Identification and characterization of a lipase gene from Antrodia cinnamomea

Fang-Hua CHUa,*, Sheng-Yang WANGb, Li-Chiun LEEc, Jei-Fu SHAWb,c

a School of Forestry and Resource Conservation, National Taiwan University, Taipei 106, Taiwan
b Department of Forestry, National Chung-Hsing University, Taichung 402, Taiwan
c Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei 115, Taiwan

A R T I C L E  I N F O

Article history:
Received 23 March 2007
Received in revised form 12 May 2008
Accepted 11 June 2008

Corresponding Editor:
Paul Hooley

Keywords:
Basidiomes
Fungal enzymes
Medicinal mushrooms

A B S T R A C T

A partial (634 bp) cDNA clone, AF1229, obtained from expressed sequence tags (ESTs) of solid-cultured basidiomes of Antrodia cinnamomea is homologous to the lipase gene in Rhizomucor miehei. 5’-rapid amplification of cDNA ends (RACE) and 3’-RACE amplification showed that the full-length lipase gene, Ac-LIP, has a 912 bp open reading frame (ORF), a 183 bp 5’ non-coding region, and a 144 bp 3’ non-coding region. Ac-LIP contains the lipase consensus sequence, VTVVGHSLGA, and encodes a 303-amino acid polypeptide that appears to be an extracellular protein with a calculated molecular mass of 31.8 kDa. RT-PCR analysis suggested that Ac-LIP was strongly expressed during the basidiomatal formation stage of A. cinnamomea. When over-expressed in Escherichia coli, Ac-LIP yielded a protein that was capable of performing hydrolysis of trilinolein by gas chromatography/mass spectrometry (GC/MS) analysis. A. cinnamomea lipase represents the first enzyme of the lipase family from a basidiomycetous fungus, which has been characterized at the molecular level.

© 2008 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.

Introduction

Microbial lipases (EC 3.1.1.3) are able to catalyse a wide variety of reactions in both aqueous and non-aqueous media and have thus become important biocatalysts (Saxena et al. 2003). They are widely used in medical and industrial applications, such as industrial reagents, cleaners, cosmetics, and food additives (Falch 1991; Ghosh et al. 1996; Gandhi 1997; Olempska-Beer et al. 2006). In addition, there is increasing evidence that extracellular lipases are a group of enzymes that are important during the development of fungus fruiting bodies (Goodrich-Tanrikulu et al. 1998; Thines et al. 2000; Sunagawa & Magae 2005).

Antrodia cinnamomea (basidiomycete) is an endemic fungal species, which has traditionally been used as a folk medicine in Taiwan. Much scientific evidence has shown that A. cinnamomea possesses a wide range of biological activities, such as antioxidative activity (Hseu et al. 2002; Song & Yen 2002; Hsiao et al. 2003; Shen et al. 2006; Yang et al. 2006), vasorelaxatory activity (Wang et al. 2003), anti-inflammatory activity (Shen et al. 2004; Hseu et al. 2005), anti-angiogenic activity (Chen et al. 2005; Cheng et al. 2005a), anti-tumour activity (Liu et al. 2004; Nakamura et al. 2004) anti-hepatitic effects (Lee et al. 2002), and hepatoprotective activity (Han et al. 2006). However, A. cinnamomea is rare and expensive because it only grows on the inner surface of the heartwood cavity of the evergreen tree Cinnamomum kanehirai, an endangered species endemic in Taiwan (Chang & Chou 2004). Although mycelia of A. cinnamomea are easily cultured on artificial media, it is difficult to produce fruit bodies in an artificial culture system.
Additionally, artificially cultured mycelia did not produce certain specific triterpenoids that are isolated only from basidiomes of *A. cinnamomea* and are considered to be the medically effective compounds (Shen et al. 2004). Thus, developing an effective method to culture compounds from *A. cinnamomea* is potentially important. In this paper, we identify and characterize a putative lipase abundant in natural basidiomes of *A. cinnamomea*.

**Materials and methods**

**Strains and culture conditions**

*Antrodia cinnamomea* strain TFRIB 479 was identified and provided by Tun-Tschu Chang (Taiwan Forestry Research Institute) and cultured as described in Chang & Wang (2005). The natural basidiomes were obtained from the infested wood of *Cinnamomum kanehirai*. Liquid-cultured mycelia, solid-cultured mycelia, solid-cultured basidiomes, and natural basidiomes were frozen in liquid nitrogen and stored at −80 °C until used.

**RNA preparation and genomic DNA isolation**

Total RNA of natural basidiomes was isolated as described by Chang et al. (1993) and modified according to Chen et al. (2004). Genomic DNA was isolated from the liquid-cultured mycelia, and harvested and ground in liquid nitrogen. The powder was transferred to a centrifuge tube and mixed gently and thoroughly with N-Cetyl-N,N,N-trimethyl-ammoniumbromide (CTAB), as per the method described in Rogers & Bendich (1994).

**Cloning of the sequence of the specific cDNA fragment**

Partial sequences of the putative lipase gene, which included the partial coding region, were obtained from expressed sequence tags (ESTs) of solid-cultured basidiomes (Chu & Chang 2007). To acquire the full-length sequence of the putative lipase gene, 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE was performed using the SMART RACE cDNA Amplification Kit (BD Biosciences, San Jose, CA) using primer 5′CATTGCCCTCCTTGATGCTGTCTACCT –3′ for RACE amplification. The complete genome sequences were obtained using a pair of specific primers: RT-1229n (5′-GTCTGACCTCGCCATTGTCTC-3′) and RT-1229c (5′-GCCGTCGACCTGACCCATCTC-3′), which corresponded to 18S rRNA of *Antrodia cinnamomea* (Chu et al. 2008). Twenty-five cycles of PCR were run using a program of 1 min at 94 °C, 30 s at 58 °C, and 30 s at 72 °C.

**Protein expression and purification**

In order to confirm that the cDNA obtained from natural basidiomes of *Antrodia cinnamomea* did encode lipase (lipolytic acyl hydrolase), the clone was over-expressed as a fusion protein in *Escherichia coli*. This allowed purification of the expression product, which was used for assaying enzymatic activity. The lipase cDNA was subcloned into the fusion protein expression vector, pGEX4T-1 (Pharmacia), by using EcoRI and Xhol cloning sites, and the resultant construct was expressed in E. coli BL-21 (DE3). Soluble glutathione S-transferase (GST) fusion proteins were purified using GST-Bind kits (Novagen, Madison, WI).

**Determination of optimal pH and temperature**

The effects of temperature and pH were assessed by using p-nitrophenyl butyrate as a substrate. The optimal pH was investigated in the pH range of 3–9 using Good’s buffer (50 mM each of Bicine, CAPS, sodium acetate, and Bis-Tris propane) at 37 °C. The optimal temperature for the esterase reaction was examined in the range 30–60 °C at pH 8.

**Lipase assays**

Lipase activity was measured in vitro by gas chromatography/mass spectrometry (GC/MS) analysis as described by Hong et al. (2000) with slight modification. Trilinolein was used as the substrate. The reaction mixture contained 100 mM Tris-HCl (pH 8), 2.5 mM substrate, and enzyme protein (100 μg) in a final volume of 100 μl. The substrates were emulsified in 5 % gum arabic before being added to the reaction mixture. To achieve this, the substrates were dissolved in chloroform, added to the gum arabic solution, and then emulsified by sonication for 30 s. The reaction was carried out at 25 °C for 2 h. The reaction mixture was then methylated by adding 10 μl methanol-H2SO4 (2.5 % v/v) and left at 80 °C for 1 h. After methylation, the reaction was extracted with 100 μl hexane. After centrifugation at 1500g for 10 min, the reaction mixtures were analysed on a Trace GC – Polaris Q mass spectrometer (Finnigan-spectronex), equipped with a DB-5 column (30 m × 0.25 mm i.d., 0.25 film thickness, J & W Scientific, Folsom, CA). The temperature program was as follows: maintenance at 100 °C for 1 min, then increased by 10 °C/min to 240 °C and maintained for 15 min. Other parameters were as follows: inject temperature 270 °C; ion source temperature 280 °C; EI 70 eV; carrier gas and flow rate, He at 1 ml/min; split ratio 1:50; and mass range 45–425 m/z. Quantification was measured by percentage peak area. Identification of individual compounds was carried out using the Wiley/NBS Registry of Mass Spectral Data and National Institute of Standards and Technology (NIST) search and authentic reference compounds. Chromatographic results that are expressed as area percentages were calculated with a response factor of 1.
Results and discussion

Primary structure analysis of the putative lipase

A partial length (634 bp) of cDNA clone AF1229 obtained from ESTs of solid-cultured basidiomycetes of Antrodia cinnamomea, contained the lipase gene family consensus sequence, ITTAGHSLGA. After 5’-RACE and 3’-RACE amplification, the full-length lipase, Ac-LIP, which has a 912 bp open reading frame (ORF), a 183 bp 5’ non-coding region, and a 144 bp 3’ non-coding region, was obtained, (GenBank accession no. EF088667). Comparing the genomic sequence with the cDNA clone showed that Ac-Lip contains six introns (Table 1). The lengths of the introns were 59, 55, 56, 51, 67, and 66 bp, as shown in Fig 1. The ORF encodes a 303 amino acid protein. The predicted molecular weight (mol. Wt) of the polypeptide was 31.8 kDa and the theoretical isoelectric point (pI) was 4.17. The signal peptide of the Ac-LIP predicted by PSORT WWW Sever (http://psort.nibb.ac.jp/) is in the N-terminal 20 amino acids and the predicted localization site is at the extra-cellular position. The Ac-LIP protein contains the lipase consensus sequence, as well as a putative motif for an N-glycosylation site, a protein kinase C phosphorylation site, a casein kinase II phosphorylation site, and an N-myristoylation site (Fig 1).

Using the Needleman–Wunsch (Needleman & Wunsch 1970) global algorithm to find the optimum alignment (including gaps) of two sequences when considering their entire length, the protein sequence of Ac-LIP exhibits 32 % identity and 47 % similarity with a cerol lipase precursor (CAC28687), 27 % identity and 42 % similarity with an esterase A protein (GenBank accession no. BAA92937), 28 % identity and 45 % similarity with an EF088667). Comparing the genomic sequence with the cDNA clone, the variation of 50 intron 1 5 –TGAGTGCACCATCATCAATAGTGGAACCCCTTGCTCCCAGCTCATGAGCGGCGTCAAGG-3’ 458 | 459 intron 2 5’-TGATGGTGCTTTGTAAGCCGACCTGCTCCGCTAGCCG-3’ 527 | 528 intron 3 5’-GAGACCCGACCCGCCTTGCCCGCTGGC-3’ 642 | 643 intron 4 5’-TCTGTTTCTCCGCTGAGATAATCCGAAATCTGCTGACCTGACCTGACCTAG-3’ 728 | 729 intron 5 5’-CCGGTTCCGACCCCGCCTTGCCCGCTGGC-3’ 818 | 819 intron 6 5’-AGGAGTGCCTGCTGAGCTTCCAACTTCGATGAGTGGGGC-3’ 987 | 988
Expression of the Ac-LIP

Ac-LIP was over-expressed as a fusion protein (lipase fused with GST-binding protein) in Escherichia coli. In this study, the full-length and the mature protein-coding region of the Ac-LIP gene were cloned into pGEX4T-1 expression vector and then transformed into E. coli BL21 (DE3) and induced in 0.4 mM IPTG at 25°C. The soluble GST fusion proteins were purified by GST-bind resin; a 68 and a 66 kDa protein were determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Fig 3). Additionally, a protein of a smaller molecular mass also appeared in each construction; both were visualized on a Western blot probed with GST antibody (Fig 3), suggesting that the C-terminal region of Ac-LIP was not stable and degraded easily. pGEX4T-1 transformed into E. coli BL21(DE3) was used as a negative control and was visualized as a 26 kDa protein by Western blot.

Lipase activity was determined at different temperatures under standard assay conditions. The reaction rate was

Fig 1 – Nucleotide and inferred amino acid sequences of Ac-LIP. Amino acid sequence: lipase consensus motif, double-line boxed region; N-glycosylation site, single-line boxed region; protein kinase C phosphorylation site, broken-line boxed region; casein kinase II phosphorylation site, single underline; N-myristoylation site, double solid underline. The black triangle indicates the position of intron. The order of intron is shown in the right of the triangle.

Table 2 – Similarity analysis of Ac-LIP with other reported lipase homologues

<table>
<thead>
<tr>
<th></th>
<th>Ac</th>
<th>At</th>
<th>Lm</th>
<th>Nc</th>
<th>Gz</th>
<th>Rm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac</td>
<td>–</td>
<td>32</td>
<td>31</td>
<td>28</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>At</td>
<td>45</td>
<td>–</td>
<td>30</td>
<td>33</td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>Lm</td>
<td>47</td>
<td>46</td>
<td>–</td>
<td>32</td>
<td>32</td>
<td>31</td>
</tr>
<tr>
<td>Nc</td>
<td>46</td>
<td>48</td>
<td>50</td>
<td>–</td>
<td>45</td>
<td>37</td>
</tr>
<tr>
<td>Gz</td>
<td>43</td>
<td>46</td>
<td>50</td>
<td>51</td>
<td>–</td>
<td>30</td>
</tr>
<tr>
<td>Rm</td>
<td>42</td>
<td>45</td>
<td>50</td>
<td>51</td>
<td>46</td>
<td>–</td>
</tr>
</tbody>
</table>

Results from pairwise amino acid sequence comparisons are shown as percent identity (right upper) and percent similarity (left lower) for Ac (lipase of Antrodia cinnamomea), At (feruloyl esterase A of Aspergillus tubingensis), Lm (lipase of Leishmania major), Nc (triacylglycerol lipase of Neurospora crassa), Gz (lipase of Gibberella zeae), and Rm (lipase of Rhizomucor miehei).
increased from 30 to 37 °C, and the highest reaction rate was obtained at 37 °C, using p-nitrophenyl butyrate as the substrate; the reaction rate decreased at above 40 °C (Fig 4). Interestingly, the lipase was active at pH 8 and could tolerate pH 9 (Fig 5). Thus, the following reaction was conducted at pH 8 (37 °C). In order to further characterize the function of purified lipase fusion protein, the end product of this lipase was measured in vitro by GC/MS. Trilinolein was used as the exogenous substrate. For GST-binding protein alone, which served as a control, only trilinolein (retention time: 14.37 min) was detected in the GC/MS chromatogram (Fig 6), i.e. there was no detectable lipase activity with trilinolein as the substrate. As the full-length protein of Ac-LIP was not easily purified and there was no detectable lipase activity with trilinolein as a substrate, only matured protein, which lacks the signal peptide, was assayed in this experiment. When the truncated version of recombinant Ac-LIP reacted with trilinolein, a new product, linoleic acid, was formed in the GC/MS chromatogram (retention time: 14.29 min; Fig 6). The mean activity was $37.2 \pm 1.28 \mu g \text{mg}^{-1} \text{protein}$ for three repeat experiments. These results indicated that when the matured lipase fusion protein was used as a source of enzyme, linoleic acid was de-esterified from trilinolein.

### Lipase activity during fungal development

Several different lines of evidence suggest that fatty acids or related lipids are important to the sexual development of filamentous fungi. In Neurospora, unsaturated fatty acids, especially α-linolate (18:2), dramatically stimulate subsequent production of fruiting bodies if the unsaturated fatty acids are applied before fertilization (Nukina et al. 1981). In cultures that are competent to undergo sexual development, α-linolate was the predominant fatty acid in N. crassa (Goodrich-Tanrikulu et al. 1998). Because the medium composition affects the production of lipase dramatically, it is important to understand the influences of the various factors and to determine the optimum cultivation conditions (Lin et al. 2006). Furthermore, evaluation of the substrate spectrum of new enzymes in hydrolytic and synthesis reactions may help develop the wide field of applications of microbial lipases in biotechnology (Pandey et al. 1999).

In addition, there is increasing evidence that extracellular lipases are important enzymes during the development of fungus fruiting bodies (Goodrich-Tanrikulu et al. 1998; Thines...
et al. 2000; Sunagawa & Magae 2005). Based on the results observed by Goodrich-Tanrikulu and his co-workers, triacylglycerol is the predominant acyl lipid at the sexual development stage in N. crassa (Goodrich-Tanrikulu et al. 1998). Also, as described with Magnaporthe grisea, triacylglycerol lipase activity increased during appressorium maturation (Thines et al. 2000). Moreover, triacylglycerol lipase was expressed during the fruiting body development of Pleurotus ostreatus as detected by differential display of RAPD screening (Sunagawa & Magae 2005). Although some lipases have been purified from Antrodia cinnamomea (Lin & Ko 2005; Lin et al. 2006; Shu et al. 2006), the nucleotide sequences of these lipases are still unknown. However, all of them were alkaline-resistant and thermostable, we did not know whether these lipases are the same or not. In this study, we have reported the isolation and characterization of a cDNA clone from a fruiting body that encodes a lipase exhibiting lipolytic acyl hydrolase activity. This lipase contains a ten amino acid consensus sequence that characterizes animal, plant, and yeast lipases, although the homologies of the amino acids were quite different. The role of this lipase in development of the fruiting body and its metabolism correlation should be further studied.

Acknowledgements

We thank Shang-Tzen Chang and Chi-Lin Wu for their valuable help and suggestions. We are also grateful for expert technical assistance from Shang-Fen Chi and Yi-Ru Lee. We thank the Council of Agriculture Executive Yuan (94AS-5.2.1-ST-a1) and the National Science Council of Taiwan (97NSC-2317-B-002-009) for financial support and the Taiwan Forestry Research Institute for providing the mycelia and basidiomes of Antrodia cinnamomea.

References


