Cloning and characterization of two cellulase genes from *Lentinula edodes*

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Abstract

*Lentinula edodes* has traditionally been grown on fallen logs. It produces a wide array of enzymes to digest the lignocellulolytic substrate for nutrients. Thus, this organism represents a rich source of potentially potent lignocellulolytic enzymes that can be harnessed for conversion of biomass to simple sugars. These sugars can then be used as feedstock for ethanol production or other chemical syntheses. We have cloned two cellulase genes from *L. edodes* grown on a wood substrate without the use of genomic or cDNA libraries by using a PCR-based strategy employing degenerate primers directed at the cellulose-binding domain. *cel7A* encoded a 516-amino acid protein that belonged to glycosyl hydrolase family 7 and had sequence similarities to *cbhI* genes from other fungi. *cel6B* encoded a 444-amino acid protein that belonged to glycosyl hydrolase family 6 and had sequence similarities to *cbhII* genes from other fungi. We demonstrated that *cel7A* and *cel6B* transcript levels were positively correlated to *L. edodes* growth in the presence of crystalline cellulose.

Keywords: Cellulose; Cellulase; mRNA; Rapid amplification of cDNA ends-PCR; Cloning; *Lentinula edodes*

1. Introduction

*Lentinula edodes*, commonly referred to as the Shiitake mushroom, is an economically important crop and has experienced increasing popularity due to both its flavor and purported medicinal value [1]. The culturing of *L. edodes* is the world’s largest wood bioconversion process [2]. *L. edodes* has traditionally been grown on freshly cut logs [3]. It is a white rot fungus that decays for nutrients all the major polymers (cellulose, hemicellulose, and lignin) found in wood lignocellulose. Thus, *L. edodes* produces a wide variety of enzymes that may have high activities against lignocellulosic biomass.

With the growing motivation to use renewable resources in manufacturing processes, there has been an increased interest in degrading lignocellulosic biomass to commercially useful feedstocks. Cellulose is the main component of lignocellulose. It is a linear glucose polymer in which the subunits are linked through 1,4-β-glycosidic bonds. The cellulose polymers coalesce to produce microfibrils that have a crystalline structure.

Cellulase enzymes digest the glycosidic bonds of the cellulose polymer. In general, cellulase enzymes have a modular structure composed of a catalytic domain, a cellulose-binding domain (CBD), and a linker region that attaches the two domains [4,5]. The catalytic domain hydrolyzes the 1,4-β-glycosidic bonds using an acid–base pair in a single- or double-displacement mechanism corresponding to an inverting or retaining action, respectively [6,7]. Cellulases can be categorized into families based upon amino acid sequence similarities of the catalytic domain [8]. Members of a particular family are thought to share the same general protein structure and mechanism (i.e. retaining or inverting enzymatic attack).

The CBD can be located at either the N- or C-terminus of the cellulase enzyme. The presence of a CBD is critical for the efficient digestion of crystalline cellulose. The CBD could increase enzyme-catalyzed degradation of crystalline cellulose by increasing the local concentration of the enzyme at the substrate. The CBD might also disrupt non-covalent interactions of the cellulose microfibrils and make the substrate more accessible to the enzyme [9]. This domain can be classified into different families based on sequence analysis [10].

The isolation and characterization of cellulase enzymes from *L. edodes* will provide additional reagents that may
increase the efficiency of biomass conversion to useful chemical feedstocks. In addition, a better understanding of the repertoire of *L. edodes* cellulases may lead to improvements in Shiitake mushroom crop yields. We used a PCR strategy involving degenerate oligonucleotides to clone two cellulase genes, *cel7A* and *cel6B*, from *L. edodes* without the use of a genomic or cDNA library. We characterized the expression of these genes on different carbon sources and demonstrated that both are activated in the presence of crystalline cellulose.

2. Materials and methods

2.1. Fungus and growth conditions

*L. edodes* strain Stamets CS-2 (Fungi Perfecti, Olympia, WA, USA) was cultured at room temperature on either alder sawdust blocks or liquid media. From the sawdust block, mycelium was harvested with a scalpel at various stages (pinning, button, and veil break) of mushroom development. The mycelium samples were pooled and frozen in liquid nitrogen and stored at −80°C until further processing.

Medium used for liquid culture was composed of 3 mM (NH₄)₂SO₄, 3 mM L-asparagine, 7 mM KH₂PO₄, 0.7 mM CaCl₂, and 0.05% yeast extract [11]. Medium was additionally supplemented with either 0.2% glucose or 0.2% microcrystalline cellulose (Avicel).

2.2. RNA isolation

Total RNA was collected from mycelium grown in liquid culture. The mycelium was harvested and then ground to a fine powder in liquid nitrogen with a mortar and pestle. One hundred mg of tissue powder was then lysed and total RNA was collected with the RNeasy Plant kit (Qiagen, Valencia, CA, USA). The RNA was treated with DNaseI and again purified with the RNeasy Plant kit. Total RNA was also collected from the mycelium grown on the alder sawdust. The total RNA was isolated as described above. Then, the Oligotex mRNA kit (Qiagen) was used to purify poly-A mRNA from the total RNA.

2.3. Cloning cellulase genes

The mRNA isolated from the sawdust-grown mycelium was used as the starting material to isolate the cellulase genes. The GeneRacer kit (Invitrogen, Carlsbad, CA, USA) was used to conduct RACE-PCR (rapid amplification of cDNA ends-PCR). In brief, the mRNA was treated with calf intestinal phosphatase followed by tobacco acid pyrophosphatase to remove the cap structure. An RNA oligonucleotide linker was ligated to the 5’ end of the decapped mRNA. cDNA was then generated by reverse-transcribing the mRNA with a poly-dT oligonucleotide linker localized to the 3’ end. cDNA resulting from 6.25 ng of mRNA was used as a template in PCR reactions using both a primer directed against the known 5’ or 3’ linker ends of the mRNA and degenerate primers that were designed based upon cloned CBD sequences from other fungal cellulases. PCR was carried out with PfuTurbo Hotstart DNA polymerase (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. Products of the PCR reaction were cloned into pCR4Blunt-TOPO vector (Invitrogen) and sequenced. This sequencing information was then used to design another set of internal primers against the putative cellulase genes. These new primers were used in conjunction with the 5’ and 3’ primers (directed against the known linker regions) in another round of PCR using the cDNA as a template. These final PCR reactions produced the 5’ and 3’ halves of the cellulase gene that were then assembled into full-length constructs in the pCR4Blunt-TOPO vector.

2.4. Reverse transcription (RT)-PCR to determine transcript levels

Total RNA isolated from mycelium grown in liquid culture supplemented with either glucose or cellulose was used as template for RT reactions. Two µg of RNA and 10 pmol of RT primer specific to either the *cel7A* or *cel6B* gene were heated to 70°C for 5 min and then chilled on ice. Reaction buffer, nucleotides, and M-MLV reverse transcriptase (Promega, Madison, WI, USA) were added, and the reaction was incubated at 42°C for 1 h. The resulting cDNA was used as template in PCR reactions using primers (RT and FOR, below) specific to either *cel7A* or *cel6B* genes. The sequences of the oligonucleotides used are shown. *cel7A*-RT 5’-GAGCAGCACCTGAGGCCGAGGTTTTG-3’, *cel7A*-FOR 5’-CTACGGCCAGGGCTC-ACAGTCCG-3’, *cel6B*-RT 5’-GCGTTCCGAATCCAGC-ACCACA-3’, *cel6B*-FOR 5’-CGGCAACCCCTTCACTG-GTTACG-3’. The PCR products were then subjected to agarose gel electrophoresis and visualized by ethidium bromide staining.

3. Results and discussion

3.1. Cloning of cellulase genes

In order to clone cellulase genes from *L. edodes* without the use of genomic or cDNA libraries, we employed a RACE-PCR strategy (see Section 2). This procedure required the use of internal primers specific to the genes of interest. Because there was no *L. edodes* cellulase gene sequence available at the time, we designed degenerate oligonucleotides based on an alignment of CBDS from...
cellulase genes that had been cloned from Agaricus bisporus [12–14] and Volvariella volvacea [15]. Using these degenerate oligonucleotides and L. edodes cDNA in RACE-PCR, several gene fragments were obtained. These fragments were sequenced, and oligonucleotides were synthesized based on the new sequence. These oligonucleotides were then used in a second round of RACE-PCR to clone the 5' and 3' halves of the cellulase genes. Two full-length cellulase genes were isolated, cel7A and cel6B.

3.2. The cel7A gene

cel7A was a 1551-bp gene that encoded a 516-amino acid protein with a predicted molecular mass of 53.5 kDa and a pI of 4.1 (GenBank accession number AF411250) (Fig. 1). As would be expected of an enzyme that is secreted, there was a signal peptide at the N-terminus that was predicted to be cleaved between Gly18 and Gln19 [16]. The cel7A enzyme had a 430-amino acid cata-
lytic domain (residues 20–449) that categorized the protein as a member of the glycosyl hydrolase family 7 [8]. When compared to other family 7 fungal cellulases, the cel7A enzyme had high sequence similarities to enzymes that had been classified as cellobiohydrolase I (Fig. 1). A serine/threonine-rich linker sequence (residues 451–476) connected the catalytic domain to a 32-amino acid fungal-type CBD (residues 485–516) at the C-terminus of the protein. The L. edodes cel7A polypeptide also contained two conserved residues (Glu232 and Glu237) that were implicated as the catalytic acid–base pair based on detailed studies of the Trichoderma reesei cellobiohydrolase I (CEL7A) [17,18].

3.3. The cel6B gene

The second cellulase gene that we cloned encoded a 444-amino acid protein with a predicted molecular mass of 46.4 kDa and a pI of 4.1 (Fig. 2). This protein had a catalytic domain (residues 103–410) that classified as...
zyme as a glycosyl hydrolase family 6 member [8]. Another
*L. edodes* family 6 cellulase gene (*cbhII-1*) had been deposed
into GenBank (accession number AF244369) after the
initiation of our work by a different laboratory, and thus
we named our gene *celbB* (GenBank accession number
AF411251). The *cbhII-1* and *celbB* genes are very similar
and differ by three amino acid substitutions (Gly26Ala,
Gly96Asp, and Tyr97Gln) at the predicted protein level.
In addition, the *celbB* gene contains an extra amino acid
(Asp98). It is unknown whether these differences would
result in different activities between these two proteins. It
is possible that these genes are alleles since different strains of
*L. edodes* (L54 for *cbhII-1* vs. Stamets CS-2 for *celbB*)
were used in the isolation of the genes.

Like *cel7A*, *celbB* contained a signal peptide that was
predicted to be cleaved between Gly20 and Gin21 (Fig. 2)
[16]. A fungal-type CBD was located at the N-terminus
(residues 25–56). A serine/threonine-rich linker region (residues
59–82) connected the CBD to the C-terminal catalytic
domain. Alignment of the predicted *celbB* polypeptide
to other fungal family 6 cellulase enzymes showed that
the protein had homology to cellobiohydrolase II enzymes
(Fig. 2). Residues that are believed to comprise the acidic-
base pair critical for catalytic activity in the *T. reesei*
cellobiohydrolase II (CEL6A) protein were conserved in
*L. edodes* *celbB* (Asp223 and Asp399) [19,20].

3.4. Regulation of *cel7A* and *celbB* transcription

To determine whether or not the *cel7A* and *celbB* genes
are regulated by growth in cellulose, *L. edodes* mycelium
was cultured in liquid media supplemented with either
glucose or cellulose as the sole carbon source (see Section
2). RNA was subjected to RT-PCR using primers that were
specific to either the *celbA* or *celbB* genes. Electrophoresis of the RT-PCR
products demonstrated that the levels of *cel7A* and *celbB*
did not differ to any significant extent (Fig. 3). This pattern of
expression supports the theory that *cel7A* and *celbB* are involved
in cellulose degradation.

In summary, we used degenerate oligonucleotides
directed against the conserved CBD to clone cellulase genes
from *L. edodes*. The *cel7A* and *celbB* genes had high ho-

mologies to cloned fungal cellobiohydrolase I and cel-
lobiohydrolase II genes, respectively. Both genes also re-
tained the amino acid residues that were previously
determined to be critical to catalytic activity in the other
cellulases. *cel7A* and *celbB* are likely to be involved in
cellulose degradation since these two genes are activated
upon growth in cellulose. This result is consistent with
those of other cloned cellulase genes that are also induced
upon cell growth in cellulose [13,15,21,22].

We were able to clone the cellulase genes without the
use of genomic or cDNA libraries. This cloning strategy
should be very useful to other groups trying to clone novel
cellulase genes. The creation of genomic or cDNA libraries
is a time-consuming and costly procedure. The process of
screening clones by filter lifts and DNA probe hybridizations
can also be very expensive with respect to time and
materials. These steps have been eliminated with our PCR-
based strategy. Although our protocol relies on the pre-

erence of a CBD in the cloned genes, degenerate oligonu-

cleotides directed against other conserved portions of the
cellulase enzymes could also be used.

Future work will include the biochemical characteriza-
tion of the protein products of these two genes. Based on
sequence analysis, both the *cel7A* and *celbB* protein prod-

ucts may be cellobiohydrolase enzymes. Cellobiohydrolase
enzymes function as ‘exo-’acting enzymes by digesting the
ends of the cellulose polymer [23]. This is in contrast to the
‘endo-’activity of the endoglucanase enzymes which digest
the internal portions of the cellulose polymer. The exo-
and endo-acting enzymes will work synergistically to di-
gest crystalline cellulose [24]. One theory is that the endo-
glucanases generate more free ends that can be attacked
by the cellobiohydrolases. However, synergistic cellulose
degradation between two *T. reesei* cellobiohydrolases has
also been demonstrated [25]. The mechanism of this exo–
exo synergism is unclear. The *L. edodes* *cel7A* and *celbB*
enzymes may also be verified to be exo-acting enzymes and
may work synergistically to digest cellulose. Overall, these
studies may lead to an improved understanding of the mechanism of cellulolytic action as well as better enzymes. We may also gain insights into the biochemistry and physiology of *L. edodes* to improve cultivation.

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**References**


