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Identification of Extracellular Phospholipase B, Lysophospholipase, and Acyltransferase Produced by *Cryptococcus neoformans*

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We recently identified phospholipase activity as a potential virulence factor of *Cryptococcus neoformans*. We have now defined the nature of the phospholipase activity produced by a clinical isolate of *C. neoformans* var. *neoformans*, under native conditions, by ^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy and thin-layer chromatography (TLC) of radiolabelled substrates. Glycerophosphocholine was identified by NMR spectroscopy as the sole phospholipid degradation product of the reaction between substrate phosphatidylcholine (PC) and cryptococcal culture supernatants indicating the presence of phospholipase B (PLB). No lysophosphatidylcholine (lyso-PC) or products indicative of phospholipase C, phospholipase D, or other lipase activity were identified. Use of PC and lyso-PC containing radiolabelled acyl chains and separation of products by TLC confirmed the PLB and lysophospholipase (LPL) activities. Lysophospholipase transacylase (LPTA) activity was identified by the formation of radioactive PC from lyso-PC. Extracellular enzyme production was maximal after 6 to 10 h in fresh medium. Assay conditions were optimized for pH, linearity with time, enzyme concentration, and saturation by substrates to allow comparison with phospholipases from other organisms. LPL activity was 10- to 20-fold greater than PLB activity, with mean (± standard deviation) specific activities of 34.9 ± 7.9 and 3.18 ± 0.2 μmol of substrate hydrolyzed per min per mg of protein, respectively. The response of PLB to increasing substrate concentrations was bimodal, whereas inhibition of LPL and LPTA activities occurred at concentrations of substrate lyso-PC greater than 200 μM. Enzyme activities were stable at acid pH (3.8), with pH optima of 3.5 to 4.5. Activities were unchanged in the presence of exogenous serine protease inhibitors, divalent cations, and EDTA. We conclude that *C. neoformans* produces highly active extracellular PLB, LPL, and LPTA under native conditions.

Cryptococcus neoformans is an important opportunistic pathogen in patients with AIDS and is the most common cause of fungal meningitis worldwide (9, 14). In addition, a significant proportion of infections occurs in immunocompetent hosts (20). Pathogenic strains of cryptococci are characterized by the presence of a polysaccharide capsule, production of phenol oxidase, and growth at 37°C (8, 16, 23). Capsule production enables the organism to evade phagocytosis, and capsular polysaccharide suppresses both cellular and humoral immunity (5, 8). Acapsular variants of *C. neoformans* are less virulent for mice, as are melanin-negative mutants, which lack phenol oxidase activity (10, 16, 23). Other features of *C. neoformans* may also contribute to virulence. Tropism for lung and brain tissue has been demonstrated in vitro, where *C. neoformans* has been observed to adhere to rat lung epithelial and glial cells by means of an adhesion protein(s) (18). An as-yet-undefined factor is apparently linked to the α-mating type locus (15), and secreted proteinases, which may contribute to the breakdown of host tissue, have been described (6). None of these putative virulence factors has been linked unequivocally with pathogenicity.

Extracellular phospholipases have been implicated in the pathogenesis of bacterial infections by causing damage to host cell membranes and allowing tissue invasion (27). Phospholipases are produced by certain fungi, including *Candida albicans* and *Penicillium notatum* (3, 12, 26). Three types of phospholipase activities have been described in *C. albicans*, namely, lysophospholipase (LPL; EC 3.1.1.5), lysophospholipase transacylase (LPTA; EC 3.1.1.5), and phospholipase B (PLB; EC 3.1.1.5) (2, 12, 19). The ability of *C. albicans* to secrete these enzymes has been considered integral to its pathogenicity. Barrett-Bee et al. (3) correlated phospholipase production by *C. albicans* with adherence to buccal epithelial cells. Furthermore, it was observed in a murine model of candidiasis that mortality was increased in mice infected with isolates producing the highest phospholipase activity (3, 12).

A simple and rapid assay to detect extracellular phospholipase activity of *C. albicans* by measurement of the zone of precipitation around colonies cultured on egg yolk agar has been described previously (22, 28). We recently demonstrated phospholipase production in 50 clinical and environmental isolates of *C. neoformans* by this technique and observed a correlation between phospholipase activity and virulence in mice (7). In the present study, we sought to characterize and determine the relative activities of phospholipases produced by one strain of *C. neoformans* by use of the independent techniques of proton (^{1}H) and phosphorus (^{31}P) nuclear magnetic resonance (NMR) spectroscopy and radiometric analysis employing thin-layer chromatography (TLC). We chose to study enzyme activities in the native (unpurified) state since the activity of phospholipases can be differentially affected by purification (13, 26) and study of cryptococcal culture supernatants may therefore more closely mimic the interaction between cryptococci and host cells in vivo. So that the phospholipase activities of *C. neoformans* might be compared with those of other fungi,

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we sought to optimize the experimental conditions with respect to pH, time, enzyme, and substrate concentrations used for assay.

MATERIALS AND METHODS

Reagents. 1-[1-^{14}C]palmitoyl-2-lysophosphatidylcholine (1-[1-^{14}C]palmitoyl-2-lyso-PC; 56.7 mCi/mmol) and 1,2-di[1-^{14}C]palmitoyl-PC (112 mCi/mmol) were purchased from Amersham Life Science, Buckinghamshire, England. Dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]PC (97 Ci/mmol) was obtained from Dupont NEN (Boston, Mass.), and 1-palmitoyl-lyso-PC, dipalmitoyl-PC (DPPC), egg yolk PC type XI-E, and imidazole were from Sigma Chemical Co. (St. Louis, Mo.). Sabouraud dextrose agar (SDA) and yeast nitrogen base broth with amino acids (YNB broth) were obtained from Difco Laboratories (Detroit, Mich.); unless specified otherwise, additional reagents were purchased from Sigma and were of the highest available purity.

Organisms and enzyme preparation. The isolate BL-1, obtained from the culture collection at Westmead Hospital, New South Wales, Australia, was selected for study since it consistently produced large zones of precipitation on egg yolk medium (7). It was recovered from a patient with pulmonary cryptococcal infection and identified as *C. neoformans* var. *neoformans* by standard methods (14). Working cultures were stored at 4°C on SDA and subcultured before use to ensure purity.

(i) Enzyme activity in the presence of cells. Isolate BL-1 was grown on SDA for 72 h at 30°C in air. Cells were scraped off and washed twice with isotonic saline and once with imidazole buffer (10 mM imidazole, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 56 mM D-glucose in isotonic saline [pH 5.5]). One milliliter of this buffer was then added to the washed, packed cells; portions of the suspension (350 μl) were distributed into sterile Eppendorf tubes. For NMR experiments, 50 μl of $^{2}H_2O$ (deuterium oxide) was added to the 350-μl aliquots of cell suspension with the further addition of either 100 μl of egg yolk PC in buffer (final concentration, 5 mM) or 100 μl of imidazole buffer (as controls). The resulting suspension was incubated at 37°C up to 24 h prior to analysis by NMR. Samples were assayed in the presence of cells or as cell-free supernatants (see "Extracellular enzyme activity" below). For TLC analysis, aliquots of cell suspensions were incubated with substrate (PC or lyso-PC) for stated periods of time (see below). The suspensions were then centrifuged at 12,000 × *g* for 5 min, and the supernatant was assayed.

(ii) Extracellular enzyme activity. Samples were prepared as described above with the exception that cellular suspensions were incubated in the absence of substrate for 20 to 24 h at 37°C. The cell-free medium was separated by centrifugation at 12,000 × *g* for 5 min; the centrifugation process was repeated twice. The supernatant was found to contain fewer than 10 cells per ml by plate count. Supernatants were assayed for phospholipase activity by NMR spectroscopy or TLC at various times after addition of substrate (see below).

To estimate the degree of cell lysis, cells which had been harvested, washed, and suspended in either imidazole buffer or YNB broth with 0.5% glucose were incubated at 37°C, and cell numbers were determined in a hemocytometer at 0, 4, 6, 10.5, and 22.5 h. Aliquots of the cell-free supernatant were assayed for phospholipase activity as described below.

For some experiments, supernatants were stored for up to 2 weeks at −70°C prior to assay. Experiments were also performed in which cells were grown and assayed in the presence of the serine protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and leupeptin (10 mM) and in EDTA (2 mM).

(iii) Extraction of phospholipase activity from agar. Cells and agar from five plates, each containing isolate BL-1 after 72 h of growth on SDA, were homogenized by hand with a glass homogenizer with a Teflon pestle in 50 ml of 0.1 M Tris HCl (pH 4.5) and centrifuged at 2,000 × *g* for 15 min. The supernatant was filtered through a 0.22-μm-pore-size membrane (Sartorius AG, Göttingen, Germany), filtered again, and then concentrated to a volume of 3 to 5 ml with a Centriplus-10 concentrator with a 10,000-molecular-weight cutoff (Amicon, Australasian Medical and Scientific, Sydney, New South Wales, Australia). The retentate was stored at 4°C overnight prior to assay.

NMR spectroscopy. (i) Preparation of samples. For NMR spectroscopy, 100 μl of egg yolk PC (5 mM final concentration) or 100 μl of buffer (controls) was added to cryptococcal cell suspensions or supernatants and incubated at 37°C for 0 to 24 h. One milliliter of isotonic NaCl was then added, and the mixture was centrifuged at 12,000 × *g* for 5 min. For ^{1}H NMR, 0.45 ml of supernatant was dispensed into a 5-mm-outer-diameter NMR tube with 50 μl of $^{2}H_2O$. For ^{31}P NMR, 1 ml of supernatant was dispensed into a 10-mm-outer-diameter NMR tube containing 0.5 ml of $^{2}H_2O$ and 1.5 ml of water.

(ii) Acquisition of NMR spectra. Spin echo spectra were acquired with a Bruker AMX-600 NMR spectrometer operating at a frequency of 600.14 MHz. The number of transients per spectrum was 128, the acquisition time was 2 s, the water presaturation time was 2 s, and the spin echo delay time (τ) was 68 ms. One-dimensional ^{1}H-NMR spectra were acquired on a Bruker AMX-400 NMR spectrometer operating at a frequency of 400.13 MHz (number of transients per spectrum, 128; presaturation time, 1 s; spectral width, 5,000 Hz). All spectra were processed with no line broadening and referenced internally to TSP (2,2,3,3,-tetradeutero-3-trimethylsilyl-1-propionate) at δ 0.00.

^{31}P-NMR spectra were obtained with the Bruker AMX-400 NMR spectrometer at 161.98 MHz in the Fourier transform mode, with the probe temperature maintained at 25°C. Spectra were generated with rapid pulsing by the method of Homer and Roberts (11). Other parameters were as follows: transient acquisition time, 0.5 s; number of transients per spectrum, 1,600 digitized into 16,384 points; spectral width, 2,000 Hz; pulse angle, 43°; duration, 11.5 μs; pulse delay, 1 ms. Samples were spun at 10 Hz, and spectra were processed with 1-Hz line broadening. Chemical shift data, δ, were expressed as parts per million. Resonances were assigned by comparison with methylenediphosphonic acid standards at δ 16.89 and confirmed by the addition of relevant authentic compounds.

Radiometric assays of phospholipase activity in culture supernatants. (i) Preparation of buffers and pH measurement. The effect of pH on phospholipase production was studied by determining enzyme activity with a citrate-sodium dihydrogen phosphate buffer (50 mM) from pH 3.0 to 7.5 and an imidazole-sodium acetate (50 mM) buffer from pH 3.0 to 8.0. The buffers were sterilized by filtration before use, and the final pH of the incubation mixture (buffer plus cryptococcal supernatant) was verified in all instances before incubation at 37°C. Preliminary experiments showed that the pH of the cryptococcal supernatant suspensions in the absence of buffer was 3.8.

(ii) Measurement of phospholipase activity. For the determination of PLB activity, the carrier DPPC (204 nmol; final concentration, 800 μM) and either 1,2-di[1-^{14}C]palmitoyl-PC (30,000 dpm) or dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)] PC (30,000 dpm) were dried under nitrogen and suspended in imidazole buffer (final pH, 4.5) by sonication. The reaction was initiated by the addition of culture supernatant (2 to 4 μg of total protein; final volume, 0.25 ml). After incubation at 37°C for 5 min to 3 h, the reaction was stopped by the addition of chloroform-methanol (see below). The rate of loss of radiolabelled PC substrates was measured. Lysophospholipase (LPL) activity was measured by the rate of loss of 1-[^{14}C]palmitoyl-lyso-PC. LPTA was estimated from the rate of production of labelled PC from 1-[^{14}C]palmitoyl-lyso-PC. Fifty nanomoles (final concentration, 200 μM) of carrier lyso-PC and radiolabelled lyso-PC (25,000 dpm) were suspended in imidazole buffer (final pH, 4.5), and enzyme solution was added to give a final volume of 0.25 ml. The reaction was carried out at 37°C for 15 s to 15 min and assayed at regular time intervals. Phospholipase activity in culture supernatants was also tested by use of different enzyme and substrate concentrations; the proportion of labelled to unlabelled substrate was maintained at a level constant during these experiments.

For all assays, the reactions were stopped by adding 1.0 ml of a chloroform-methanol solution (2:1, vol/vol), and the reaction products were extracted by the method of Bligh and Dyer (4). These were evaporated, and the residue was redissolved in a small volume of chloroform-methanol (6:1, vol/vol) and applied to a silica gel 60 thin-layer plate (Merck, Darmstadt, Germany) which was developed with chloroform-methanol-water (65:25:4, vol/vol/vol). Lipids were visualized by iodine staining and compared with authentic standards (DPPC, 1-palmitoyl-lyso-PC, palmitic acid, and phosphatidic acid). The silica gel areas corresponding to individual lipids were scraped off, and the radioactivity was measured in a scintillation counter. PLB and LPL activity were expressed as micromoles of substrate hydrolyzed per minute per milligram of protein.

Protein estimations. Protein concentrations were measured by use of the Bio-Rad protein assay kit (Bio-Rad, Ryde, New South Wales, Australia) with bovine serum albumin as the standard.

RESULTS

Estimation of cell lysis as a contributor to enzyme activity in culture supernatants. The initial cell count, as measured in a hemocytometer, of an aliquot of cryptococcal suspension in imidazole buffer was approximately 1.4×10^7 cells per ml. This remained constant throughout the time period of study. After 22.5 h of incubation, cell numbers averaged 1.76×10^7 cells per ml; cells were noted to be intact at each time point, with budding of cryptococci observed at 22.5 h. There was no evidence of cell lysis on microscopy. PLB activity in aliquots of centrifuged culture supernatants was minimal at zero time and near maximal after 4 h (approximately 60% of substrate PC hydrolyzed). Approximately 67% of substrate PC was hydrolyzed at 10 h, after which there was no further increase (Fig. 1). The results indicate that a cryptococcal phospholipase(s) was secreted into the supernatant. The extracellular nature of the enzyme activities is supported by the observation of white zones of precipitate diffusing out from cryptococcal colonies on egg yolk agar with increasing time (7). Results similar to those shown in Fig. 1 were obtained when cryptococci were harvested, suspended, and incubated in YNB broth containing 0.5% glucose, indicating that nutritional insufficiency was not a prerequisite for phospholipase secretion.

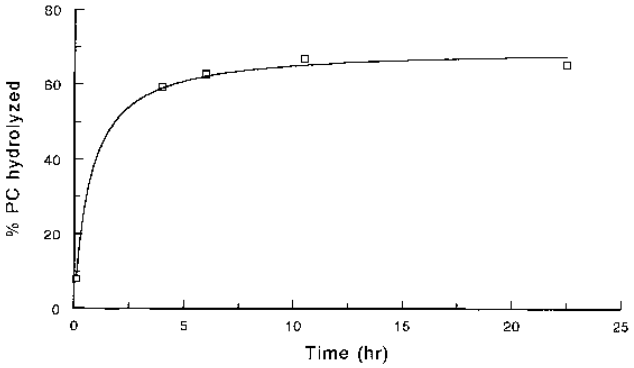

FIG. 1. PLB activity in culture supernatants of *C. neoformans* obtained from a suspension of cryptococcal cells in imidazole buffer incubated at 37°C for 0, 4, 6, 10.5, and 22.5 h. Results are expressed as the percentage of substrate PC hydrolyzed.

Identification of PLB activity in cell suspensions and cell-free supernatants by NMR spectroscopy. ^{1}H-NMR spectra acquired from cryptococcal cell suspensions incubated with egg yolk PC for up to 21 h are shown in Fig. 2A. A progressive increase in resonance intensity at δ 3.216 was observed, indicating the formation of glycerophosphocholine (GPC). No spectral changes were noted in cell suspensions incubated in the presence of buffer alone. In addition, incubation of PC in the absence of cells or culture supernatants showed that spontaneous breakdown of substrate had not occurred.

The findings by ^{1}H-NMR spectroscopy were confirmed by ^{31}P-NMR spectroscopy of cryptococcal cell suspensions after the addition of egg yolk PC. Figure 2B demonstrates an increase in the resonance at δ 0.53 corresponding to the presence of GPC after 24 h of incubation. In the spectra resulting from the presence of PC, this phospholipid appeared as a broad resonance (δ 0.2), rendering it undetectable above the background spectrum. This is a result of the short T_2 (transverse relaxation time) value of PC, which in turn is due to its relatively large size. Any decrease in PC accompanying the increase in GPC thus could not be observed. Figure 2C illustrates the spectra obtained after 24 h of incubation of culture supernatants with PC in the absence of cells. The large resonance arising from the formation of GPC indicates the presence of extracellular PLB or of both phospholipase A_1 (PLA_1) and PLA_2. The absence of detectable lyso-PC in any of the NMR spectra supports the former possibility. In no experiment was choline phosphate, indicative of phospholipase C (PLC) activity, nor free choline, indicative of phospholipase D (PLD) activity, detected under the conditions of assay.

Identification of secreted PLB, LPL, and LPTA by radiometric analysis. Assay of culture supernatants for the activities of specific phospholipases showed that the isolate BL-1 possessed PLB, LPL, and LPTA activities (see below).

(i) Effect of enzyme concentration on phospholipase activity. Hydrolysis of the substrate DPPC, labelled in the 2-acyl position, was accompanied by the release of radiolabelled free fatty acid. This was due to the presence of PLB since no radiolabelled lyso-PC was detected at either low or high concentrations of enzyme. The reaction velocity, estimated by the loss of radioactivity from the substrate DPPC, was linear ($r^2 = 0.98$) with respect to mass of protein (up to 3.5 μg) until approximately 56.2% of the substrate was hydrolyzed (Fig. 3A). Maximal hydrolysis of DPPC (approximately 75%) was achieved after incubation with 12 to 15 μg of protein, after which no further increase occurred.

To check that both acyl chains were removed by the enzyme, we also investigated the hydrolysis of DPPC radiolabelled in both acyl chains. The kinetics were similar to those of the substrate labelled in the 2-acyl position (the rate was linear up to 4 μg of protein, with a maximum of 50% substrate hydrolysis; data not shown). Again, no radiolabelled lyso-PC was detected, confirming the activity as PLB. In all experiments, the total radioactivity recovered from the TLC plates was 90 to 95% of that added to the reaction mixture. Labelled products comigrating with either authentic diglyceride or phosphatidic acid were not detected, excluding PLC or PLD activity.

LPL activity of *C. neoformans* was measured by the rate of hydrolysis of 1-[^{14}C]palmitoyl-2-lyso-PC (Fig. 3B). Both radiolabelled free fatty acid (hydrolase activity) and radiolabelled

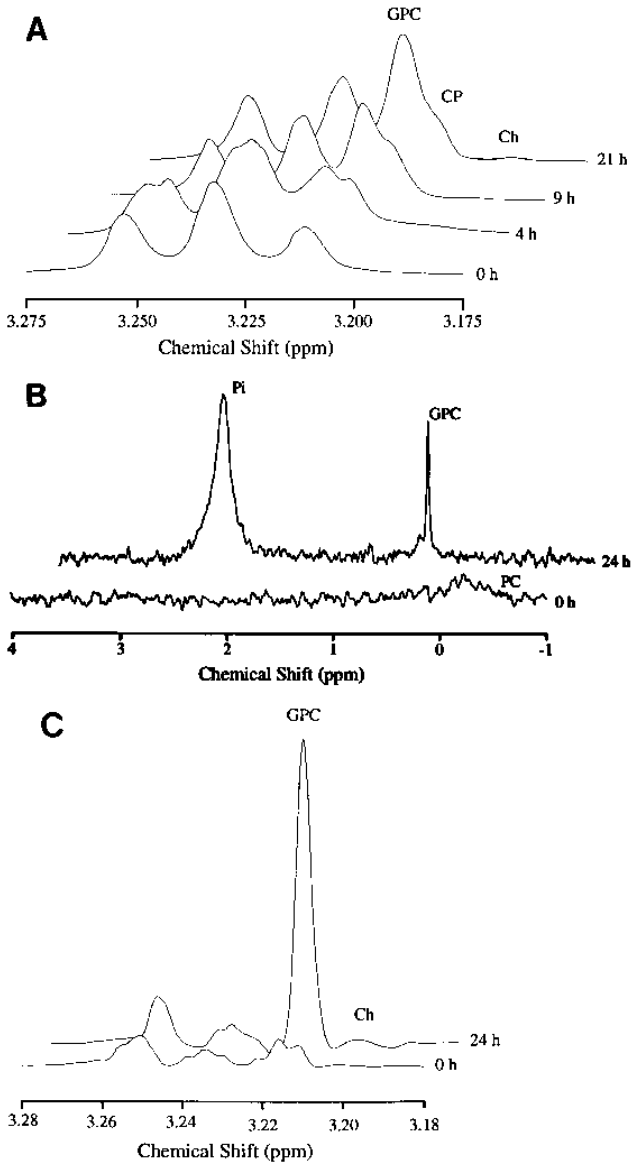

FIG. 2. (A) One-dimensional ^{1}H-NMR spectra of a cryptococcal cell suspension incubated for 0, 4, 9, and 21 h at 37°C with egg yolk PC. (B) ^{31}P-NMR spectra illustrating formation of GPC after incubation of a cryptococcal cell suspension with egg yolk PC for 24 h at 37°C. (C) Detection of GPC by ^{1}H spin echo NMR after incubation of cryptococcal culture supernatant with egg yolk PC for 24 h at 37°C. CP, choline phosphate; Ch, free choline; Pi, inorganic phosphate.

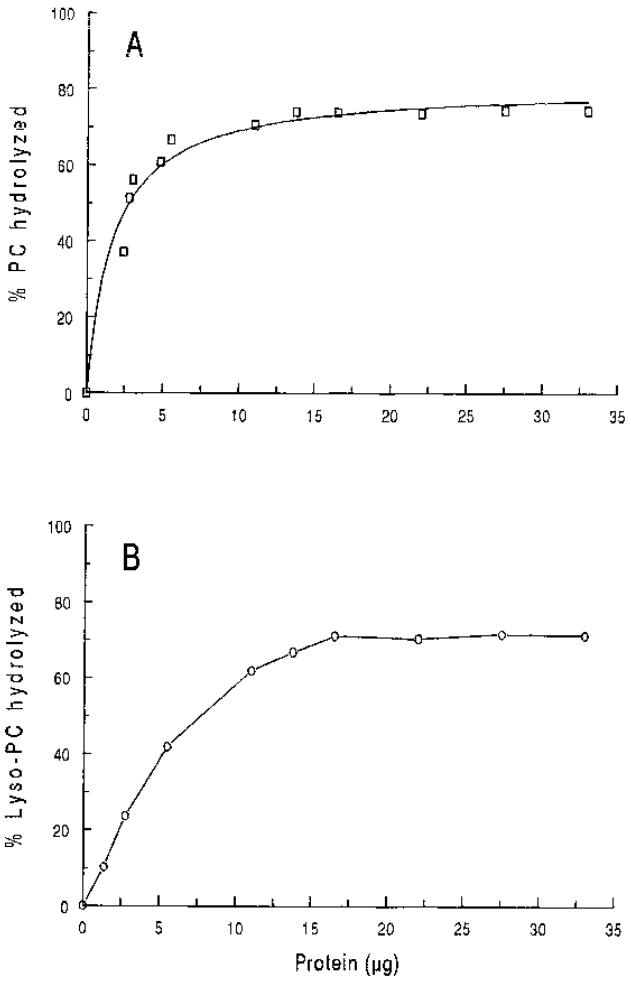

FIG. 3. (A) Effect of protein (enzyme) concentration on PLB activity in cryptococcal culture supernatants after incubation with the carrier DPPC and dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]PC. Results are expressed as the percentage of PC hydrolyzed. Each point represents the mean from duplicate experiments, with the range varying from ±2.4 to ±6.3. (B) Effect of protein (enzyme) concentration on LPL (hydrolase) activity in cryptococcal culture supernatants after incubation with carrier 1-palmitoyl-lyso-PC and 1-[1-^{14}C]palmitoyl-2-lyso-PC. Results are expressed as the percentage of substrate lyso-PC hydrolyzed. Each point represents the mean from triplicate experiments, with standard deviations ranging from ±0.5 to ±10.3.

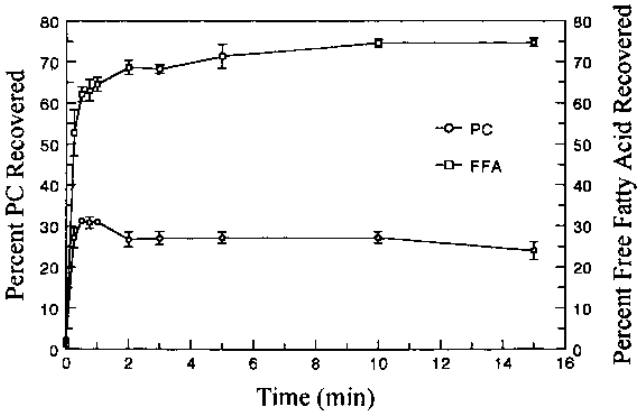

FIG. 4. Time course of the relative production of radiolabelled fatty acid (LPL activity) and radiolabelled PC (LPTA activity) after incubation of cryptococcal supernatants with carrier 1-palmitoyl-lyso-PC and 1-[1-^{14}C]palmitoyl-2-lyso-PC. Results are expressed as the percentage of total radioactivity recovered in the PC and fatty acid fractions (FFA). Each point represents the mean ± standard deviation values from triplicate experiments.

DPPC (LPTA activity) were detected (Fig. 4). The reaction velocity was linear ($r^2 = 0.959$) with respect to mass of protein (up to 11 μg), until approximately 61% of the substrate was hydrolyzed.

Control samples without enzyme or in which enzyme was exposed to temperatures of 100°C (5 min) or 60°C (10 min) demonstrated no PLB or LPL activities. Supernatants which had been stored at 4, −20, and −70°C for a maximum of 14 days retained full enzyme activity despite the acidity of the enzyme preparation. Results were independent of whether phospholipase activity was measured in culture supernatants, cell suspensions, or agar extracts. Lower specific activities were evident with agar extracts, which were also more time-consuming to prepare. Culture supernatants were studied in subsequent experiments. No detectable differences in enzyme activities were noted when the cells were grown, and assays were performed, in the presence of the serine protease inhibitors leupeptin and phenylmethylsulfonyl fluoride.

(ii) Time course of enzyme activity. Progressive hydrolysis of DPPC, labelled in the 2-acyl position, occurred over a period of 3 h with a mean loss of 60% radioactivity at 30 min. The rate of hydrolysis was linear from 5 to 15 min (50% loss of radioactivity), after which it remained constant for up to 3 h (data not shown). Hydrolysis of DPPC, labelled in both acyl chains, occurred more slowly; the rate was linear for 22 min, with only 35 to 40% maximal substrate degradation achieved after 45 min of incubation, suggesting that cleavage of both acyl fatty acid chains did not occur at the same time. Similar observations were made by Banno et al. in their study of the hydrolysis of radiolabelled substrate PC by *C. albicans* phospholipases (2).

LPL activity was rapid, with most substrate degraded within the first minute (80% of radiolabelled lyso-PC hydrolyzed) (Fig. 4); the rate of hydrolysis was linear only to about 15 s. Formation of DPPC from 1-acyl-2-lyso-PC (acyltransferase activity) was a relatively minor activity catalyzed by culture supernatants, as compared with the LPL activity (Fig. 4); most of the ^{14}C-labelled product comigrated with palmitic acid, with only approximately 20% comigrating with DPPC. Since DPPC would be hydrolyzed by PLB as it is forming, its rate of production from lyso-PC could not be measured accurately by this method.

(iii) Enzyme activity versus substrate concentration. By use of enzyme concentrations and assay times over the linear ranges established above, the phospholipase activities were studied as a function of substrate concentration.

When the carrier DPPC and dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]PC were used, a bimodal response in the rate of PLB activity with increasing substrate concentration was observed (Fig. 5). The approximate K_m for the first step of the curve was about 100 μM. Hydrolysis of PC increased substantially at substrate concentrations higher than 400 μM; the approximate K_m for this part of the curve was about 360 μM. A maximal rate (approximately 52% of substrate hydrolyzed) was achieved at a substrate concentration of 700 μM, above which the response appeared to be saturated.

The initial velocities of both LPL and LPTA activities, measured at 15 s of incubation, peaked at approximately 200 μM lyso-PC. At concentrations above 200 μM, the rates of both enzyme activities declined significantly (Fig. 5). The approximate K_m for hydrolase activity was 100 μM. Although the ratio

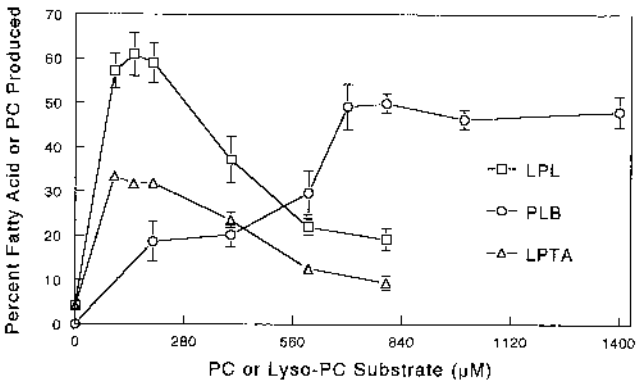

FIG. 5. Effects of substrate concentration on PLB, LPL, and LPTA activities. Each point represents the mean ± standard deviation values. Symbols: △, LPTA activity (percent PC formed); □, LPL (hydrolase) activity (percent fatty acid formed); ○, PLB activity (percent fatty acid formed).

of hydrolase to transacylase activity varied, in all instances, the hydrolase activity predominated (Fig. 5).

(iv) pH requirements. Figure 6 illustrates that the pH optima for fatty acid release by PLB were pH 3.5 and pH 4.5 when tested in the citrate-phosphate and imidazole-acetate buffer systems, respectively. Enzyme activity in the imidazole-acetate buffer fell sharply below pH 4.0 and at pH values of greater than 5. Similar pH optima were demonstrated for LPL (hydrolase) and LPTA activities, with maximum substrate hydrolysis occurring between pH 4.0 and pH 5.0 (data not shown). The ratio of hydrolase to transacylase activities remained constant at approximately 2.71 over the pH range tested.

(v) Cation requirements. Culture supernatants that were prepared and assayed in the absence of exogenous Ca^{2+} and/or Mg^{2+} indicated that neither was essential for PLB, LPL, or LPTA activity. In addition, enzyme activities were independent of Zn^{2+} and of added EDTA (2 mM).

(vi) Comparison of specific enzyme activities. The specific enzyme activities for PLB determined at pH 4.5 in imidazole buffer were 3.2 ± 0.2 μmol/min per mg of protein (measured at 15 min) and 1.8 ± 0.3 μmol/min per mg of protein (measured at 22 min) where dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]-PC and 1,2-di[1-^{14}C]palmitoyl-PC were used, respectively. In both instances, a concentration of 800 μM substrate PC was chosen for the estimation of PLB activity. These parameters were selected to provide optimal amounts of substrate, and PLB was determined at a time when the rate of hydrolysis of PC was linear. The specific activity of LPL, measured as loss of substrate lyso-PC at 15 to 30 s in imidazole buffer (pH 4.5), was 34.9 ± 7.9 μmol/min per mg of protein. The data also indicate that the substrate concentration of 200 μM lyso-PC used for the estimation of the specific activity of LPL was appropriate. The specific activity of this enzyme component was approximately 10 to 20 times higher than that of PLB.

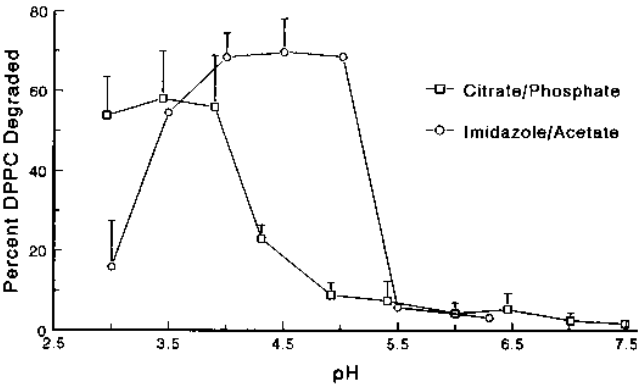

FIG. 6. Effects of pH on degradation of substrate 1,2-di[1-^{14}C]palmitoyl-PC by PLB.

DISCUSSION

This study is the first to characterize extracellular phospholipase production by *C. neoformans*. By NMR spectroscopy and radiometric analysis, we identified PLB, LPL, and LPTA activities in culture supernatants from a clinical isolate of *C. neoformans* var. *neoformans*. Under native conditions, the LPL component had the highest specific activity. Neither PLC, PLD, nor other lipase activity was detected by radiometric analysis or by NMR spectroscopy. We have previously been unable to detect PLA_2 activity as determined by inoculation of the organism onto egg yolk agar (7). Although extracellular phospholipases have been demonstrated in a number of fungi (12, 26, 30), phospholipase activity in *C. neoformans* has not been reported by other investigators. Secretion of other fungal phospholipases has been reported to occur in response to carbohydrate deprivation (21, 28), in contrast to the cryptococcal enzymes which are secreted in both complete yeast growth medium and a simple buffer containing isotonic saline and glucose.

LPL activity produced by *C. neoformans* was 10 to 20 times greater than PLB activity. This contrasts with reports of *P. notatum* phospholipases, in which PLB and LPL activities (1.54 μmol of phosphorus liberated per min per mg of protein) were comparable, when similar amounts of enzyme were used (1 to 4 μg), prior to purification (13, 26). PLB activity of *P. notatum* decreased substantially following the initial stage of purification of the enzyme(s) as a result of the action of endogenous proteinases and through the concentration process, with preservation of LPL activity (13, 26), emphasizing the importance of comparing extracellular enzyme activities in the native state in studies of putative virulence characteristics. Purified forms of *Saccharomyces cerevisiae* phospholipase also display PLB activity, at only 1 to 3% of the level of LPL activity (17, 30). Cryptococcal phospholipase activities were independent of added serine protease inhibitors.

In a study of ammonium sulfate-precipitated culture filtrates of *C. albicans*, LPTA specific activity (293 nmol/ml/min) was observed to be 70-fold greater than that of PLB (4.5 nmol/ml/min), and LPL (hydrolase) activity was minimal (12). However, enzyme activities were not expressed with reference to the protein content, and therefore, direct comparisons with the results of others cannot be made. Of note, although PLB and LPL activities in culture supernatants of *C. neoformans* appeared to be substantially greater than that described for relatively unpurified enzyme extracts of *C. albicans* (12), in both *C. neoformans* and *C. albicans*, these levels of phospholipase production correlated with virulence in the mouse model (7, 12). Secreted phospholipase activity in *C. neoformans* also appears to be greater than that reported for unfractionated cellular extracts of *Candida* spp. (range, 4.0 ± 0.45 to 33 ± 1.4 pmol/mg/h) (3).

Our data indicate that LPL (hydrolase) activity in *C. neoformans* was greater than LPTA activity; both LPL and LPTA activities in crude enzyme preparations were inhibited at concentrations greater than 200 μM, and hydrolase activity was

consistently higher than that of transacylase. The apparent reduction in hydrolase activity at higher concentrations of the substrate lyso-PC could be a result of end product or substrate inhibition, while the reduction of DPPC formation, at substrate lyso-PC concentrations of around 400 μM, may be explained by degradation of formed DPPC by PLB.

The bimodal response in cryptococcal PLB activity observed when the concentration of substrate PC was varied raises several possibilities; for example, there may be high- and low-affinity binding sites on the one enzyme molecule, as has been proposed for the PLB of *P. notatum* (25). Alternatively, there may be two forms of PLB produced by *C. neoformans*—one with a small and one with a large Michaelis constant; in *S. cerevisiae*, two cell-associated forms and an additional secretory form of PLB have been described (30). However, the substrates lyso-PC and DPPC, as well as their free fatty acid products, act as detergents in aqueous media, and altering their concentrations may affect their physical forms. For example, monomers may aggregate to form micelles, or the proportions of vesicles and mixed micelles may vary, with consequent effects on enzyme activity. Detergents such as Triton X-100 have been reported to stimulate PLB and to inhibit LPL in *P. notatum* (26). Detergent effects of substrates and/or reaction products could therefore also account for the results of increasing the substrate concentrations on the enzyme activities in Fig. 4.

Phospholipase activity in *C. neoformans* was not influenced by exogenous divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}) or EDTA, consistent with observations for phospholipases produced by other yeasts (19, 26). Cryptococcal phospholipases in their native state were not significantly inhibited by exogenous serine proteinases, in contrast to the enzyme(s) of *P. notatum* (26). Activity in *C. neoformans* was maximal at pH 3.5 to 4.5, compared with a pH optimum of 6.0 for one of the described forms of *C. albicans* LPTA and with pH optima of 4.5 to 5.0 for *P. notatum* phospholipases (13, 26). We recently demonstrated, by ^{1}H-NMR spectroscopy, that large amounts of acetate are produced by cryptococci in broth culture (31). If this also occurs in vivo, an acidic microenvironment favorable to cryptococcal phospholipase activity would ensue. The stability of cryptococcal phospholipases at a low pH (3.8) also contrasts with those described for *P. notatum* (26).

The utility of ^{1}H- and ^{31}P-NMR spectroscopy for the detection of products of phospholipase activity is demonstrated in the study. Characteristics of the carbon backbone of the phospholipid (GPC in the case of PLB) were adequately identified by NMR, which is an alternative to more time-consuming methods such as the assay of glycerophosphate esters (29). The presence of GPC as the only degradation product of PC, without any evidence of choline, choline phosphate, or lyso-PC production, excluded PLC, PLD, and independent PLA_1 and PLA_2 activities and was confirmed by TLC, where only radiolabelled free fatty acid was formed from substrate PC. Although choline phosphate, GPC, and choline all have *N-methyl*-^{1}H resonances in the chemical shift region δ 3.1 to 3.3, they are sufficiently well resolved to be distinguished by ^{1}H NMR (1). In addition, these compounds have easily identifiable chemical shifts on the ^{31}P-NMR spectrum (24). Kinetic studies and characterization of LPL activities by NMR are more difficult because of the high rate of the enzyme reactions.

In conclusion, we have demonstrated that significant amounts of PLB, LPL, and LPTA are produced by *C. neoformans* and have developed optimal assay conditions which allow comparison of these activities with those from other organisms. The enzymes are secreted into the growth medium, have high specific activity in the native state, and exhibit some characteristics which differ from phospholipases secreted by other fungi. Purification and further characterization of these enzymes are in progress to help clarify their role in the pathogenesis of infections due to *C. neoformans* and to determine if they are part of one protein complex or exist as separate entities.

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