ELSEVIER

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/mycres

Identification and characterization of a lipase gene from *Antrodia cinnamomea*

Fang-Hua CHU[a,*], *Sheng-Yang WANG*[b], *Li-Chiun LEE*[c], *Jei-Fu SHAW*[b,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*

ARTICLE INFO

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

ABSTRACT

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.

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 *Cinnamonum 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.

* Corresponding author. Tel.:+886 2 33665261; fax: +886 2 23654520.
E-mail address: fhchu@ntu.edu.tw
0953-7562/$ – see front matter
doi:10.1016/j.mycres.2008.06.006

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 *Cinnamonum 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′AF1229 (5′-TACTGTATCGAGTCGCCATTGCCACC-3′) and 3′AF1229 (5′-C CATTGCCCTCCTTGATGCTGTCTACCT –3′) for PCR amplification. The complete genome sequences were obtained using PCR using the specific primers AF1229N (5′-ATGGGCCCTTTCG TACTAGGTGCT-3′) and AF1229C (5′-CTAGCTAGCAACCCATCT CATTGCCATTG-3′). The PCR products were cloned using pGEM-T Easy vector system (Promega, Madison, WI) and sequenced using an ABI 377 automatic sequencer (Perkin Elmer, Boston, MA).

RT-PCR analysis

For detection of differential expression by RT-PCR, total RNA was reverse-transcribed into first-strand cDNA using the protocol for SMART cDNA synthesis using the SMART cDNA library construction kit (Clontech, Mountain View, CA). RT-PCR analysis was performed using a pair of specific primers: RT-1229n (5′-TTCGCGAATTACGTCGACGCGCA-3′) and RT-1229c (5′-CCCGCTCAGCTAGCAACCCATCTC-3′), which corresponded to the partial coding region and 3′ non-coding region. The internal control was analysed using a pair of specific primers as previously studied: RT-18Sn (5′-ACTGTGAAACTGCGAATG GCTC-3′) and RT-18Sc (5′-GACTTGCCCTCCAATTGTTCCTC-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 *Eco*RI and *Xho*I 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–H_2SO_4 (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 1500*g* 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^{-1} 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^{-1}; spilt ratio 1:50; and mass range 45–425 m z^{-1}. 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 basidiomes of *Antrodia cinnamomea*, contained the lipase gene family consensus sequence, ITFAGHSLGA. 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 (*p*I) 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 extracellular 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 45 % similarity with an *Aspergillus tubingensis* feruloyl esterase A protein (GenBank accession no. BAA92937), 28 % identity and 46 % similarity with a *Neurospora crassa* triacylglycerol lipase precursor (CAC28687), 27 % identity and 42 % similarity with a *Rhizomucor miehei* lipase (CAA00250), 31 % identity and 47 % similarity with a *Leishmania major* lipase (CAJ08271), and 29 % identity and 43 % similarity with a *Gibberella zeae* lipase (AAQ23181; Table 2). These results indicate that Ac-LIP is very different from the lipases of fungi published to date.

The substrate-binding consensus sequence for the lipase gene family is [HYWV]-S-{YAG}-G-[GSTAC], and the conserved serine is thought to be an active site residue (PROSITE). Ac-LIP possesses this conserved motif in common with other organisms, including human biphenyl hydrolase (GenBank accession no. CAA57137), *Drosophila melanogaster* hydrolase (CAA04153), *Chenopodium album* chlorophyllase (BAA93635), *Citrus sinensis* chlorophyllase (AAF59834), and *R. miehei* lipase (CAA00250). The predicted secondary and tertiary structures of Ac-LIP protein were constructed by using SCRATCH servers (http://www.igb.uci.edu/tools/scratch/) (Cheng *et al.* 2005b) and SWISS-MODEL Protein Modeling Server (http://swissmodel.expasy.org/) (Schwede *et al.* 2003). It revealed the structure of Ac-LIP is similar to the crystal structure of *R. miehei* triacylglycerol lipase (GenBank accession no. 4TGL). The movement of a 15 amino acid long 'lid' (residues 82–96) is a hinge-type rigid-body motion in *R. miehei*, triacylglycerol lipase, and it is a reasonable inference that the general stereochemistry of lipase activation is at the oil–water interfaces (Derewenda *et al.* 1992). Consistent with the crystal structures of *R. miehei* triglyceride lipase the protein possesses the α-helix 85–92 (Ile-Arg-Asn-Trp-Ile-Ala-Asp-Leu) between the native and inhibited forms of the enzyme, Ac-LIP also possesses the α-helix 115–122 (Ile-Leu-Pro-Leu-Ile-Thr-Asp-Ala-Asp-Ile-Glu-Lys-Thr-Asp-Ala) in the structure of the central part of the lid. At the N-terminal end, each of the 'lids' in Ser-113 and Glu-114 residues undergo a conformational change. Identification of the full-length gene will enable future study of the effect on development of the fruiting body and its metabolism.

Expression of the Ac-LIP *during different stages*

The variation of *Ac-LIP* expression in different *Antrodia cinnamomea* samples, such as liquid-cultured mycelia, solid-cultured mycelia, solid-cultured basidiomes, and natural basidiomes, was analysed using RT-PCR with specific primers. A 273 bp DNA fragment, which corresponds to the partial coding region and 3′ non-coding region of the *Ac-LIP* cDNA, was clearly discernible in the agarose gel. The results of RT-PCR analysis indicated that the *Ac-LIP* gene was strongly expressed during the basidiomatal formation stage, especially in natural basidiomes (Fig 2). The gene is also expressed during the fruiting body development of *Pleurotus ostreatus*, *Neurospora crassa*, and *Magnaporthe grisea* (Sunagawa & Magae 2005). As triacylglycerol is known as an energy dense substance (Gibbons *et al.* 2000), it is reasonable that triacylglycerol is used as an energy source for the rapid development of fruiting bodies in fungi in which triacylglycerol lipases play a role in lipid degradation. Additionally, extracellular lipases are also important microbial virulence factors (Stehr *et al.* 2003). A secreted lipase of *Fusarium graminearum* was also proved to be the virulence factor required for infection of cereals (Nagao *et al.* 1994). Based on our knowledge, *A. cinnamomea* only grows on the heartwood cavity of *Cinnamonum kanehirai*. Future phytopathogenic investigations will provide a better understanding of whether this extracellular lipase of *A. cinnamomea* is virulent to *C. kanehirai*.

Table 1 – The sequence and position of intons in the *Ac-LIP* locus

	Sequence	Position
intron 1	5′-TGAGTGCACCATCATCAATAGTGGAACCCTTGTGTCCCCGCTCATGAGCGGCGTCAAGG-3′	458↓459
intron 2	5′-GTAAGTTGGTGTATTGCTGCGGCATGCCTACGACCTCAGAACTCTCGCGCCTCAG-3′	527↓528
intron 3	5′-TGAGTGTTTGATAGGCGATCTGATCTCAACATGCTAGAACTAATGAATTCCGTAGG-3′	642↓643
intron 4	5′-TCTGTTCCTTCGCGATGAAATTCCGAACTCTGCCTGACTACTATCTACAGG-3′	728↓729
intron 5	5′-CCGTTTGCGCTCGAGCCGTCTGAATCTTGCTACCGGTACTGAACTGCTATGTCCGTGCGTCGTAGGT-3′	818↓819
intron 6	5′-ACGTGTGCTCGTGACTTGATACATTCACTTGATGGCGGTTCTGATGGCGGCATCATGTGGTTAGGT-3′	987↓988

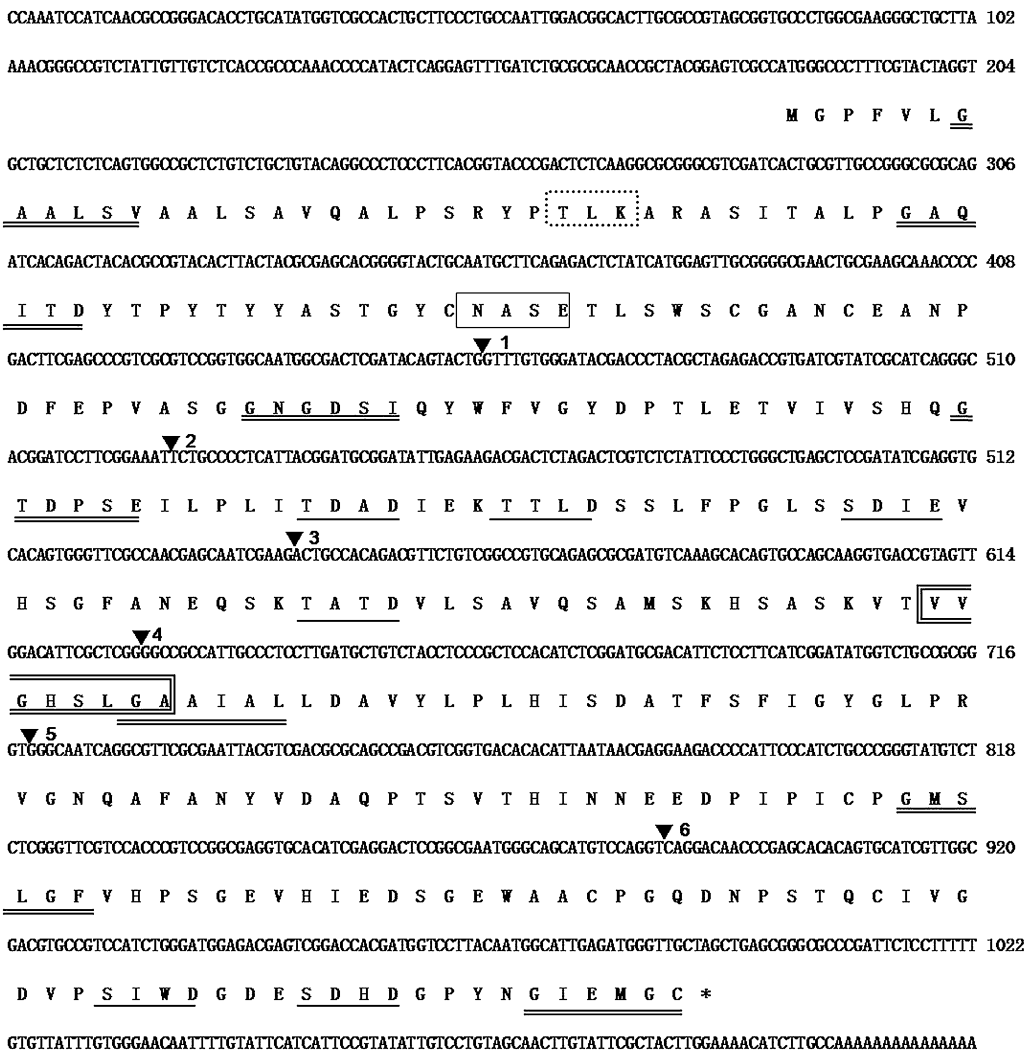

Fig 1 – Nucleotide and inferred amino acid sequences of *Ac-LIP*. Amino acid sequence: lipase consensus motif, double-line boxed region; N-glycosylation site, single-line boxed region; protein kinase C phosphorylation site, broken-line boxed region; casein kinase II phosphorylation site, single underline; 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

	Ac	At	Lm	Nc	Gz	Rm
Ac	–	32	31	28	29	27
At	45	–	30	33	31	32
Lm	47	46	–	32	32	31
Nc	46	48	50	–	45	37
Gz	43	46	51	62	–	30
Rm	42	45	50	51	46	–

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*).

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

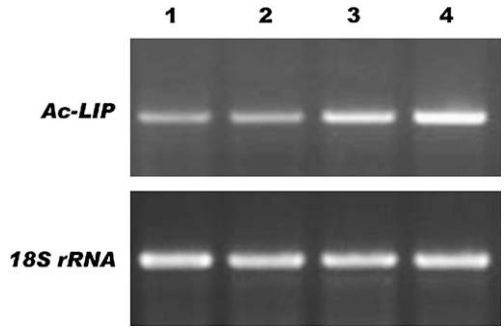

Fig 2 – RT-PCR analysis of total RNA isolated from different samples. Lane 1: liquid-cultured mycelium; lane 2: solid-cultured mycelium; lane 3: solid-cultured basidiomes; lane 4: natural basidiomes.

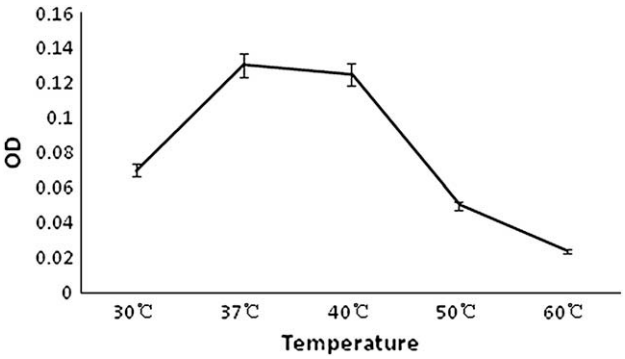

Fig 4 – Effect of temperature on esterase activity of Ac-LIP protein. The enzyme reaction was carried out in 50 mM Tris–HCl buffer (pH 8) for 8 min with 0.03 mg purified protein. The bars indicate standard errors.

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 $37.2 \pm 1.28\ \mu g\ mg^{-1}$ 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.

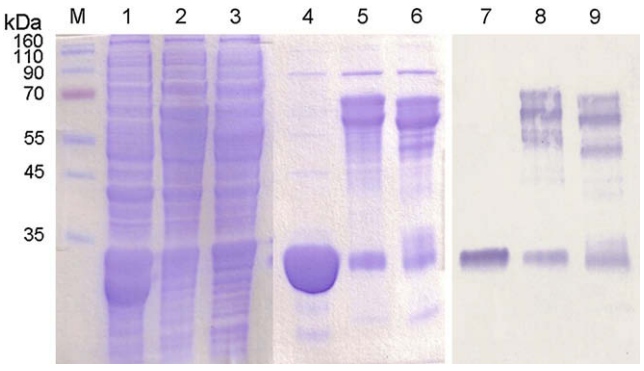

Fig 3 – Coomassie blue stained SDS-PAGE and Western blot immunoanalysis of the proteins expressed in *Escherichia coli* pGEX system by IPTG induction. Lane 1–3: crude protein. Lane 4–6: purified protein. Lane 7–9: Western blot against GST-Ab. Lane 1, 4 and 7: pGEX 4T-1. Lane 2, 5 and 8: full-length *Ac-LIP* gene. Lane 3, 6 and 9: matured coding region of *Ac-LIP* gene.

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-linoleate (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-linoleate 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

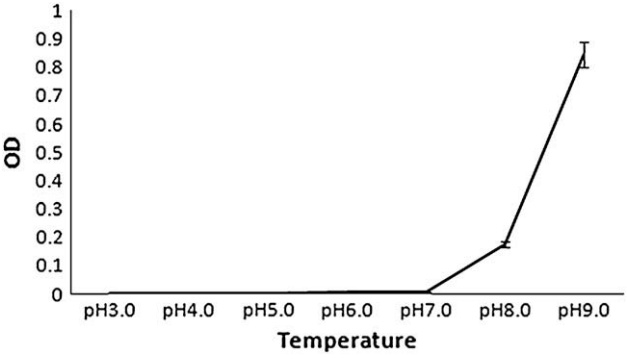

Fig 5 – Effect of pH on esterase activity of Ac-LIP protein. The enzyme reaction was carried out at 37 °C in Good's buffer. The bars indicate standard errors.

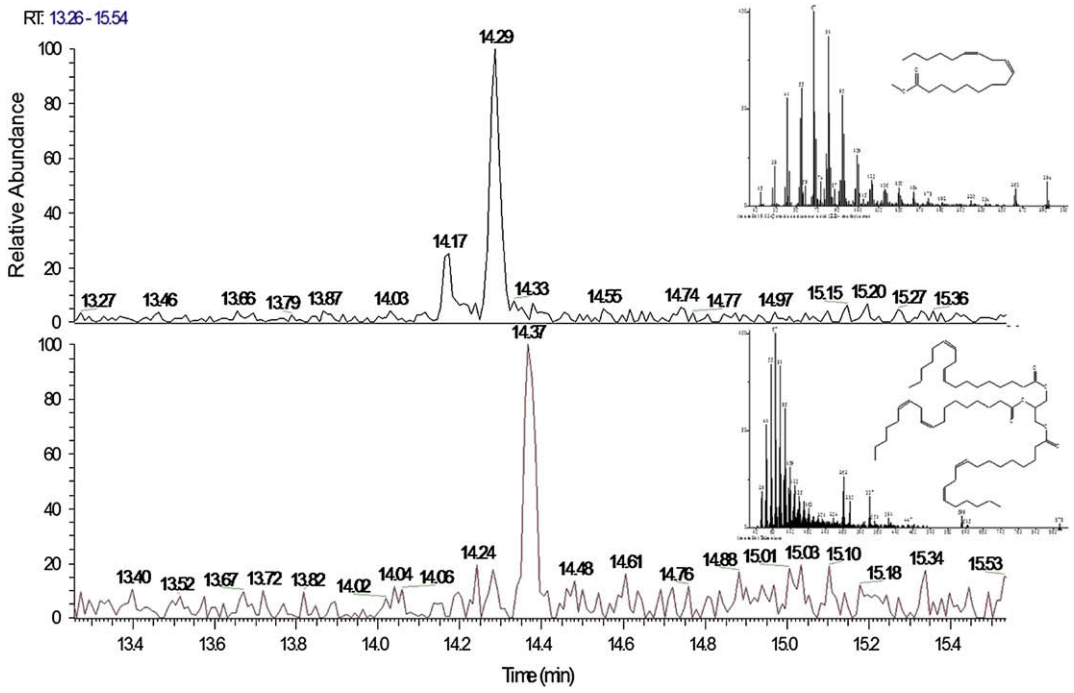

Fig 6 – GC-MS analysis of the product formed by the truncated version of recombinant Ac-LIP with trilinolein as substrate. The GC profile (left) and mass fragmentation patterns (right) of GST-binding protein alone (bottom) and Ac-LIP (top) are illustrated. Trilinolein was used as substrate (retention time: 14.37 min) and a new product, linoleic acid, was formed by enzyme digestion (retention time: 14.29 min).

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

Chang S, Puryear J, Cairney J, 1993. A simple and efficient method for isolation RNA from pine trees. *Plant Molecular Biology Report* **11**: 113–116.

Chang TT, Chou WN, 2004. *Antrodia cinnamomea* reconsidered and *A. salmonea* sp. nov. on *Cunninghamia konishii* in Taiwan. *Botanical Bulletin of Academia Sinica* **45**: 347–352.

Chang TT, Wang WR, 2005. Basidiomatal formation of *Antrodia cinnamomea* on artificial agar media. *Botanical Bulletin of Academia Sinica* **46**: 151–154.

Chen SC, Lu MK, Cheng JJ, Wang DL, 2005. Antiangiogenic activities of polysaccharides isolated from medicinal fungi. *FEMS Microbiology Letters* **249**: 247–254.

Chen YR, Lee YR, Wang SY, Chang ST, Shaw JF, Chu FH, 2004. Establishment of expressed sequence tags from Taiwania (*Taiwania cryptomerioides* Hayata) seedling cDNA. *Plant Science* **167**: 955–957.

Cheng JJ, Huang NK, Chang TT, Wang DL, Lu MK, 2005a. Study for anti-angiogenic activities of polysaccharides isolated from *Antrodia cinnamomea* in endothelial cells. *Life Science* **76**: 3029–3042.

Cheng J, Randall A, Sweredoski M, Baldi P, 2005b. SCRATCH: a protein structure and structural feature prediction server. *Nucleic Acids Research* **33**: 72–76.

Chu FH, Chang TT, 2007. Basidiomatal formation in *Antrodia cinnamomea* from the perspective of gene expression. *Botanical Studies* **48**: 387–396.

Chu FH, Lee YR, Chou SJ, Chang TT, Shaw JF, 2008. Isolation and analysis of genes specifically expressed during basidiomatal development in *Antrodia cinnamomea* by subtractive PCR and cDNA microarray. *FEMS Microbiology Letters* **280**: 150–159.

Derewenda U, Brzozowski AM, Lawson DM, Derewenda ZS, 1992. Catalysis at the interface: the anatomy of a conformational change in a triglyceride lipase. *Biochemistry* **31**: 1532–1541.

Falch EA, 1991. Industrial enzymes – developments in production and application. *Biotechnology Advances* **9**: 643–658.

Gandhi NN, 1997. Applications of lipase. *Journal of American Oil Chemists' Society* **74**: 621–634.

Ghosh PK, Saxena RX, Gupta R, Yadav RP, Davidon S, 1996. Microbial lipase: production and applications. *Science Progress* **79**: 119–157.

Gibbons GF, Islam K, Pease RJ, 2000. Mobilisation of triacylglycerol stores. *Biochimica et Biophysica Acta* **1483**: 37–57.

Goodrich-Tanrikulu M, Howe K, Stafford A, Nelson MA, 1998. Changes in fatty acid composition of *Neurospora crassa* accompany sexual development and ascospore germination. *Microbiology* **144**: 1713–1720.

Han HF, Nakamura N, Zuo F, Hirakawa A, Yokozawa T, Hattori M, 2006. Protective effects of a neutral polysaccharide isolated from the mycelium of *Antrodia cinnamomea* on *Propionibacterium acnes* and lipopolysaccharide induced hepatic injury in mice. *Chemical & Pharmaceutical Bulletin* **54**: 496–500.

Hong Y, Wang TW, Hudak KA, Schade F, Froese CD, Thompson JE, 2000. An ethylene-induced cDNA encoding a lipase expressed at the onset of senescence. *Proceedings of the National Academy of Sciences, USA* **97**: 8717–8722.

Hseu YC, Chang WC, Hseu Y, Lee CY, Yech YJ, Chen PC, Chen JY, Yang HL, 2002. Protection of oxidative damage by aqueous extract from *Antrodia camphorata* mycelia in normal human erythrocytes. *Life Science* **71**: 469–482.

Hseu YC, Wu FY, Wu JJ, Chen JY, Chang WH, Lu FJ, Lai YC, Yang HL, 2005. Anti-inflammatory potential of *Antrodia camphorata* through inhibition of NOS, COX-2 and cytokines via the NF-kappa B pathway. *International Immunopharmacology* **5**: 1914–1925.

Hsiao G, Shen MY, Lin KH, Lan MH, Wu LY, Chou DS, Lin CH, Su CH, Sheu JR, 2003. Antioxidative and hepatoprotective effects of *Antrodia camphorata* extract. *Journal of Agricultural and Food Chemistry* **51**: 3302–3308.

Lee IH, Huang RL, Chen CT, Chen HC, Hsu WC, Lu MK, 2002. *Antrodia camphorata* polysaccharides exhibit anti-hepatitis B virus effects. *FEMS Microbiology Letters* **209**: 63–67.

Lin ES, Ko HC, 2005. Glucose stimulates production of the alkaline-thermostable lipase of the edible basidiomycete *Antrodia cinnamomea*. *Enzyme and Microbial Technology* **37**: 261–265.

Lin ES, Wang CC, Sung SC, 2006. Cultivating conditions influence lipase production by the edible basidiomycete *Antrodia cinnamomea* in submerged culture. *Enzyme and Microbial Technology* **39**: 98–102.

Liu JJ, Hung TS, Hsu ML, Chen CC, Lin WS, Lu FJ, Chang WH, 2004. Antitumor effects of the partially purified polysaccharides from *Antrodia camphorata* and the mechanism of its action. *Toxicology and Applied Pharmacology* **201**: 186–193.

Nagao T, Shimada Y, Sugihara A, Tominaga Y, 1994. Cloning and nucleotide sequence of cDNA encoding a lipase from *Fusarium heterosporum*. *Biochemistry* **116**: 536–540.

Nakamura N, Hirakawa A, Gao JJ, Kakuda H, Shiro M, Komatsu Y, Sheu CC, Hattori M, 2004. Five new maleic and succinic acid derivatives from the mycelium of *Antrodia camphorara* and their cytotoxic effects on LLC tumor cell line. *Journal of Natural Products* **67**: 46–48.

Needleman SB, Wunsch CD, 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *Journal of Molecular Biology* **48**: 443–453.

Nukina M, Sassa T, Ikeda M, Takahasi K, Toyota S, 1981. Linoleic acid enhances perithecial production in *Neurospora crassa*. *Agricultural and Biological Chemistry* **45**: 2371–2373.

Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ, 2006. Food-processing enzymes from recombinant microorganisms—a review. *Regulatory Toxicology and Pharmacology* **45**: 144–158.

Pandey A, Benjamin S, Soccol GR, Nigam P, Krieger N, Soccol VT, 1999. The realm of microbial lipases in biotechnology. *Biotechnology and Applied Biochemistry* **29**: 119–131.

Rogers SO, Bendich AJ, 1994. Extraction of total cellular DNA from plants, alage and fungi. In: Gelvin SB, Schilperoort RA (eds), *Plant Molecular Biology Manual*. Kluwer Academic Publishers, Dordrecht, pp. 1–8.

Saxena RK, Sheoran A, Giri B, Davidson WS, 2003. Purification strategies for microbial lipases. *Journal of Microbiological Methods* **52**: 1–18.

Schwede T, Kopp J, Guex N, Peitsch MC, 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Research* **31**: 3381–3385.

Shen CC, Shen YC, Wang YH, Lin LC, Don MJ, Liou KT, Wang WY, Hou YC, Chang TT, 2006. New lanostanes and naphthoquinones isolated from *Antrodia salmonea* and their antioxidative burst activity in human leukocytes. *Planta Medica* **72**: 199–203.

Shen YC, Wang YH, Chou YC, Chen CF, Lin LC, Chang TT, Tien JH, Chou CJ, 2004. Evaluation of the anti-inflammatory activity of Zhankuic acids isolated from the fruiting bodies of *Antrodia camphorata*. *Planta Medica* **70**: 310–314.

Shu CH, Xu CJ, Lin GC, 2006. Purification and partial characterization of a lipase from *Antrodia cinnamomea*. *Process Biochemistry* **41**: 734–738.

Song TY, Yen GC, 2002. Antioxidant properties of *Antrodia camphorata* in submerged culture. *Journal of Agricultural and Food Chemistry* **50**: 3322–3327.

Stehr F, Kretschmar M, Kröger C, Hube B, Schäfer W, 2003. Microbial lipases as virulence factors. *Journal of Molecular Catalysis, B: Enzymatic* **22**: 347–355.

Sunagawa M, Magae Y, 2005. Isolation of genes differentially expressed during the fruit body development of *Pleurotus ostreatus* by differential display of RAPD. *FEMS Microbiology Letters* **246**: 279–284.

Thines E, Weber RWS, Talbot NJ, 2000. MAP kinase and protein kinase A-dependent mobilization of tricylglycerol and glycogen during appressorium turgor generation by *Magnaporthe grisea*. *Plant Cell* **12**: 1703–1718.

Wang GJ, Tseng HW, Chou CJ, Tasi TH, Chen CT, Lu MK, 2003. The vasorelaxation of *Antrodia camphorata* mycelia: involvement of endothelial Ca^{2+}-NO-cGMP pathway. *Life Science* **73**: 2769–2783.

Yang HL, Chen CS, Chang WH, Lu FJ, Lai YC, Chen CC, Hseu TH, Kuo CT, Hseu YC, 2006. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*. *Cancer Letters* **231**: 215–227.