J Am Oil Chem Soc (2012) 89:1435–1448
DOI 10.1007/s11746-012-2050-x

ORIGINAL PAPER

Phospholipases A_1 from *Armillaria ostoyae* Provide Insight into the Substrate Recognition of α/β-Hydrolase Fold Enzymes

Martin Dippe · Mathias Q. Müller · Andrea Sinz · Renate Ulbrich-Hofmann

Received: 16 August 2011 / Revised: 15 January 2012 / Accepted: 2 March 2012 / Published online: 18 March 2012

Abstract Four enzymes with phospholipase A_1 (PLA_1) activity were purified from the fruiting bodies of the basidiomycete *Armillaria ostoyae*. The enzymes (PLA_1-1, -2, -3 and -4) showed similar isoelectric points (4.3, 3.9, 4.0 and 4.0) and apparent molecular masses in the range of 35–47 kDa. Mass spectrometric analyses of proteolytic fragments revealed sequences homologous to α/β-hydrolase fold enzymes. The enzymes share one conserved region with fungal phospholipases B and the active site sequence with bacterial esterases and PLA_1s. PLA_1-1 cleaves phospholipids and lysophospholipids with an optimum activity at pH 5.3. In contrast, PLA_1-2, -3 and -4 are characterized by broad pH optima in the slightly acidic to neutral range and are additionally capable of hydrolyzing mono- and diglycerides as well as fatty acid methyl esters. All enzymes favor glycerol-based lipids with a single medium-sized fatty acid moiety in the *sn*-1 position but show reduced activity towards the corresponding 1,2-diacyl derivatives with bulky long-chain or inflexible saturated fatty acid moieties in the *sn*-2 position. The enzymes prefer zwitterionic phospholipid substrates and are unable to hydrolyze triglycerides. From the selectivity of these broad-spectrum α/β-hydrolase fold enzymes towards the different classes of their substrates a regiospecific steric hindrance and a head group recognition are concluded.

Keywords *Armillaria ostoyae* · Fatty acid specificity · α/β-Hydrolase fold enzyme · Phospholipase A_1 · Substrate selectivity

Abbreviations

CID	Collision-induced dissociation
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
HEPES	*N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid)
LPPC	1-palmitoyl-*sn*-glycero-3-phosphocholine
LTQ	Linear quadrupole ion trap
MES	2-Morpholinoethanesulfonic acid
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PIPES	Piperazine-*N*,*N*′-bis(ethanesulfonic acid)
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLB	Phospholipase B
PMSF	Phenylmethanesulfonyl fluoride

Data from mass-spectrometric peptide sequencing are available in the PRIDE database (http://www.ebi.ac.uk/pride/) under the accession numbers 17662–17665.

Electronic supplementary material The online version of this article (doi:10.1007/s11746-012-2050-x) contains supplementary material, which is available to authorized users.

M. Dippe (✉) · R. Ulbrich-Hofmann
Institute of Biochemistry and Biotechnology, Martin-Luther University Halle-Wittenberg, Kurt-Mothes-Str. 3, 06120 Halle, Germany
e-mail: martindippe@gmx.de

M. Q. Müller · A. Sinz
Institute of Pharmacy, Martin-Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, 06120 Halle, Germany

PNGase F	Peptide-*N*-glycosidase F
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PS	Phosphatidylserine
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
U	Unit

Introduction

Acyl ester hydrolases (EC 3.1.1) are ubiquitous enzymes catalyzing the hydrolytic cleavage of carboxylic acid esters. They are involved in many physiological processes from digestion to signal transduction [1, 2]. Due to their importance in preparative chemistry and in industrial processes, many acyl ester hydrolases have been isolated from microbial sources such as bacteria and filamentous fungi [3]. Most of these enzymes are characterized by a catalytic serine nucleophile and a typical α/β-fold [4]. Despite these mechanistic and topological similarities, acyl ester hydrolases differ widely in their substrate utilization. With respect to their lipid substrates, esterases, mono-, di-, and triglyceride lipases, phospholipases A (PLA) and lysophospholipases are distinguished. The phospholipases A are further subdivided into phospholipases A_1 (PLA_1s, EC 3.1.1.32) and phospholipases A_2 (PLA_2s, EC 3.1.1.4) according to their regiospecificity in the cleavage of the *sn*-1 or *sn*-2 acyl moieties in glycerophospholipids. Enzymes which act on both positions are called phospholipases B (PLB, EC 3.1.1.5). The assignment of phospholipases to PLB, however, must be considered with caution because they are difficult to differentiate from PLA_1s due to the acyl migration from the *sn*-2 to the *sn*-1 position in 1-lysophospholipids.

However, a great number of enzymes cannot be clearly assigned to one or other of the groups because they hydrolyze a broad range of substrates. For example, the calcium-independent cytosolic PLA_2s catalyze the hydrolysis of *sn*-1-bound fatty acid moieties in phospholipids if no acyl moiety at the *sn*-2-position is present and they are also active towards lysophospholipids [5]. Endothelial lipases acting on triglycerides also cleave phospholipids [6]. The lipolytic enzyme from guinea pig pancreas shows PLA_1- [7] but also mono-, di- and triglyceride lipase activity [8]. Additionally, it converts galactolipids [9]. Moreover, many enzymes that hydrolyze lysophospholipids function additionally as acyltransferases by transferring the fatty acid moiety of one lysophospholipid molecule to another, an activity exhibited by lysophospholipase from rabbit lung [10].

Extensive mutational studies on acyl ester hydrolases have shed light on the molecular basis of the multifaceted selectivity towards different classes of lipids. Interestingly, in some enzymes the selectivity is determined by only a few amino acid positions as shown for the esterase from *Bacillus subtilis*. The enzyme can be converted to a monoacylglycerol lipase by a single amino acid exchange [11]. A variant of the *Staphylococcus hyicus* PLA_1 created by introduction of defined amino acid residues of the closely related triglyceride lipase from *Staphylococcus aureus* is characterized by an increased activity towards triglycerides and, vice versa, a strongly reduced affinity for phospholipids [12]. However, additional regulatory elements such as the so-called lid segment may also contribute to substrate selectivity [13].

Recently, PLA_1 activity has been found in fruiting bodies of the basidiomycete *Armillaria ostoyae*, which was shown to be responsible for phospholipid degradation during sporogenesis [14]. In this paper we report on the purification and characterization of this enzymatic activity yielding four new enzymes with PLA_1 activity but uncommon substrate selectivity. The enzymes show similarities to both fungal PLB and microbial and plant PLA_1s. While one of these PLA_1s is specific for phospholipids, the three other enzymes are also capable of converting neutral lipids. From kinetic analyses, a strong dependence of the activity on the chain length and saturation of fatty acids and on the head group of phospholipids is concluded. Based on the results, a model for the control of substrate selectivity is proposed.

Experimental Procedures

Materials

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidic acid (PA), all from soybean, and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were products from Lipoid (Ludwigshafen, Germany). Sphingomyelin from bovine brain was obtained from Enzo (Farmingdale, USA). 2-Lysophospholipids and 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine were received from AvantiPolar (Alabaster, USA). 1-Palmitoyl-2(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine was from Invitrogen (Carlsbad, USA) and Triton X-100 from Applichem (Darmstadt, Germany). Sequencing grade trypsin was purchased from Roche Diagnostics (Mannheim, Germany). All other phospholipids, fatty acids, fatty acid esters, enzyme inhibitors and peptide-*N*-glycosidase F (PNGase F) from *Elizabethkingia meningoseptica* were from Sigma (St. Louis, USA). Soybean fatty acids were prepared from PC as described elsewhere [14]. All other chemicals were of the highest purity commercially available.

Preparation of Crude Extracts

Fruiting bodies from *A. ostoyae* were collected from infected spruce trees (*Picea abies*) in the Harz mountains, Germany. After grinding in liquid nitrogen, the resulting powder was suspended in 20 mM sodium acetate buffer, pH 6.5, containing 1 mM ascorbic acid, 1 mM ethylenediaminetetraacetic acid (EDTA) (2 l kg^{-1} of fruiting bodies). The suspension was homogenized with a hand blender and centrifuged for 20 min at 16,000×*g*. For the removal of spores, the supernatant was subjected to one freeze–thaw cycle with subsequent centrifugation (20 min, 27,000×*g*).

Hydrophobic Interaction Chromatography

Ammonium sulfate (4 M) was added to 450 ml of crude extract to yield a final concentration of 500 mM. The extract was applied to 20 ml of Phenyl Sepharose 6 Fast Flow (high substitution, GE Healthcare, Little Chalfont, United Kingdom) on an Äkta FPLC system (GE Healthcare), and proteins were eluted with 20 mM sodium acetate buffer, pH 6.5, containing 1 mM EDTA and subsequently with a gradient of the same buffer containing 0–20 % (v/v) acetonitrile. According to the profile of eluted PLA_1 activity, fractions were combined to three main fractions (I–III), which were dialyzed against 20 mM sodium acetate buffer, pH 6.5, containing 1 mM EDTA.

Ion Exchange Chromatography

Anion exchange chromatography was performed on Source 15Q matrix (GE Healthcare). Samples obtained from hydrophobic interaction chromatography (Supporting information, Fig. S1) were loaded onto 3.6 ml of matrix material and the proteins were eluted by a gradient of 0–500 mM NaCl in 20 mM sodium acetate, pH 6.5, containing 1 mM EDTA.

Samples obtained from hydroxyapatite chromatography (Fig. S1) were applied to 1.5 ml of matrix material and the proteins were eluted by a gradient of 0–300 mM NaCl in 20 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA. In both cases, fractions containing PLA_1 activity were dialyzed against 20 mM sodium acetate buffer, pH 6.5, containing 1 mM EDTA.

Hydroxyapatite Chromatography

Preparations from ion exchange chromatography were supplemented with 10 mM piperazine-*N*,*N*′-bis(ethanesulfonic acid) (PIPES)/NaOH, pH 6.5, and 3 mM $MgCl_2$ and applied to 3.5 ml of hydroxyapatite BioGel HTP (Bio-Rad, Hercules, USA). The matrix was washed with 10 mM PIPES/NaOH, pH 6.5, and proteins were eluted by a gradient from 0 to 150 mM sodium phosphate buffer, pH 6.5. Fractions with PLA_1 activity were pooled and dialyzed against 20 mM sodium acetate buffer, pH 6.5, containing 1 mM EDTA.

For purification of PLA_1-1 under high salt conditions, the procedure was modified by addition of 200 mM NaCl to the buffers used for washing and elution.

Determination of PLA_1 Activity During Purification

Reactions (20 µl) contained 12.5 mM PC from soybean, 5 mM sodium dodecyl sulfate (SDS) and 25 mM Triton X-100 in 100 mM sodium acetate buffer, pH 5.6. Due to the absence of Ca^{2+}-inducible phospholipase activity in extracts of *Armillaria ostoyae*, this ion was not included in the assay. Residual EDTA deriving from the buffers used in enzyme purification did not cause any disturbance. After incubation in microplates for 0–90 min under shaking (400 rpm) at 30 °C, fatty acid concentrations were determined by the NEFA C kit according to Hoffmann et al. [15] using a standard curve of soybean fatty acids. Enzyme activity was obtained from the linear fit of the fatty acid concentration as a function of the reaction time by the software SigmaPlot 11.0 (Systat Software, USA). One unit (U) represents the amount of enzyme releasing 1 µmol of fatty acid per minute.

Protein Determination

For crude extracts, the method by Bradford [16] was used because of the presence of ascorbic acid. In all other cases, protein concentration was determined by the bicinchoninic acid kit from Pierce (Rockford, USA), according to the manufacturer's instructions. Protein concentrations were calculated using standard curves of bovine serum albumin.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed in 12.5 % (w/v) polyacrylamide gels according to Laemmli [17]. The gels were stained with silver [18] or with Coomassie Brilliant Blue G250 [19]. Apparent molecular masses were estimated from two independent experiments according to [20] using molecular markers from Fermentas (Burlington, Canada).

Isoelectric Point (*p*I) Determination

Proteins were focused in a PhastGel IEF 3-9 applying the Phast Electrophoresis System (GE Healthcare, Little Chalfont, UK). *p*I values were estimated using molecular markers (*p*I 3–10) from Serva (Heidelberg, Germany).

Deglycosylation

Enzymes were digested with PNGase F (80 U per mg of PLA_1 protein) according to the protocol of the supplier.

Samples of the digested proteins were analyzed by SDS-PAGE as described above.

Nano-HPLC/Nano-ESI-LTQ-Orbitrap Mass Spectrometry

The purified enzymes (4 μg each) were applied to SDS-PAGE as described above and stained by Coomassie Brilliant Blue G250. PLA_1 bands were excised from the gel and treated with PNGase F according to the manufacturer's instruction. After reduction with 10 mM dithiothreitol and carbamidomethylation with 55 mM iodoacetamide, the proteins were *in-gel* digested with 0.2 μg of trypsin overnight [21]. For mass spectrometric analyses according to [22], the proteolytic peptide mixtures were fractionized by reversed-phase chromatography on an Ultimate nano-HPLC system (Dionex, Idstein, Germany), including a concentration and desalting step (C18 precolumn: Acclaim PepMap, 300 μm × 5 mm, 5 μm, 100 Å) before separation (C18 separation column: Acclaim, 75 μm × 250 mm, 3 μm, 300 Å). The tryptic peptides were eluted using a 90-min linear gradient of 5–80 % (v/v) acetonitrile in 0.1% formic acid at a flow rate of 300 nl min^{-1}, followed by washing with 80 % acetonitrile (15 min). ESI–MS/MS analysis was performed in positive ionization mode using a LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, Bremen, Germany), which was directly coupled to the nano-HPLC system via a nano-ESI source (Proxeon, Odense, Denmark). Each high-resolution mass spectrum (m/z 300–2,000, $R = 60{,}000$) was followed by collision-induced dissociation tandem mass spectrometry (CID–MS/MS) of the five most intense signals in the linear ion trap (LTQ). Fragment ions were either analyzed in the orbitrap ($R = 7{,}500$) or in the LTQ. For data acquisition, the software XCalibur 2.0.7 (ThermoFisher) was combined with DCMS link 2.0 (Dionex).

Amino acid sequences were identified by Mascot MS/MS ion search [23]. Sequences showing significant similarity to GXSXG-lipase patterns, which are typical for the active site of serine hydrolases [24], were identified using the BLAST tool [25]. Furthermore, highly conserved PLB regions were used as templates for the alignment. These regions were identified by comparing amino acid sequences of the six fungal PLB from *Cryptococcus neoformans* var. *neoformans* (GenBank ID: AAF61964.1), *Candida albicans* (GenBank ID: XP_713822.1), *Neurospora crassa* (GenBank ID: O42790.2), *Penicillium chrysogenum* (GenBank ID: CAA42906.1), *Saccharomyces cerevisiae* (GenBank ID: CAA88523.1) and *Aspergillus fumigatus* (GenBank ID: Q9P8P4.2), corresponding to the motifs Y40-S49, P96-T112, S140-A151, A257-W283, F323-L337, L390-L406, W428-R440, F463-N478, P483-Y502, W531-S543 and C555-G566 in the PLB from *Cryptococcus neoformans* var. *neoformans*.

Kinetic Measurements

Reactions were performed in microplates by mixing 20 μl of substrate solution (0.1–20 mM phospholipid, acylglycerol or fatty acid methyl ester, and Triton X-100 in the molar ratio of 1:2) with 20 μl of purified enzyme (0.1 mU in 100 mM 2-morpholinoethanesulfonic acid (MES)/NaOH, pH 5.6). After incubation for 0–90 min under shaking (400 rpm) at 30 °C, 10 μl of 500 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES)/NaOH, pH 7.0, were added to adjust the reaction mixture to neutral pH. The release of free fatty acids was determined by the NEFA C kit as described in the previous section using standard curves of the corresponding fatty acids. From the initial rates as a function of the substrate concentration, the maximum rate (V_{max}), the substrate concentration at half-maximum saturation ($S_{0.5}$) and the Hill coefficient h were calculated by the Hill equation using the software SigmaPlot 11.0 (Systat Software, USA). All experiments were performed in triplicate.

pH Dependence

PLA_1 activity was determined towards mixed micelles consisting of 5 mM 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 10 mM Triton X-100 as described in the previous section using the following buffers: 50 mM sodium formate buffer (pH 3.0), sodium acetate buffer (pH 3.5–5.3), MES/NaOH (pH 5.6–6.75), HEPES/NaOH (pH 7.0–7.5) or Tris/HCl (pH 8.0–9.0). The ionic strength was adjusted with NaCl to 50 mM. The experiments were performed in triplicate.

Inhibitor Studies

Purified enzyme (10 mU ml^{-1} in 20 mM HEPES/NaOH, pH 7.0) was incubated with 1 mM of phenylmethanesulfonyl fluoride (PMSF), iodoacetamide, 4-bromophenacyl bromide or 5 mM ethylene glycol tetraacetic acid (EGTA) at 25 °C. After 12 h the remaining enzyme activity was assayed towards 5 mM DPPC/10 mM Triton X-100 at pH 5.6 as described above. Samples without inhibitor treatment were used as references. The experiments were performed in triplicate.

Esterase Assay with 4-Nitrophenyl Acetate

Hydrolysis of 4-nitrophenyl acetate (2 mM in 50 mM MES/NaOH, pH 5.6, 10 mM Triton X-100) by the enzymes (7.5 mU ml^{-1}) was analyzed in glass cuvettes at 30 °C by continuous determination of the absorbance at 360 nm. The concentration of the product 4-nitrophenol was calculated on the basis of an experimentally determined extinction

coefficient ($\varepsilon_0 = 2.43 \times 10^3$ M^{-1} cm^{-1}). The experiments were performed in duplicate.

Fluorimetric Determination of Regiospecificity

Hydrolysis of 1-palmitoyl-2(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine was assayed according to the method of Radvanyi et al. [26].

Results

Purification of PLA_1 Enzymes from *A. ostoyae* Fruiting Bodies

In contrast to many enzymes produced by filamentous fungi [27], there is little information about acyl ester hydrolases from basidiomycetes. Of several basidiomycetes tested, the fruiting bodies of the species *A. ostoyae* were shown recently to contain high PLA_1 activity [14], therefore this species was chosen for protein purification and characterization.

During the purification of the protein(s) showing PLA_1 activity, surprisingly, four enzymes could be distinguished. The purification scheme comprised hydrophobic interaction chromatography on Phenyl Sepharose, anion exchange chromatography at varying pH on Source 15Q, and hydroxyapatite chromatography (Supporting information, Fig. S1). In the initial step of purification on Phenyl Sepharose, the activity could be partitioned into three distinct peaks representing enzymes with different hydrophobicity (Fig. 1a). The three active fractions (corresponding to the elution volumes 18–34, 40–58, and 80–120 ml in Fig. 1a) were separately subjected to three further chromatographic steps (anion exchange at pH 6.5, hydroxyapatite, anion exchange at pH 5.0), yielding four enzymes called PLA_1-1, PLA_1-2, PLA_1-3 and PLA_1-4. Interestingly, PLA_1-3 and PLA_1-4 co-eluted during the first

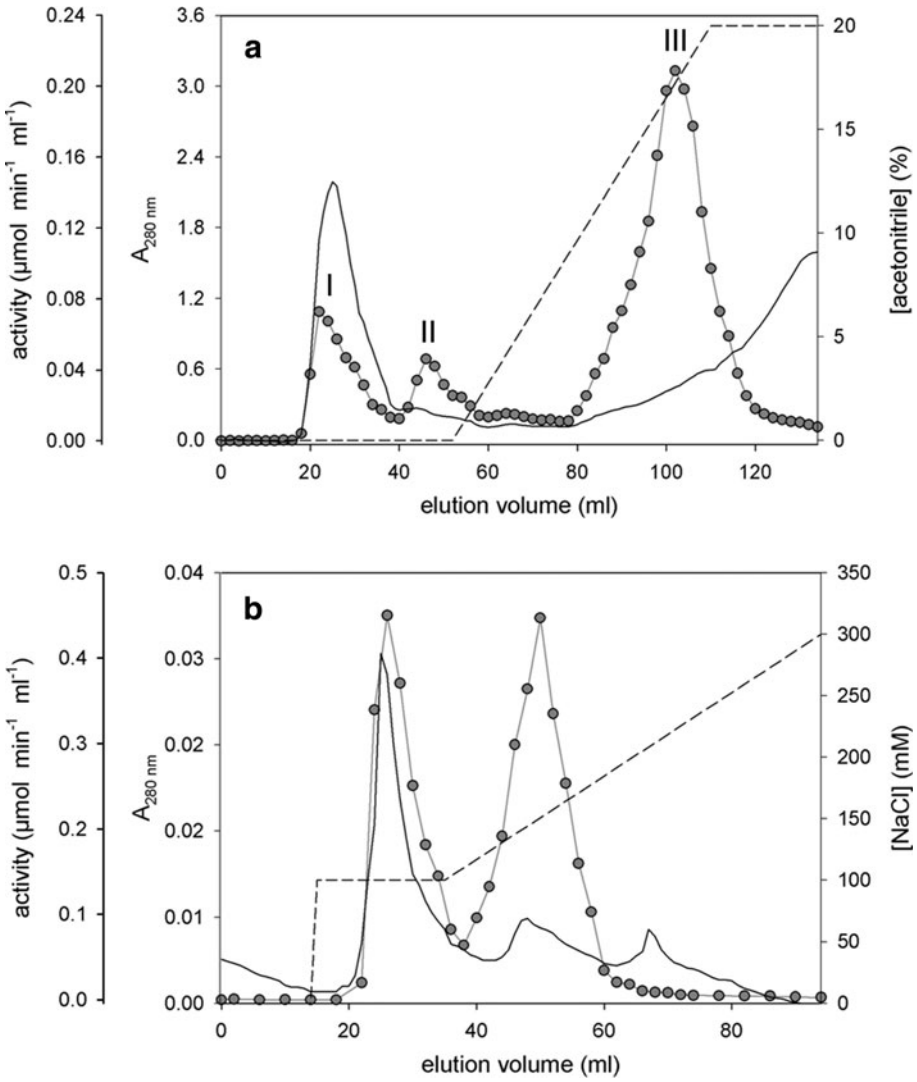

Fig. 1 Elution profiles of PLA_1s from *A. ostoyae*. **a** Three active fractions in hydrophobic interaction chromatography. The crude extract was applied to Phenyl Sepharose as described in "Experimental Procedures". The active peaks represent PLA_1-1 (I), PLA_1-2 (II), and PLA_1-3 + PLA_1-4 (III). **b** Separation of PLA_1-3 and PLA_1-4 by anion exchange chromatography on Source 15Q at pH 5.0. Peak III of the initial hydrophobic interaction chromatography (Fig. 1a), which was further purified on Source 15 Q at pH 6.5 and hydroxyapatite as described in the "Experimental Procedures" section, was applied to Source 15 Q at pH 5.0. *Filled circles* PLA_1 activity towards soybean PC/Triton X-100/SDS; *solid lines* absorbance at 280 nm; *dashed lines* acetonitrile or NaCl concentration of the eluent

three steps of purification, but could be separated by anion exchange at pH 5.0 (Fig. 1b). Therefore, these two proteins seem to be very similar in their molecular properties. In the case of PLA_1-2, -3 and -4, the proteins were homogeneous in SDS-PAGE after the anion exchange step at pH 5.0, whereas for PLA_1-1 an additional hydroxyapatite chromatography step at high salt conditions was necessary to obtain pure protein (Fig. 2a–c). The data of the purifications are summarized in Table 1. From 1 kg of fruiting bodies, 11 (PLA_1-1), 25 (PLA_1-2), 171 (PLA_1-3) and 139 (PLA_1-4) μg of pure protein were obtained. The purification factors were 5,650- (PLA_1-1), 2,610- (PLA_1-2), 1,250- (PLA_1-3) and 2,400 (PLA_1-4).

Molecular Masses, Isoelectric Points and Glycosylation of the PLA_1s

The molecular masses of PLA_1-1, PLA_1-2, PLA_1-3 and PLA_1-4 derived from SDS-PAGE (Fig. 2) were 46.7 ± 0.5, 32.9 ± 0.9, 39.2 ± 0.6 and 35.4 ± 0.4 kDa. Upon treatment with PNGase F (Supporting information, Fig. S2), which cleaves the bond between asparagine and glucosamine residues in glycoproteins [28], the electrophoretic mobilities of PLA_1-2, PLA_1-3 and PLA_1-4 revealed lower apparent molecular masses of 30.1 ± 0.4, 35.2 ± 0.5 and 33.0 ± 0.8 kDa. Therefore, these three proteins seem to be glycosylated. In contrast, no significant difference indicating an asparagine-linked carbohydrate moiety was detected for PNGase F-treated PLA_1-1 (46.9 ± 0.6 kDa). The pI values of the proteins were 4.3, 3.9, 4.0, and 4.0 for PLA_1-1, PLA_1-2, PLA_1-3, and PLA_1-4, respectively.

Mass Spectrometry-Based Amino Acid Sequence Alignment

To obtain initial information on the similarity of the new PLA_1s to characterized acyl ester hydrolases, the PLA_1s were subjected to proteolytic digestion by trypsin. The resulting peptides were analyzed by MS and MS/MS and compared to amino acid sequences of known proteins. For each PLA_1, 10 peptides were identified on average. The PLA_1 peptides identified showed high similarity to amino acid sequences of serine hydrolases. For example, the fragment GRAPGETTVPDR, which is present in all four PLA_1s is partially conserved in fungal PLBs (Fig. 3) but cannot be found in other PLA_1s, bacterial esterases or fungal triglyceride lipases. On the other hand, PLA_1-1, PLA_1-3 and PLA_1-4 strongly differ from common fungal PLB, which possess an A/SGLSGGXW consensus core. In contrast, the PLA_1 enzymes are characterized by amino acid sequences, which are highly similar to the catalytic motifs of bacterial esterases and lysophospholipases

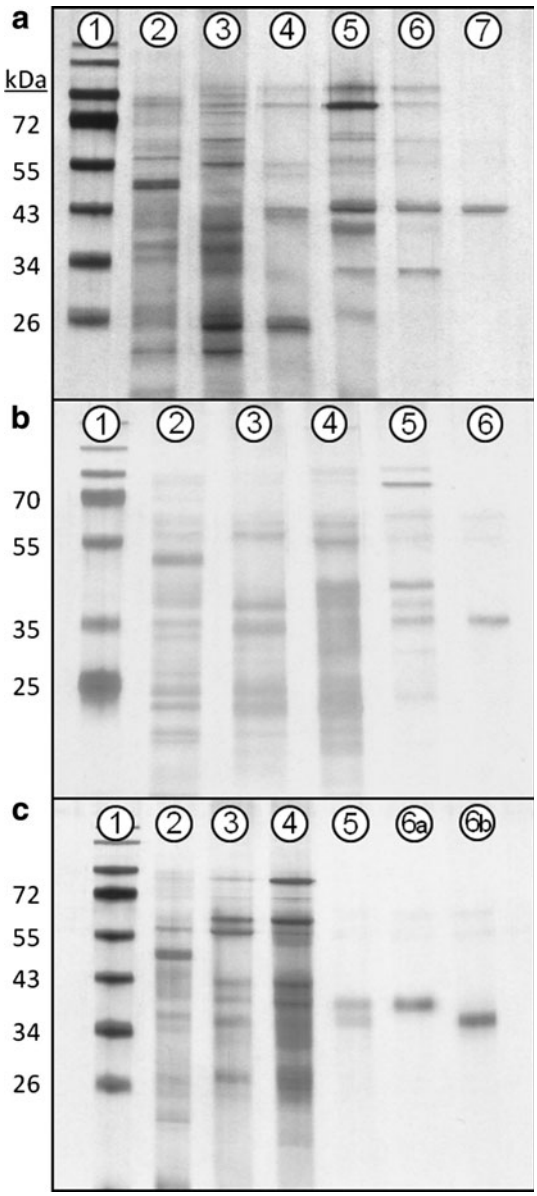

Fig. 2 SDS-PAGE of PLA_1s from *A. ostoyae* during purification. Silver-stained SDS-PAGE gels of enzyme fractions of the various purification steps yielding PLA_1-1 (**a**), PLA_1-2 (**b**), PLA_1-3 and PLA_1-4 (**c**) were produced as described in "Experimental Procedures". The lanes represent the molecular markers with molecular masses as indicated in the figure (*lane 1*), the crude extract (*lane 2*), the fractions I (**a**), II (**b**), and III (**c**) after hydrophobic interaction chromatography corresponding to Fig. 1a (*lane 3*), the corresponding active enzyme fractions after anion exchange at pH 6.5 (*lane 4*), hydroxyapatite chromatography (*lane 5*), anion exchange at pH 5.0 (*lane 6*), and hydroxyapatite purification under high-salt condition (*lane 7*). PLA_1-3 and PLA_1-4, which co-eluted in the first three steps of purification and were separated only by anion exchange at pH 5.0 (Fig. 1b), are shown in *lanes 6a* and *6b* in **c**

(Fig. 3). According to its AXSXG motif, PLA_1-1 also displays similarity to yeast lipase 2 and PLA_1 from maize (Fig. 3) and to the lipase from *Bacillus subtilis* [29].

Table 1 Purification of PLA_1 enzymes from *A. ostoyae* fruiting bodies

Purification step	Specific activity (μmol min^{-1} mg^{-1})/activity yield (%)			
	PLA_1-1	PLA_1-2	PLA_1-3	PLA_1-4
Phenyl Sepharose	0.01/14.0	0.07/9.0	0.27/75.0	
Source 15Q, pH 6.5	0.12/10.4	0.24/5.2	1.17/70.6	
Hydroxyapatite	1.04/9.4	3.64/4.8	11.8/45.2	
Source 15Q, pH 5.0	50.5/2.5	26.1/2.2	12.5/7.1	24.0/11.0
Hydroxyapatite (high salt)	56.5/2.1			

Specific activities towards PC/Triton X-100/SDS were determined according to "Experimental Procedures". The activity yield was related to the activity of the crude extract (10.6 μmol min^{-1} l^{-1}). The specific activity of the crude extract was 0.01 μmol min^{-1} mg^{-1}

Enzyme	Sequence	Mr_{exp}	Mr_{theo}	ppm
PLA_1-1	GRAPGETTVPDR	1254.6565	1254.6317	20
PLA_1-2	GRAPGETTVPDR	1254.6565	1254.6317	20
PLA_1-3	GRAPGETTVPDR	1254.6582	1254.6317	21
PLA_1-4	GRAPGETTVPDR	1254.6579	1254.6317	21
Aspfu2	260 DGRAPGEILVPANTTVFEFNPWEFGSW 286			
Canal1	249 DGRAPDTTIINLNSTVIELTPYEFGSW 275			
Neucr	286 DGRAPGDTIISLNATNYEFNPFETGSW 312			
Pench	255 DGRNPGELVIGSNSTVYEFNPWEFGTF 281			
Sacce1	265 DGRYPGTTVINLNATLFEFNPFEMGSW 291			
Cryne1	257 AEREAGELVIAENATVWEFTPYEFGSW 283			
PLA_1-1	LVAHSMGGLVVR	1254.6545	1253.6914	768
Bacli	378 LVAHSMGGLLVR 389			
Sacce2	140 LIAHSMGGLDCR 151			
Zeama	70 LISHSMGGLLVR 83			
PLA_1-3	EMNNIVMVGHSMGGLIT-R	1992.2298	1989.9434	1149
Baccl	82 IVLVGHSMGGLISIR 96			
Strsv	98 LVLVGHSMGGLIA 110			
PLA_1-4	TGPQETGIAGASLGGLISVYAGFQMPEK	2777.2452	2778.3898	412
Bacpu	121 LIGASLGGLVSMYA 134			

Fig. 3 Alignment of PLA_1 fragments with motifs of α/β-hydrolases. Sequences of peptides obtained by digestion of *A. ostoyae* PLA_1s with trypsin were identified by tandem mass spectrometry according to "Experimental Procedures". The deviation of the experimental (Mr_{exp}) from the theoretical masses (Mr_{theo}) is given in ppm. Underlined methionine residues were oxidized. PLA_1 fragments shown are homologous to conserved regions of fungal PLBs (*Aspfu2*, *Aspergillus fumigatus* PLB2; *Canal1*, *Candida albicans* PLB1; *Neucr*, *Neurospora crassa* PLB; *Pench*, *Penicillium chrysogenum* PLB; *Sacce1*, *Saccharomyces cerevisiae* PLB1; *Cryne1*, *Cryptococcus neoformans* var. *neoformans* PLB1) or catalytic motifs of bacterial esterases (Bacli, *Bacillus licheniformis* esterase/acyl transferase, GenBank ID: AAA79183.1; Baccl, *Bacillus clausii* lysophospholipase, GenBank ID: YP_176371.1; Strsv, *Streptomyces sviceus* lysophospholipase, GenBank ID: ZP_06921311.1; Bacpu, *Bacillus pumilus* esterase/lipase, GenBank ID: YP_001488906.1) and other serine hydrolases (Sacce2, *Saccharomyces cerevisiae* triglyceride lipase2, GenBank ID: CAY78566.1; Zeama, *Zea mays* PLA_1, GenBank ID: ACG31809.1). Dark grey: amino acid positions characteristic for serine hydrolase consensus cores; light gray: positions identical in the found sequences and in sequences from peptide libraries; framed: positions conserved in fungal PLBs (identical in ≥80 % of the selected sequences)

Influence of pH and Effectors on Enzyme Activities

The pH dependence of the activity towards DPPC in mixed micelles showed strong differences for the four PLA_1s (Fig. 4). PLA_1-1 is active in the range from pH 4.0 to 6.0 with a distinct optimum at pH 5.3, whereas the other enzymes were most active under less acidic or neutral conditions. In contrast to PLA_1-1, they exhibited optimum activity over a broad range of pH (PLA_1-2: pH 6.0–7.25; PLA_1-3: pH 5.3–6.0; PLA1-4: pH 5.0–6.0). Under the conditions used, the maximum activities of PLA_1-2, -3 and -4 were 51, 16 and 28 % compared to PLA_1-1, which had the highest activity of all the enzymes.

The enzymes did not show any dependence on metal ions, which was tested at pH 5.6 for $MgCl_2$ (0–40 mM), $CaCl_2$ (0–40 mM) and KCl (0–120 mM) and at pH 7.0 for $CaCl_2$ (0–40 mM). The enzymes were not inhibited by EGTA, demonstrating the absence of tightly bound metal ions. The PLA_1s also proved to be relatively insensitive to serine- or cysteine-modifying reagents. Thus, treatment

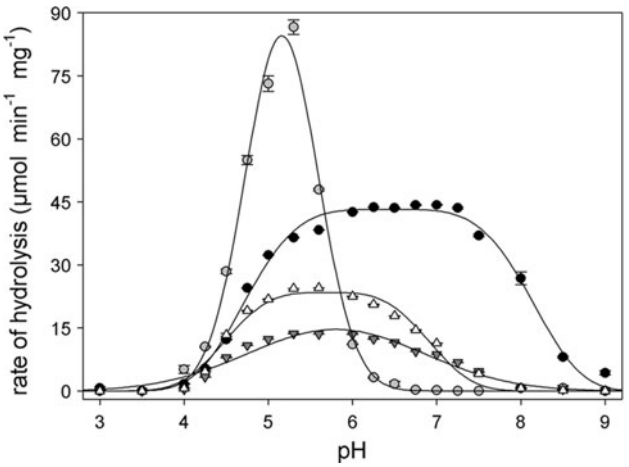

Fig. 4 pH-activity profiles of *A. ostoyae* PLA_1s. The hydrolysis of 5 mM DPPC in micelles containing 10 mM Triton X-100 by PLA_1-1 (*open circles*), PLA_1-2 (*filled circles*), PLA_1-3 (*filled inverted triangles*), and PLA_1-4 (*open triangles*) was assayed according to "Experimental Procedures"

with PMSF or iodoacetamide for 12 h resulted in only a small loss of activity (≥80 % residual activity). In contrast, 4-bromophenacyl bromide significantly inhibited PLA_1-1 (8 % residual activity) and, to a certain extent, also PLA_1-2 (55 % residual activity) but did not affect PLA_1-3 or PLA_1-4.

Regiospecificity in the Cleavage of Phospholipids

With regard to the regiospecificity of the purified *A. ostoyae* phospholipases, the hydrolytic rates for the diacylphospholipid POPC were compared with those for the structurally related 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine. In the presence of 5 mM phospholipid and 10 mM Triton X-100, POPC was readily hydrolyzed (122.6 ± 1.1, 61.5 ± 0.6, 18.9 ± 0.3, 23.1 ± 0.3 µmol min^{-1} mg^{-1} by PLA_1-1, PLA_1-2, PLA_1-3, PLA_1-4, respectively), whereas the lack of a cleavable *sn*-1 ester linkage in 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine [6, 30] prevented the hydrolytic degradation of this lipid. The rate of hydrolysis was ≤0.3 µmol min^{-1} mg^{-1} for all four enzymes. Moreover, the enzymes were shown to be unable to cleave the fluorogenic 1-palmitoyl-2(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine which is another typical PLA_2-specific substrate [26] (data not shown), indicating therefore that they act specifically as PLA_1s.

Substrate Selectivity Towards Different Classes of Lipids

In order to probe the substrate spectrum of the PLA_1s, compounds representing different classes of natural lipids were selected. Mono- and diacylglycerophospholipids (1-palmitoyl-*sn*-glycero-3-phosphocholine (LPPC) and DPPC), sphingolipid (sphingomyelin), mono-, di- and triacylglycerols (1-monoolein, 1,2-diolein, triolein) and fatty acid alkyl ester (methyl oleate) were applied in the presence of Triton X-100. The glycerophospholipids LPPC and DPPC were hydrolyzed by all four enzymes. Surprisingly, however, a remarkable hydrolytic degradation of 1-monoolein, 1,2-diolein and methyl oleate was also observed for PLA_1-2, -3 and -4. However, no significant reaction rate (≤0.3 µmol min^{-1} mg^{-1}) was detectable with triolein or the fatty acid amide in sphingomyelin. The missing conversion of the triolein by all of the enzymes is in accordance with our findings that the crude enzyme extracts from *A. ostoyae* do not show any lipase activity toward triglycerides in the absence of detergents as well as in the presence of Triton X-100 or taurocholate [14].

For a detailed comparison, kinetic studies with the cleavable lipids were performed (Fig. 5). With only few exceptions, the reaction rates as a function of the substrate concentration were characterized by sigmoid kinetics, which could be fitted by the Hill function. The resulting kinetic parameters are shown in Table 2. PLA_1-1 is restricted to the conversion of glycerophospholipids and exhibited only very low activity towards the acylglycerols and methyl oleate even at high substrate concentrations. Under conditions of substrate saturation, the hydrolysis rate of LPPC exceeded that of DPPC by a factor of 3.8 while the $S_{0.5}$ value for LPPC was nearly tenfold higher. For the other enzymes a similar preference for the monoacyl phospholipid LPPC at substrate saturation compared to the diacyl compound DPPC was observed; however, the $S_{0.5}$ values were in the same range. Based on the Hill coefficients, the hydrolysis reaction showed positive cooperativity in the case of DPPC ($h = 1.7$–2.1) but not in the case of LPPC ($h \leq 1.0$).

The enzymes PLA_1-2, PLA_1-3, and PLA_1-4 showed a similar trend in the conversion of neutral lipids. Compared to 1,2-diolein, the activity towards 1-monoolein was strongly favored. Moreover, the kinetics also showed a higher degree of positive cooperativity for the diacyl compound ($h = 2.2$–3.2) than for the monoglyceride ($h = 1.4$–2.0). The $S_{0.5}$ values for 1,2-diolein were in the millimolar range (≥2.2 mM) and higher by a factor of 5–8 than for 1-monoolein (Table 2).

Interestingly, the simple fatty acid ester methyl oleate was also degraded by PLA_1-2, PLA_1-3, and PLA_1-4. The maximal hydrolytic rates, however, were at least threefold lower compared to both monoacyl glycerolipids (LPPC and 1-monoolein) (Fig. 5). Moreover, higher $S_{0.5}$ values (>1.2 mM) and a decrease in cooperativity ($h = 1.0$–1.2) were observed (Table 2). 1-Monoolein and DPPC showed a substrate inhibition at high concentrations (>5 mM) (Fig. 5).

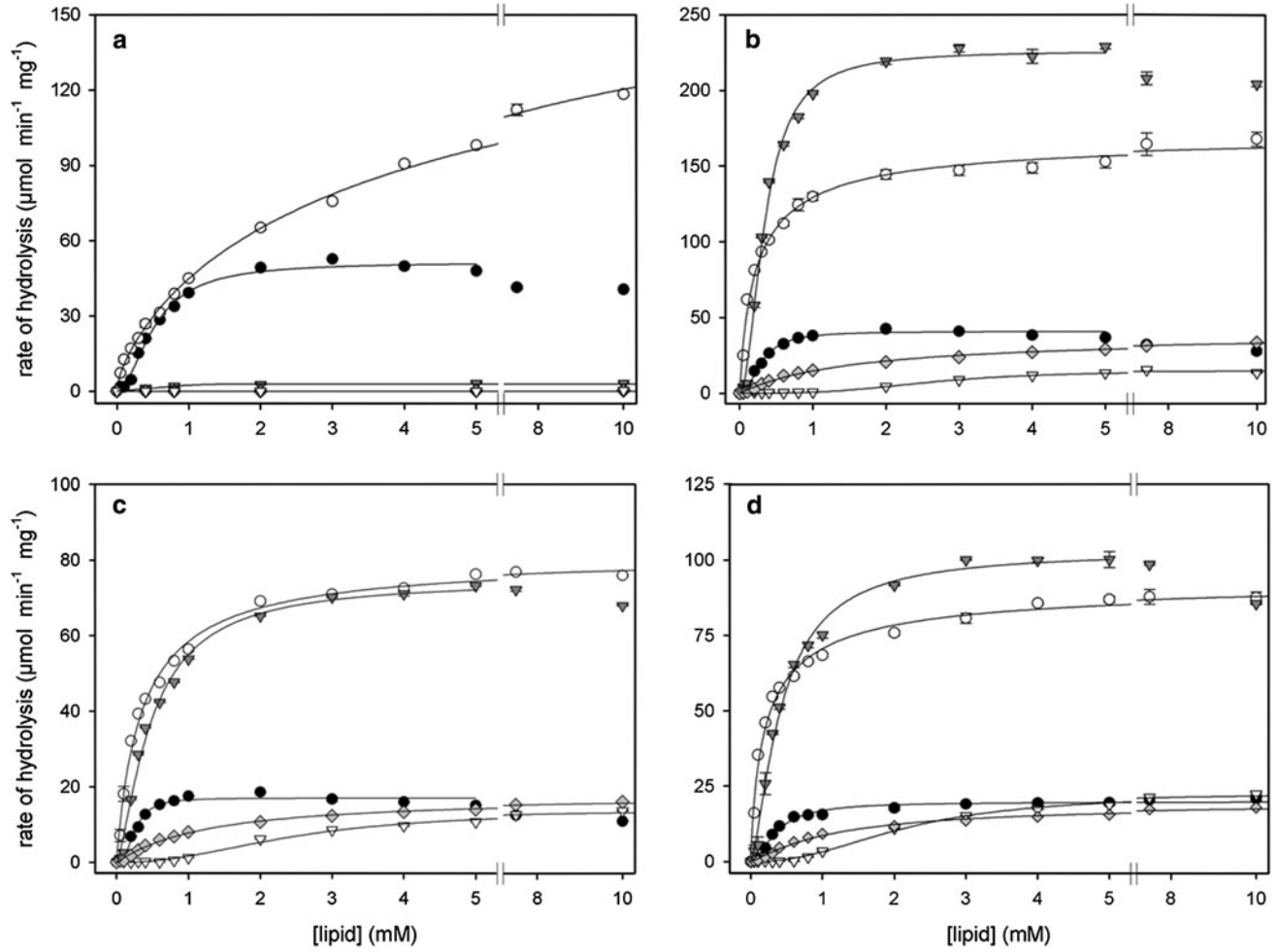

Fig. 5 Rates of hydrolysis of different lipids catalyzed by PLA_1-1 (**a**), PLA_1-2 (**b**), PLA_1-3 (**c**) or PLA_1-4 (**d**) as a function of the substrate concentration. The hydrolysis rates of LPPC (*open circles*), DPPC (*filled circles*), 1-monoolein (*filled inverted triangles*), 1,2-diolein (*open inverted triangles*) or methyl oleate (*filled diamonds*) were determined as described in "Experimental Procedures". Curves were fitted according to the Hill model not considering data points in the range of substrate inhibition

Substrate Selectivity in Phospholipid Hydrolysis

The hydrolytic rates of the fungal PLA_1s were compared for a series of diacylphospholipids differing in molecular properties such as in the length and saturation of the hydrophobic acyl moieties, as well as in the type of the polar head group. Kinetic analyses of the conversion of short-chain (1,2-dioctanoyl-*sn*-glycero-3-phosphocholine) and unsaturated (DOPC) phosphatidylcholines confirmed that the reactions were performed at substrate saturation (data not shown). Figure 6a demonstrates the strong dependence of the PLA_1 reaction rates on the chain length of the fatty acid moieties in the 1,2-diacyl-*sn*-glycero-3-phosphocholines. Hydrolysis proceeded at least two orders of magnitudes faster for all four enzymes with the short-chain 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine than with the corresponding diarachidoyl compound.

The influence of the degree of fatty acid unsaturation in long-chained phosphatidylcholines is demonstrated in Fig. 6b. Compared to the hydrolysis of the rigid DSPC, the hydrolysis of DOPC was accelerated by a factor of ≥4.9. Interestingly, even slightly higher reaction rates were obtained for POPC having one oleoyl moiety in the *sn*-2 position only. Otherwise, phospholipids carrying two polyunsaturated fatty acids such as the natural soya-PC, which mainly contains linoleic and linolenic acid [31], were degraded with the same rate as POPC.

As shown in the previous section, the presence of a phosphoalcoholic head group in the substrate was crucial for PLA_1-1 but not for the other enzymes. Nevertheless, the phosphocholine moiety strongly influenced the kinetic constants and cooperativity in PLA_1-2, -3 and -4 as concluded from a comparison of DOPC and 1,2-diolein (Table 2). Accordingly, the kind of head group also proved

Table 2 Kinetic parameters for lipid hydrolysis catalyzed by the PLA_1 enzymes

Substrate	Enzyme	$S_{0.5}$ (mM)	V_{max} (μmol min^{-1} mg^{-1})	*h*
LPPC	PLA_1-1	5.11 ± 1.09	194.2 ± 14.6	0.7 ± 0.1
	PLA_1-2	0.24 ± 0.02	169.1 ± 4.8	0.8 ± 0.1
	PLA_1-3	0.30 ± 0.03	80.0 ± 1.8	1.0 ± 0.1
	PLA_1-4	0.22 ± 0.02	92.4 ± 2.7	0.8 ± 0.1
DPPC	PLA_1-1	0.52 ± 0.03	51.4 ± 1.4	1.9 ± 0.2
	PLA_1-2	0.32 ± 0.02	43.0 ± 1.5	1.9 ± 0.2
	PLA_1-3	0.26 ± 0.02	17.0 ± 0.5	2.1 ± 0.4
	PLA_1-4	0.36 ± 0.02	19.8 ± 0.5	1.7 ± 0.2
1-Monoolein	PLA_1-1	b.d.	b.d.	b.d.
	PLA_1-2	0.34 ± 0.01	226.2 ± 3.6	2.0 ± 0.1
	PLA_1-3	0.48 ± 0.03	74.8 ± 2.2	1.4 ± 0.1
	PLA_1-4	0.43 ± 0.02	103.0 ± 2.5	1.5 ± 0.1
1,2-Diolein	PLA_1-1	b.d.	b.d.	b.d.
	PLA_1-2	2.63 ± 0.12	14.8 ± 0.5	3.2 ± 0.4
	PLA_1-3	2.47 ± 0.20	13.7 ± 0.5	2.2 ± 0.2
	PLA_1-4	2.17 ± 0.09	22.4 ± 0.6	2.3 ± 0.2
Methyl oleate	PLA_1-1	n.d.	3.2 ± 0.3	n.d
	PLA_1-2	1.57 ± 0.20	38.0 ± 1.8	1.0 ± 0.1
	PLA_1-3	1.21 ± 0.13	17.3 ± 0.6	1.1 ± 0.1
	PLA_1-4	1.16 ± 0.10	18.9 ± 0.8	1.2 ± 0.1
DOPC	PLA_1-1	0.73 ± 0.05	102.5 ± 2.5	1.4 ± 0.1
	PLA_1-2	0.52 ± 0.04	51.1 ± 1.3	1.4 ± 0.1
	PLA_1-3	0.47 ± 0.02	15.8 ± 0.3	1.6 ± 0.2
	PLA_1-4	0.57 ± 0.04	21.1 ± 0.5	1.3 ± 0.1

Parameters were obtained as described in "Experimental Procedures"

b.d. below detection limit, *n.d.* not determined

to be important for phospholipid turnover. All phospholipid compounds analyzed were degraded by all four enzymes (Fig. 6c). Throughout, the zwitterionic phospholipids PC and PE were preferred to the anionic species, with PC as the more favored substrate. For the anionic lipids, the order in the rates of hydrolysis was PG > phosphatidylinositol (PI) > phosphatidylserine (PS) > PA for PLA_1-1, PG > PA > PI > PS for PLA_1-2, PA > PG = PI > PS for PLA_1-3 and PG > PI > PA > PS for PLA_1-4.

Cleavage of Monoacyl Substrates

In the hydrolysis of lysophosphatidylcholines, the dependence of PLA_1 activity on the chain length as well as the saturation of the acyl moiety (Fig. 7a) significantly differed from that of the corresponding diacyl phospholipids (Fig. 6a, b). The activity was optimal for lysophosphatidylcholine with 10 (PLA_1-2, PLA_1-3, PLA_1-4) or 12 (PLA_1-1) carbon atoms. The influence of unsaturation, which was probed for 1-stearoyl-*sn*-glycero-3-phosphocholine and its unsaturated oleoyl analogue, was minimal.

Comparable results were obtained for fatty acid methyl esters, which were significantly cleaved by PLA_1-2, PLA_1-3, and PLA_1-4 only (Fig. 7b). However, the PLA_1s exhibited a less distinct selectivity for the chain length of the fatty acids. In accordance with the results obtained for lysophosphatidylcholines, methyl oleate as an unsaturated ester was not preferred to the corresponding saturated compound methyl stearate. However, activities of all enzymes were marginal (0.001–0.196 μmol min^{-1} mg^{-1}) towards the very short-chain 4-nitrophenyl acetate, which is a typical esterase substrate.

Discussion

PLA_1 Enzymes from *A. ostoyae* are New Representatives of the α/β-Hydrolase Fold Enzymes

To the best of our knowledge this study presents the first report on the isolation and characterization of PLA_1s from fungal fruiting bodies. Chromatographic methods, such as hydrophobic interaction, anion exchange and hydroxyapatite chromatography, which are commonly used in the isolation of fungal or mammalian PLB-type enzymes [32–34], were also successfully employed in this study (Fig. 1, Table 1) and yielded four different enzymes as homogeneous proteins (Fig. 2). Due to their activity toward POPC

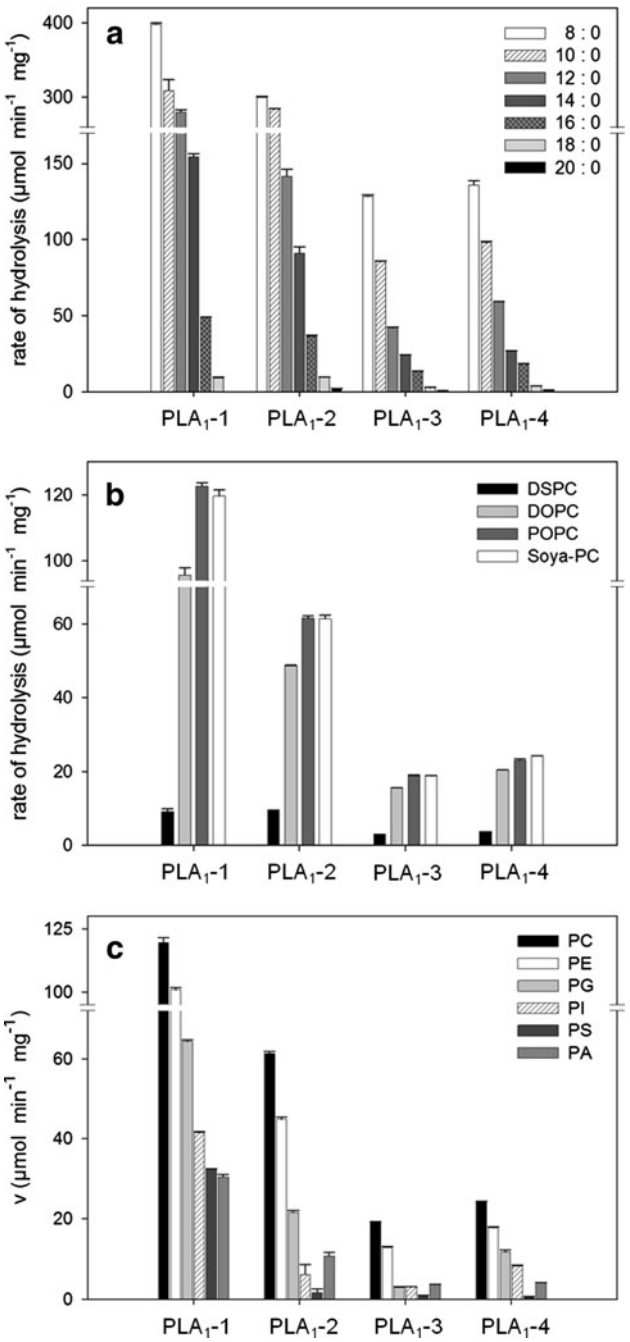

Fig. 6 Selectivity in phospholipid hydrolysis by the *A. ostoyae* PLA_1s. The hydrolysis rates of phosphatidylcholines with fatty acid moieties of varying chain length (**a**), long-chain phosphatidylcholines with different saturation (**b**) and phospholipids from soybean with different head groups (**c**) were determined at conditions of substrate saturation (5 mM phospholipid/10 mM Triton X-100) as described in "Experimental Procedures"

but inactivity towards 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine, the enzymes were attributed to the PLA_1 family of phospholipases.

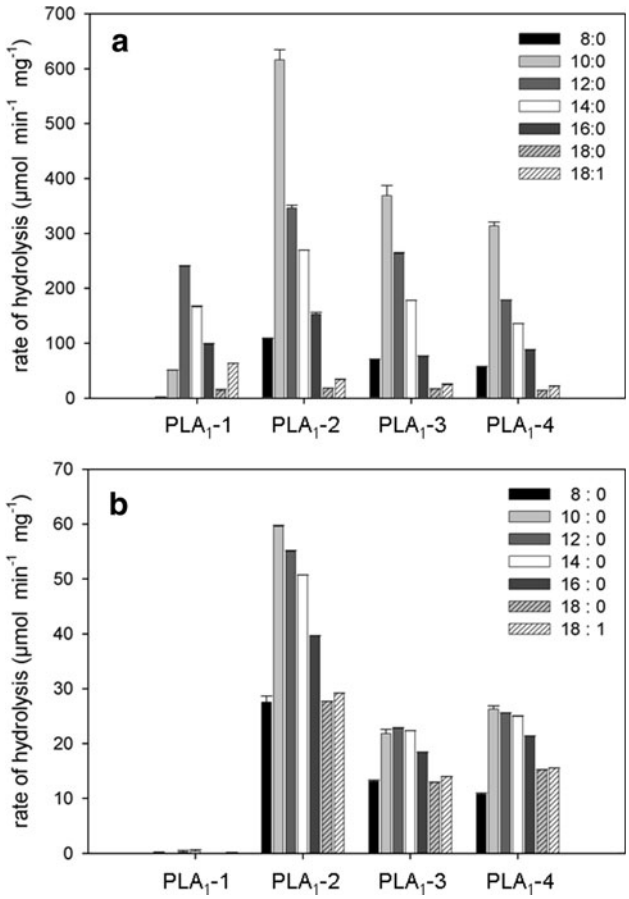

Fig. 7 Selectivity in the hydrolysis of monoacyl substrates by the *A. ostoyae* PLA_1s. The hydrolysis rates of 2-lysophospholipids (**a**) and fatty acid methyl esters (**b**) of varying chain length and different saturation were determined at conditions of substrate saturation (5 mM monoacyl substrate/10 mM Triton X-100) as described in "Experimental Procedures"

Hitherto, the most abundant type of phospholipid-hydrolyzing enzymes described in fungi is PLB, which belongs to the protein family of α/β-hydrolase fold enzymes [27]. For example, the production of multiple PLB enzymes was reported for *Candida albicans* [35] and *Saccharomyces cerevisiae* [36]. Apart from the dominant group of PLBs, there are only a few reported examples of phospholipid-specific enzymes from fungi, such as the lipase-related PLA_1s from *Mucor javanicus* [37] and *Aspergillus oryzae* [38], which also belong to the structural family of α/β-hydrolases. The enzymes presented in this paper (PLA_1-1, PLA_1-2, PLA_1-3 and PLA_1-4) share several features with both types of fungal α/β-hydrolase fold enzymes, such as their low pI values [38–40] and the N-linked glycosylation [40–42] found for PLA_1-2, -3 and -4. In particular, PLA_1-1 resembles the PLBs from *Saccharomyces cerevisiae* [43] or cryptococci [44] due to its acidic pH optimum (Fig. 4) and independence of metal ions. In contrast, the other PLA_1s (especially PLA_1-2)

exhibit a broad-range activity from slightly acidic to neutral pH (Fig. 4) and seem, therefore, to be more similar to PLA_1 from *Aspergillus oryzae* [38].

The similarity between the *A. ostoyae* PLA_1s and fungal PLBs was additionally confirmed by the identification of proteolytic peptide fragments with a high degree of similarity to an amino acid sequence, which is unique to the latter group of enzymes (Fig. 3). Otherwise, PLA_1-1, PLA_1-3 and PLA_1-4 clearly differ from the conventional PLBs by a sequence that it is highly similar to the catalytic motifs of microbial and plant PLA_1s and esterases (Fig. 3). In fungal phospholipid-cleaving enzymes, a related motif is only reported for the uncommon PLB from the fungus *Anthrodia cinnamomea* [45] and the PLA_1 from *Aspergillus oryzae* [38]. Comparable to these two enzymes, the molecular masses of the *A. ostoyae* PLA_1s are much lower (33–47 kDa) than those of common fungal PLBs, which are in the range of 63–95 kDa [40–44, 46–48]. Also the insensitivity towards serine- and cysteine-modifying reagents is uncommon for mammalian and fungal PLBs [49, 50]. Instead, the inhibition of PLA_1-1 and -2 by 4-bromophenacyl bromide points to the presence of a catalytically important histidine residue, which has also been shown to be crucial for the catalytic activity of some other phospholipases [51, 52]. In combination with their strong preference for the *sn*-1 position, the *Armillaria* enzymes are more similar to lipase-related PLA_1s than to conventional PLBs.

The Substrate Selectivity of the PLA_1 Enzymes from *A. ostoyae* Reflects Steric Restraints and Head Group Recognition

The results described on *A. ostoyae* PLA_1s allow us to draw conclusions on the relationship between enzyme activity and the molecular details of the substrate structure. The conversion rates of the structurally diverse lipid substrates (Table 2) can be explained by a combined recognition of the hydrophobic and hydrophilic regions in the respective substrate molecule.

The hydrolysis of different lysophospholipids and fatty acid methyl esters (Fig. 7a, b) provides information about the role of the binding site of the scissile acyl moiety. Clearly, this site strongly favors hydrophobic medium chain acyl substrates. Likewise, the non-acceptance of the very short chain ester 4-nitrophenyl acetate seems to be caused by this chain length selectivity, although its turnover may also be prevented by the non-natural secondary alcohol moiety. On the other hand, the state of saturation of acyl chains only marginally influenced the substrate conversion. For the docking of a second acyl moiety in glycerol-based lipids to the corresponding *sn*-2 site, its molecular properties are even more significant. Compared to the monoacyl substrates, the presence of the additional acyl residues in diacyl compounds (phosphatidylcholines as well as diglycerides) strongly reduces the enzyme activity. In phosphatidylcholines, this effect is alleviated by a decrease in chain-length (Fig. 6a) or an increased flexibility due to an introduction of double bounds in the *sn*-2 positioned acyl moiety (Fig. 6b). Therefore, the *sn*-2 pocket seems to play a key role in the regulation of substrate acceptance. Like the *A. ostoyae* enzymes, other α/β-hydrolase fold enzymes favor lysophosphatidylcholines over their diacyl derivatives [39, 53] or phospholipids with unsaturated fatty acids over the saturated analogues [54].

Besides the fatty acid moieties, the other molecular constituents of the phospholipids also proved to be important for the substrate turnover. The preference for monoglycerides and lysophospholipids to the corresponding fatty acid methyl ester (Fig. 5; Table 2) indicates the recognition of the glycerol backbone. Moreover, the head group of phospholipids is essential for the hydrolysis by PLA_1-1. The other enzymes do not need this charged group (Fig. 5; Table 2) but also show an effect of the kind of head group on PLA_1 activity (Fig. 6c). This effect may arise from a decreased interaction of the acidic PLA_1 enzymes with negatively charged lipid aggregates due to electrostatic repulsion. However, together with the non-acceptance of triglycerides as substrates, the presence of a small, hydrophilic and charge-sensitive *sn*-3 binding pocket is suggested for all four PLA_1s. A similar influence of the phospholipid head group on activity was described for yeast [43] and mammalian lysophospholipases [55].

Another mode as to how the substrate structure influences the catalytic efficiency is reflected by the cooperativity of substrate binding as expressed in the Hill coefficients (Table 2). This cooperative behavior might be caused by regulatory substrate binding sites, as has been discussed for several monomeric PLA_2s [56, 57] or the formation of enzyme oligomers as described for many fungal phospholipases [40, 44].

In conclusion, the PLA_1s seem to combine two different mechanisms of substrate recognition. Firstly, size-control mechanisms similar to that which determines the specificity for fatty acid chain length in lipases [58] or esterases [59] clearly generate (I) the selectivity of the *sn*-1 site and (II) the discrimination of substrates bearing bulky acyl moieties in the *sn*-2 position. Secondly, the dependence of PLA_1 activity on the phospholipid head group resembles the mechanism of the lipase-related PLA_1 from *Streptococcus hyicus*, which favors phospholipid turnover due to a small and charged *sn*-3 binding cavity [60].

Acknowledgments We thank the LIPOID GmbH (Ludwigshafen, Germany) for the gift of phospholipids, Christa Kuplens and Katja Fröhlich for their excellent technical assistance, Gary Sawers for reading the manuscript and Cornelia Dippe, Frank Dippe and Emil Hinze for their help in collecting fungal fruiting bodies. The financial

support by the State of Sachsen-Anhalt and by the Graduiertenkolleg 1026 (Deutsche Forschungsgemeinschaft, Bonn, Germany) is gratefully acknowledged.

References

1. Miled N, Canaan S, Dupuis L, Roussel A, Riviere M, Carriere F, de Caro A, Cambillau C, Verger R (2000) Digestive lipases: from three-dimensional structure to physiology. Biochimie 82:973–986
2. Gosh M, Tucker DE, Burchett SA, Leslie CC (2006) Properties of the group IV phospholipase A_2 family. Prog Lipid Res 45: 487–510
3. Bornscheuer UT, Kazlauskas RJ (2005) Hydrolases in organic synthesis: regio- and stereoselective biotransformations, 2nd edn. Wiley-VCH, Weinheim
4. Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, Franken SM, Harel M, Remington SJ, Silman I, Schrag J (1992) The α/β hydrolase fold. Protein Eng 5:197–211
5. Burke JE, Dennis EA (2009) Phospholipase A_2 structure/function, mechanism, and signaling. J Lipid Res 50(Suppl):S237–S242
6. Chen S, Subbaiah PV (2007) Phospholipid and fatty acid specificity of endothelial lipase: potential role of the enzyme in the delivery of docosahexaenoic acid (DHA) to tissues. Biochim Biophys Acta 1771:1319–1328
7. Fauvel J, Chap H, Roques V, Douste-Blazy L (1984) Substrate specificity of two cationic lipases with high phospholipase A_1 activity purified from guinea pig pancreas. II. Studies on glycerophospholipids. Biochim Biophys Acta 792:72–78
8. Fauvel J, Chap H, Roques V, Sarda L, Douste-Blazy L (1984) Substrate specificity of two cationic lipases with high phospholipase A_1 activity purified from guinea pig pancreas. I. Studies on neutral glycerides. Biochim Biophys Acta 792:65–71
9. Withers-Martinez C, Carrière F, Verger R, Bourgeois D, Cambillau C (1996) A pancreatic lipase with a phospholipase A_1 activity: crystal structure of a chimeric pancreatic lipase-related protein 2 from guinea pig. Structure 4:1363–1374
10. Martín J, Pérez-Gil J, Acebal C, Arche R (1990) Theoretical approach to the steady-state kinetics of a bi-substrate acyltransfer enzyme reaction that follows a hydrolysable-acyl-enzyme-based mechanism. Application to the study of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung. Biochem J 266:47–53
11. Eggert T, Pencreach G, Douchet I, Verger R, Jaeger KE (2000) A novel extracellular esterase from *Bacillus subtilis* and its conversion to a monoacylglycerol hydrolase. Eur J Biochem 267:6459–6469
12. van Kampen MD, Verheij HM, Egmond MR (1999) Modifying the substrate specificity of staphylococcal lipases. Biochemistry 38:9524–9532
13. Carrière F, Withers-Martinez C, van Tilbeurgh H, Roussel A, Cambillau C, Verger R (1998) Structural basis for the substrate selectivity of pancreatic lipases and some related proteins. Biochim Biophys Acta 1376:417–432
14. Dippe M, Ulbrich-Hofmann R (2011) Phospholipid acylhydrolases trigger membrane degradation during fungal sporogenesis. Fungal Genet Biol 48:921–927
15. Hoffmann GE, Schmidt D, Bastian E, Guder WG (1986) Photometric determination of phospholipase A. J Clin Chem Clin Biochem 24:871–875
16. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
17. Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685
18. Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99
19. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Rhighetti PG (2004) Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25:1327–1333
20. Hames BD, Rickwood D (1990) Gel electrophoresis of proteins: a practical approach, 2nd edn. Oxford University Press, New York
21. Schulz DM, Kalkhof S, Schmidt A, Ihling C, Stingl C, Mechtler K, Zschörnig O, Sinz A (2007) Annexin A2/P11 interaction: new insights into annexin A2 tetramer structure by chemical cross-linking, high-resolution mass spectrometry, and computational modeling. Proteins 69:254–269
22. Müller MQ, Dreiocker F, Ihling CH, Schäfer M, Sinz A (2010) Cleavable cross-linker for protein structure analysis: reliable identification of cross-linking products by Tandem MS. Anal Chem 82:6958–6968
23. Matrix Science. Mascot MS/MS Ion Search. http://www.matrixscience.com. Accessed Nov 2010
24. Kourist R, Jochens H, Bartsch S, Kuipers R, Padhi SK, Gall M, Böttcher D, Joosten HJ, Bornscheuer UT (2010) The α/β-hydrolase fold 3DM database (ABHDB) as a tool for protein engineering. ChemBioChem 11:1635–1643
25. National Center for Biotechnology Information. BLAST. http://www.ncbi.nlm.nih.gov. Accessed Nov 2010
26. Radvanyi F, Jordan L, Russo-Marie F, Bon C (1989) A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. Anal Biochem 177:103–109
27. Köhler GA, Brenot A, Haas-Stapleton E, Agabian N, Deva R, Nigam S (2006) Phospholipase A_2 and phospholipase B activities in fungi. Biochim Biophys Acta 1761:1391–1399
28. Tarentino AL, Gómez CM, Plummer TH Jr (1985) Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. Biochemistry 24:4665–4671
29. Dartois V, Baulard A, Schanck K, Colson C (1992) Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis* 168. Biochim Biophys Acta 1131:253–260
30. El Tamer A, Record M, Fauvel J, Chap H, Douste-Blazy L (1984) Studies on ether phospholipids. I. A new method of determination using phospholipase A_1 from guinea pig pancreas: application to Krebs II ascites cells. Biochim Biophys Acta 793:213–220
31. Schneider M (2008) Major sources, composition and processing. In: Gunstone FD (ed) Phospholipid technology and applications. The Oily Press, Bridgwater, pp 21–40
32. Oishi H, Morimoto T, Watanabe Y, Tamai Y (1999) Purification and characterization of phospholipase B from *Kluyveromyces lactis*, and cloning of phospholipase B gene. Biosci Biotechnol Biochem 63:83–90
33. Fujino S, Akiyama D, Akaboshi S, Fujita T, Watanabe Y, Tamai Y (2006) Purification and characterization of phospholipase B from *Candida utilis*. Biosci Biotechnol Biochem 70:377–386
34. Xu S, Zhao L, Larsson A, Venge P (2009) The identification of a phospholipase B precursor in human neutrophils. FEBS J 276:175–186
35. Sugiyama Y, Nakashima S, Mirbod F, Kanoh H, Kitajima Y, Ghannoum MA, Nozawa Y (1999) Molecular cloning of a second phospholipase B gene, caPLB2 from *Candida albicans*. Med Mycol 37:61–67
36. Witt M, Schweingruber ME, Mertsching A (1984) Phospholipase B from the plasma membrane of *Saccharomyces cerevisiae*.

Separation of two forms with different carbohydrate content. Biochim Biophys Acta 795:108–116
37. Ishihara H, Okuyama H, Ikezawa H, Tejima S (1975) Studies on lipase from *Mucor javanicus*. I. Purification and properties. Biochim Biophys Acta 388:413–422
38. Watanabe I, Koishi R, Yao Y, Tsuji T, Serizawa N (1999) Molecular cloning and expression of the gene encoding a phospholipase A_1 from *Aspergillus oryzae*. Biosci Biotechnol Biochem 63:820–826
39. Ichimasa M, Morooka T, Niimura T (1984) Purification and properties of a membrane-bound phospholipase B from baker's yeast (*Saccharomyces cerevisiae*). J Biochem 95:137–145
40. Wright LC, Payne J, Santangelo RT, Simpanya MF, Chen SC, Widmer F, Sorrell TC (2004) Cryptococcal phospholipases: a novel lysophospholipase discovered in the pathogenic fungus *Cryptococcus gattii*. Biochem J 384:377–384
41. Turner KM, Wright LC, Sorrell TC, Djordjevic JT (2006) N-linked glycosylation sites affect secretion of cryptococcal phospholipase B1, irrespective of glycosylphosphatidylinositol anchoring. Biochim Biophys Acta 1760:1569–1579
42. Masuda N, Kitamura N, Saito K (1991) Primary structure of protein moiety of *Penicillium notatum* phospholipase B deduced from the cDNA. Eur J Biochem 202:783–787
43. Merkel O, Fido M, Mayr JA, Prüger H, Raab F, Zandonella G, Kohlwein SD, Paltauf F (1999) Characterization and function in vivo of two novel phospholipases B/lysophospholipases from *Saccharomyces cerevisiae*. J Biol Chem 274:28121–28127
44. Chen SC, Wright LC, Golding JC, Sorrell TC (2000) Purification and characterization of secretory phospholipase B, lysophospholipase and lysophospholipase/transacylase from a virulent strain of the pathogenic fungus *Cryptococcus neoformans*. Biochem J 347:431–439
45. Hsu KH, Wang SY, Chu FH, Shaw JF (2010) Characterization and heterologous expression of a novel lysophospholipase gene from *Antrodia cinnamomea*. J Appl Microbiol 108:1712–1722
46. Mirbod F, Banno Y, Ghannoum MA, Ibrahim AS, Nakashima S, Kitajima Y, Cole GT, Nozawa Y (1995) Purification and characterization of lysophospholipase-transacylase (h-LPTA) from a highly virulent strain of *Candida albicans*. Biochim Biophys Acta 1257:181–188
47. Shen DK, Noodeh AD, Kazemi A, Grillot R, Robson G, Brugère JF (2004) Characterisation and expression of phospholipases B from the opportunistic fungus *Aspergillus fumigatus*. FEMS Microbiol Lett 239:87–93
48. Watanabe Y, Yashiki Y, Sultana GN, Maruyama M, Kangawa K, Tamai Y (1994) Cloning and sequencing of phospholipase B gene from the yeast *Torulaspora delbrueckii*. FEMS Microbiol Lett 124:29–34
49. Hou WY, Long DX, Wu YJ (2009) The homeostasis of phosphatidylcholine and lysophosphatidylcholine in nervous tissues of mice was not disrupted after administration of tri-o-cresyl phosphate. Toxicol Sci 109:276–285
50. Santangelo RT, Nouri-Sorkhabi MH, Sorrell TC, Cagney M, Chen SC, Kuchel PW, Wright LC (1999) Biochemical and functional characterisation of secreted phospholipase activities from *Cryptococcus neoformans* in their naturally occurring state. J Med Microbiol 48:731–740
51. Roberts MF, Deems RA, Mincey TC, Dennis EA (1977) Chemical modification of the histidine residue in phospholipase A_2 (*Naja naja naja*). A case of half-site reactivity. J Biol Chem 252:2405–2411
52. Brok RG, Dekker N, Gerrits N, Verheij HM, Tommassen JA (1995) A conserved histidine residue of *Escherichia coli* outermembrane phospholipase A is important for activity. Eur J Biochem 234:934–938
53. Kuwabara Y, Maruyama M, Watanabe Y, Tanaka S, Takakuwa M, Tamai Y (1988) Purification and some properties of membrane-bound phospholipase B from *Torulaspora delbrueckii*. J Biochem 104:236–241
54. Jackson RL, Demel RA (1985) Lipoprotein lipase-catalyzed hydrolysis of phospholipid monolayers: effect of fatty acyl composition on enzyme activity. Biochem Biophys Res Commun 128:670–675
55. Toyoda T, Sugimoto H, Yamashita S (1999) Sequence, expression in *Escherichia coli*, and characterization of lysophospholipase II. Biochim Biophys Acta 1437:182–193
56. Mansfeld J, Brandt W, Haftendorn R, Schöps R, Ulbrich-Hofmann R (2011) Discrimination between the regioisomeric 1,2- and 1,3-diacylglycerophosphocholines by phospholipases. Chem Phys Lipids 164:196–204
57. Boegeman SC, Deems RA, Dennis EA (2004) Phospholipid binding and the activation of group IA secreted phospholipase A_2. Biochemistry 43:3907–3916
58. Pleiss J, Fischer M, Schmid RD (1998) Anatomy of lipase binding sites: the scissile fatty acid binding site. Chem Phys Lipids 93:67–80
59. Hasenpusch D, Bornscheuer UT, Langel W (2011) Simulation of the structure of pig liver esterase. J Mol Model 17:1493–1506
60. Tiesinga JJ, van Pouderoyen G, Nardini M, Ransac S, Dijkstra BW (2007) Structural basis of phospholipase activity of *Staphylococcus hyicus* lipase. J Mol Biol 371:447–456