Purification and characterization of secretory phospholipase B, lysophospholipase and lysophospholipase/transacylase from a virulent strain of the pathogenic fungus *Cryptococcus neoformans*

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Infection caused by the fungus *Cryptococcus neoformans* is potentially fatal. A highly active extracellular phospholipase, demonstrating phospholipase B (PLB), lysophospholipase (LPL) and lysophospholipase/transacylase (LPTA) activities, was purified to homogeneity from *C. neoformans* using (NH₄)₂SO₄ fractionation, and hydrophobic-interaction, anion-exchange and gel-filtration chromatography. All three enzyme activities co-purified as a single protein with an apparent molecular mass of 70–90 kDa by SDS/PAGE and 160–180 kDa by gel filtration. The ratio of the three activities remained constant after each purification step. The amino acid composition, as well as the sequences of the N-terminus and of five internal peptide fragments were novel. The protein was an acidic glycoprotein containing N-linked carbohydrate moieties, with pI values of 5.5 and 3.5. The apparent $V_{\max}$ values for PLB and LPL activities were 12.3 and 870 $\mu\text{mol}/\text{min}$ per mg of protein respectively; the corresponding

K_m values were approx. 185.3 and 92.2 μM . The enzyme was active only at acidic pH (pH optimum of 4.0 for PLB and 4.0–5.0 for LPL and LPTA). Enzyme activity did not require added cations, but was inhibited by Fe³⁺. LPL and LPTA activities were decreased by 0.1% (v/v) Triton X-100 to 50% of the control value. Palmitoylcarnitine (0.5 mM) inhibited PLB (97% inhibition) and LPL and LPTA activities (35% inhibition) competitively. All phospholipids except phosphatidic acid were degraded by PLB, but dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylcholine were the preferred substrates. This is the first complete description of the purification and properties of a phospholipase, which may be involved in virulence, from a pathogenic fungus.

Key words: extracellular phospholipase, cryptococci, yeast.

INTRODUCTION

Cryptococcus neoformans is an important fungal pathogen which most commonly affects the central nervous system. The majority of infections in humans are caused by *C. neoformans* var. *neoformans* serotype A. Although patients with cell-mediated immunodeficiency are at particular risk, significant neurological and pulmonary morbidity also occur in immunocompetent individuals [1,2].

It is generally accepted that *C. neoformans* enters the host by the respiratory route and then disseminates to other body site(s) [1]. Several virulence determinants and their corresponding genes have been described [1]. It is likely, however, that the virulence of a given isolate is dependent on the expression of a critical number of factors working in concert [3]. The ability to grow at 37 °C, produce a polysaccharide capsule and express laccase activity are essential for survival of *C. neoformans* in the host [1,4,5]. Potential tissue-invasive properties such as the production of proteinases and adhesion factors have also been identified [6,7], and secretion of the metabolic product, mannitol, has been reported to correlate with increased pathogenicity [8].

Secretory phospholipases, well-known for their destructive effects on biological tissue, have been implicated in the pathogenesis of bacterial infections [9,10]. Studies of fungal phos-

pholipases, however, have mainly focused on the characterization of phospholipase B (PLB; EC 3.1.1.5) from non-pathogenic fungi, including *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Schizosaccharomyces pombe* and *Penicillium notatum* [11–16]. In some instances, lysophospholipase (LPL; EC 3.1.1.5) and lysophospholipase/transacylase (LPTA; EC 3.1.1.5) activities have also been described [11,13,16], but the biological functions of these phospholipases remain undetermined. Furthermore, the relative activities of PLB, LPL and LPTA vary between different fungal genera. The terms PLB, LPL and/or LPTA have been used interchangeably [13,16], making attempts at comparison of enzyme properties difficult. Saito et al. proposed that LPL is an artifact produced by proteolysis of PLB, since PLB activity was lost almost completely, with the preservation of LPL activity, during purification of phospholipases from *P. notatum* [11]. In most studies, cell-associated or membrane-bound forms of PLB have been characterized. If PLB were to have a role as a virulence factor, it is more likely that it would be a secreted product.

Of the medically important fungi, extracellular phospholipase activities have been described in *Candida albicans* and *Aspergillus fumigatus*, but characterized only in the former [17,18]. In *C. albicans*, LPL, LPTA and PLB activities were identified in crude culture filtrates of the organism, but only enzymes with LPTA

Abbreviations used: ANGIS, Australian National Genomic Information Service; C₁₈-PAF, platelet-activating factor; DOPC, dioleoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DOPS, dioleoyl phosphatidylserine; DPPC, dipalmitoyl phosphatidylcholine; DTT, dithiothreitol; IEF, isoelectric focusing; LPL, lysophospholipase; LPTA, lysophospholipase/transacylase; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; lyso-PI, lysophosphatidylinositol; lyso-PS, lysophosphatidylserine; PA, phosphatidic acid; PAF, platelet-activating factor; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA₂, phospholipase A₂; PLB, phospholipase B; SDA, Sabouraud dextrose agar; PNGase F, peptide N-glycosidase F.

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activity were purified [18–20]; no PLB activity was detected in purified enzyme preparations [18,21]. Candidal phospholipase activity has been correlated with virulence in mice and may contribute to pathogenicity by damaging host cell membranes [21]. It is unclear which of the phospholipase activities are physiologically relevant for this purpose.

We previously reported extracellular phospholipase production in a large number of *C. neoformans* isolates and identified the enzyme activities in one clinical isolate (strain BL-1) as LPL, LPTA and PLB by applying radiometric analysis using TLC and NMR spectroscopy [22,23]. No other phospholipase or lipase activities were identified. Analysis of four cryptococcal strains with various levels of phospholipase activity revealed a correlation between phospholipase activity and virulence in BALB/c mice [22]. We also showed that phospholipid components of lung surfactant and cellular membranes were targets for *C. neoformans* phospholipases in their natural state [24]. Vidotto et al. subsequently suggested that phospholipase production by *C. neoformans* may be linked to capsule size [25]. The mechanisms by which cryptococcal phospholipases influence pathogenicity are not known.

In the present study we have, for the first time, purified one protein containing PLB, LPL and LPTA activities from a pathogenic fungus, *C. neoformans*. We determined the biochemical and kinetic properties of the enzyme and performed partial sequence analysis on the protein. The protein appears to differ from mammalian and other fungal phospholipases and is therefore worthy of more detailed study regarding its mechanism of action and as a potential target for anti-cryptococcal therapy.

EXPERIMENTAL

Reagents and materials

1-[1-¹⁴C]Palmitoyl 2-lysophosphatidylcholine (1-[1-¹⁴C]palmitoyl 2-lyso-PC; 56.7 mCi/mmol) and 1,2-di[1-¹⁴C]palmitoyl phosphatidylcholine (1,2-di[1-¹⁴C]palmitoyl PC; 112 mCi/mmol) were purchased from Amersham Life Science, Amersham, Bucks., U.K. Carrier lipids, 1-palmitoyl-*sn*-glycero-3-phosphocholine (1-palmitoyl lyso-PC), dipalmitoyl phosphatidylcholine (DPPC) and substances used as substrates were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). These included 1-oleoyl *sn*-glycero-3-phosphocholine (1-oleoyl lyso-PC), 1-palmitoyl *sn*-glycero-3-phosphoethanolamine (1-palmitoyl lyso-PE), 1-palmitoyl-*sn*-glycero-3-phosphoinositol (1-palmitoyl lyso-PI), dioleoyl phosphatidylcholine (DOPC), 1-palmitoyl 2-oleoyl-PC, dioleoyl phosphatidylserine (DOPS), phosphatidylinositol (PI, from bovine liver), phosphatidylglycerol (PG), phosphatidic acid (PA), sphingomyelin (from egg yolk), dioleoyl phosphatidylethanolamine (DOPE), trioleoylglycerol, C₁₈-PAF (platelet-activating factor; 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) and palmitoylcarnitine chloride. Corresponding radiolabelled phospholipids were purchased from Amersham Life Science, with the exception of PAF, 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine [octadecyl-9,10-³H(N)] (60–180 Ci/mmol) and [palmitoyl-1-¹⁴C]palmitoylcarnitine chloride (40–55 mCi/mmol) which were from DuPont–NEN (Boston, MA, U.S.A.). Sabouraud dextrose agar (SDA) was obtained from Difco Laboratories (Detroit, MI, U.S.A.). Unless specified otherwise, additional reagents were purchased from Sigma and were of analytical grade or the highest available purity. Phenyl-Sepharose CL-4B resin, Superose 12-HR 10/30, Superdex 200 PC 3.2/30 columns and PD-10 desalting columns were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden) and the UNO[®] Q-1 anion-exchange column was from Bio-Rad (Hercules, CA, U.S.A.).

Organism and enzyme preparation

A clinical isolate of *C. neoformans* var. *neoformans*, BL-1, from the culture collection at Westmead Hospital was studied. Extracellular phospholipase production by this isolate has been previously identified [22,23]. Working cultures were stored at 4 °C on SDA.

The isolate was grown to confluence on SDA in 16 cm-diameter Petri dishes for 72 h at 30 °C in air. Cells scraped from 80–100 dishes were washed sequentially with isotonic saline and imidazole buffer (10 mM imidazole/2 mM CaCl₂/2 mM MgCl₂/56 mM D-glucose, made up in isotonic saline, pH 5.5), resuspended in 50–60 ml of this buffer and incubated at 37 °C for 24 h. The cell-free supernatant was separated as previously described [23] and stored at –70 °C.

Purification of PLB, LPL and LPTA

The purification schedule is shown in Table 1 below. Unless otherwise stated, all procedures were performed at 4 °C. Cryptococcal supernatants were dialysed against buffer A (50 mM sodium citrate/2 mM EDTA, pH 6.3) for 16 h, and solid (NH₄)₂SO₄ was added to achieve 50 % saturation, stirred for 4 h and left overnight. The protein precipitate was pelleted by centrifugation at 20000 *g* for 50 min. The supernatant, containing 100 % of PLB, and > 90 % of LPL and LPTA activities, was applied to a Phenyl-Sepharose CL-4B (2.5 cm × 6 cm) column equilibrated with buffer A containing 50 % (w/v) saturated (NH₄)₂SO₄. The column was eluted with a linear gradient of (NH₄)₂SO₄, from 50–0 % saturation, at a flow rate of 1 ml/min. Fractions (2 ml) were collected and enzyme activity was measured as described below. Active fractions were pooled and dialysed against buffer B (50 mM Mes/2 mM EDTA, pH 6.3).

The dialysed solution was loaded on to a UNO[®] Q-1 anion-exchange column (7 mm × 35 mm) equilibrated with buffer B, washed to remove unbound protein and eluted using a linear concentration gradient of 0–0.6 M NaCl in 30 ml of buffer B at a flow rate of 0.5 ml/min. Fractions containing enzyme activity were pooled, desalted with PD-10 columns and concentrated by lyophilization.

The lyophilized material was reconstituted in buffer C (50 mM Mes/2 mM EDTA/0.15 M NaCl, pH 6.3) and applied to a Superose 12 HR 10/30 column equilibrated with the same buffer. Proteins were eluted at a flow rate of 0.5 ml/min. Active fractions were dialysed against distilled water, lyophilized, re-dissolved in a small volume of buffer D (50 mM Mes/2 mM EDTA/0.6 M NaCl, pH 6.3) and applied to a Superdex 200 PC 3.2/30 column using a SMART HPLC system (Pharmacia–LKB Biotechnology). Elution was performed at 25 °C in the same buffer at a flow rate of 40 µl/min. Active fractions were collected and re-chromatographed on the same column. The purified enzyme was dialysed against water, lyophilized and stored at 25 °C in a desiccator. The protein concentration at each step was estimated by the A₂₈₀ and/or the Coomassie Blue-binding assay (Pierce Chemical Co., Rockford, IL, U.S.A.) using BSA as standard, and purification was monitored using SDS/PAGE (see below).

Measurement of enzyme activities

Radiometric assay

Enzyme activities were measured as described previously [22,23] in a final volume of 0.25 ml, using 1–2 µg of total protein, at 37 °C. For the determination of PLB activity, carrier DPPC (final concentration 400 µM) and 1,2-di[1-¹⁴C]palmitoyl PC (20000 d.p.m.) were dried under nitrogen and suspended in 125 mM imidazole/acetate buffer (assay buffer, pH 4.0) by

sonication using a Branson 450 sonifier. The reaction time was 22 min and PLB activity was measured by the rate of decrease of radiolabelled PC substrate. LPL and LPTA activities were measured simultaneously in a reaction mixture containing 1- ^{14}C palmitoyl lyso-PC (25 000 d.p.m.) and carrier lyso-PC (final concn. 200 μM) in assay buffer. The reaction time was 15 s and LPL activity was measured by the rate of loss of 1- ^{14}C palmitoyl lyso-PC with release of radiolabelled fatty acids. LPTA activity was estimated from the rate of formation of radiolabelled DPPC. All reactions were terminated by adding 1.0 ml of chloroform/methanol (2:1, v/v). The reaction products were extracted by the method of Bligh and Dyer [26], separated by TLC and quantified as previously described [23]. PLB and LPL activities were expressed as μmol of substrate hydrolysed/min per mg of protein. LPTA activity was estimated as the number of μmol of DPPC formed/min per mg of protein.

Characterization of enzyme activities

All experiments were performed in duplicate using 0.1–0.2 μg of pure enzyme. DPPC or 1-palmitoyl lyso-PC were used as substrates unless otherwise specified. Heat-stability was determined by measurement of residual activity at 37 °C after 10 min incubation at various temperatures. The effect of pH (range 3.0–7.6) was determined as previously described, using 0.1 M citric acid/0.2 M NaH_2PO_4 [23].

Modifying agents and cations were added to reaction mixtures at the desired concentration following sonication of the substrate. Cation solutions were prepared in deionized water. Other agents were prepared as stock solutions in imidazole/acetate buffer, pH 4.0, with the exception of palmitoylcarnitine, which was prepared in methanol. The final concentration of methanol added was < 0.1 % (v/v) and was present in controls.

Determination of released free fatty acid by colorimetric assay

Where suitable radiolabelled lysophospholipid substrates were not available, reaction products of LPL and LPTA activity were extracted by the method of Bligh and Dyer [26] and evaporated. The relative levels of non-esterified fatty acid in each sample were determined using the acyl-CoA oxidase system assay kit (Boehringer Mannheim, Mannheim, Germany).

Amino acid sequencing and composition

The N-terminal amino acid sequence was determined by the method of Matsudaira [27]. Briefly, 15–20 μg of pure protein was separated by SDS/PAGE. The band of interest (70–90 kDa) was extracted from the gel by mixing overnight in 100 mM sodium acetate/0.1 % SDS/10 mM dithiothreitol (DTT), and passive adsorption on to PVDF membrane. Sequencing was performed with an ABI model 494 gas-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The sequence of internal peptide fragments was obtained using an 'in-gel' protease-digestion method as modified by Hellman et al. [28]. The 70–90 kDa band was excised following SDS/PAGE, washed, dehydrated under nitrogen, rehydrated in 2–5 μl of 0.2 M NH_4HCO_3 and digested overnight with 0.5 μg of lysyl endopeptidase (from *Achromobacter lyticus*; Wako Chemicals, Richmond, VA, U.S.A.) at 30 °C after full hydration of the gel. Resulting peptides were recovered by extraction with 60 % acetonitrile and 0.1 % trifluoroacetic acid and separated by reverse-phase HPLC using the SMART system as previously described [28]. Amino acid sequences were determined using the Procise cLC Protein Sequencer and data analysed with the B10 Analysis software (Applied Biosystems).

Amino acid composition was determined after hydrolysis at 110 °C in 6 M HCl for 24 h and analysed by derivatization chemistry using the Waters AccQ Tag system [29]. Amino standard H preparations were used as standards (Pierce Chemical Co.).

Other methods

Analytical SDS/PAGE was performed by the method of Laemmli [30] under reducing conditions in 4–20 % linear gradient polyacrylamide gels (Novex, San Diego, CA, U.S.A.). Gels were stained with Coomassie Brilliant Blue R-250 and silver (Novex Silver Express; Novex) for proteins, and with thymol/ H_2SO_4 stain for glycoproteins [31]. The apparent molecular mass was estimated by SDS/PAGE and gel-filtration chromatography using the Superdex 200 PC 3.2/30 column. In the latter case the column was calibrated with molecular-mass marker-protein standards from Bio-Rad: thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B-12 (1.35 kDa) and a standard curve constructed. Pure protein was also electrophoresed under native-PAGE conditions using the Laemmli buffer system, but without SDS or DTT. It was then subjected to electroelution with the Mini Whole Gel Eluter (Bio-Rad), according to the manufacturer's specifications, in a continuous buffer system using 50 mM imidazole adjusted to pH 6.3 with acetic acid. Aliquots of eluted protein solution were assayed for enzyme activity and the active fractions re-analysed by SDS/PAGE.

Isoelectric focusing (IEF)/PAGE (IEF/PAGE) was performed by focusing on a non-linear 7 cm Immobiline DryStrip, pH 3.0–10.0 (Pharmacia Biotech) using carrier ampholytes of 0.5 % pH 3.0–10.0 (BioLytes; Bio-Rad) for a total of 12 kV·h. Proteins were then run on a 10–20 % gradient mini-gel (Bio-Rad) and visualized by staining with diamine silver according to the method of Glasson et al. [32].

Deglycosylation experiments were performed using the GlycoPro[®] deglycosylation kit (ProZyme Inc., San Leandro, CA, U.S.A.). Pure protein (15–20 μg) was incubated overnight at 37 °C with one or more of the enzymes, peptide N-glycosidase F (PNGase F), sialidase A and endo-O-glycosidase in accordance with the manufacturer's protocols. Bovine fetuin was used as the control. After treatment, samples were boiled for 2 min in buffer containing 0.125 M Tris/HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol and 3 % (w/v) DTT and run on a 4–20 % gel (Novex). Bands were revealed by staining with Coomassie Blue R-250. Experiments were also performed under non-denaturing conditions, and aliquots of the resulting mixture were assayed for phospholipase activity as described above.

Total protein estimations were performed using a Coomassie Blue-binding assay (Pierce Chemical Co.) with BSA as standard.

Analysis of enzyme kinetic data

The KaleidaGraph Software package (version 3.0; Synergy Software, PCD Inc., Reading, PA, U.S.A.) was used to estimate the Michaelis constant (K_m) and V_{max} values for PLB and LPL activities, and to construct Lineweaver–Burk plots for substrate-inhibition studies.

RESULTS

Purification of extracellular phospholipase(s) from *C. neoformans*

Experimental conditions were optimized for maximal yield and purity by utilization of $(\text{NH}_4)_2\text{SO}_4$ fractionation, then hydrophobic-interaction, anion-exchange and gel-filtration chroma-

Table 1 Purification of PLB, LPL and LPTA from *C. neoformans*

Purification was performed and enzyme activities were determined by radiometric analysis as described in the Experimental section. Results in the Table are representative of five independent preparations, each giving similar results. A unit of enzyme activity is defined as the amount of enzyme hydrolysing 1 μ mol of substrate/min at 37 °C.

Step	Protein (mg) yield	Total activity (units) [PLB (LPL)*]	Specific activity (units/mg) [PLB (LPL)*]	Recovery (%) [PLB (LPL/LPTA)]	Purification (fold) [PLB (LPL/LPTA)]
Culture supernatant	9.6	7731 (267637)	0.4 (17.0)	100 (100)	1.0 (1.0)
(NH ₄) ₂ SO ₄	7.4	13160 (391275)	0.8 (45.0)	170 (146)	2.0 (2.6)
Phenyl-Sepharose CL-4B	5.0	6379 (191363)	0.9 (90.0)	83 (72)	2.5 (5.3)
Uno Q-1	1.7	1450 (46880)	2.5 (162.1)	18.7 (17.5)	6.5 (9.5)
Superose 12 HR 10/30	0.4	1440 (45600)	6.8 (422.2)	18.6 (17.0)	17.9 (24.8)
Superdex 200 PC 3.2/30	0.04	120 (7920)	10.5 (559.0)	1.6 (3.0)	27.6 (32.9)

*The corresponding total and specific activities of LPTA at each step were 0.4–0.5 of the value calculated for LPL.

tography. This five-step procedure resulted in the purification of cryptococcal phospholipase to homogeneity whilst preserving enzyme activity (see Table 1).

Following (NH₄)₂SO₄ fractionation, the recovery of PLB was approx. 170%, whereas that of LPL and LPTA activities was 146% (Table 1). This suggests that endogenous enzyme inhibitors had been removed from the crude enzyme preparation. Following centrifugation, recovery of PLB activity in the supernatant fraction was complete, but 5–10% of total LPL and LPTA activity remained in the precipitate.

The enzyme in the supernatant adsorbed to the phenyl-Sepharose CL-4B resin under conditions of high ionic strength, and the activities were eluted predominantly with buffer containing no (NH₄)₂SO₄ (from approx. 20–0% saturation). Enzyme activities were eluted from the UNO™ Q-1 column with 0.15–0.43 M NaCl (results not shown). Further purification was achieved by gel filtration using Superose 12 HR 10/30; a broad activity peak was reproducibly eluted between 158 kDa (bovine γ -globulin) and 44 kDa (ovalbumin; results not shown). A relatively large increase in the enzyme specific activities was noted (Table 1). The final chromatographic step using the Superdex 200 (HPLC) column produced a single symmetrical peak, corresponding to the major protein peak, for all three enzyme activities.

No separation of PLB from LPL, or of LPL from LPTA activities, was observed during purification of five independent enzyme preparations. The ratio of LPL to PLB activity was 47–53 and the ratio of LPL to LPTA activity was constant at 2.0–2.5 after each purification step. Approx. 3.0% of LPL and LPTA, and 1.7% of PLB activities were recovered. The specific activity of the purified enzyme for PLB was 10.5 ± 2.3 μ mol/min per mg of protein and that for LPL was 559.0 ± 42.1 μ mol/min per mg of protein. The purified material was stable at 25 °C for several weeks, but only in lyophilized form.

Initial experiments were performed with the addition of protease inhibitors using the 'Complete, Mini' preparation (Boehringer Mannheim) according to the manufacturer's instructions and 1 μ M pepstatin A. Similar recoveries of enzyme activities were obtained with or without the addition of these inhibitors during the purification procedure.

Molecular mass

SDS/PAGE analysis of fractions containing enzyme activity from successive purification stages showed the appearance, with increasing relative intensity, of a broad band of approximate molecular mass 70–90 kDa. Separation by SDS/PAGE after

Figure 1 SDS/PAGE analysis of purified cryptococcal phospholipase

The pure protein was separated by SDS/PAGE as described in the Experimental section. The protein solution obtained after electroelution following native PAGE was assayed for enzyme activity and the active aliquots were re-analysed by SDS/PAGE. Lanes 2 and 3, molecular-mass markers as shown on the left: β -galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; α -lactalbumin, 14 kDa; Lane 5, aliquot of pure protein (10–15 μ g) after Superdex 200 chromatography; Lane 6, fraction containing activity after electroelution.

chromatography on the Superdex 200 column showed that each enzyme activity co-purified with this protein (lane 5, Figure 1). The band stained strongly with thymol/H₂SO₄, indicating that it is a glycoprotein. The molecular mass of the non-denatured, pure protein was estimated to be about 160–180 kDa by gel filtration, on the basis of its elution profile when compared with standard markers. To confirm that this polypeptide was associated with phospholipase activity, material eluted from the Superdex 200 column was separated by native PAGE; this produced a broad band corresponding to a mass of 140–232 kDa by Coomassie Blue staining (results not shown). After electroelution, protein aliquots containing enzyme activity, when re-analysed by SDS/PAGE, gave the same 70–90 kDa band described above (lane 6, Figure 1).

Amino acid sequencing and composition

To prove that only one phospholipase-containing enzyme protein was present, the 70–90 kDa band (Figure 1) was cut into three

Table 2 Total amino acid composition of purified cryptococcal phospholipase

Amino acid composition was determined, after hydrolysis in HCl, using the water AccQ Tag system as described in the Experimental section. Abbreviation: BLD, below the limits of detection.

Amino acid	Composition (mol %)
Asx	14.1
Ser	12.0
Thr	11.2
Ala	10.8
Gly	9.3
Glx	8.3
Val	7.0
Leu	5.7
Pro	5.4
Ile	3.7
Phe	3.4
Tyr	2.8
Lys	1.9
Arg	1.8
Met	1.7
His	1.4
Cys	BLD

serial sections after Coomassie Blue staining. The *N*-terminal sequence was determined as TAPDPNSNDYMSYYSSPPSQT and was identical for all three sections. The sequences of five internal peptide fragments designated L1–L5, generated after digestion by lysyl endopeptidase, were determined as LATVN-LPNVPVATPDGRR, NVADAFNMEXYLNSGK, NYEN-VLGYY, ELTEYATV and KAVGNK respectively. Comparison of these six peptide sequences against the non-redundant protein database using the SWISS-PROT, BLASTP and TBLASTN programs [33], accessed via the Australian National Genomic Information Service (ANGIS), revealed that they were novel peptides.

The results of total amino acid analysis obtained after acid hydrolysis showed that the protein was rich in Asp/Asn, Ser, Thr, Ala, Glu/Gln and Val (Table 2). Lys and Met each comprised less than 2.0 mol %. Again, the composition of the pure protein appeared to be unique (SWISS-PROT database comparison).

pI

On two-dimensional electrophoresis the pure protein migrated reproducibly as a series of forms corresponding to pI values of about 4.5 and 3.5 respectively (results not shown).

Kinetic characteristics of enzyme activities

To examine the kinetic characteristics of the purified enzyme, substrate–activity profiles were compared. Although a continuous assay was not used, preliminary experiments established that reaction velocities for PLB and LPL (and LPTA) activities were linear over the incubation times used, namely 5–35 min and 10–30 s respectively and over the entire range of substrate concentrations investigated (results not shown). Hence, velocities measured were close to the initial rates of reaction. The velocity of the pure enzyme was linear with respect to mass of protein for 1-palmitoyl lyso-PC until approx. 70 % of substrate was hy-

Figure 2 Effect of protein concentration on (A) LPL and LPTA activities and (B) PLB activity in a pure enzyme preparation

LPL, LPTA and PLB activities were measured by radiometric analysis in the presence of various amounts of enzyme as described in the Experimental section. Results are expressed as the percentage of 1-palmitoyl lyso-PC hydrolysed (A) or DPPC hydrolysed (B). Typical curves generated from such an analysis are shown and the data represent the means for duplicate experiments.

drolysed, and for DPPC until 40 % was hydrolysed (Figures 2A and 2B).

All three enzyme activities displayed non-Michaelis–Menten kinetics and were best fitted using a Hill plot (results not shown). The Hill coefficients were 1.7 and 1.5 for LPL and PLB activities respectively. Linearity was observed with increasing substrate concentrations up to 400 µM for PLB activity and up to 200 µM for LPL and LPTA activities. Inhibition of enzyme activity was apparent at substrate concentrations above 600 µM for PLB and 200 µM for LPL and LPTA. When the reaction product palmitic acid (> 30 nmol) was added together with 600 µM DPPC or 200 µM 1-palmitoyl lyso-PC, enzyme inhibition was also apparent; the addition of the reaction product glycerophosphocholine at similar substrate concentrations had no effect on either of the enzyme activities (results not shown). An apparent maximum velocity ($V_{\max}$) of 12.3 µmol/min per mg with an apparent K_m of approx. 185.3 µM was calculated for PLB. An apparent $V_{\max}$ of 870 µmol/min per mg and apparent K_m of 92.2 µM was calculated for both fatty acid release (hydrolase) and PC production (transacylase, from lyso-PC) catalysed by LPL and LPTA activities respectively.

Effects of pH and thermal stability

The optimum pH for fatty acid release (LPL) and PC production (LPTA) from lyso-PC was approx. pH 4.0 (Figure 3). The optimum pH range for fatty acid release by PLB was narrower, but also peaked at pH 4.0. LPL and LPTA activities fell sharply at pH values of greater than 5.5, although 50–60 % of maximum activity was detected between pH 3.0 and 4.0 compared with that

Figure 3 Effect of pH on the activities of purified cryptococcal phospholipase

Enzyme activities were measured by radiometric analysis at various pH values as described in the Experimental section. Results are expressed as the percentage of substrate hydrolysed. ●, Hydrolysis of 1-palmitoyl lyso-PC; ■, hydrolysis of DPPC. The data represent the means for duplicate experiments.

Table 3 Effect of metal ions or reagents on cryptococcal PLB, LPL and LPTA activities

Assays for enzyme activities were performed at pH 4.0 as described in the Experimental section using DPPC and 1-palmitoyl lyso-PC as substrates. Solutions of metal ions or reagents were added to the reaction mixture at the final concentration as shown in the Table. Experiments were performed twice with similar results.

Metal ion/reagent	Concentration	Relative activity (%)	
		PLB	LPL/LPTA†
None	—	100*	100
CaCl ₂	10 mM	151	108
FeCl ₃	1 mM	5.0	100
	10 mM	3.3	17.5
EDTA	10 mM	98.6	100
EGTA	10 mM	99.2	97.8
DTT	10 mM	50.8	72.8
Palmitoylcarnitine	100 µM	60.3	90.0
	500 µM	3.0	65.1
Carnitine	10 mM	30.2	80.4
SDS	0.05%	0	10.0
Triton X-100	0.1%	102	50.1

*The control value (100%) represents the specific activity of 10.5 ± 2.3 µmol/min per mg of protein for PLB and 559 ± 42.1 µmol/min per mg of protein for LPL.

†Percentages refer to effects on both LPL and LPTA activities.

at pH 4.0. The ratio of LPL to LPTA activities remained constant over the pH range tested.

The thermal stabilities of PLB, LPL and LPTA were, in general, similar. While it was apparent that enzyme activity declined significantly after exposure to temperatures above 42 °C, maximal LPL and LPTA activities decreased by approx. 45% after pre-incubation at 50 °C compared with PLB, where the activity was decreased by almost 100% (results not shown). The optimum temperature was at or near 37 °C for PLB activity, and ranged from 20 to 42 °C for LPL and LPTA. Activity was

Figure 4 Lineweaver-Burk plot of (A) LPL activity and (B) PLB activity

Enzyme activities were measured as described in the Experimental section at substrate concentrations ranging from 67 to 400 µM inclusive, in the absence or presence of different concentrations of palmitoylcarnitine as illustrated. (A) ●, 0 mM; ■, 0.1 mM; ▲, 0.5 mM; ○, 1 mM. (B) ●, 0 mM; ■, 0.05 mM; ▲, 0.1 mM; □, 0.4 mM palmitoylcarnitine.

lost completely by incubation at or above 50 °C for PLB and 60 °C for LPL and LPTA.

Modulation of enzyme activities by various agents

The effects of selected metal ions and chemical reagents on enzyme activities are shown in Table 3. Whilst cryptococcal PLB exhibited some differences from LPL (hydrolase) and LPTA (transacylase) in response to modifying agents, LPL and LPTA activities were always affected similarly; the effects of the agents tested on these two activities are thus summarized together in Table 3. None of the enzyme activities was affected by exogenous Mg²⁺, Zn²⁺, Mn²⁺ and Fe²⁺ ions. Fe³⁺ ions (10 mM) caused 83 and 97% inhibition of LPL (and LPTA) and PLB activities respectively. Enzyme activities were also independent of added EDTA and EGTA. Exogenous Ca²⁺ ions increased PLB activity slightly; this effect was abolished by the further addition of 1 mM EGTA. None of the enzyme activities was affected by phenylglyoxal, *p*-bromophenacyl bromide, *N*-ethylmaleimide and *N*-bromosuccinimide at concentrations of up to 10 mM. Of the detergents tested, Triton X-100 inhibited LPL and LPTA (to about 50% of the control), but not PLB. PLB activity was most affected by palmitoylcarnitine, with 40 and 97% decreases in activity occurring in the presence of 0.1 mM and 0.5 mM concentrations of this agent respectively, while the corresponding LPL and LPTA activities were decreased by 10 and 35%. The competitive nature of this inhibition is shown in the Lineweaver-Burk plots in Figures 4(A) and 4(B).

Table 4 Substrate specificity of cryptococcal phospholipase activities

PLB and LPL activities of the pure enzyme were measured by the radiometric and colorimetric assays respectively as described in the Experimental section. The reactions were performed using 400 μ M of the various phospholipids, or 200 μ M of the lysophospholipid substrates. Results are expressed as a percentage of the control value (100%), where this value represents a specific activity of 10.5 ± 2.3 mol/min per mg of protein for PLB (DPPC as substrate) and 559 ± 42.1 mol/min per mg of protein for LPL (1-palmitoyl lyso-PC as substrate). Abbreviation: BLD, below the limits of detection.

Substrate	Relative activity (%)	
	PLB	LPL
Phospholipids		
DPPC	100	—
DOPC	129	—
PG	94.2	—
1-Palmitoyl-2-oleoyl phosphatidylcholine	36.1	—
DOPS	22.5	—
DOPE	35.5	—
PI	15.6	—
PAF	8.9	—
PA	BLD	—
Sphingomyelin	BLD	—
Trioleoylglycerol	BLD	—
Palmitoylcarnitine	BLD	—
Lysophospholipids		
1-Palmitoyl lyso-PC	—	100
1-Oleoyl lyso-PC	—	194
Lyso-PI	—	77.9
1-Palmitoyl lyso-PE	—	29
1-Palmitoyl lyso-PS	—	3.8

Substrate specificity

The hydrolytic activity of purified PLB and LPL was examined using different substrates (see Table 4). At acidic pH, there was, in general, broad specificity for lysophospholipids, with 1-palmitoyl lyso-PC and 1-oleoyl lyso-PC being the preferred substrates. The enzyme demonstrated low specificity for 1-palmitoyl lyso-PS (3.8%). Lysophospholipids were hydrolysed at 40–60 times higher rates than the corresponding phospholipids.

PLB hydrolysed all the phospholipids examined, except for phosphatidic acid, with DOPC and DPPC hydrolysed most rapidly. No detectable hydrolysis of trioleoylglycerol, sphingomyelin or of palmitoylcarnitine was observed, nor was accumulation of lysophospholipids noted during hydrolysis of the phospholipids.

Deglycosylation experiments

Figure 5 shows the results of SDS/PAGE analysis following treatment of the pure protein with deglycosylation enzymes. There was a decrease in the apparent molecular mass of the protein, from 70 to 90 kDa to approx. 55–70 kDa, representing a molecular-mass shift of about 30% after digestion with PNGase F, indicating removal of *N*-linked sugar moieties. The deglycosylated protein contained at least three distinct bands; the *N*-terminal amino acid sequences of two of these bands were identical with each other and with that of the untreated protein. Sequencing of the third (lower) band was unsuccessful, as it was present in inadequate quantity. Treatment of the protein with sialidase or endo-*O*-glycosidase alone resulted in no detectable molecular-mass shift (Figure 5), and digestion with either enzyme in combination with PNGase F resulted in no further shift

Figure 5 SDS/PAGE of the purified protein after deglycosylation

Pure protein (15 μ g) was deglycosylated and analysed by SDS/PAGE as described in the Experimental section. Lane 1, undigested phospholipase protein; lanes 2 and 3, PNGase F-digested protein; lane 4, sialidase-treated protein; lane 6, PNGase F-digested bovine fetuin; lane 7, bovine fetuin control; lane 8; molecular-mass markers (Promega: phosphorylase *b*, 97.4 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; α -lactalbumin, 14 kDa).

(results not shown). Deglycosylation of the protein by PNGase F under non-denaturing conditions resulted in the complete loss of PLB, LPL and LPTA activities relative to controls (activities of the undigested protein under identical test conditions).

DISCUSSION

A highly active secretory phospholipase, with PLB, LPL and LPTA activities, from the pathogenic fungus *C. neoformans*, was purified to homogeneity. The study is the first to identify and characterize the properties of all three enzyme activities, which were found in association with a single fungal protein. On the basis of a comparison of the *N*-terminal amino acid sequence, sequences of five internal peptide fragments and the amino acid composition with other fungal PLBs, the protein appears to be novel. In addition, differences in preferred substrate requirements and response to modifying agents between cryptococcal phospholipase and known fungal phospholipases were identified.

The apparent molecular mass of purified cryptococcal phospholipase was 160–180 kDa by gel filtration and 70–90 kDa by SDS/PAGE, suggesting that it is an oligomeric protein comprising two (or three) subunits. This is consistent with the hypothetical multimeric structure of cell-associated PLB purified from *S. pombe* and *T. delbrueckii* [15,16], but unlike that of the water-soluble form of PLB from *S. cerevisiae* [34]. The apparent molecular mass of *C. neoformans* phospholipase on SDS/PAGE compares with 84 kDa for *C. albicans*, 100–150 kDa for *S. pombe*, 145–280 kDa for *S. cerevisiae*, and 140–190 kDa for *T. delbrueckii* [12,14–16,18], suggesting the possibility of structural differences between PLB enzymes of pathogenic fungi and industrial yeasts.

Cryptococcal phospholipase is an acidic glycoprotein and, in this respect, resembles phospholipases from *T. delbrueckii* and *P. notatum* [11,14]. The broadness of the protein band noted on SDS/PAGE has also been observed for other fungal PLBs [12,15,16,35] and is believed to be due to heterogeneity of the carbohydrate moiety linked to the enzyme. The bands revealed on SDS/PAGE (Figure 5) after enzymic removal of *N*-linked carbohydrate from purified cryptococcal phospholipase pre-

sumably represent forms of the same enzyme which differ only in the degree of glycosylation (the *N*-terminal sequence of two of these bands was identical with that of the undigested protein), an interpretation supported by two pI values obtained for the protein. Although the motif Asn-Xaa-Ser (Thr), characteristic of potential *N*-glycosylation sites, was not present within the sequenced peptide fragments, Ser, Thr and Asn residues were relatively abundant. All fungal phospholipases studied thus far have been glycosylated [13,14,16,21]. Deglycosylation of the protein by PNGase F resulted in almost total loss of enzyme activity, indicating that the *N*-linked carbohydrate moiety is important in the catalytic capability of the protein.

Attempts to separate cryptococcal PLB, LPL and LPTA activities were unsuccessful, suggesting that they reside on the same protein. Glycoproteins generally exist as populations of glycosylated variants (glycoforms) of a single polypeptide [36]. While we have not proved conclusively that all parts of the sequence of the 70–90 kDa band are identical, it is unlikely that there are several proteins with different K_m values contributing to the results of the kinetic studies. There was no evidence for this in the Lineweaver–Burk plots, where one would expect deviations from linearity. The only deviation from linearity occurred at high substrate concentrations, where inhibition of cryptococcal phospholipase activities was observed; this may be explained by product (fatty acid) inhibition (see the text) and/or the formation of large multilamellar substrate vesicles restricting access of the enzyme to the substrate. Although the extent of glycosylation may influence the activity of the enzyme, it is unlikely that separate substrate specificities would be present on different glycoforms. Differences in temperature-stability, pH optima, and the effect of cations and selected modifying agents on the activities support the hypothesis that there are at least two active sites on the same protein.

Several differences between the enzymic properties of purified cryptococcal phospholipase and those of *C. albicans*, as well as non-pathogenic fungi, are worthy of comment. Both cryptococcal and candidal enzymes were optimally active at physiological temperatures, but the highly acidic pH optimum (pH 4.0) of cryptococcal phospholipase was substantially lower than that of a highly active secretory LPTA ('h-LPTA') of *C. albicans* (pH 6.0; the pH optimum of candidal PLB is unknown) [18]. We have recently shown that *C. neoformans* secretes millimolar quantities of acetic acid *in vitro* [37] and, in a rat model, acetic acid was demonstrable in lung and brain cryptococcomas by PMR spectroscopy (U. Himmelreich, T. Dzendrowskyj, C. Allen, S. Dowd, R. Malik, P. Shehan, C. Mountford and T. Sorrell, unpublished work). Thus a microenvironment favourable for cryptococcal phospholipase activity could be created by secretion of acetic acid by the fungus itself. In addition, Levitz et al. have reported that *C. neoformans* is well-adapted to intracellular survival in the acid environment (pH 5.0) of phagolysosomes in human macrophages [38].

Purified cryptococcal phospholipase was unaffected by *N*-bromosuccinimide (a tryptophan-modifying agent) and phenylglyoxal (an arginine-modifying compound). In contrast, both compounds inhibited PLB of *P. notatum* [39] and phospholipase A₂ (PLA₂) from secreted venoms [40]. *p*-Bromophenacyl bromide, an effective inhibitor of mammalian secretory PLA₂ and of *P. notatum* PLB, did not inhibit the activity of cryptococcal phospholipase [41,42]. Most major membrane phospholipids (including DPPC and PG, the most abundant components of lung surfactant) and lysophospholipids were hydrolysed by the pure enzyme, whereas the less accessible, acidic inner-leaflet phospholipids, PS, PI and PA were preferred substrates for PLBs from non-pathogenic fungi [11,12,16]. Palmitoylcarnitine, which

has a similar chemical structure to DPPC, was not hydrolysed by cryptococcal phospholipase. Instead, this agent was a potent competitive inhibitor of PLB activity, whereas LPL and LPTA activities were only modestly inhibited. In contrast, palmitoylcarnitine enhanced LPL activity in *C. albicans* and was an essential component of the reaction mixture in the assay for PLB activity in crude culture filtrates [18,21]. The present study indicates that extracellular cryptococcal phospholipase activities are distinct from the candidal enzymes.

Cryptococcal PLB, LPL and LPTA exhibited, in general, 10–200-fold higher specific activities than the corresponding enzymes from non-pathogenic fungi [11–13,15,16]. LPL and LPTA activities were 100–500 fold greater than those of mammalian lysophospholipases [43,44]. Notably, unlike other fungal phospholipases [11,15,18], all three enzyme activities were preserved at each step of the purification, indicating that LPL is not an artifact of PLB degradation [11], nor is secretory PLB unstable during purification as reported for *T. delbrueckii* [15]. In *C. albicans*, the only other medically important fungus studied thus far, LPTA activity was purified and characterized, but no PLB activity was detected in purified preparations of the enzyme [18]. Candidal LPTA appears to be integral to the pathogenesis of infection with *C. albicans* [21]. Retention of PLB, LPL and LPTA activities was reported during the purification of phospholipases from *S. pombe*, but only PLB was characterized, and LPTA activity estimated by the conversion of lyso-PC substrate into PC was only 2.2 % [16]. The relative importance of the three different phospholipase activities in various fungi is unknown. The respective roles of extracellular PLB, LPL and LPTA activities in human cryptococcal infection may vary in specific microenvironments within the host. We have previously shown that the LPL and LPTA activities of the native enzyme can reacylate lysophospholipids and reverse damage caused to neutrophils by other components secreted by cryptococci [24]. The ability to degrade (PLB) membrane phospholipids may promote invasion of lung tissue, whereas the ability to repair (LPL/LPTA) the phospholipids may enable cryptococci to parasitize phagocytes without eliciting an immune response.

In conclusion, purified secretory cryptococcal phospholipase is a single, oligomeric, acidic glycoprotein associated with three enzyme activities. We propose that it is important in the initiation of infection in the phospholipid-rich environment of the human lung. This is the subject of ongoing investigation in our laboratory.

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