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Molecular cloning, gene expression analysis and structural modelling of the cellobiohydrolase I from *Penicillium occitanis*

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ABSTRACT

The filamentous fungus *Penicillium occitanis* produces a complete set of cellulolytic enzymes needed for efficient solubilization of native cellulose. Cellobiohydrolase I (CBHI), the most abundant cellulolytic enzyme produced by this micro-organism, has been purified and characterized.

In this report, the cDNA encoding this enzyme, isolated from a cDNA bank of *P. occitanis*, and the equivalent genomic sequence have been cloned. DNA sequencing revealed that the *cbh1* gene is intronless and has an open reading frame of 1587 bp encoding a putative polypeptide of 529 amino acids. This polypeptide has a predicted molecular mass of 52.5 kDa and consists of a fungal cellulose binding module (CBM) and a catalytic module, linked together by a serine–threonine-rich region.

Northern blot analysis showed that *cbh1* mRNA expression is partially constitutive since, besides being highly induced by cellulose, it is slightly repressed by glucose.

Comparative investigation of different cellobiohydrolases I 3D structures by molecular modelling showed that poor hydrogen bonding, together with a more open configuration of the active site account for the weak binding and the relative insensitivity of *P. occitanis* CBHI to product inhibition.

1. Introduction

Cellulose is an insoluble polysaccharide composed of long linear chains of β-1,4-linked glucose units. It is the most abundant renewable biomass on earth since its microbial breakdown creates the potential for the production of energy [1]. Cellulolytic enzymes can be divided into three types: endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-glucanase (cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). They are collectively known as cellulases and act in a synergistic manner to facilitate complete cleavage of β-1,4-glycosidic bonds of the cellulose to produce glucose [1].

Cellulases are used in waste recycling processes and in the processing of cellulose-rich raw materials for food, detergent, paper and textiles industries. Recently, cellulases gained significant commercial importance due to their potential applications in biofuel production [2]. Because of the low activity of endo-β-1,4-glucanases to hydrolyze crystalline cellulose, exo-type cellulases such as cellobiohydrolases (CBHs) are necessary in hydrolyzing crystalline cellulose. These exo-acting enzymes possess tunnel-like active sites, which can only accept a substrate chain via its terminal regions [3]. Thus, CBH enzymes act by threading the cellulose chain through the tunnel, where successive cellobiose units are removed in a sequential manner.

Cellobiohydrolases I (CBHI) are modular enzymes consisting of a minimum of one catalytic module and one cellulose binding module (CBM) connected by a proline/serine/threonine-rich linker [3,4]. CBHs from different microbial sources belong to families 6 and 7 of glycoside hydrolases [5]. The most characterized members of the family 7 are cellobiohydrolase Cel7A from *Trichoderma reesei* and cellobiohydrolase Cel7D from *Phanerochaete chrysosporium*. The structure of both CBHs consists of two β-sheets that pack face-to-face to form a β-sandwich [6,7]. The cellobiohydrolase Cel7A from *T. reesei* is composed of long loops, on one face of the sandwich, that form a cellulose binding tunnel of 50 Å. The catalytic residues are glutamate 212 and 217, which are located on opposite sides of the active site, separated by an intervening distance consistent with a double-displacement retaining mechanism [3]. The mechanism of action, the kinetic parameters and the enzyme–ligand interactions of enzymes belonging to family 7 of glycoside hydrolases are well characterized [6].

The fungus *Penicillium occitanis* has been shown to possess a high capacity for the production of cellulases [8,9] having high cel-

Abbreviations: *cbh1*, cellobiohydrolase I; CBM, cellulose binding module; GHF, glycoside hydrolase family.

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lulose degradation efficiency [10]. Two cellobiohydrolases (CBHI and CBHII) and two β-glucosidases have been purified from this fungus and their properties were characterized [11]. Compared with other *P. occitanis* cellulases, the amount of CBHI secreted is much higher (50% of total proteins). CBHI of *P. occitanis* was described as an enzyme producing cellobiose from cellulose. This enzyme shares some biochemical properties with the cellobiohydrolases of glycoside hydrolases family 7 [11]. CBHI was also reported to act synergistically with cellobiohydrolases II and to be inhibited by cellobiose [11]. But, compared to *P. chrysosporium*, *T. reesei* and *Talaromyces emersonii* cellobiohydrolases I, *P. occitanis* CBHI exhibited less pronounced product inhibition [11–13]. Interestingly, this enzyme showed a mannanase activity using the Locust Bean Gum as substrate (unpublished data).

Cellobiohydrolases genes have been cloned and characterized from a variety of fungal sources [14–17]. However, there are no reports of gene sequences coding for extracellular cellulolytic enzymes from *P. occitanis*.

As an initial step toward elucidating the genetic basis for the production of cellulases from this organism, we report here the cloning and the characterization of the *cbh1* cDNA and its corresponding gene from a genomic bank of *P. occitanis*. We also describe the similarity between the deduced CBHI protein and other fungal cellobiohydrolases.

In order to explain the origin of the resistance to cellobiose inhibition, exhibited by this enzyme, structural modelling of the *P. occitanis* CBHI has been performed based on the X-ray crystallographic structure of *T. emersonii* cellobiohydrolase I.

2. Materials and methods

2.1. Strains

P. occitanis CL100 and Pol6 were provided by Professor Tiraby, CAYLA Company, Toulouse—France. The Pol6 strain is a hypercellulolytic mutant selected by Jain et al. [8] after eight rounds of mutagenesis from the CL100 mother strain. The CT1 strain is a hyperpectinolytic and a fully constitutive mutant selected after a single round of mutagenesis from the same parental strain [18]. *Escherichia coli* strain: Top 10 F′ ((F′ *lacI*^{q} Tn*10* (Tet^R)) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R)*end*A1 *nup*G; Invitrogen) was used as a host for the pUC18 and pMOS*blue* T cloning vectors.

2.2. Vectors

pMOS*blue* T-vector (Amersham) was used for the cloning of PCR fragments; λMOS *Elox* plasmid (Amersham) was used for the construction of the cDNA library; pMOS *Elox* derived from the excision of the λMOS *Elox* after infection of the BM 25.8 strain and pUC18 was used for the construction of the genomic library.

2.3. Media and growth conditions

Potato dextrose agar (PDA, Merck Co.) was used for the propagation and the storage of the fungal strains. The liquid medium of Mandels and Weber [19] modified by Ellouz Chaabouni et al. [9] was also used. The carbon source is 2% cellulose (Avicel PH 101 Fluka, Switzerland) or 2% glucose. The cultures were grown in Erlenmeyer flasks (100/500 ml) at 30 °C. Luria broth medium was used for the cultivation of bacterial strains.

2.4. Amplification of the cbh1 cDNA

Reverse transcription was performed for 60 min at 37 °C on cellulose-induced polyA$^+$ mRNA as a template with an oligo-dT primer [5′-GGGATCCGCGGCCGC(T_{15})]. The mRNA extracted from glucose grown culture, was used as a control.

Based on the sequences of fungal cellobiohydrolases I present in the database, primers were designed for the amplification of the *cbh1* cDNA. The primer sequences are as follows: P1: 5′-TGCGGTCTCAACGGCGCCCTCTA-3′, P2: 5′-ATGGACGCCGACGGTGG-3′ and P3: 5′-GAGATGGATATCTGGGAGGCCAA-3′ (sense primers corresponding to the peptides CGLNGALY, MDADGG and EMDIWEAN, respectively), P4: 5′-GGGATAGGTGCTGTCGAGCCACAACA-3′, P5: 5′-CCICCA/GCAT/CTGICC-3′ and P6: 5′-AAGGCATTGCGAGTAGTAGTCGTT-3′ (antisense primers corresponding to the peptides MLWLDSTYP, GQCGG and YYSQCL, respectively). The amplification protocol consisted of an initial denaturing cycle of 30 s at 94 °C followed by a 90 s annealing step at 55 °C and finally a 4 min polymerisation cycle at 72 °C.

2.5. Construction and screening of the cDNA library

The cDNA library was constructed using 4 μg of mRNA according to the manufacturer's instructions of the cDNA Synthesis System kit (Amersham). Library screening was done with the RT-PCR amplified fragment described previously, as a probe.

2.6. Construction and screening of the genomic library

Total genomic DNA of CL100 was prepared according to Aifa et al. [20], partially digested with Sau3AI and fractionated by sucrose gradient (10–40%) according to Hopwood et al. [21]. The fragments sizing from 6 to 9 kb length were isolated and cloned in pUC18 vector linearized at the BamH1 site. The recombinant clones were selected on Luria broth plates containing 100 μg/ml of ampicillin and 12.5 μg/ml of tetracycline.

2.7. Northern and Southern blots

RNA, prepared according to Aifa et al. [20], was denatured with formamide and size fractionated by electrophoresis in 1.5% agarose formaldehyde gel. Restriction enzyme digestions, Northern and Southern hybridizations were performed as described by Sambrook et al. [22] using N$^+$-Hybond.

2.8. Primer extension

Poly(A)$^+$ RNA were isolated as described in the Quick Prep Micro mRNA Purification Kit (Amersham). Two picomoles of a 5′γ^{32}P labelled primer (5′-GGTTTCAGCAGTATAAGT-3′) located at 87 nucleotides from the initiating ATG of the *cbh1* gene, were mixed with 4.5 μg of polyA$^+$ cellulose-induced RNA in the presence of 5× annealing buffer (25 mM Tris, pH 8.3; 375 mM KCl and 5 mM EDTA), denatured at 75 °C for 2 min, incubated at 45 °C for 30 min and then gradually cooled down to 37 °C.

After an ethanol precipitation step, the extension was performed in 20 μl final volume containing 15 units of AMV (Amersham) in 1× reverse transcriptase buffer (50 mM Tris–HCl, pH 8.3; 50 mM NaCl; 8 mM $MgCl_2$ and 1 mM DTT), 5 mM DTT, 1.5 mM dNTP. After 1 h at 42 °C, the reaction was stopped by addition of a solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF.

2.9. Sequencing and sequence analysis

The nucleotide sequence was carried out on both strands with both Thermosequenase Cycle Sequencing Kit (Amersham) and the BigDye Terminator version. 3.1 Cycle Sequencing Kit using an automated ABI Prism 3100-Avant Genetic Analyser (Applied Biosystems Inc.).

The *cbh1* gene sequence reported in this article has been submitted to the GenBank under accession number AY690482.

2.10. Amino acid sequence analysis and homology modelling

Multalin software was used to generate the alignment of CBHI sequences [23]. Rendering of the alignment figure including the prediction of the secondary structures was performed with the ESPript program [24]. Putative signal sequences were identified using the SignalP prediction software [25].

2.11. Computer-aided model building of the tertiary structure of the CBHI

The automated protein structure homology-modelling server, SWISS-MODEL [26] was used to generate the 3D model. The Deep View Swiss PDB Viewer software from EXPASY server (available at http://www.expasy.org/spdbv) was used to visualize and analyze the atomic structure of the model. Molecular modelling of *P. occitanis* CBHI was analyzed based on the X-ray crystallographic structure of the cellobiohydrolase of *T. emersonii* (pdb accession code 1Q9H). Finally, PyMOL [27], the Molecular Graphics System was used to render figures.

3. Results and discussion

3.1. Isolation of the cbh1 cDNA and analysis of its sequence

In order to clone the *cbh1* cDNA of *P. occitanis*, we performed RT-PCR reactions on poly(A)$^+$ RNA extracted from the hypercellulolytic Pol6 mutant grown on cellulose and glucose using different primer combinations: P1–P4, P2–P4, P3–oligo-dT, P2–P5 and P2–P6 (Fig. 1B).

The amplified fragments were tested in Southern blot by a CBHI probe obtained by PCR from a *Trichoderma* species. Only the cellulose-induced RNA allowed the amplification of 1 kb fragment using the P3 and the oligo-dT primers, which strongly hybridized