Molecular characterization of a glycoside hydrolase family-51 \(\alpha\)-L-arabinofuranosidase from *Auricularia auricula*

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**ABSTRACT**

The cDNA encoding \(\alpha\)-L-arabinofuranosidase was cloned from the edible fungus *Auricularia auricula* for the first time. The open reading frame of the \(\alpha\)-L-arabinofuranosidase gene *abf* was 1953 bp encoding 650 amino acids, with a predicted protein molecular weight of 71.19 kDa and a theoretical isoelectric point of 5.23. The putative protein was predicted to belong to the glycoside hydrolase family-51. In addition, *abf* was cloned into the pET-32a vector and then expressed in *Escherichia coli* BL21. The recombinant protein, with an expected molecular weight, was observed in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, the transcription levels of *abf* in response to different carbon sources were investigated in this study. The results showed that the expression of *abf* was mostly up-regulated when the mycelia were grown in different carbon sources, and L-arabinose or maltose induction had a significant effect on the expression of *abf*, which was 5.13- and 4.58-fold higher than that in the untreated control sample, respectively. In addition, the highest transcript levels induced by glucose and sucrose appeared on the third day and the levels were 2.47- and 3.11-fold higher compared to the control. These results laid a foundation for further studies on the \(\alpha\)-L-arabinofuranosidase from *A. auricula*.

**Introduction**

China is an agricultural country that produces large quantities of hemicellulose waste every year, such as corn cobs, straw, sugarcane bagasse, rice bran, etc. Due to improper handling, this agricultural and forest waste may be a source of environmental pollution. However, it is encouraging that the hemicellulose waste could be digested by fungi. *Auricularia auricula*, a white-rot fungus, absorbs its nutrients through degrading lignin, cellulose and hemicellulose. It is one of the most cultivated mushrooms in the world due to its high nutritive, economic and medicinal value [1]. It has potential anti-tumour, anti-inflammatory, hypoglycemic, hypolipidemic and anticoagulant characteristics [2–6]. Furthermore, its extracts have been reported to possess antioxidant and radical scavenging properties [7–9]. Despite these important properties of *A. auricula*, the cultivation of this black fungus has been poorly explored, especially the degradation mechanism of cellulose or hemicellulose at the molecular level.

Xylan, the major component of plant hemicelluloses, found in the cell walls of monocots and hard woods, represents one of the most abundant biomass resources [10]. Xylan is a heteroglycan with a backbone of \(\beta\)-\((1\rightarrow4)\)-linked D-xylopyranose residues that can be partially substituted with \(\alpha\)-L-arabinofuranose as side chains [11]. The hydrolysis of xylan is a complex process with the participation of a series of enzymes including \(\alpha\)-L-arabinofuranosidase (E.C. 3.2.1.55), which hydrolyzes arabinose residues in the alpha configuration linked at positions C-2 and/or C-3 of the xylose [12]. \(\alpha\)-L-arabinofuranosidases are currently found in glycoside hydrolase (GH) families 2, 3, 10, 43, 51, 54 and 62 on the basis of amino-acid sequence similarities (http://afmb.cnrs-mrs.fr/CAZY/) [13].

**Keywords**

Auricularia auricula; \(\alpha\)-L-arabinofuranosidase; glycoside hydrolase family-51; SDS-PAGE; quantitative real-time PCR

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In this study, to the best of our knowledge, we described for the first time the structure of a GH family-51 α-L-arabinofuranosidase gene (abf) from A. auricula and its encoded product ABF. Moreover, we also reported the transcript levels of abf in response to different carbon sources.

Materials and methods

Strain and growth conditions

The A. auricula strain DL202 was collected from Quercus mongolica at Liangshui Nature Reserve, Lesser Xing’an Mountains in Yichun city, Heilongjiang Province, China, in August 2008, and was identified based on internal transcribed spacer sequence alignment. The mycelia of A. auricula were maintained on potato dextrose agar plates and kept in the dark at 25 °C for 7 d. Then they were inoculated into liquid potato dextrose (PD) medium and frozen at −80 °C until RNA extraction. Total RNA was extracted using TRizol reagent according to the manufacturer’s directions (Invitrogen, Carlsbad, CA, USA); afterwards, the samples were treated with RNase-free DNasel. The quality and quantity of RNA were assessed by agarose gel electrophoresis and checked by BioPhotometer D30 (Eppendorf, Hamburg, Germany). cDNA synthesis was carried out using PrimeScript™ 1st strand cDNA synthesis kit (TaKaRa, Japan). cDNA synthesis was performed using the manufacturer’s instructions. Afterwards, the cloning of 3′ and 5′ ends of the abf by RACE-PCR was performed according to the manufacturer’s directions. The primers of abf-GSP3 and abf-GSP5 listed in Table 1 were used for 3′ and 5′ RACE-PCR, respectively. The 3′ RACE-PCR reactions were carried out under the following conditions: 5 min at 94 °C, 35 cycles (30 s at 94 °C; 30 s at 68 °C; 30 s at 72 °C) and 7 min at 72 °C. Meanwhile, 5′ RACE-PCR amplification procedure was set as follows: 5 min at 94 °C, 35 cycles (30 s at 94 °C; 30 s at 65 °C; 30 s at 72 °C) and 7 min at 72 °C. The amplified fragments of both 3′ and 5′ RACE were cloned into pMD18-T vector (TaKaRa, China) and transformed into Escherichia coli DH5α cells (TaKaRa, China). Based on the blue–white screening, positive clones were selected and sequenced. After assembling the sequences of 3′ and 5′ RACE products, a full-length cDNA sequence of abf was obtained.

RNA extraction and cDNA synthesis

The A. auricula mycelia were harvested after 8 d of growth in the liquid PD medium, and frozen at −80 °C until RNA extraction. Total RNA was extracted using TRizol reagent according to the manufacturer’s directions (Invitrogen, Carlsbad, CA, USA); afterwards, the samples were treated with RNase-free DNasel. The quality and quantity of RNA were assessed by agarose gel electrophoresis and checked by BioPhotometer D30 (Eppendorf, Hamburg, Germany). cDNA synthesis was carried out using PrimeScript™ 1st strand cDNA synthesis kit (TaKaRa, Japan). cDNA synthesis was performed using the manufacturer’s instructions. Afterwards, the cloning of 3′ and 5′ ends of the abf by RACE-PCR was performed according to the manufacturer’s directions. The primers of abf-GSP3 and abf-GSP5 listed in Table 1 were used for 3′ and 5′ RACE-PCR, respectively. The 3′ RACE-PCR reactions were carried out under the following conditions: 5 min at 94 °C, 35 cycles (30 s at 94 °C; 30 s at 68 °C; 30 s at 72 °C) and 7 min at 72 °C. Meanwhile, 5′ RACE-PCR amplification procedure was set as follows: 5 min at 94 °C, 35 cycles (30 s at 94 °C; 30 s at 65 °C; 30 s at 72 °C) and 7 min at 72 °C. The amplified fragments of both 3′ and 5′ RACE were cloned into pMD18-T vector (TaKaRa, China) and transformed into Escherichia coli DH5α cells (TaKaRa, China). Based on the blue–white screening, positive clones were selected and sequenced. After assembling the sequences of 3′ and 5′ RACE products, a full-length cDNA sequence of abf was obtained.

Cloning of full-length abf

A putative α-L-arabinofuranosidase gene fragment was found in the A. auricula transcriptome database (unpublished). A pair of degenerate oligonucleotide primers, abf-F1 and abf-R1 (Table 1), were designed according to the fragment sequences and then used for the amplification of the cDNA fragment of abf. Using cDNA as a template, the polymerase chain reaction (PCR) amplification procedure was set as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 40 s, and final extension at 72 °C for 10 min (Bio-Rad, Hercules, CA, USA). A 1954 bp DNA fragment was obtained, cloned into the pMD18-T vector (TaKaRa, China) and subjected to nucleotide sequencing (Boshi, Harbin, China). This fragment was subsequently used for designing gene-specific primers (GSP) for the cloning of 3′ and 5′ ends of the abf by RACE (rapid amplification of cDNA ends) PCR.

Next, 3′ and 5′-RACE-Ready cDNA synthesis was carried out using a SMARTer RACE cDNA amplification kit (Clontech, Carlsbad, CA, USA), following the manufacturer’s instructions. Afterwards, the cloning of 3′ and 5′ ends of the abf by RACE-PCR was performed using Advantage 2 PCR kit (Clontech, USA), according to the manufacturer’s directions. The primers of abf-GSP3 and abf-GSP5 listed in Table 1 were used for 3′ and 5′ RACE-PCR, respectively. The 3′ RACE-PCR reactions were carried out under the following conditions: 5 min at 94 °C, 35 cycles (30 s at 94 °C; 30 s at 68 °C; 30 s at 72 °C) and 7 min at 72 °C. Meanwhile, 5′ RACE-PCR amplification procedure was set as follows: 5 min at 94 °C, 35 cycles (30 s at 94 °C; 30 s at 65 °C; 30 s at 72 °C) and 7 min at 72 °C. The amplified fragments of both 3′ and 5′ RACE were cloned into pMD18-T vector (TaKaRa, China) and transformed into Escherichia coli DH5α cells (TaKaRa, China). Based on the blue–white screening, positive clones were selected and sequenced. After assembling the sequences of 3′ and 5′ RACE products, a full-length cDNA sequence of abf was obtained.

Sequence analysis

The coding region of abf was obtained using ORF Finder (http://www.ncbi.nlm.nih.gov/orf/gorf/gorf.html). The molecular weight, theoretical pl, amino-acid composition and hydrophobicity analysis of the predicted protein were estimated using ProtParam (http://www.expasy.ch/tools/protparam.html) [20]. Signal peptide prediction was carried out using SignalP software (http://www.cbs.dtu.dk/services/SignalP/). Structural regions were identified in deduced protein sequence by the SMART tool (http://smart.embl-heidelberg.de/) and NCBI (National Center for Biotechnology Information) Conserved Domains. Also, secondary structure was determined by Predictprotein tool (http://www.predictprotein.org/) and the three-dimensional structure of ABF was predicted using the Phyre web tool (http://www.sbg.bio.ic.ac.uk/~phyre/index.cgi).
Phylogenetic analysis
Sequence similarity searches were performed in GenBank through the BLAST (basic local alignment search tool) algorithm (http://www.ncbi.nlm.nih.gov/blast) at NCBI, using our abf sequence as a query. Then similar sequences were downloaded and aligned with the abf sequence using the ClustalX software; subsequently, a neighbour-joining tree was constructed with MEGA 5.0 software [21] and bootstrap analysis with 1000 replicates was also conducted in order to obtain confidence levels for the branches.

Heterologous expression of abf in E. coli
Using cDNA as a template, the entire open reading frame (ORF) containing the start codon and the stop codon regions was amplified by abf-B and abf-H primers, which harbour BamHIII and HindIII restriction sites, respectively (Table 1). The amplification procedure was as follows: 5 min at 94 °C, 35 cycles (30 s at 94 °C; 30 s at 57 °C; 30 s at 72 °C) and 7 min at 72 °C. Afterwards, the amplified product was double digested with BamHIII and HindIII enzymes and subsequently ligated into a pET-32a vector (Novagen, Darmstadt, Germany) which was digested with the same restriction enzymes. The pET-32a-abf construct was transformed into E. coli BL-21 (DE3) cells.

An overnight culture of E. coli BL-21(DE3) cells containing the pET-32a-abf construct was grown in a rotary shaker incubator (180 r/min) at 37 °C in Luria–Bertani (LB) medium. Then, 2% of the overnight culture was inoculated into fresh LB medium to obtain an exponentially growing culture (optical density at 600 nm (OD600) of 0.5–0.8). Afterwards, 1 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the exponentially growing cells. Induced cultures of 1 mL each were harvested at 2 h time span for 10 h. Then 1 mL of harvested culture was centrifuged at 12,000 x g for 10 min and the pellet was re-suspended in 100 μL of 4× protein buffer (Solarbio, Beijing, China). Cells were subsequently disrupted by boiling water bath for 10 min. Finally, the lysate was loaded on an 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Transcription analysis of abf in A. auricula
In this study, five different carbon sources (glucose, sucrose, maltose, D-xylose and L-arabinose) were separately added into the basal medium in which A. auricula was grown. The induction medium was composed of 200 g potatoes/L, 20 g carbon source/L, 3 g KH2PO4/L, 1.5 g MgSO4/L and 10 mg VB/L.

The seed culture was incubated using a rotary shaker incubator (160 r/min) at 25 °C for 8–10 d in liquid PD medium. The second set of experiments was performed in 500 mL flasks containing 250 mL of induction medium after inoculation with 10% (v/v) of the seed culture. The flasks were then cultured at 160 r/min at 25 °C. Induction experiments were performed in triplicate (three flasks per inducing medium). The mycelia were harvested at 0, 1, 2, 3, 4 and 5 d, respectively, and then frozen at −80 °C until RNA extraction.

Total RNA was extracted using an RNAPrep pure Plant Kit (Tiangen, Beijing, China) in accordance with the manufacturer’s protocols. Quality and quantity of RNA were assessed as described earlier. Subsequently, total RNA (1 μg) was reverse-transcribed to cDNA using a PrimeScript™ RT reagent Kit (TaKaRa, China), according to the manufacturer's instructions. Finally, the synthesized cDNA (20 μL) was diluted to 200 μL with deionized water and used as the template for the molecular experiments.

Primers abf-qF and abf-qR were used to evaluate the transcript levels of abf, and GAPDH was used as a reference gene (Table 1). Then quantitative real-time PCR (qRT-PCR) was performed with the Mx3000P Sequence Detection System (Agilent Technologies, Santa Clara, CA, USA), and the amplification procedure was set as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The mycelia that were harvested at 0 d served as the reference sample against which all other genes were compared. Transcript levels were evaluated by qRT-PCR according to the 2−ΔΔCT method described by Livak and Schmittgen [22].

Results and discussion
Cloning and sequence analysis of the abf gene
A 1954 bp fragment was obtained and sequenced based upon our transcriptome data of A. auricula, and the gene fragment was confirmed to be an abf fragment due to its high similarity with known sequences. Then 5′- and 3′-cDNA ends were amplified by the RACE technique, depending on the specific fragment obtained. The 2520 bp full-length cDNA of the abf gene was isolated from A. auricula by RACE, including a 426 bp 5′-untranslated region (UTR), a 141 bp 3′ UTR and a 1953 bp ORF encoding 650 amino-acid residues (Figure 1). The cDNA sequence was deposited in GenBank under the accession number KX272626.

Analysis of the deduced protein sequence
The calculated molecular weight of the deduced protein was 71.19 kDa with a theoretical pl of 5.23 and it was a
hydrophobin, according to the ProtParam tool. ABF was identified as a member of the \( \alpha \)-L-AF_C Superfamily by the SMART tool (Figure 2(A)) and NCBI Conserved Domains (Figure 2(B)). Scanning transmembrane protein topology using the TMHMM tool \[23\] revealed that there was no transmembrane region in the ABF protein. In addition, ABF has a signal peptide with a length of 15 residues predicted by the Signal 4.0 server \[24\].

Figure 1. Nucleotide sequence and deduced amino-acid sequence of abf from Auricularia auricula. Note: The ATG start codon and the TAA stop codon are highlighted.

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secondary structure analysis of ABF done using PredictProtein showed that the predicted ABF protein consists of 19.08% alpha-helix, 27.85% beta-sheet and 53.08% loop (coil). Moreover, the three-dimensional structure of ABF was predicted using the Phyre web tool, and the results are shown in Figure 3. These results further confirmed that the ABF protein from *A. auricula* was a member of the \( \alpha \)-L-AF_C Superfamily.

**Phylogenetic analysis**

Using the ABF sequence as a query, 14 homologous sequences were selected from the GenBank database through the BLAST algorithm at NCBI, and the information of homologous sequences from other species is listed in Table 2. The constructed phylogenetic tree showed that ABF from *A. auricula* was most similar to ABF from *A. subglabra* and had a larger evolutionary
distance from ABF of *Trametes versicolor* and ABF of *Phlebiopsis gigantea* (Figure 4). As a result of this search, ABF from *A. auricula* can be assigned to GH family-51. To date, several fungi have been reported to express more than one α-L-arabinofuranosidase. The two isoenzymes from *A. niger* [25], *Aspergillus awamori* [26] and *Penicillium chrysogenum* [27], belong to families 51 and 54.

**Bacterial expression of abf**

In order to observe the expression of *abf* in *E. coli*, the entire protein-coding cDNA of *abf* was cloned into the expression vector pET-32a. Then the pET-32a-*abf* construct was transferred into *E. coli* BL-21 cells. Compared to the control transformant BL21-pET-32a, the recombinant transformant BL21-pET-32a-*abf* produced a clear protein band with a molecular weight of approximately 71.2 kDa in an 8% SDS-PAGE gel (Figure 5). The results indicated that the ABF protease had been successfully synthesized in the *E. coli* cells. The biochemical properties of ABF such as optimal temperature, pH etc. will be investigated in further studies.

### Transcription of *abf* in response to different carbon sources

To investigate the transcription of *abf* in response to different carbon sources (glucose, sucrose, maltose, D-xylose and L-arabinose), qRT-PCR was performed. The real-time PCR results indicated that the transcription of *abf* was differentially regulated in the presence of different carbon sources (Figure 6). The transcript peak of *abf* was obtained in response to glucose, sucrose, maltose and L-arabinose induction within 5 d, except for D-xylose. Among them, the highest transcript levels induced by glucose and sucrose appeared on the third day and were 2.47- and 3.11-fold higher than that in the control, respectively. As for maltose and L-arabinose induction, the highest expression values were observed on the fourth day. In addition, the transcript levels of *abf* induced by D-xylose were up-regulated at 2–5 d. In summary, the results indicated that L-arabinose or maltose induction had a significant effect on the expression of *abf*, which was 5.13-and 4.58-fold compared to the control, respectively. However, L-arabinose is much more expensive than maltose in the cultivation of *A. auricula*. Therefore, we could recommend the use of maltose instead of L-arabinose to increase the expression of *abf* in *A. auricula*.

### Conclusions

In this work, we successfully cloned the full-length cDNA of *abf* from *A. auricula*. The *E. coli* BL-21 was selected as a host for heterologous expression of *abf*, and its product, the

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**Table 2. Origins of homologous amino-acid sequences from other species.**

| Species                        | Accession ID | Kingdom (class)          |
|-------------------------------|--------------|--------------------------|
| Botryobasidium botryosum      | KDQ21500     | Botryobasidium           |
| Auricularia subglabra         | XP_007352099 | Auricularia              |
| Paxillus rubicundulus         | KIK93171     | Paxillus                 |
| Schizopora paradoxa           | KLO12264     | Schizopora               |
| Neosartorya fischeri          | XP_001260980 | Neosartorya              |
| Fomitiporia mediterranea      | XP_007263520 | Fomitiporia              |
| Aspergillus fumigatus         | KMK61512     | Aspergillus              |
| Phlebiopsis gigantea          | KIP05651     | Phlebiopsis              |
| Galerina marginata            | KDR85498     | Galerina                 |
| Rhizoctonia solani            | EUC57383     | Rhizoctonia              |
| Fomitiporia pinicola          | EPS96452     | Fomitiporia              |
| Aspergillus flavus            | KOC10082     | Aspergillus              |
| Pleurotus sp.                 | CCC33068     | Pleurotus                |
| *Trametes versicolor*         | XP_008041371 | *Trametes*               |

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**Figure 4.** Phylogenetic analysis of ABF of *Auricularia auricula* and ABF of other species. Note: The phylogenetic tree is drawn on the basis of the deduced amino-acid sequences.
ABF enzyme, was observed in SDS-PAGE. Moreover, the transcript levels of abf were investigated in fungal growth under different carbon sources. These results provide theoretical basis for abf functional research and may be of significant interest to understand the regulatory role of abf in the degradation mechanism of hemicellulose.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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