A Comparison of Phospholipase Activity, Cellular Adherence and Pathogenicity of Yeasts

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Phospholipase A and lysophospholipase activities were measured in the culture fluid and in the blastospores of Candida albicans. When phospholipase activity was measured in six yeasts (four strains of C. albicans and a single strain each of Candida parapsilosis and Saccharomyces cerevisiae) a correlation was found between this activity and two potential parameters of pathogenicity. The C. albicans isolates which adhered most strongly to buccal epithelial cells and were most pathogenic in mice had the highest phospholipase activities. Non-pathogenic yeasts, including C. albicans isolates which did not adhere and did not kill mice, had lower phospholipase activities.

INTRODUCTION

Phospholipases, which degrade phospholipids, are common in all forms of life. They are frequently found associated with cell membranes and membrane-bound vesicles, in the structure and function of which they are believed to play a major role (see Van den Bosch, 1980). Since the products of phospholipase A and B activity, lysophospholipids, are readily able to lyse biological membranes, it is not surprising that phospholipases are active components of many bacterial toxins, arthropod poisons and snake venoms (Dennis, 1983).

Phospholipases have been found in many fungi, and it has been suggested that they play a role in pathogenicity. Phospholipase activity was first demonstrated in C. albicans by Costa et al. (1968). Subsequently Price & Cawson (1977) demonstrated the presence of phospholipase A and lysophospholipase activity in C. albicans cell extracts. In liquid cultures production of phospholipase and lysophospholipase activities was found to be associated with bud formation in rapidly growing C. albicans. This finding suggests the involvement of phospholipase in growth and recsculpturing of cell membranes during the development of yeasts. In stationary phase cultures phospholipase was localized at the cell periphery and was secreted into the surrounding medium (Pugh & Cawson, 1975). Samaranayake et al. (1984) have recently shown that in agar-grown C. albicans, phospholipase production takes place only within a limited pH range of 3.6–4.7.

Pugh & Cawson (1976), using cytochemical techniques have also localized phospholipase A and lysophospholipase activities in blastospores and hyphae of C. albicans infecting chick chorioallantoic membranes. During tissue invasion, a path was cleared by yeast cells with high levels of phospholipase activity which underwent autolysis in the process. Other cells followed and proceeded to invade and colonize the tissues with the production of hyphae. Very high enzyme activities were demonstrated at the hyphal tip where it was in contact with the host cytoplasm.

In this study we have investigated and measured the amounts of phospholipase activity in different isolates of C. albicans and attempted a correlation with known parameters of pathogenicity.
METHODS

Yeast isolates and culture conditions. Candida parapsilosis and Saccharomyces cerevisiae were obtained from Dr E. G. V. Evans, Regional Mycology Laboratory, Leeds General Infirmary, C. albicans B2630 from Janssen Pharmaceutica, Beerse, Belgium, and C. albicans A (NCPF 3153) from Professor D. W. R. Mackenzie, Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine. C. albicans N3 and N6 were obtained from the mouth of an asymptomatic carrier. Organisms were maintained on Sabouraud's dextrose agar slopes containing 10 g Mycological Peptone (Oxoid) 1⁻¹ and 40 g glucose 1⁻¹; they were subcultured bi-weekly. For liquid cultures, a slope was washed with water, and 1 litre of Sabouraud's dextrose broth was inoculated with 10⁶ cells ml⁻¹. Cultures were incubated in 2 litre conical flasks in an orbital shaker at 37 °C (30 °C for S. cerevisiae) and 200 r.p.m. for 16 h for S. cerevisiae, by which time they were in mid-exponential phase. All yeasts were used in this phase of growth.

Enzyme preparation. Cultures were harvested by centrifuging at 1000 g for 10 min at 4 °C, and the cell-free supernatant was removed. The yeast pellet was then washed twice with cold water and resuspended in buffer solution (50 mm-phosphate buffer pH 7). The cells were broken by shaking with glass beads (0.4 mm; 50%, by vol.) in a Braun disintegrator (Braun, Melsungen, FRG) for 3 min. The preparation was then centrifuged at 2000 g for 20 min at 4 °C. The resultant pellet was termed the 2000 g pellet and was resuspended in an equal weight of buffer. The pellet and supernatant fractions were stored at −20 °C until required; during this storage the activity did not decline. The initial culture medium was dialysed against many changes of water for 48 h, and then concentrated 400-fold by dialysing against polyethylene glycol 4000 (30% w/v). The final concentrate was stored at −20 °C.

Enzyme assay conditions. Phospholipid or lysophospholipid substrate was prepared by sonicating 1,2-phosphatidylcholine, dipalmitoyl (Sigma) together with L-α-phosphatidylcholine, dipalmitoyl (Amersham) in buffer in a closed vessel for 10 min to give a final specific activity of 0.02 μCi mol⁻¹ (74 kBq mol⁻¹). For lysophospholipase assays, similar substrates were prepared using radiolabelled lysolecithin. For routine assays, the enzyme preparation (0.07 ml) was incubated with substrate suspension (0.07 ml) in a plastic tube. The reaction was stopped by addition of 0.56 ml of cold chloroform/methanol (2:1, v/v) followed by shaking. The chloroform layer contained any unreacted phospholipid plus any products of hydrolysis. The chloroform extract was applied to a silica gel TLC plate (Polygram Sil N-HR/UV254) and the plate eluted with chloroform/methanol/glacial acetic/water (65:25:2:2, by vol.). The position of compounds on the plate was visualized by using the spray reagents Oil Red O and Zinzadze reagent (Dittman & Lester, 1964). The nature of the compounds was determined by comparison with authentic standards (dipalmitoyl lecithin, palmitoyl lysolecithin, palmitic acid, phosphatidic acid, diglyceride, choline and phosphorylcholine). The bands were then cut from the plate and radioactivity was counted in PCS scintillant (Amersham) in a liquid scintillation counter.

Silicic acid extraction method. The reaction mixture was extracted with chloroform/methanol as before, the extracts were dried in glass tubes under vacuum and the resulting residues resuspended in 1 ml chloroform containing palmitic acid (10 g l⁻¹). Freshly activated silicic acid (0.1 g) was added to the solution and mixed in. The silicic acid was sedimented in a bench centrifuge and 0.7 ml of the supernatant fluid removed to scintillation vials and evaporated to dryness under vacuum. The residue was dissolved in toluene (1 ml) plus PCS scintillant (3 ml) and the radioactivity was determined by liquid scintillation counting.

Adherence to buccal cells. The adherence assay was similar to that of Kimura & Pearsall (1978). Briefly, C. albicans yeast cells (10⁶ in 1 ml) and human buccal epithelial cells (10⁴ in 1 ml), obtained by gently scraping the inside of donor cheeks, were incubated together for 50 min at 37 °C in 0·1 M-phosphate buffer pH 7·2. After incubation the suspensions were filtered through 12 μm polycarbonate filters (Nucleopore Corp., Pleasanton, Calif., USA) and the filters stained with crystal violet. The filters were examined microscopically and the number of yeast cells adhering to epithelial cells was counted; the results were expressed as adhering yeast cells per 100 buccal cells together with standard deviations. The variability in the measurement of adhering yeast cells was in keeping with that found by some other workers (see Kearns et al., 1983).

Mouse virulence test. The pathogenicity of yeasts in mice was determined by injecting 10⁶ agar-grown yeast cells in 0·2 ml saline intravenously into mice (30 g, Alderley Park strain). Mortality was recorded daily and results were expressed as the percentage mortality by day 21.

RESULTS AND DISCUSSION

Initial enzyme studies. Initial attempts to detect phospholipase activity in broken cells of C. albicans B2630 by incubating enzyme preparations with dipalmitoyl lecithin radiolabelled in the choline moiety, and separating the products by thin layer chromatography, failed to show a build up of lysolecithin, even when the substrate concentration decreased with time. Using the same substrate, but commercial pancreatic phospholipase, a buildup of lysolecithin was observed with time. The reason for this discrepancy was found to be the presence in the cell
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The 2000 g pellet material was diluted in assay buffer and incubations continued for 1 h at 37 °C, pH 4.5, using the appropriate substrates (lecithin and lysolecithin, 5 mg ml⁻¹). Results are expressed as the percentage of initial radioactivity not adhering to the silicic acid.

Fig. 2. Comparison of the pH dependence of (a) phospholipase activity and (b) lysophospholipase activity. O, Extracellular enzyme activity; ●, intracellular (2000 g pellet) activity. Activity is defined as the percentage of total radioactivity released from phospholipid. Initial substrate concentrations 5 mg ml⁻¹. Incubation 37 °C for 1 h.

lysat e of a lysophospholipase. This enzyme completed the hydrolysis of lysolecithin to fatty acids and glyceryl choline.

Using phospholipid radiolabelled in the acyl moiety it was not possible to detect any radiolabelled diglyceride, indicating that phospholipase C activity was very low in the broken cell preparations. Similarly no phosphatidic acid was detected, indicating that there was little phospholipase D activity.

In order to circumvent the problem imposed by the presence of large amounts of lysophospholipase activity in the assay of phospholipase A (see Fig. 1), a technique was developed which utilized the adherence of phospholipids to silicic acid, whilst free fatty acids remained in solution. The presence of excess lysophospholipase doubled the amount of fatty acids released. Lysophospholipase activity was measured using lysolecithin as substrate.

Optimization of phospholipase assay conditions. The amount of enzyme preparation was varied and the activity of phospholipase A and lysophospholipase was measured (Fig. 1). Clearly in this preparation the lysophospholipase activity was much greater than the phospholipase A activity. Hence much lower enzyme concentrations had to be used for the measurement of lysophospholipase activity. In subsequent experiments, the amount of enzyme preparation was adjusted to give less than 10% conversion of substrate, so that the rate of hydrolysis was linear with time.

Effect of incubation temperature. The temperature optimum for the phospholipase activity for external and internal enzymes was 55 °C. Lysophospholipase had an optimum of 40 °C for the internal enzyme and 55 °C for the external enzyme. All enzyme activities were lost as the temperature approached 70 °C. This high temperature optimum is common amongst small hydrolytic enzymes, e.g. the chitinase of C. albicans (Barrett-Bee & Hamilton, 1984).
Table 1. Correlation of phospholipase activity with measures of pathogenicity in yeasts

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Phospholipase activity (pmol h(^{-1}) mg(^{-1}))</th>
<th>Mortality of mice*</th>
<th>Adhering yeast cells per 100 buccal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>3.7 ± 0.38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>4.0 ± 0.45</td>
<td>0</td>
<td>13.6 ± 10</td>
</tr>
<tr>
<td>C. albicans N3</td>
<td>4.7 ± 0.29</td>
<td>2</td>
<td>117 ± 68</td>
</tr>
<tr>
<td>C. albicans N6</td>
<td>20.8 ± 3.2</td>
<td>8</td>
<td>1002 ± 294</td>
</tr>
<tr>
<td>C. albicans A</td>
<td>33 ± 1.4</td>
<td>16</td>
<td>1174 ± 544</td>
</tr>
<tr>
<td>C. albicans B2630</td>
<td>29.7 ± 0.85</td>
<td>18</td>
<td>386 ± 138</td>
</tr>
</tbody>
</table>

* No. out of 20 that had died after 21 d.

Effect of pH. Intracellular phospholipase had a fairly sharp optimum at pH 4.2 whilst the external enzyme had a broader optimum (Fig. 2a) – which is what might be expected from an enzyme whose role was to act on host cells nearer to neutrality. Internal lysophospholipase showed a similar pH-dependence profile to phospholipase, but the pH optimum of the external lysophospholipase activity was very broad (Fig. 2b).

Variation of substrate concentration. Plotting the reciprocal of the enzyme velocity against the reciprocal of the substrate concentration gave linear plots, indicative of Michaelis–Menten kinetics, for all the enzyme activities. The apparent \( K_m \) varied from one substrate batch to another, which is what would be expected, since the sonicated liposome substrate may not always be identical in terms of vesicle size and hence surface area. Sarda & Desnuelle (1954) have shown with pancreatic lipase that enzyme activity is proportional to the surface area of micelles in ester emulsions; similar results have also been obtained with C. albicans chitinase preparations (Barrett-Bee & Hamilton, 1984).

Distribution of enzyme activities. In liquid culture 40% of the phospholipase activity was free in the medium whilst 29% was present in the 2000 g pellet and 31% in the supernatant. The distribution of lysophospholipase in the medium, the pellet and the supernatant was 0.7%, 50% and 49.3% respectively, implying that lysophospholipase was essentially intracellular. We propose that these distributions represent a defence mechanism. Within the cell the need for lysophospholipase may be high; any nascent lysophospholipids released by phospholipase activity during membrane remodelling or membrane fusion could be deleterious to the cell, since they may damage cell membranes. Outside the cell the need for lysophospholipase activity is minimal. If phospholipase activity is indeed essential for the invasion of host cells, its products, lysophospholipids, would be the damaging agents and there would hence be no requirement for lysophospholipase to remove them.

Correlation of phospholipase activity with pathogenicity. Having determined optimum conditions for the assay of phospholipase A, we performed experiments to measure the activity in several strains and species of yeast; the adhesion of the same yeasts to buccal epithelial cells and their ability to kill mice following intravenous inoculation were also measured (Table 1). There was a correlation between phospholipase activity, pathogenicity and adherence to epithelial cells. S. cerevisiae and C. parapsilosis showed low phospholipase activity and low adherence and were non-pathogenic. Of the two isolates of C. albicans obtained from a single individual, N3 showed low enzyme activity, mild pathogenicity and low adherence, whilst N6 showed much greater enzyme activity along with moderate pathogenicity and high adherence. The two remaining pathogenic isolates of C. albicans showed high enzyme activity and good adherence. These observations support the earlier suggestion (Pugh & Cawson, 1977) that phospholipases are important in the pathogenicity of C. albicans.
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REFERENCES


