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Functional and structural analyses of a 1,4-β-endoglucanase from *Ganoderma lucidum*

Guizhi Liu[a,1], Qian Li[b,1], Na Shang[b,1], Jian-Wen Huang[c,d], Tzu-Ping Ko[e], Weidong Liu[b], Yingying Zheng[b], Xu Han[b], Yun Chen[f], Chun-Chi Chen[b,*], Jian Jin[f,**], Rey-Ting Guo[b,*]

[a] *School of Biotechnology, Jiangnan University, Wuxi 214122, China*
[b] *Industrial Enzymes National Engineering Laboratory, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China*
[c] *Genozyme Biotechnology Inc., Taiwan 196, Taiwan*
[d] *AsiaPac Biotechnology Co., Ltd., Dongguan 523808, China*
[e] *Institute of Biological Chemistry, Academia Sinica, Taipei 11529, Taiwan*
[f] *School of Pharmaceutical Sciences, Jiangnan University, Wuxi, Jiangsu 214122, China*

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ABSTRACT

Ganoderma lucidum is a saprotrophic white-rot fungus which contains a rich set of cellulolytic enzymes. Here, we screened an array of potential 1,4-β-endoglucanases from *G. lucidum* based on the gene annotation library and found that one candidate gene, GlCel5A, exhibits CMC-hydrolyzing activity. The recombinant GlCel5A protein expressed in *Pichia pastoris* is able to hydrolyze CMC and β-glucan but not xylan and mannan. The enzyme exhibits optimal activity at 60 °C and pH 3–4, and retained 50% activity at 80 and 90 °C for at least 15 and 10 min. The crystal structure of GlCel5A and its complex with cellobiose, solved at 2.7 and 2.86 Å resolution, shows a classical $(\beta/\alpha)_8$ TIM-barrel fold as seen in other members of glycoside hydrolase family 5. The complex structure contains a cellobiose molecule in the +1 and +2 subsites, and reveals the interactions with the positive sites of the enzyme. Collectively, the present work provides the first comprehensive characterization of an endoglucanase from *G. lucidum* that possesses properties for industrial applications, and strongly encourages further studying in the cellulolytic enzyme system of *G. lucidum*.

1. Introduction

Cellulose is the most abundant renewable resource on earth. The polysaccharide is composed of β-1,4-glycosidic bond-linked glucose units. Cellulose constitutes the primary structure of plant cell wall and its decomposition holds great potentials in many commercial applications including biofuel production, food and feed industry, and textile processing [1]. Complete cellulose decomposition requires a concerted action of endo-1,4-β-D-glucanases (endoglucanase, EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) [2]. Among these enzymes, endoglucanase which catalyzes the hydrolysis of β-1,4-glycosidic bonds and cleaves the macromolecular cellulose into smaller fragments is the most widely applied cellulolytic enzyme product. Therefore, searching for endoglucanases with higher efficacy from various cellulolytic organisms and tissues has been an important research direction.

A number of endoglucanases have been identified from various organisms and microorganisms. Based on protein sequence similarity and catalytic domain structure, endoglucanases are classified by the Carbohydrate-Active Enzyme database (CAZy) into 13 glycoside hydrolase (GH) families including 5–9, 12, 44, 45, 48, 51, 74, 124, and 131 [3] (http://www.cazy.org). These enzymes exploit carboxyl amino acids (asp or glu) as catalytic residues to hydrolyze substrates via retaining (GH5, GH7, GH12, GH44, and GH51) or inverting mechanism (GH6, GH8, GH9, GH45, GH48, GH74, and GH124, and GH131). Generally, hydrophilic and aromatic amino acids are utilized to bind carbohydrate substrates [4].

Ganoderma lucidum is a white-rot macrofungus which has been used as a medicinal mushroom in Asia for centuries. As a saprotrophic organism, *G. lucidum* produces an array of lignocellulolytic enzymes to decompose lignin and cellulose. A number of

* Corresponding authors at: 32 XiQiDao, Tianjin Airport Economic Park, Tianjin 300308, China. Fax: +86 22 84861926.
** Corresponding author at: 1800 Lihu Avenu, Wuxi, Jiangsu 214122, China. Fax: +86 510 85918219.
E-mail addresses: ccckate0722@gmail.com (C.-C. Chen), jinjian31@126.com (J. Jin), guo_rt@tib.cas.cn (R.-T. Guo).
[1] These authors contributed equally.

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0141-0229/

No.	Gene code
1	GL29421
2	GL23664
3	GL24196
4	GL28282
5	GL26499
6	GL25471
7	GL19331
8	GL23260
9	GL23097
10	GL19332

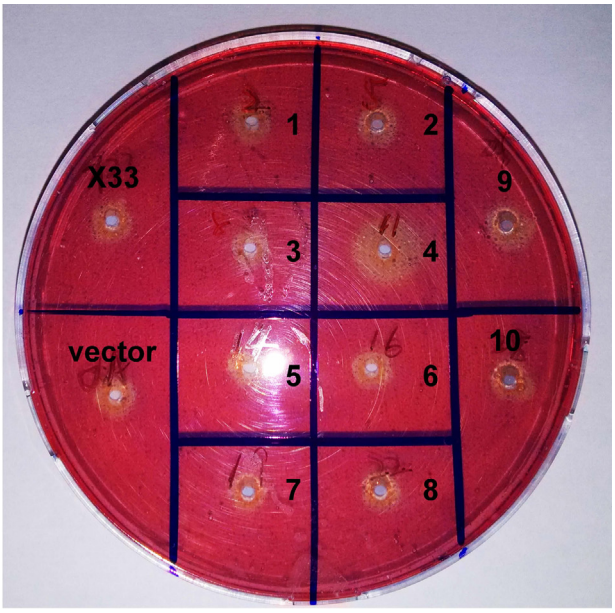

Fig. 1. Cellulolytic enzyme screening with Congo red staining method. Ten potential endoglucanase genes from *G. lucidum* are listed on the left table. At least three single colonies for each gene transformants were selected for screening, and a representative result is shown. X33, the *P. pastoris* without transformation; vector, *P. pastoris* transformed with vector without gene insertion.

studies have reported identification and characterization of lignin-modifying enzymes from *G. lucidum* [5–7], while only two studies reported the characterization of cellulose-degrading enzymes from this fungus [8,9]. In these reports, the enzymes with cellulolytic activity were directly analyzed for the liquid culture of *G. lucidum*. The experimental procedure takes a long time due to the slow growth rate of the fungus, and the following analyses and applications might be hampered by the lack of biomolecular information.

Recently, the full genome sequence of *G. lucidum* has been reported [10,11]. Up to 13,000 protein-coding genes are predicted and more than 80% of them can be functionally annotated. It is suggested that the *G. lucidum* genome encodes a rich set of wood-degradation enzymes. In the present study, potential endoglucanase coding-genes in *G. lucidum* were sought out from the annotation library, expressed in an industrial strain of *Pichia pastoris*, and examined for the CMC-hydrolytic activity. One candidate gene that showed significant enzymatic activity was further characterized, and its crystal structures including a cellobiose complex were determined. The present work reports the first comprehensive analysis of an endoglucanase from *G. lucidum*, and it shall shed light on further characterization of the cellulolytic enzyme system of the fungus.

2. Materials and methods

2.1. Selection of potential endoglucanase encoding genes in G. lucidum

By using the key word, endoglucanase; to search the *G. lucidum* gene annotation at the *G. lucidum* Genome database (GaLuDB) (http://www.medfungi.org/20141010/galu/analyzer/home); a collection of endoglucanase candidates in *G. lucidum* was retrieved. The catalytic domain and signal peptide of the candidates were analyzed by the protein blast tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and by SignalP 4.1 Server at CBS (http://www.cbs.dtu.dk/services/SignalP/); respectively. The sequences of catalytic domain without signal peptide of the candidates were synthesized adapting *P. pastoris* codon usage and cloned into pPICZαA vector (Novagen). The recombinant plasmids were linearized and transformed to *P. pastoris* X33 strain by electroporation. The transformants were selected by YPD (1% yeast extract; 2% peptone; 2% dextrose; 2% agar) plates containing 100 μg/ml Zeocin at 30 °C for two days. Single colonies were picked and inoculated to 5 ml BMGY medium (1% yeast extract; 2% peptone; 100 mM potassium phosphate; pH 6.0; 1.34% YNB; 4×10^{-5}% biotin; 1% glycerol) at 30 °C for two days. Cells were collected by centrifugation at 3500 rpm for 10 min and resuspended in 2 ml BMMY medium (1% yeast extract; 2% peptone; 100 mM potassium phosphate; pH 6.0; 1.34% YNB; 4×10^{-5}% biotin; 0.5% methanol) at 30 °C for five days. A final concentration of 0.5% methanol was added every day to induce protein expression.

2.2. Screening cellulase activity with Congo red staining method

10 μl of 5-day *P. pastoris* culture supernatants were applied to the spot on the carboxymethylcellulose (CMC) agar plate (1% CMC–Na, 2% agar, 0.1 M HAc–NaAc, pH 5.0) and incubated at 25 °C for 30 min. The plate was covered with Congo red solution (100 mg/ml, dissolved in water, Chengdu Kelong Chemical Reagent Company) and incubated at 50 °C for 30 min. The unbound Congo red was washed away with 1 M NaCl solution to reveal the opaque circle surrounding the protein spot.

2.3. Protein expression and purification

GlCel5A expression was induced with 1% (v/v) methanol in 50 ml of BMMY medium at 30 °C for 5 days. The proteins were harvested by centrifugation at 4,000 rpm for 15 min and dialyzed against a buffer containing 25 mM MES pH 6.0. The proteins were purified by FPLC using a DEAE column (GE Healthcare) with 25 mM MES buffer, pH 6.0, containing gradient of 0–500 mM NaCl. The protein solution was collected and dialyzed against a buffer containing 25 mM MES, pH 6.0, 150 mM NaCl, and then subjected to endoglycosidase H (endoH) digestion as previous described [12]. The protein quality was validated by SDS-PAGE analysis.