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Cloning and Sequence Analysis of the Cellobiohydrolase I Genes from Some Basidiomycetes

Ekachai Chukeatirote[1,2]*, Sajeewa S. N. Maharachchikumbura[1,2], Shannaphimon Wongkham[3], Phongeun Sysouphanthong[1,2], Rungtiwa Phookamsak[1,2] and Kevin D. Hyde[1,2]

[1]*Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[2]*School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[3]*Scientific & Technological Instruments Centre, Mae Fah Luang University, Chiang Rai 57100, Thailand*

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Genes encoding the cellobiohydrolase enzyme (CBHI), designated as *cbhI*, were isolated from the basidiomycetes *Auricularia fuscosuccinea, Pleurotus giganteus, P. eryngii, P. ostreatus*, and *P. sajor-caju*. Initially, the fungal genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) protocol and used as a DNA template. The *cbhI* genes were then amplified and cloned using the pGEM-T Easy Vector Systems. The sizes of these PCR amplicons were between 700~800 bp. The DNA sequences obtained were similar showing high identity to the *cbhI* gene family. These *cbhI* genes were partial consisting of three coding regions and two introns. The deduced amino acid sequences exhibited significant similarity to those of fungal CBHI enzymes belonging to glycosyl hydrolase family 7.

KEYWORDS : Basidiomycetes, *cbh*, Cellobiohydrolase, Cellulase, Gene cloning

Introduction

Cellulose, one of the most abundant renewable biopolymer, is a linear insoluble polysaccharide of β-D-glucose (~100 to 20,000 residues) joined by β-1,4-glucosidic bond. As a major component of plant cell wall, cellulose degradation provides several benefits considered from environmental and bio-energy issues [1, 2]. Decomposition of plant litter is also an important process to release nutrients into the soil [3]. In nature, cellulose can be hydrolysed by enzymatic means, the enzymes mainly being derived from a wide range of microbes. Cellulase enzymes are generally composed of a multienzyme system [4] including cellobiohydrolase (CBH, E.C. 3.2.1.91), endo-1,4-β-glucanase (EG, E.C. 3.2.1.4), and β-glucosidase (BGL, E.C. 3.2.1.21). The CBH and EG enzymes function cooperatively and synergistically, and depolymerise cellulose to cellobiose and oligosaccharides. The BGL enzyme then converts these small molecules to glucose [5]. The CBH enzymes are known as the key component of cellulase system that display an exo-type of digesting biopolymers, and the major product of their action on cellulose is cellobiose [6]. Based on amino acid sequence similarity, the CBH enzymes can be grouped into glycoside hydrolase families: GH6, GH7, and GH48 [7]. Of these, only the GH7 type is thought to be exclusively of fungal origin and this family includes the CBHI and EGI enzymes from both ascomycetes and basidiomycetes [8].

Several fungi can utilize cellulose as an energy source by secreting a complex of cellulase enzymes. These enzymes are of great interest considered from the viewpoint of industrial and environmental application [2]. The fungal CBH in particular is of great interest as potent cellulolytic fungi are capable of producing two different forms of the CBH enzymes: CBHI and CBHII [6]. It should be noted however that the CBHI is the principal component of the cellulase enzymes as reported in *Trichoderma reesei* [9] and in *Volvariella volvacea* [10]. Considered a key role of fungi in degrading plant biomass in ecosystems, a programme was initiated to investigate the nature of the cellulolytic system with an emphasis on the CBHI of some basidiomycetes. In this paper, we report the presence of a gene encoding the cellobiohydrolase enzyme from *Auricularia fuscosuccinea*, *Pleurotus giganteus*, *P. eryngii*, *P. ostreatus*, and *P. sajor-caju*.

Materials and Methods

Fungal culture. The fungal strains used in this study were *A. fuscosuccinea* MG01, *P. eryngii* MG02, *P.*

*Corresponding author <E-mail : ekachai@mfu.ac.th>

giganteus MFLU100154, *P. ostreatus* MG03, and *P. sajor-caju* MG04. All was maintained on potato dextrose agar at 28°C for 10 days.

DNA extraction. The genomic DNA was extracted from the fungal mycelium using a modified cetyltrimethyl ammonium bromide (CTAB) protocol [11]. Fifty mg of fungal mycelium were collected and resuspended in 500 µL of the extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid [pH 8.0], 0.5% SDS). Three hundred µL of phenol and 150 µL of chloroform were added and the cell suspension was mixed by inverting. The cell debris were then pelleted at 13,000 rpm for 20 min. The supernatant was transferred to a new microfuge tube and 5 µL of 10 mg/mL RNaseA was added; the reaction mixture was incubated at 37°C for 10 min. The solution was then extracted with an equal volume of chloroform. The treated DNA was precipitated by adding 250 µL of isopropanol by incubating at −20°C overnight. The DNA was then pelleted by centrifugation at 13,000 rpm for 15 min at 4°C. Two hundred fifty µL of ice-cold ethanol was then added and the mixture was mixed by inverting and centrifuged at high speed (13,000 rpm) for 5 min. The DNA pellet was then aspirated and resuspended in 50 µL of TE.

PCR, cloning and sequence analysis. Approximately 100 ng of the genomic DNA was used as a template for PCR amplification. A PCR reaction was set up in a final volume of 25 µL containing 1 mM $MgCl_2$, PCR buffer, each dNTPs at 200 µM, 20 pmol of the IB (5'-TGYGAYTCICARTGYCCICGIGA-3') and the IC (5'-GARTCIAGCCAIAGCATRTTIGC-3') primers [10] and 1 U of *Taq* DNA polymerase. The PCR amplification was carried out in a T-Gradient thermocycler (Biometra, Gottingen, Germany) using the following incubation cycles: 94°C for 3 min, 52°C for 1 min, and 72°C for 1 min followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min. After completion of the PCR, 10 µL of the samples was electrophoresed on a 0.8% agarose gel to determine whether the DNA of interest was amplified. The amplified products were then purified using the Nucleospin Extract II column (Macherey-Nagel Inc., Düren, Germany) and cloned using the pGEM-T Easy Vector Systems (Promega, Madison, WI, USA). The recombinant plasmids were transformed into the competent *Escherichia coli* DH5α cells prepared in accordance with the protocol of Sambrook *et al*. [12]. The cloned *cbhI* genes were then sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The nucleotide sequence data obtained were deposited in the GenBank database with the following accession Nos.: JQ717249 for *A. fuscosuccinea* MG01, JQ717250 for *P. giganteus* MFLU100154, JQ717251 for *Pleurotus eryngii* MG02, JQ717252 for *P. sajor-caju* MG04, and JQ717253 for *P. ostreatus* MG03. These DNA sequences were then analysed using the Clustal W and BLAST programmes [13, 14]. To obtain more information, the *cbhI* genes were also deduced after alignment against the known *cbhI* mRNA sequences (*P. ostreatus* sp. 'Florida' [AM262873.1 and AM262871.1], *Flammulina velutipes* Fv-1 [AB540999.1], and *Irpex lacteus* [AB019376.1]). Partial protein sequences were then predicted by translation after removal of introns. The putative CBHI proteins were assessed with BLAST P software [13].

Results and Discussion

Class I cellobiohydrolases (also known as CBHI or family 7 glycosyl hydrolases) are the principal enzymes of the cellulase complexes. In this work, we aimed to extend previous knowledge which has described the presence of the *cbhI* gene in mushrooms [10, 15-17]. Initially, the genomic DNA samples were extracted from the mushroom mycelia (Fig. 1) and thus used as template for PCR. It was found that a partial sequence of the *cbhI* genes (~750 bp) were amplified successfully using the IB and IC primers (Fig. 2). These amplified DNA fragments were then subcloned into the plasmid pGEM-T. It was confirmed, by a PCR method, that the *cbhI* gene was successfully cloned (data not shown) and these amplicons were subjected to nucleotide sequencing.

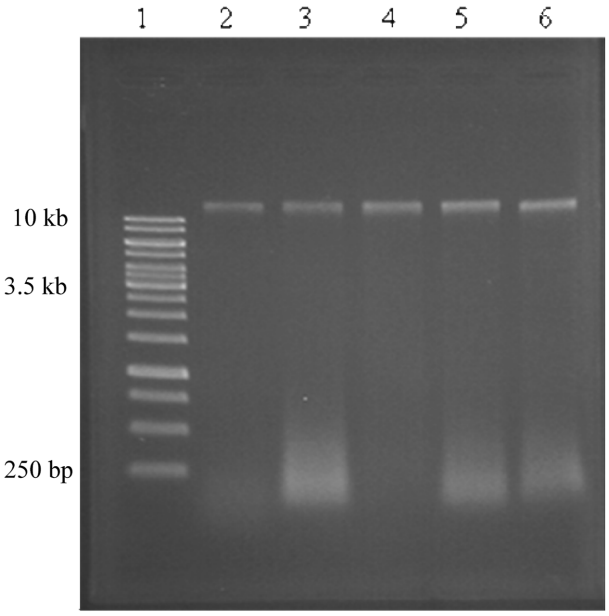

Fig. 1. Electrophoresis of genomic DNA isolated from mushroom mycelia. DNA samples were separated on a 0.8% agarose gel in 1 × TAE buffer. Lane 1, 1 kb DNA marker; lane 2-6, genomic DNAs of *Auricularia fuscosuccinea*, *Pleurotus giganteus*, *P. eryngii*, *P. ostreatus*, and *P. sajor-caju*, respectively.

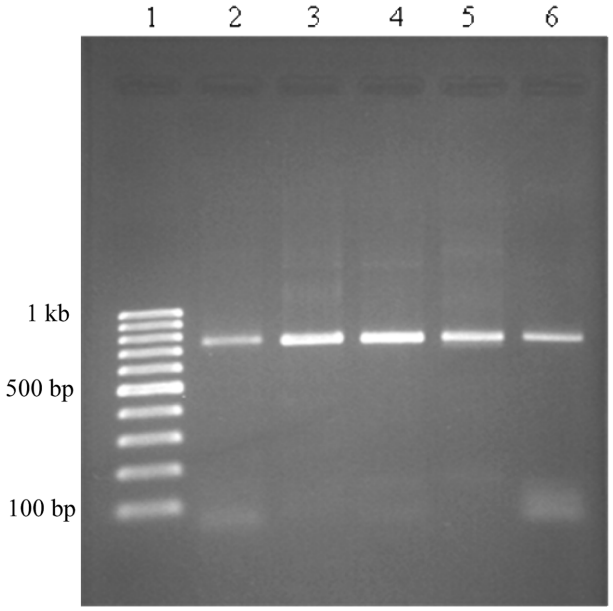

Fig. 2. PCR amplicons of the *cbh* gene from mushroom genomic DNA. Lane 1, 100 bp DNA marker; lane 2-6, genomic DNAs of *Auricularia fuscosuccinea*, *Pleurotus giganteus*, *P. eryngii*, *P. ostreatus*, and *P. sajor-caju*, respectively.

Each DNA sequence obtained was subsequently submitted as a query in a BLASTN search of the GenBank database. This homology search was performed resulting that all DNA sequences were closely related to those of the *cbhI* gene family of either *P. ostreatus* or *F. velutipes* (Table 1). The similarity of these mushroom *cbhI* genes was determined using the Clustal W software. Based on this analysis, their similarity was very high ranging from 71 to 99% (data not shown).

The translation of these *cbhI* genes (excluding introns) into the CBHI proteins was also performed using the Translate tool (http://web.expasy.org/translate/). The analysis revealed five putative amino acid sequences similar to the cellobiosidase of *P. ostreatus* sp. 'Florida' and the cellobiohydrolase of *Phanerochaete chrysosporium* (see Table 2). It is evident that these mushroom CBHI proteins showed high homology with the enzymes belonging to glycoside hydrolase family 7 [8].

There are several studies on fungal cellulolytic systems but these are limited to a few key species such as *Trichoderma reseei* and *P. chrysosporium*. The cellulase activity has also been reported in some mushrooms including *Agaricus bisporus* [18], *Lentinula edodes* [15], and *V. volvacea* [10]. Our data provide additional information on the presence of the *cbhI* genes in several mushrooms including the recently recollected species *P. giganteus* [19]. It should be also noted that there were several forms of the *cbhI* genes. For example, the *cbhI* gene of *P. ostreatus* MG03 (in this study) was distinct although it showed high homology to the *cbhI-II* of *P. ostreatus* sp. 'Florida' (96.9%). This finding is in agreement with previous work in which multiple forms of the *cbhI* gene have been reported [20, 21]. This is possibly due to a combination of alleic variation. However, the existence of different forms of the *cbhI* gene may be advantageous considered from their ability in protein expression when exposed to different environmental conditions.

In summary, we successfully cloned the genes encoding the CBHI proteins from five mushrooms. This present work was undertaken with an expectation that future research will be explored including the expression of the *cbhI* gene as well as the factors affecting the CBHI activity.

Table 1. Cellobiohydrolase genes (*cbhI*) recovered from genomic DNA of five mushrooms and best BLAST N matches

Mushroom *cbhI* genes (accession No.)	Length of amplicon (bp)	BLAST N match (% identity) (accession No.)
Auricularia fuscosuccinea (JQ717249)	748	*P. ostreatus cbhI-II* gene (96.93) (AM262873.1)
Pleurotus eryngii (JQ717251)	746	*P. ostreatus cbhI-III* gene (91.73) (AM262871.1)
P. giganteus (JQ717250)	745	*Flammulina velutipes cel7A* gene (77.67) (AB540999.1)
P. ostreatus (JQ717253)	747	*P. ostreatus cbhI-II* gene (96.9) (AM262873.1)
P. sajor-caju (JQ717252)	748	*P. ostreatus cbhI-II* gene (97.42) (AM262873.1)

Table 2. Cellobiohydrolase enzymes (CBHI) of five mushrooms deduced from their *cbhI* gene counterparts and best BLAST P matches after intron removal and translation

Mushroom CBHI	Length of protein (amino acids)	BLAST P match (% identity) (accession No.)
Auricularia fuscosuccinea	214	Cellobiosidase of *P. ostreatus* sp. 'Florida' (97.66) (CAK18800.1)
Pleurotus eryngii	98	Cellobiosidase of *P. ostreatus* sp. 'Florida' (95.88) (CAK18798.1)
P. giganteus	140	Cellobiohydrolase of *Phanerochaete chrysosporium* (69.39) (1GPI_A)
P. ostreatus	134	Cellobiosidase of *P. ostreatus* sp. 'Florida' (92.93) (CAK18800.1)
P. sajor-caju	134	Cellobiosidase of *P. ostreatus* sp. 'Florida' (92.93) (CAK18800.1)

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