Journal of Theoretical Biology 374 (2015) 107–114

Contents lists available at ScienceDirect

Journal of Theoretical Biology

journal homepage: www.elsevier.com/locate/yjtbi

Thermostable chitinase II from *Thermomyces lanuginosus* SSBP: Cloning, structure prediction and molecular dynamics simulations

Faez Iqbal Khan [a,b], Algasan Govender [b], Kugen Permaul [b], Suren Singh [b], Krishna Bisetty [a,*]

[a] Department of Chemistry, Durban, Steve Biko Campus, Durban University of Technology, Durban, South Africa
[b] Department of Biotechnology and Food Technology, Steve Biko Campus, Durban University of Technology, Durban, South Africa

HIGHLIGHTS

- Chitinase gene cloned and expressed from *Thermomyces lanuginosus* SSBP.
- 3D structure predicted and analyzed by docking and molecular dynamics simulations.
- Chitinase was found to be stable and functionally active at higher temperatures.

ARTICLE INFO

Article history:
Received 20 January 2015
Received in revised form
2 March 2015
Accepted 27 March 2015
Available online 8 April 2015

Keywords:
Chitin
TIM-barrel
MD simulations
Stability
Molecular docking

ABSTRACT

Thermomyces lanuginosus is a thermophilic fungus that produces large number of industrially-significant enzymes owing to their inherent stability at high temperatures and wide range of pH optima, including thermostable chitinases that have not been fully characterized. Here, we report cloning, characterization and structure prediction of a gene encoding thermostable chitinase II. Sequence analysis revealed that chitinase II gene encodes a 343 amino acid protein of molecular weight 36.65 kDa. Our study reports that chitinase II exhibits a well-defined TIM-barrel topology with an eight-stranded α/β domain. Structural analysis and molecular docking studies suggested that Glu176 is essential for enzyme activity. Folding studies of chitinase II using molecular dynamics simulations clearly demonstrated that the stability of the protein was evenly distributed at 350 K.

1. Introduction

Chitin is a natural biopolymer composed of repeating units of *N*-acetyl-β-D-glucosamine and primarily forms the structural component of protective biological matrices such as fungal cell walls and exoskeletons of insects and arthropods (Aam et al., 2010). Chitin-degrading enzymes (chitinases) play a significant role in the defense against chitin-containing parasites by hydrolyzing the β-1,4-linkages in chitin (Bucolo et al., 2011; Lobo et al., 2013). Chitinases have been a focus of research in the past few years due to their vast array of biotechnological applications, especially in the field of agriculture for bio-control of fungal phytopathogens (Hamid et al., 2013). Since the effectiveness of conventional insecticides is increasingly compromised by the occurrence of resistance, chitinases offer a potential alternative to the use of chemical fungicides as well as anti-biofouling agents (Herrera-Estrella and Chet, 1999). The thermostable enzymes isolated from thermophilic microorganisms have gained widespread attention in industrial, medical, environmental and biotechnological applications due to their inherent stability at high temperatures and wide range of pH optima (Maheshwari et al., 2000; Meng Zhang et al., 2014; Stephens et al., 2014). However, despite their huge potential, the precise three-dimensional structure of most of the chitinases, including those isolated from *Thermomyces lanuginosus* (Duo-Chuan, 2006) is not fully characterized. Hence, the main focus of the present study was to gain a better understanding of the structural features of chitinases obtained from this thermostable fungus using both experimental and computational techniques, and their relationship with their activity profiles. In this work, a novel chitinase II gene from *T. lanuginosus* was expressed in *E. coli*. The structure of chitinase II was predicted using homology modeling and molecular dynamics (MD)

Abbreviations: LB, Luria Bertani; SSBP, Suren Singh Bernard Prior; cDNA, complementary deoxyribonucleic acid; RMSD, root mean square deviation; SignalP, signal peptide; MD, molecular dynamics; GROMACS, GROningen MAchine for Chemical Simulations; OPLS-AA/L, optimized potential for liquid simulations/all atoms; DS, Discovery Studio

* Correspondence to: Department of Chemistry, Durban University of Technology SB Campus, POBOX 1334 Durban 4000.
E-mail address: bisettyk@dut.ac.za (K. Bisetty).

http://dx.doi.org/10.1016/j.jtbi.2015.03.035
0022-5193/

simulations methods. The generated (3D) structures were refined and the best reliable models were selected and subjected to MD simulations to better understand the details of protein conformation and stability as a function of time.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

T. lanuginosus SSBP (Singh et al., 2000) was grown at 50 °C on potato dextrose medium (Merck), sub-cultured every two weeks and stored at 4 °C. *E. coli* BL21 (Stratagene) was grown and maintained on Luria Bertani (LB) medium (10 g/l peptone, 5 g/l yeast extract powder, 10 g/l NaCl and 15 g/l technical agar). Transformed *E. coli* was maintained on LB medium supplemented with 100 μg/ml ampicillin and grown at 37 °C. For short term storage, sub-culturing was done every two weeks, whilst for long term storage cultures were supplemented with 15% glycerol and stored at −70 °C. The pET21c cloning vector (Novagen) was used in this study.

2.2. Chitinolytic plate enzyme assays

Chitinase activity was assayed with glycol chitin as a substrate. Glycol chitin was prepared according to the methods of Trudel and Asselin (1989) and Lee et al. (2007). Glycol chitin at different concentrations was added to melted 1% agarose and poured into petri plates. Wells were punched into the agarose medium after solidification. 400 μl of supernatant was loaded into different wells in the plate with the negative control being chitinase production medium, which was then incubated for 2 h at 50 °C. The plate was then stained with 2% Calcofluor White M2R stain (Fluka) for 1.5 h and washed with distilled water for 2 h, then observed under UV light to determine whether clear zones can be observed around the wells.

2.3. Total RNA isolation from Thermomyces lanuginosus SSBP and first-strand cDNA synthesis

1 ml of an aqueous spore suspension (1×10^6 spores/ml) was inoculated into chitinase production medium [10 g/l colloidal chitin; K_2HPO_4, 0.87 g/l; KH_2PO_4, 0.68 g/l; KCl, 0.2 g/l; NH_4NO_3, 1 g/l; $MgSO_4$, 0.2 g/l; yeast extract, 4 g/l; pH 6.5] (Guo et al., 2005) and incubated at 50 °C on a rotary shaker at 150 rpm. Mycelia were harvested by centrifugation following 12, 24 and 48 h incubation and stored at −70 °C. Total RNA was isolated using the TRIzol reagent and the integrity was confirmed by electrophoresis (0.8% agarose gel) and NanoDrop 1000 spectrophotometer (OD260 > 1.8) analysis. The first-strand cDNA was synthesized from 5 μg total RNA using the iScriptTM cDNA synthesis kit (Bio-Rad).

2.4. PCR amplification and cloning

Primers were designed using Oligo version 6.0 software (Rychlik, 2007), based on chitinase genes identified in the currently available annotated genome sequence data of *T. lanuginosus* SSBP (GenBank Accession no. KJ740647) and included the *Nhe*I and *Xho*I restriction sites. PCR amplification was done under the following conditions: initial denaturation at 98 °C for 30 s, cyclic denaturation at 98 °C for 10 s, primer annealing at 55 °C for 20 s, extension at 72 °C for 30 s (30 cycles) and final extension at 70 °C for 7 min. The PCR product was confirmed by 1% agarose gel electrophoresis and purified using the DNA Clean and Concentrator Kit (Zymo Research). Purified DNA was digested with restriction enzymes *Nhe*I and *Xho*I (Fermentas) and once again purified using the DNA Clean and Concentrator Kit (Zymo Research). This was then ligated into the pET21c vector by overnight incubation of ligation mixture at 4 °C. The ligated product was transformed into competent *E. coli* BL21 and selected on LB agar plates containing ampicillin using standard protocols. Positive clones were confirmed by colony PCR.

2.5. Sequence analysis

The 343 residue long amino acid sequence of chitinase II from *T. lanuginosus* SSBP was analyzed using several available bioinformatics tools such as BLAST (Altschul et al., 1990), HMMER (Finn et al., 2011) and FASTA server (Pearson and Lipman, 1988). BLAST searches the non-redundant protein sequences (nr) database and hits with an e-value (< 0.0005) and sequence identity (> 20%) were considered as matches. The domain analysis was performed using InterProScan 5 (Jones et al., 2014), SMART (Schultz et al., 2000), SYSTERS (Schultz et al., 2000) and ProtoNet (Rappoport et al., 2012) in order to identify the function more precisely. InterProScan 5 searches the similar signature in the interpro consortium databases. PTMcode (Minguez et al., 2013) was used to detect presence of any possible post-translational modification sites in the protein sequence. SMART was further used to annotate the genetically mobile domains in the sequence of chitinase II and SYSTERS use the clustering based algorithms for the function prediction of proteins. The Classify Your Protein module of ProtoNet was used to analyze the protein family of chitinase II. The sequence based secondary structure was analyzed using Psipred (McGuffin et al., 2000). The presence of a signal peptide in the sequence framework of chitinase II was predicted using artificial neural network algorithm of SignalP 4.0 (Petersen et al., 2011) and Signal-CF (Chou and Shen, 2007; Shen and Chou, 2007). Signal-CF performed the best among the existing signal peptide predictors, particularly for long signal peptides (Hiss and Schneider, 2009). Protein glycosylation is significant for secretion, localization and stability of protein. Thus, glycosylation sites were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.6. Structure prediction and evaluation

The knowledge of protein three-dimensional (3D) structures is vitally important for rational drug design (OuYang et al., 2013). Although X-ray crystallography is a powerful tool in determining protein 3D structures, it is time-consuming and expensive, and not all proteins can be successfully crystallized (Berardi et al., 2011). Membrane proteins are difficult to crystallize and most of them will not dissolve in normal solvents. Therefore, so far very few membrane protein structures have been determined (Call et al., 2006, 2010). NMR is indeed a very powerful tool in determining the 3D structures of membrane proteins, but it is also time-consuming and costly. To acquire the structural information in a timely manner, a series of 3D protein structures were developed by means of the homology technique (Chou, 2004, 2005; Wang et al., 2007) and from a comprehensive review (Chou, 2004), it was found very useful for drug development. In view of this, the homology technique was also adopted to develop the relevant protein 3D structures for the current study. The 3D structure of chitinase II was predicted using homology and *ab initio* methods. The structural homolog search was performed in the Protein Data Bank (PDB) (Berman et al., 2000) using PSI-BLAST module in Discovery Studio (DS) 4.0 (Accelrys Software Inc. 2013), HHpred (Soding et al., 2005), HMMER (Finn et al., 2011) and Phyre (Kelley and Sternberg, 2009). A BLAST (Altschul et al., 1990) search with identity > 30% was considered as the suitable template for the structure prediction of the chitinase II. The HHpred identified structural homologs in the PDB, structural classification of proteins (SCOP) and CATH. Furthermore, HMMER were used to detect reliable homologs in the databases by exploring the modules such as hmmsearch, hmmscan, jackhammer and phemmer. Similarly, Phyre a server based on profile–profile matching algorithm was used to enhance the