U/mg, is comparable to other published chitinases (Table 1). Reports show that recombinant chitinases establish higher activity if the enzymes are purified. Nevertheless, *T. virens* UKM1 Cht2 activity is relatively comparable to other chitinases that are partially purified, such as Chi46 of *T. ressei* (Ike et al., 2006) and Chit36 of *T. harzianum* (Viterbo et al., 2002). The partially purified *T. reesei* Chi46 shows an activity of 1.30 U/mg but this activity increases to 18.1 U/mg after the enzyme is purified. The activity of the partially purified *T. harzianum* Chit36 is 1.2 U/mg, whereas the activity of the purified protein is 2 fold higher (Table 1).

Substrate specificity

2 substrates, glycol chitin and colloidal chitin, were tested for substrate affinity of Cht2. The experiments were performed at 35°C and pH 6.0. From the analysis, Cht2 had low K_M in colloidal chitin and a higher K_M in glycol chitin. The low K_M value of Cht2 with colloidal chitin reflects the high affinity of Cht2 towards this substrate, and the high K_M value of Cht2 with glycol chitin reflects the low affinity of Cht2 towards this substrate. This result showed that Cht2 activity is more favorable towards degradation of colloidal chitin. Colloidal chitin was prepared by acid hydrolysis, so all proteins and lipids were removed. This provides an easier substrate for the enzyme to hydrolyse. Chitinase Chit42 of *T. harzianum* (de la Cruz et al., 1992) and Ech1 of *T. virens* UKM-1 (Alias et al., 2009) also exhibited higher activity towards colloidal chitin.

Conclusion

The endochitinase gene *cht2* of *T. virens* was

isolated, and the recombinant Cht2 was successfully expressed in *P. pastoris* with a molecular weight of - 35 kDa. Domain analysis of this protein showed that it belongs to class III of glycosyl hydrolase family 18, and the presence of a signal peptide suggested that it may be an extracellularenzyme. Enzymatic characterization of Cht2 suggested that its highest activity is at 35°C and pH 6.0 and that it is stable in the temperature range of 30 - 55°C and pH range of 5.0 - 7.0. This is the first bio-chemical properties described for a class III edochitinase from *T. virens* and this study has showed that Cht2 demonstrates similar pH and temperature stability to the recombinant Ech1, a class V *T. virens* endochitinase, and to the majority of the charaterised *Trichoderma* endo-chitinases. This findings is in agreement with the idea that multiple chitinases produced by a fungus have a mutually synergistic and complementary effect between them and their collective actions are required to hydro-lyse different sources of chitinolytic substrates effectively. Future work can be carried out to study this synergistic effects and the specific role of Cht2 in biocontrol activity of *T. virens* against pathogen-containing chitin. Similarly, the potential of Cht2 in the degradation of complex chitin waste, such as crustaceans' shells and fungal cell wall, into useful products could be explored.

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