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ORIGINAL ARTICLE

Characterization and heterologous expression of a novel lysophospholipase gene from *Antrodia cinnamomea*

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Abstract

Aims: A novel lysophospholipase (LysoPL) from the basidiomycetous fungi *Antrodia cinnamomea* named ACLysoPL was cloned, heteroexpressed in *Escherichia coli* and characterized.
Methods and Results: The gene encoding ACLysoPL was obtained from expressed sequence tags from *A. cinnamomea*. The full length of this gene has a 945 -bp open reading frame encoding 314 amino acids with a molecular weight of 35·5 kDa. ACLysoPL contains a lipase consensus sequence (GXSXG) motif and a Ser–His–Asp catalytic triad. A putative peroxisomal targeting signal type 1 was found in the C-terminal. Heterologous expression of ACLysoPL in *E. coli* showed that the enzyme preferentially hydrolyses long-chain acyl esterases at pH 7 and 30°C. ACLysoPL is a psychrophilic enzyme about 40% of whose maximum activity remained at 4°C. The LysoPL activities with lysophospholipids as substrate were analysed by gas chromatography/mass spectrometry.
Conclusion: We have identified and characterized a gene named ACLysoPL encoding a protein performing LysoPL and esterase activities.
Significance and Impact of the Study: This is the first LysoPL of *A. cinnamomea* identified and characterized at the molecular level.

Introduction

Phospholipases are ubiquitous enzymes that are involved in a wide range of biological functions such as membrane homeostasis, nutrient acquisition and generation of bioactive molecules. They are of considerable commercial interest because of their potential use in a wide variety of applications in the food, oil refinement and pharmaceutical industries (Saffer and Schwartzman 1991; Songer 1997; Winter *et al.* 1998; De Maria *et al.* 2007). Phospholipases are classified either as phospholipase A_1 (PLA_1), A_2 (PLA_2), B (PLB), C (PLC) or D (PLD) or as lysophospholipase (LysoPL) depending on the site of the hydrolysed ester linkage. LysoPL has been implicated in the regulation of the level of lysophospholipids that participate in many cellular functions such as proliferation, apoptosis, cell migration, cell transformation, nutrition retraction and cancer cell invasion in mammals (Hayashi *et al.* 2001; Mills and Moolenaar 2003; Tigyi and Parrill 2003; Moolenaar *et al.* 2004); however, to date LysoPL has been little studied in fungi.

Antrodia cinnamomea is a unique basidiomycetous fungus, which is resupinate to effused-reflexed. It has a porous hymenium that grows in the inner cavity of the *Cinnamomum kanehirai Hay*, a tree species endemic to Taiwan (Chang and Chou 2004). *Antrodia cinnamomea* is most well known for its use as a traditional Chinese medicine that was first discovered by aboriginal Taiwanese. Over the past few years, scientific evidence has accumulated showing that *A. cinnamomea* possesses a wide range of biological functions, such as anti-oxidative (Hseu *et al.* 2002; Song and Yen 2002; Hsiao *et al.* 2003; Shen *et al.* 2006; Yang *et al.* 2006), vasorelaxatory (Wang *et al.* 2003), anti-inflammatory (Shen *et al.* 2004; Hseu *et al.* 2005), anti-angiogenic (Chen *et al.* 2005; Cheng *et al.* 2005), anti-tumour (Liu *et al.* 2004; Nakamura *et al.*

2004), anti-hepatitic (Lee *et al.* 2002; Phuong *et al.* 2009) and hepatoprotective activities (Han *et al.* 2006). However, the physiological regulation of *A. cinnamomea* is not well understood. Comparative expression profiles of the artificially cultured mycelia and natural basidiomes of *A. cinnamomea* have been studied using expressed sequence tags (ESTs) from a cDNA library, subtractive PCR and cDNA microarray (Chu and Chang 2007; Chu *et al.* 2008a). Genes involved in metabolism were found to be most abundant in natural basidiomes, which are considered to contain numerous medically effective compounds (Chu and Chang 2007; Chu *et al.* 2008a,b). In the present study, we identified and characterized a gene encoding a protein performing LysoPL and esterase activities.

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 by Chang and Wang (2005). Natural basidiomes were obtained from the infested wood of *C. 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 the natural basidiomes was isolated as described by Chang and Chou (1993) and modified according to Chen *et al.* (2004). Genomic DNA was isolated from the liquid-cultured mycelia, 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 (Rogers and Bendich 1994).

Cloning of the sequence of the specific cDNA fragment

Partial sequences of the putative lipase gene, which included the carboxyl-terminal partial coding region and the 3′ untranslated region (UTR), were obtained from ESTs of natural basidiomes (Chu and Chang 2007). To acquire the full-length sequence of the putative lipase gene, 5′-rapid amplification of cDNA ends (RACE) was performed using a SMART RACE cDNA Amplification kit (BD Biosciences, San Jose, CA, USA) using primer AT1201-1R (5′-AAGCTCATGATAAGCATCCGGATATA-3′) for PCR amplification. The PCR products were cloned using pGEM-T Easy vector system (Promega, Madison, WI, USA) and sequenced using an ABI 377 automatic sequencer (Perkin Elmer, Boston, MA, USA).

Southern and Northern blot analyses

Genomic DNA was digested overnight with *Eco*RI, *Eco*RV and *Pst*I and separated in 0·8% (w/v) agarose gel. The purified genomic DNA was transferred to GeneScreen hybridization transfer membrane (Perkin Elmer). Hybridization was performed at 42°C in a hybridization buffer consisting of 5× SSC, 50% (v/v) formamide, 0·02% (w/v) SDS, 0·1% (v/v) sodium-lauroylsacosine and 2% (w/v) blocking reagent (Roche, Mamnheim, Germany). The digoxigenin-labelled probes were prepared by PCR, according to the manufacturer's instructions (Roche). The hybridized membrane was washed twice with 2× SSC plus 0·1% SDS for 15 min at 65°C. The blots were incubated with the chemiluminescence substrate CDP-Star (Roche) and exposed on X-ray films.

For Northern blots, about 15 μg of total RNA was resolved on 1% formaldehyde-agarose gel and then transferred to nylon membranes as described for the Southern blots. Hybridization was performed at 65°C in a hybridization buffer containing 0·2× SSPE (0·2 mol l^{-1} sodium phosphate monobasic, pH 7·4, containing 25 mmol l^{-1} EDTA and 3 mol l^{-1} NaCl), 0·5% sodium-lauroylsacosine, 1% SDS and 1% blocking reagent. Digoxigenin-labelled probes were prepared by *in vitro* transcription according to the manufacturer's instructions. The hybridized blot was washed twice at 65°C with 0·1× SSPE/0·1% SDS for 10 min. The blots were incubated with the chemiluminescence substrate CDP-Star and exposed on X-ray films.

Protein expression and purification

The *ACLysoPL* gene was synthesized using cDNA as a PCR template. The forward primer AT1201F (5′-GACTAGTATGTCTGCGGCTCACGAG-3′) with restriction site *Spe*I and the reverse primer AT1201R (5′-CCGCTC GAGTCAGAGCTTTGACATGGGTGC-3′) with restriction sites *Xho*I were used. The product of the PCR was ligated to the fusion protein expression vector, pTYB12 (New England BioLabs, Beverly, MA) using *Spe*I and *Xho*I cloning sites. The resultant construct was expressed in *Escherichia coli* BL-21 (DE3) (Novagen, Darmstadt, Germany). The pTYB12 is an N-terminal fusion vector in which the N-terminus of the ACLysoPL is fused to the intein tag. The intein–ACLysoPL fusion protein was purified using an affinity chitin column (New England BioLabs). The intein underwent specific self-cleavage when 50 mmol l^{-1} cysteine and 10 mmol l^{-1} DTT were added at 4°C. The ACLysoPL was released from the chitin-bound intein tag

column (Chong *et al.* 1997). The eluted protein was collected and dialysed against phosphate-buffered saline (PBS buffer) (13·7 mmol l^{-1} sodium chloride, 2·7 mmol l^{-1} potassium chloride, 4·3 mmol l^{-1} Na_2HPO_4, 1·4 mmol l^{-1} KH_2PO_4, pH 7·0). The molecular mass of the purified protein was determined under denaturing conditions by SDS-PAGE, and the protein concentration was determined using a Bio-Rad assay kit.

Enzyme characterization

Enzyme activity was assayed using a spectrophotometer (U-2001; Hitachi). The hydrolysis of *p*-nitrophenyl (*p*-NP) esters was carried out at 30°C in 100 μl of 1× PBS buffer containing 0·5% Triton X-100 and a 5 mmol l^{-1} solution of the corresponding *p*-NP ester. The reactions were terminated by adding 100 μl acetone. Enzyme activity was determined by changes in absorbance at 405 nm. One unit of activity was defined as the quantity of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute under assay conditions. The effects of temperature and pH were assessed using *p*-NP palmitate. The optimum pH was investigated in the pH range 3–10 using Good's buffer (50 mmol l^{-1} each of Bicine, CAPS (N-cyclohexyl-3-aminopropanesulfonic acid), sodium acetate and Bis–Tris propane) (Chu *et al.* 2008b). The optimum temperature for the esterase reaction was examined in the range 4–60°C at pH 7·0. To analyse thermal stability, the enzyme was incubated for 10 min at various temperatures in the range 30–70°C.

LysoPL assays

LysoPL activities were measured by gas chromatography/mass spectrometry (GC/MS) using a modification of the method described by Hong *et al.* (2000). 1-Palmitoyl-*sn*-glycero-3-phosphocholine, 1-dodecanoyl-*sn*-glycero-3-phosphocholine, 1, 2-dipalmitoyl-*sn*-glycero-3-phospho choline and 1, 2-dodecanoyl-*sn*-glycero-3-phosphocholine were used as substrates. The reaction mixture contained 1× PBS buffer, 2·5 mmol l^{-1} substrate emulsified in 5% gum arabic and enzyme protein (3 μg) in a final volume of 100 μl. The emulsification was achieved by sonication before being added to the reaction mixture. The reaction was carried out at 30°C for 1 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 using a trace GC-polaris Q mass spectrometer (Finnigan-spectronex, Thermo, MA, USA), equipped with a DB-5 column (30 m × 0·25 mm i.d., 0·25 film thickness; J&W Scientific, Folsom, CA, USA). The temperature programme 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 ration 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 (National Biological Survey) Registry of Mass Spectral Data and the National Institute of Standards and Technology search and authentic reference compounds. Chromatographic results that are expressed as area percentages were calculated with a response factor of 1.

Results

Sequence analysis of ACLysoPL

A partial length (618 bp) of cDNA clone *ACLysoPL* obtained from ESTs of natural basidiomes of *A. cinnamomea*, contained the lipase gene family consensus sequence, ITFAGHSLGA. After 5′-RACE amplification, the full-length lipase, ACLysoPL, which has a 945 -bp open reading frame (ORF), a 160 bp 5′ noncoding region and a 318 -bp 3′ noncoding region, was obtained (GenBank accession no. FJ970031). The ORF encodes a 314 amino acid protein. The predicted molecular weight of the polypeptide is 35·5 kDa, and the theoretical isoelectric point (pI) is 6·06. No putative signal peptide was foundin the N-terminal region of ACLysoPL, but the peroxisomal targeting signal type 1 (PTS1) predictor (http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp) predicted the last 12 C-terminal residues to be a putative PTS (Neuberger *et al.* 2003). The ACLysoPL protein contains the lipase consensus sequence, as well as a putative motif for a protein kinase C phosphorylation site, a casein kinase II phosphorylation site, an *N*-myristoylation site, an amidation site, a leucine zipper pattern and a cAMP- and cGMP-dependent protein kinase phosphorylation site (Fig. 1). Based on the NCBI BLAST, the protein sequence of ACLysoPL is similar to those of other yeast and fungi proteins. ACLysoPL exhibits 32% identity and 48% similarity with *Schizosaccharomyces pombe* predicted serine hydrolase (accession no. O94305), 27% identity and 44% similarity with *Saccharomyces cerevisiae* serine hydrolase YJU3 (P28321) and 29% identity and 48% similarity with *Penicillium marneffei* putative alpha/beat hydrolase (XP_002151146). ACLysoPL, also exhibits 30% identity and 44% similarity with *Salinibacter ruber* LysoPL (YP_445943), 29% identity and 45% similarity with *Archaeoglobus fulgidus* lysophospholiase (NP_070581), 26% identity and 43% similarity with *Leptospira borgpetersenii* serovar Hardjobovis LysoPL (YP_799744), 25% identity and 37%

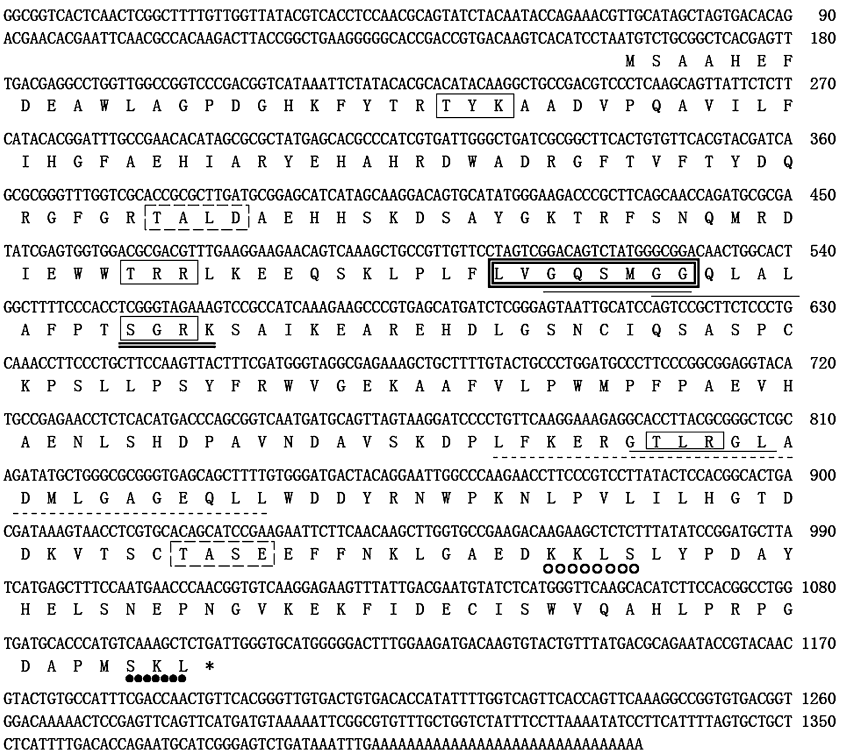

Figure 1 Nucleotide and inferred amino acid sequences of *ACLysoPL*. Amino acid sequence: lipase consensus motif, double-line boxed region; putative protein kinase C phosphorylation site, single-line boxed region; putative casein kinase II phosphorylation site, broken-line boxed region; putative *N*-myristoylation site, single underline; putative amidation site, double underline; putative leucine zipper pattern, broken single underline; putative cAMP- and cGMP-dependent protein kinase phosphorylation site, hollow circle; putative microbodies C-terminal targeting signal, solid circle; the UGA stop codon, asterisk.

similarity with human monoglyceride lipase (LysoPL-like) (Q99685) and 24% identity and 39% similarity with *Rattus norvegicus* monoglyceride lipase (Q8R431). These results indicate the putative lipase and LysoPL activity of ACLysoPL.

The ACLysoPL protein sequence included the active site serine motif, GXSXG, which is conserved among esterases, lipases and serine proteases (Brenner 1988) and a Ser–His–Asp triad, which is a well-known structural feature of the serine proteases and lipases (Schrag *et al.* 1991). Sequence alignment showed ACLysoPL to contain the catalytic triad similar to its homologous protein, human monoglyceride lipase (Fig. 2) (Karlsson *et al.* 1997). The predicted secondary and tertiary structures of ACLysoPL protein were constructed using ESyPred3D Web Server 1.0 (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/) (Lambert *et al.* 2002) and the BioinfoBank Meta Server (http://meta.bioinfo.pl/) (Ginalski *et al.* 2003). They revealed that the structure of ACLysoPL is similar to the crystal structure of *Pseudomonas fluorescens* aryl esterase (PDB ID 1va4). A total of nine α helices are surrounded by eight β strands can been seen, indicating a canonical α/β hydrolase fold (Ollis *et al.* 1992).

Characterization, expression and purification of ACLysoPL

Southern blot was performed by using the partial coding region and 3′-UTR of *ACLysoPL* as probes. The genomic DNA was isolated from liquid-cultured mycelia and digested with *Eco*RI, *Eco*RV and *Pst*I. No cleavage sites were observed within the probe sequences where the three restriction enzymes were used. This result indicates that there are multiple copies of *ACLysoPL* on the genome (Fig. 3a). Based on Northern blots, *ACLysoPL* was most highly expressed in liquid-cultured mycelium and remained at a basal expression level in solid-cultured mycelium, solid-cultured basidiomes and natural basidiomes (Fig. 3b).

To investigate the biochemical properties of the protein encoded by ACLysoPL, the full-length gene was cloned into pTYB12 expression vector and transformed into *E. coli* BL21 (DE3). The soluble intein–ACLysoPL fusion protein was purified using an affinity chitin column, after a specific on-column cleavage of intein a 35·5 kDa protein was cleaved, eluted from the column and then determined using SDS-PAGE; Fig. 4). More than 90% of the intein–ACLysoPL fusion protein was cleaved by cysteine and DTT mixture at 4°C.

Characterization of the recombinant ACLysoPL

The optimum temperature of ACLysoPL was investigated using *p*-NP palmitate as substrate at pH 7·0. ACLysoPL had an optimum temperature range of between 20 and 30°C (Fig. 5a) and retained about 50% of its maximal activity at 10°C and 40% at 4°C. The

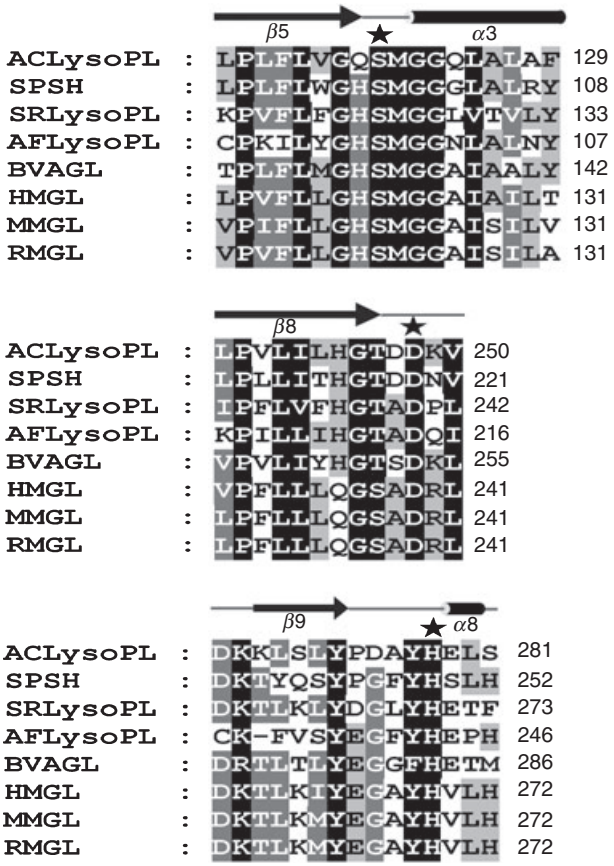

Figure 2 Amino acid sequence alignments of three conserved blocks contain catalytic triad with several related proteins similar to ACLysoPL. SPSH, *Schizosaccharomyces pombe* probable serine hydrolase (accession number O94305; 32% identity); SRLysoPL, *Salinibacter ruber* DSM 13855 lysophospholipase (LysoPL) (YP_445943; 30% identity); AFLysoPL, *Archaeoglobus fulgidus* DSM 4304 LysoPL (NP_070581; 29% identity); BCAGL, *Burkholderia vietnamiensis* G4 acylglycerol lipase (YP_001118825; 31% identity); HMGL, human monoglyceride lipase (Q99685; 25% identity); MMGL, mouse monoglyceride lipase (O35678; 24% identity); RMGL, rat monoglyceride lipase (Q8R431; 24% identity). The alignment was conducted using mega version 4 (Tamura *et al.* 2007). A secondary structure prediction was obtained for ACLysoPL with the ESyPred3D Web Server 1.0 (Lambert *et al.* 2002). The predicted secondary structure elements of ACLysoPL are shown above the sequence. The α helix is shown as a cylinder, and the β strand is shown as an arrow. The residues of the HMGL catalytic triad identified by site directed mutagenesis (Karlsson *et al.* 1997) are marked with asterisks.

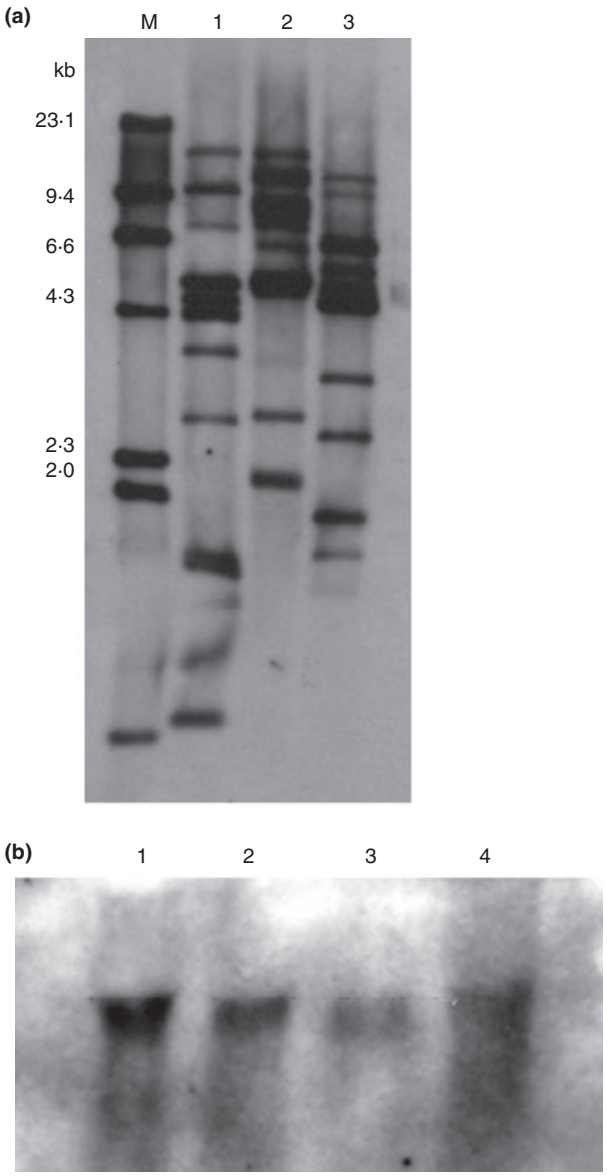

Figure 3 (a) Southern hybridization analysis employing the labelled probe. Lane M: λ DNA-*Hin*dIII digested marker. Lane 2: genomic DNA from *Antrodia cinnamomea* was digested with *Eco*RI. Lane 3: *Eco*RV. Lane 4: *Pst*I. (b) Northern blot analysis of total RNA isolated from different sample. Lane 1: liquid-cultured mycelium. Lane 2: solid-cultured mycelium. Lane 3: solid-cultured basidiomes. Lane 4: natural basidiomes.

pH dependence of ACLysoPL was studied using Good's buffer for a pH range from 3·0 to 10·0 at 30°C (Fig. 5b). The optimum pH of ACLysoPL was 7·0. Most enzyme activity was lost below pH 5·0 and above 9·0. Incubation at various temperatures between 30 and 70°C for 10 min at pH 7·0 showed that ACLysoPL was stable in incubation temperatures under 40°C (Fig. 5c). Inactivation occurred at temperatures of higher than 50°C. Hydrolysis of *p*-NP esters containing fatty acids of various chain lengths by ACLysoPL is outlined in Table 1. ACLysoPL showed a noteworthily preference for long-chain fatty acid esters (C_{16} and C_{18}). There was no activity when ACLysoPL was reacted with short-chain fatty acid esters (C_4 and C_8). Divalent metal ions (10 mmol l^{-1}), $CuCl_2$, $FeCl_2$, $HgCl_2$, $NiCl_2$ and $ZnCl_2$ significantly inhibited enzyme activity when

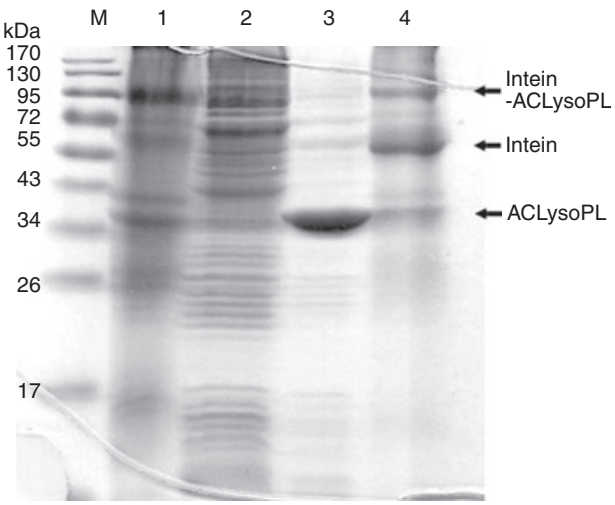

Figure 4 Expression and purification of ACLysoPL using the pTYB12 system. Lane M: protein marker. Lane 1: insoluble pellet dissolved in SDS. Lane 2: crude extract from soluble proteins. Lane 3: eluted ACLysoPL after stopping column flow and inducing a self-cleavage reaction at 4°C overnight. Lane 4: SDS stripping of remaining proteins bound to column.

compared to the control sample (Fig. 5d). The activity of ACLysoPL showed independent from metal ions like Ca^{2+} and Mg^{2+}.

LysoPL activity of ACLysoPL

To further characterize the LysoPL activity of AcLysoPl, the products of this enzyme catalysed reaction was measured *in vitro* by GC/MS. 1-Palmitoyl-*sn*-glycero-3-phosphocholine and 1-dodecanoyl-*sn*-glycero-3-phosphocholine were used as the exogenous substrate. When recombinant AcLysoPL reacted with 1-palmitoyl-*sn*-glycero-3-phosphocholine and 1-dodecanoyl-*sn*-glycero-3-phosphocholine, two new products, palmitic acid (hexadecanoic acid) (retention time: 36·10) and dodecanoic acid (retention time: 26·50), respectively, appeared in the GC/MS chromatogram (Fig. 6). There was no detectable enzyme activity when 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1, 2-dodecanoyl-*sn*-glycero-3- phosphocholine were used as substrates. These results indicate the specific LysoPL activity of AcLysoPL which

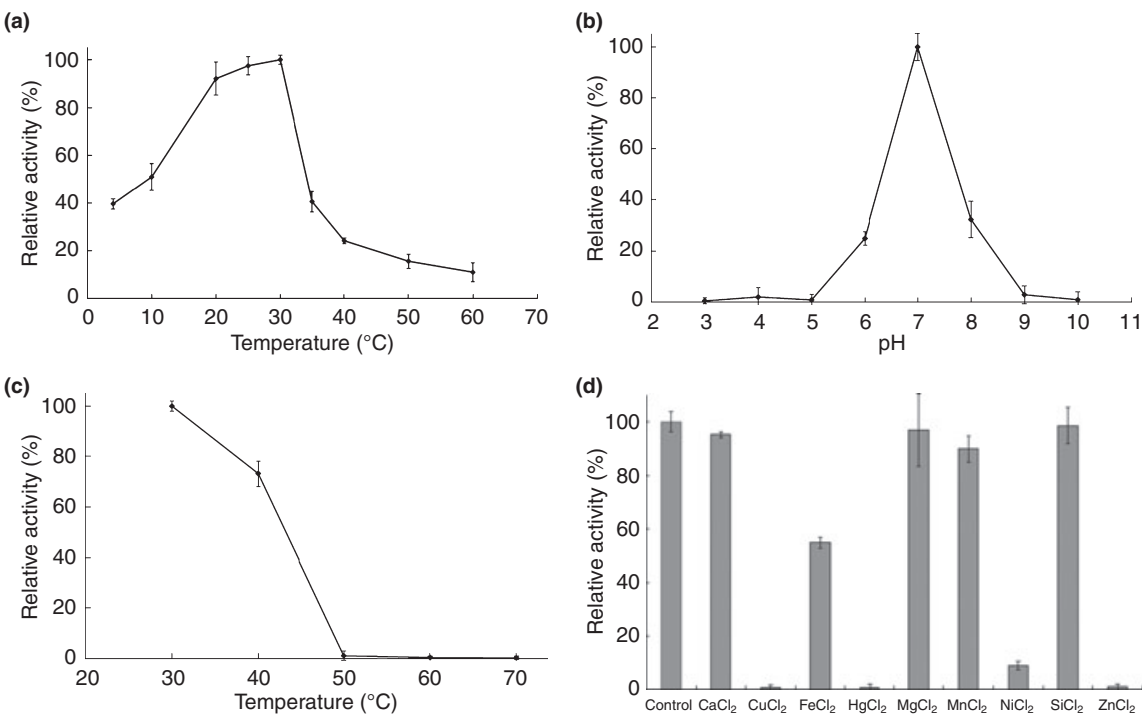

Figure 5 (a) Effect of temperature on esterase activity of ACLysoPL protein. The activity was measured by spectrophotometric method using *p*-nitrophenyl (*p*-NP) palmitate as substrate at pH 7·0. The bars indicate standard errors. (b) Effect of pH on esterase activity of ACLysoPL protein. The enzyme reaction was carried out at 30°C in Good's buffer using *p*-NP palmitate as substrate. The bars indicate standard errors. (c) Thermal stability of ACLysoPL protein. The residual activities after 10 min of incubation at different temperatures were measured using a spectrophotometric method with *p*-NP palmitate as substrate at 30°C and pH 7·0. The bars indicate standard errors. (d) Effect of metal ions on ACLysoPL. Activities were measured using a spectrophotometric method with *p*-NP palmitate as substrate at 30°C and pH 7·0 in phosphate-buffered saline buffer containing various metal ions with the concentration of 10 mmol l^{-1}. The relative activities were presented as the ratio of enzyme reaction with different metal ions compared with the control including no metal ion.

Table 1 Substrate specificity of ACLysoPL in the hydrolysis of *p*-nitrophenyl (*p*-NP) esters containing fatty acids of various chain lengths. Hydrolyses of *p*-NP esters were measured at pH 7·0 and 30°C

Substrate	Acyl group chain length	Specific activity* (units mg^{-1})	Relative activity† (%)
p-NP butyrate	C_4	0·06 ± 0·01	0·45
p-NP caproate	C_8	0·07 ± 0·02	0·53
p-NP laurate	C_{12}	1·00 ± 0·68	7·56
p-NP myristate	C_{14}	1·69 ± 0·51	12·77
p-NP palmitate	C_{16}	13·23 ± 0·19	100·00
p-NP stearate	C_{18}	7·50 ± 0·86	56·69

*The unit of specific activity is defined as the quantity of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute under assay conditions.

†The highest activity of enzyme is denoted as 100% compared with other different substrates.

removed the fatty acyl group from the monoacylglycerophospholipids.

Discussion

We have identified and characterized a novel LysoPL gene (*ACLysoPL*) from *A. cinnamomea* which codes for a cellular protein that has LysoPL and esterase activity at pH 7·0. In mammalian cells, LysoPLs have been classified into large form LysoPL (about 60 kDa) and small form LysoPL (about 25 kDa) (Wang and Dennis 1999). The ORF of *ACLysoPL* encoded 314 amino acids, had a predicted molecular weight of 35·5 kDa and a pI of 6·06. Many phospholipases previously studied in fungi were in secreted form (Wright *et al.* 2004; Kohler *et al.* 2006), but the N-terminal of ACLysoPL does not contain a signal peptide for secretion. Interestingly, a putative PTS1 was found in the last 12 C-terminal residues. ACLysoPL could be involved in various processes in peroxisomes, such as lipid metabolism and peroxisome biogenesis. Lpx1p is a peroxisomal protein with a PTS1 in *S. cerevisiae*. Lpx1p showed acyl hydrolase and phospholipase A activity *in vitro* and, in addition, its deletion mutants had an aberrant peroxisome morphology (Thoms *et al.* 2008). These catalytically active phospholipases and LysoPLs regulate a variety of cellular reactions in phospholipid metabolism and support the substrates for plasma membrane and multi intracellular organelles with phospholipid-base membrane layers. Recently, peroxisomes have been shown to play a role in sexual development of filamentous fungi (Bonnet *et al.* 2006). Moreover, a putative *N*-myristoylation site, previously studied in a cytosolic phospholipase A2 in *Aspergillus nidulans* (Kohler *et al.* 2006), which might be involved in interaction with membranes and proteins or have regulatory functions, was also found in ACLysoPL.

The putative catalytic triad consisting of the amino acids Ser–Asp–His in ACLysoPL was identical to those in the catalytic domain of LysoPL from *S. ruber* and *A. fulgidus* (Fig. 2). Little is known about the molecular structure of LysoPL. The predicted tertiary structure of ACLysoPL protein is similar to the crystal structure of *Ps. fluorescens* aryl esterase, which shows low bromoperoxidase activity and favours activated esters with small acyl groups (Cheeseman *et al.* 2004). Interestingly, ACLysoPL prefers long-chain acyl esters such as *p*-NP palmitate and *p*-NP stearate as substrates (Table 1). Because peroxisomes are the site of long-chain fatty acid β-oxidation in plants and fungi, the ACLysoPL might play an important role in the fatty acid metabolism in peroxisome. The Southern and Northern blot analyses showed high copy numbers and constitutive expression level of *ACLysoPL*. Many fungal species appear to have more than one PLB gene. *Saccharomyces cerevisiae* encodes three and *Candida albicans* encodes a phospholipase B family with five members (Kohler *et al.* 2006). These indicate the possible important role of their cellular functions such as membrane homeostasis, lipid metabolism and even signal transduction. LysoPL is considered to play an important role in regulating the cytotoxic lysophosphatidylcholine concentrations in the cell by converting lysophosphatidylcholine to glycerophosphodiester and fatty acids (Weltzien 1979). Lysophosphatidylcholine modulates multiple gene expression, promotes secretion of growth factors and induces cell adhesion. The signalling activity of lysophospholipids is mediated by the activation of transmembrane G-protein-coupled receptors (Anliker and Chun 2004). Many lysophospholipids affect fundamental cellular functions like proliferation, differentiation, survival, migration, adhesion, invasion and morphogenesis (Ishii *et al.* 2004). In *Candida tropicalis*, LysoPL has been found to mediate adhesive interactions between host cells and fungi (Prakobphol *et al.* 1994).

ACLysoPL showed its activity was not affected by metal ions like Ca^{2+} and Mg^{2+}. This result correlates with other small form LysoPLs purified and characterized from rat and *Ps. fluorescens* (Hong *et al.* 1991; Sugimoto *et al.* 1996). ACLysoPL can be considered the class of psychrophilic enzyme because its reactive temperature below 30°C and its ability to retain 40% activity at 4°C. This adaptation corresponds to the growing environment of *A. cinnamomea* at altitudes ranging from 500 to 1500 m on hillsides in Taiwan. An extracellular lipolytic enzyme from the snow mould fungus, *Typhula ishikariensis*, was most active at 30°C and retained 23·4% of its maximum activity at 4°C (Nettleman *et al.* 1997). LysoPLs could be used in industry in applications such as the degumming of edible oils and the synthesis of triglycerides enriched in polyunsaturated fatty acids from soybean lysolecithin (Heusch

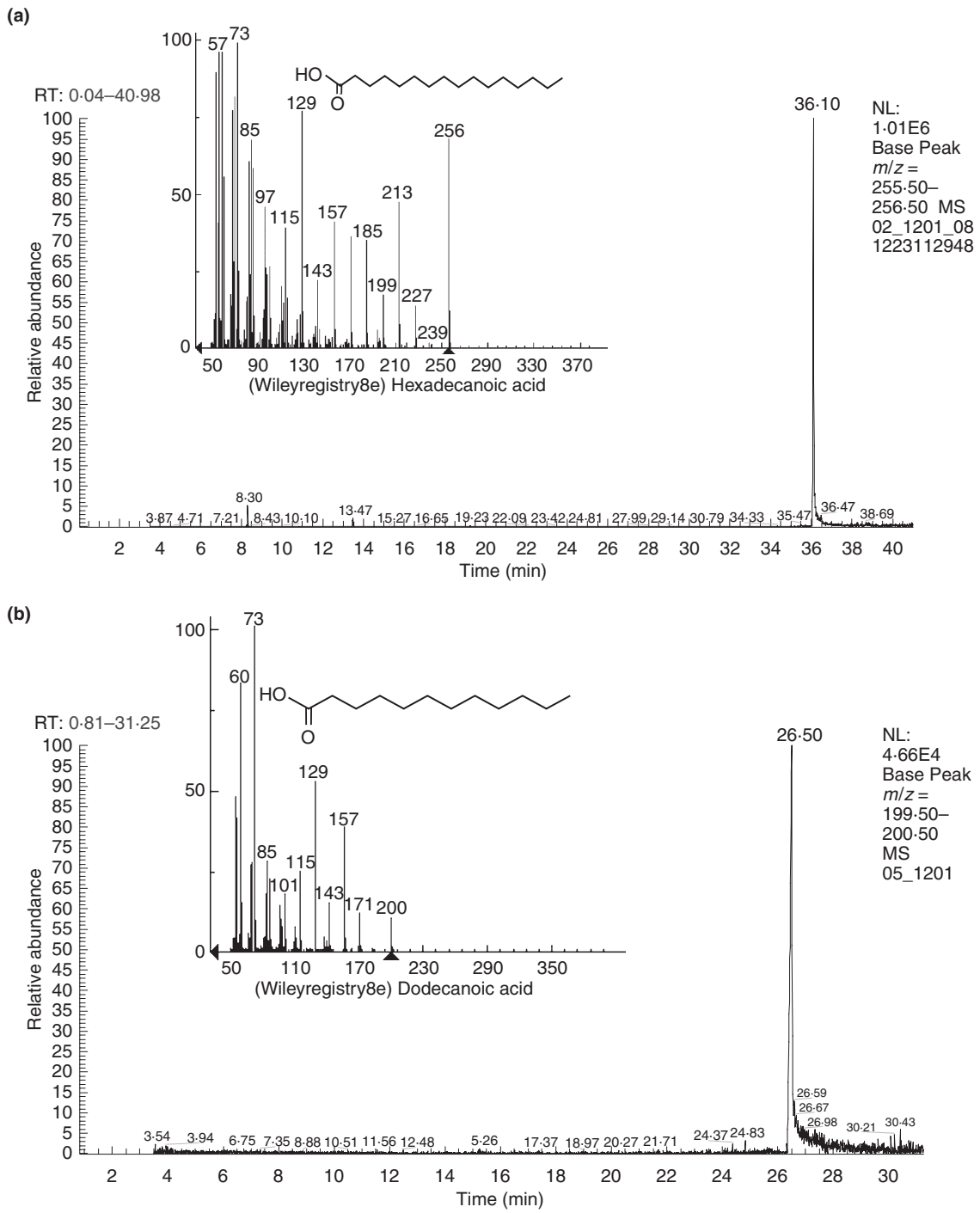

Figure 6 Gas chromatography (GC)-mass spectroscopy analysis of the product formed by the truncated version of recombinant ACLysoPL with 1-palmitoyl-*sn*-glycero-3-phosphocholine (a) and 1-dodecanoyl-*sn*-glycero-3-phosphocholine (b) as substrate. The GC profile (bottom) and mass fragmentation patterns (top) are illustrated. The new products, palmitic acid (hexadecanoic acid, retention time: 36·10) and dodecanoic acid (retention time: 26·50), were formed by enzyme digestion.

1987; Dahlke *et al.* 1995). A commercial enzymatic complex, Spezyme, containing LysoPL was applied to remove lysophospholipids from blocking the filters in the process of saccharification of wheat starch into glucose (Nebesny *et al.* 1998). The feature of ACLysoPL is its specificity for long-chain acyl group substrates, which are the

major component of the natural oil. The use of enzymes to replace chemical processes has the advantages including high catalytic efficiency, specificity and less pollution.

To our knowledge, this is the first LysoPL gene that has been identified and characterized from a brown-rot fungus. We expressed and purified the small form ACLysoPL protein heterologously in *E. coli*, and the generated recombinant soluble protein exhibited both esterase and LysoPL activities. Our previous report showed that a lipase gene, *Ac-LIP*, was strongly expressed during the basidiomatal formation stage of *A. cinnamomea* (Chu *et al.* 2008b). Although the expression level of *ACLysoPL* was constant in our study, the predicted targeting to peroxisome revealed its possible role in governing phospholipids turnover and reconstituting of membrane contents. The mechanism of the regulation and physiological function of this enzyme should be further studied.

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