Enzyme and Microbial Technology 49 (2011) 485–491

Contents lists available at SciVerse ScienceDirect

Enzyme and Microbial Technology

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

Cloning of novel cellulases from cellulolytic fungi: Heterologous expression of a family 5 glycoside hydrolase from *Trametes versicolor* in *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 15 February 2011
Received in revised form
15 September 2011
Accepted 11 October 2011

Keywords:
Endoglucanase
Heterologous expression
Lignocellulose hydrolysis
Pichia pastoris
Trametes versicolor
White rot fungi

ABSTRACT

Total cDNA isolated from cellulolytic fungi cultured in cellulose was examined for the presence of sequences encoding for endoglucanases. Novel sequences encoding for glycoside hydrolases (GHs) were identified in *Fusarium oxysporum*, *Ganoderma applanatum* and *Trametes versicolor*. The cDNA encoding for partial sequences of GH family 61 cellulases from *F. oxysporum* and *G. applanatum* shares 58 and 68% identity with endoglucanases from *Glomerella graminicola* and *Laccaria bicolor*, respectively. A new GH family 5 endoglucanase from *T. versicolor* was also identified. The cDNA encoding for the mature protein was completely sequenced. This enzyme shares 96% identity with *Trametes hirsuta* endoglucanase and 22% with *Trichoderma reesei* endoglucanase II (EGII). The enzyme, named TvEG, has *N*-terminal family 1 carbohydrate binding module (CBM1). The full length cDNA was cloned into the pPICZαB vector and expressed as an active, extracellular enzyme in the methylotrophic yeast *Pichia pastoris*. Preliminary studies suggest that *T. versicolor* could be useful for lignocellulose degradation.

1. Introduction

The study of microbial cellulases has recovered attention in the last years, mainly because of the increased interest in the optimization of processes of manufacture of bio-products derived from lignocellulosic residues, e.g. bioethanol [1]. The cellulosic bioethanol process requires basically three steps: pretreatment to loosen up the lignin-cellulose fiber entanglement, hydrolysis of cellulose and fermentation to ethanol. The cellulose hydrolysis is a complex and expensive process, due mainly to the lignocellulose structure and recalcitrance. Developments in the field are still necessary in order to decrease the impact of this stage in the whole process's economy.

Cellulose hydrolysis is carried out mostly by the action of cellulases, enzymes acting in concert to release soluble sugars from cellulose [2]. These enzymes are glycoside hydrolases breaking β-1,4-linkages in cellulose with different specificities. Endo-β-1,4-glucanase (EG; EC 3.2.1.4) hydrolyzes internal glycosidic bonds in cellulose, while exo-β-1,4-glucanase, or cellobiohydrolase (CBH; EC 3.2.1.91), releases cellobiose from the reducing or non-reducing ends of the cellulose chain; finally, cellobiase (EC 3.2.1.21) hydrolyzes cellobiose to glucose, which can be consumed by fermenting microorganisms. Cellulases are typically multidomain proteins consisting of a catalytic core domain linked to a carbohydrate-binding module (CBM) via a flexible linker region [3]. Catalytic domains of glycoside hydrolases (GHs) are classified according to their sequence in more than 120 families [4], while 61 CBMs families have been described up to now (Carbohydrate-Active enZYmes Database, http://www.cazy.org/) [5]. Now it is well understood that other enzymes are also necessary components to complete and efficient hydrolysis of lignocellulose [6].

Cellulases are produced by many different microorganisms in nature. Lignocellulolytic enzyme-producing fungi are widespread, and include species from phylum Ascomycota (e.g. *Trichoderma reesei*), Basidiomycota including white-rot fungi (e.g. *Phanerochaete chrysosporium*), brown-rot fungi (e.g. *Fomitopsis palustris*) and other anaerobic species. Historically cellulases have been derived from *Trichoderma* species, due to the great capacity of production of this fungus. *T. reesei* produces two major cellobiohydrolases belonging to GH families 6 and 7 (Cel6A and Cel7A, or CBHI and CBHII, respectively) and at least five endoglucanases classified as GH families 7, 5, 12, 61 and 45 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A or EGI, EGII, EGIII, EGIV, and EGV) [7]; these enzymes, which

Abbreviations: CBM, carbohydrate binding module; cDNA, complementary deoxyribonucleic acid; CMC, carboxymethyl cellulose; DNS, dinitrosalicylic acid; GH, glycoside hydrolase; RT-PCR, reverse transcription polymerase chain reaction.

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doi:10.1016/j.enzmictec.2011.10.003

are produced exclusively when cellulose is available as carbon source [8], act synergistically to hydrolyze the cellulose [9,10].

Investigations addressed the search and characterization of novel and more efficient cellulases have driven scientists toward fungi responsible of white rot in woods, specifically basidiomycetous fungi [6]. In this group, *Trametes versicolor* has been studied largely because its ligninolytic properties, which have potential applications in bioremediation of pulp and paper mill effluents. Activities of selected wood-degrading enzymes such as cellobiase and cellulase have been monitored in *T. versicolor* [9,10]. The existence of an endoglucanase activity in the supernatant of a *T. versicolor* culture was reported some years ago [11] but no cloning or recombinant expression of the enzyme has been informed yet. Also, a cellobiohydrolase-encoding cDNA from *T. versicolor* has been cloned and expressed in *Aspergillus niger* [9]. In summary, the potential of *T. versicolor* as producer of cellulolytic enzymes is widely recognized, but a systematic study of the functionality of the *T. versicolor* cellulases is still incomplete.

The aim of this study was the identification and cloning of novel endoglucanase genes from native Chilean cellulolytic fungi. In particular, a new *T. versicolor* endoglucanase was expressed as a recombinant protein in *Pichia pastoris*, a first step addressed to the enzyme overproduction and future characterization of the cellulolytic properties of this enzyme. The long term purpose is the isolation of cellulases having efficient hydrolytic action on wood chips of trees that are abundant in Chile. In this work we have used *Nothofagus pumilio* (Lenga beech or Lenga) as lignocellulosic substrate. Lenga, a native hardwood tree belonging to the family Nothofagaceae, comprises about 25% of native forest surface and is an important source of lignocellulosic waste in Chile.

2. Materials and methods

2.1. Nucleic acid manipulations

DNA manipulations were carried out as described [12]. PCR products were purified from agarose gel after electrophoresis using the QIAEXII kit (QIAGEN). PCR products were cloned into the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α cells. Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (QIAGEN). Primers, restriction enzymes and *Taq* DNA polymerase were supplied by Integrated DNA Technologies, New England Biolabs and Promega, respectively.

2.2. Fungal growth conditions

A battery of seven Chilean native cellulolytic fungi was supplied by the Laboratory of Biodegradation and Wood Preservation of the Faculty of Forest Sciences and Nature Conservation of the University of Chile. Cultures of *T. versicolor*, *G. applanatum*, *Poria placenta*, *Fusarium oxysporum*, *Pleurotus ostreatus*, *Lentinus edodes* and *Peniophora gigantea* were maintained on potato dextrose agar (PDA) at 4 °C. For RNA isolation, the fungi were cultured in agar plates at 28 °C for 10 days on induction media [13] including 0.5% (w/v) carboxymethyl cellulose (CMC; Megazyme) or Avicel (Merck). For induction in liquid medium the same medium was used without agar.

2.3. Evaluation of the cellulase production by cellulolytic fungi

The production of carboxymethycellulase activity (CMCase) in liquid medium supplemented with cellulose was evaluated by measuring reducing sugars released from CMC, after six days inoculation with 1 cm^2 PDA culture. The enzymatic reaction was performed as follow: a mixture of 50 μL of fungal culture supernatant and 100 μL of 1% (w/v) CMC in 50 mM sodium citrate pH 4.8 was incubated for 30 min at 37 °C; reducing sugars were quantified by reaction with dinitrosalicylic acid (DNS) [14]. Absorbance at 550 nm was measured in a microplate reader (Asys UVM 340, Eugendorf, Austria) and values were converted to enzyme units by use of a glucose calibration curve. One enzyme unit was defined as the amount of enzyme that releases 1 μmol of glucose equivalent per minute.

2.4. Hydrolysis of N. pumilio wood chips

Supernatant of fungi cultured in induction medium was concentrated by ammonium sulfate precipitation at 80% saturation. Pellets were suspended in 50 mM sodium acetate buffer pH 6 and dialyzed over night against the same buffer. The hydrolysis assays were conducted in 50 mM sodium acetate pH 5, using 50 mg mL^{-1} ionic liquid-pretreated *N. pumilio* wood chips (0.1–0.2 mm) [15], 0.25 paper filter units (FPU) per mL of concentrated fungal cellulase or Celluclast (Novozyme), equivalent to 5 FPU g^{-1} substrate, and 2.5 g L^{-1} Tween 20. Hydrolysis was conducted at 50 °C with orbital agitation (300 rpm) for 1.5, 25 or 50 h. Reducing sugars were measured as indicated in point 2.3. FPU activity was measured as described [16].

2.5. Primers design and bioinformatic analyses

cDNA sequences of fungal GH family 5, 7 and 61 endoglucanase (EC 3.2.1.4) were obtained from EMBL-EBI database and aligned using ClustalX 2.0.9 [17]. The accession numbers of the sequences used to design the degenerated primers were Q75UV6, Q5W7K4, Q9C3Z8, O74706, Q4WN62, O59951, Q5TKT6, Q2VRK9, Q8WZD7, Q12638, Q12624, Q12637 and Q04469 for GH family 5; P07981, Q12714, Q4WCM9, O13455, Q8NK01, Q4WAJ6, Q12622, P56680, P46237 and Q9HGT3 for GH family 7 and O14405, Q4WF08, A2QJX0, Q6MYM8, Q7Z9M7 and A2R5N0 for GH family 61. For identification of the partial cellulase sequences, consensus-degenerate primers were designed based on these alignments, using the CODEHOP strategy [18]. Specific primers were designed by hand in order to complete the TvEG cDNA sequence. The primer sequences are shown in Table 1.

Homology analysis was carried out using the BLAST software [19] from the National Center for Biotechnology Information (NCBI); putative functional domains identification was carried out by search the Conserved Domain Database (CDD) [20].

2.6. RNA isolation and cDNA synthesis

Total RNA was isolated from fungal mycelium grown in solid induction media. The mycelium was harvested from the plate and lysed with TRI reagent (Ambion) in liquid nitrogen using a mortar and pestle, according to the manufacturer's directions. Single strand cDNA (sscDNA) was synthesized with the Oligo$(dT)_{15}$ primer using the Reverse Transcription System kit (Promega) according to the manufacturer's directions. PCR was performed in a reaction mixture containing: 1.4 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM reverse primer, 1 μM forward primer, buffer *Taq* 1×, 1 U *Taq* DNA polymerase and 0.5 μL of template sscDNA in a final volume of 25 μL. Reactions were carried out in an Eppendorf Master Cycler Gradient; gel-purified PCR products were cloned into pGEM-T Easy vector, and sequenced by Macrogen (Korea).

2.7. Phylogenetic analysis

Multiple alignments of sequences of glycoside hydrolases from families 5, 7 and 61 obtained from the EMBL-EBI data base were performed using ClustalX 2.0.9. Information on the accession number of the sequences used as templates are available in Supplementary Material 1. Construction and visualization of a radial neighbor-joining phylogenetic tree were done with the TreeView version 1.6.6 program [21].

2.8. Expression of the endoglucanase gene in P. pastoris X-33

The cloning and recombinant expression of the full length *T. versicolor* endoglucanase gene was carried out using the Easy-Select *Pichia* expression kit (Invitrogen). Specific primers TvEGPP1_For and TvEGPP1_Rev (Table 1) were designed in order to add *Xho*I and *Xba*I restriction sites at the ends of the endoglucanase gene. The PCR product was digested with these enzymes and the fragment was cloned into the pPICZαB vector. The forward primer contained a *Xho*I recognition site, a Kex2 signal cleavage and the sequence encoding for the five first amino acids of the mature TvEG enzyme. The reverse primer contained the sequence encoding for the last four amino acids of the protein, followed by six histidine codons and a stop codon. Recombinant plasmid was introduced into *P. pastoris* X-33 by electroporation, following the supplier recommendations. Transformants were selected in YPD plates supplemented with 1 M sorbitol and 100 μg mL^{-1} Zeocin. Clones having multiple insertions in the yeast genome were selected in YPD plates supplemented with 500 μg mL^{-1} Zeocin. The correct sequence of the construction was confirmed by automatic sequencing at Macrogen. Recombinant protein production was carried out according to the kit provider directions. The confirmed clone was cultured in BMGY (Buffered complex medium containing glycerol) until OD_{600} 4. For the induction phase, cells were transferred to BMMY medium (Buffered complex medium with 0.5% (v/v) methanol), repeating the addition of methanol every 24 h. Recombinant expression was monitored every 24 h in aliquots of the culture supernatant by measuring activity on CMC and by electrophoresis. For this, a four mL aliquot of each supernatant was concentrated by ultracentrifugation using a Centricon centrifugal device YM-3 (Millipore). The concentrated fractions were analyzed by SDS–PAGE and CMC-zymogram. For the CMCase activity assays, aliquots of supernatant were analyzed directly, while the electrophoretic analysis was carried out using the concentrated fractions. SDS–PAGE was carried out in 12.5% polyacrylamide gel in Miniprotean II (BioRad), as recommended. For the zymogram analysis the protein separation was carried out in a similar protocol except that the gel contained 0.2% (w/v) CMC and the protein samples were not heated before the electrophoresis. SDS removal for protein renaturation in the gel was carried out by incubating with 0.25% (v/v) Triton X-100 for one hour at room temperature, and then the gel was incubated for one hour at 37 °C in 50 mM sodium acetate pH 5.0. The activity bands were revealed by staining with 0.1% (w/v) Congo Red and distaining with 1 M NaCl. In all the experiments *P. pastoris* X-33 transformed with the vector pPICZαB was analyzed in parallel and used as control.