EFFECTS OF DIMORPHISM ON PHOSPHOLIPASE PRODUCTION
IN CANDIDA ALBICANS

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INTRODUCTION

Phospholipases are enzymes that catalyze the hydrolysis of phosphoglycerides, which are major constituents of the cell membrane. These enzymes have been detected in animals, plants, fungi, and bacteria. The phospholipases are distinguished from one another by the site of action on the phosphoglyceride molecule (see diagram below).

The two forms of phospholipase A, phospholipase A₁ and A₂, catalyze the hydrolysis of diacylphosphoglycerides by hydrolyzing one of two ester bonds to form fatty acids. The A type of phospholipases (A₁ and A₂) are active components of snake venoms, arthropod poisons, and bacterial toxins, all of which kill or injure their victims (Kates, 1960; Dawson, 1962). These enzymes have also been found in some fungi such as the dimorphic fungus Candida albicans. In C. albicans enzyme activity is associated with the cell surface of yeast cells (blastospores) as well as hyphae, but it is concentrated on the growing tip of hyphal cells (Pugh and Cawson, 1975). The phospholipase(s) secreted by
C. albicans, which is an extracellular enzyme, could play a part in the invasion of host tissues (in lesions of candidiasis) by helping to break down the cell membrane thus facilitating access to deeper tissues. C. albicans is believed to be more virulent in the hyphal phase than in the yeast phase. This might be the result of differences in phospholipase activity.

A modified Sabouraud Dextrose agar (SDA), containing 8% sterile egg yolk, has been developed to indicate presence and relative amount of extracellular phospholipase A activity in C. albicans (Price, et al., 1982). This report describes use of this and other techniques in an attempt to correlate phospholipase production and/or amount produced with the cell type (yeast or hyphae) exhibited by the fungus.
MATERIALS AND METHODS

Isolates

Ten isolates of C. albicans were used. Eight were obtained from Ball Memorial Hospital, Muncie, Indiana, and the other two were obtained from Margaret F. Price of the Baylor College of Medicine, Houston, Texas. The stock cultures were maintained on SDA slants at 5°C and were transferred every eight weeks to fresh slants.

Medium

The cultures were grown in a liquid medium designed to allow growth of both forms of the fungus (Lee, et al., 1975). The medium contained four salts \((\text{NH}_4)_2\text{SO}_4, 2.5 \text{ g}, \text{MgSO}_4\cdot7\text{H}_2\text{O}, 0.1 \text{ g}, \text{K}_2\text{HPO}_4\text{(anhydrous)}, 1.25 \text{ g}, \text{ and NaCl, 2.5 g})\), dextrose, 6.25 g, and seven L-amino acids (alanine, 0.25 g, leucine, 0.65 g, lysine, 0.5 g, ornithine, 0.0357 g, phenylalanine, 0.25 g, proline, 0.25 g, and threonine, 0.25 g), all dissolved in 500 ml of deionized H\(_2\)O. The pH of the mixture was found to be 6.8±0.5. This was sterilized in an autoclave for fifteen minutes prior to use.

Stock solutions of biotin (100 micrograms/ml), albumin (20 mg/ml), and methionine (2.5 mg/ml) were all used. Since these cannot be autoclaved, each was dissolved in H\(_2\)O and filter sterilized.

Dimorphism

The cultures were each transferred from the SDA slants to a sterile 250 ml Erlenmeyer flask containing 25 ml of Lee’s medium enriched with biotin (1.00 microgram/ml) from the stock solution and put in a rotary water bath at 25°C for 24 hours. After this
growth period the starter cultures were checked and found, in all cases, to contain 100% yeast growth (blastospores). A haemocytometer was used to obtain a cell count. Cells were counted as cell units, which consisted of cells plus buds (if buds were present) or individual cells if buds were not present. Then $10^6$ cell units/ml were collected on a sterile membrane filter and transferred to a sterile 125 ml Erlenmeyer flask containing 25 ml of Lee's medium enriched with biotin (1.00 microgram/ml) and albumin (2.00 mg/ml) from stock solutions. These cultures were grown for three to four hours at 37°C in a rotary water bath.

During the growth period, small samples were collected with sterile Pasteur pipettes, placed on a haemocytometer, and observed microscopically for germ tube formation in order to examine dimorphism. Positive germ tube formation was indicated by the presence of germ tubes at least three yeast cell diameters in length. The shift to hyphae was also indicated by clumping together of the cells in the culture medium.

**Phospholipase Production**

Production of phospholipase was examined using SDA plates enriched with the supernatant from centrifuged sterile egg yolk (8%), 1.0 M NaCl, and 0.005 M CaCl$_2$ (Price, et al., 1982). Cultures were taken from the SDA slants and used to inoculate sterile 250 ml Erlenmeyer flasks containing 25 ml of Lee's medium enriched with biotin (1.00 microgram/ml) from stock solution. These cultures were grown for 24 hours at 25°C. They were then streaked onto the egg yolk-supplemented SDA. Plates were incubated for approximately 72 hours at 37°C. At the end of the growth period
a cloudy region could be seen around the colonies in phospholipase-producing strains. Measuring with a ruler, the ratio of the diameter of the colony to the diameter of the colony plus the cloudy region was determined (see below) and is referred to as the Pz value (Price, et al., 1982). The greater the amount of phospholipase a strain produces, the lower the Pz value.

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Pz \text{ value} = \frac{\text{colony diameter}}{\text{colony plus cloudy region}}.
\]
RESULTS

Dimorphism of Cultures

All eight of the cultures obtained from Ball Memorial Hospital and the positive sample obtained from the Baylor group were found to be dimorphic after growth in albumin-entiched Lee's medium incubated at 37°C. The negative sample obtained from the Baylor group was not found to be dimorphic.

Pz Values in Yeast Phase

All eight cultures obtained from Ball Memorial Hospital and the one already determined to be positive by Price, et al., were found to be positive for phospholipase production. The culture determined to be negative by Price, et al., was again found to be negative. The Pz values are listed in the table below.

<table>
<thead>
<tr>
<th>CULTURE NUMBER</th>
<th>SOURCE</th>
<th>DIMORPHIC</th>
<th>Pz</th>
</tr>
</thead>
<tbody>
<tr>
<td>1300</td>
<td>vaginal</td>
<td>positive</td>
<td>0.55</td>
</tr>
<tr>
<td>1965</td>
<td>vaginal</td>
<td>positive</td>
<td>0.46</td>
</tr>
<tr>
<td>1918</td>
<td>vaginal</td>
<td>positive</td>
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</tr>
<tr>
<td>1285</td>
<td>sputum</td>
<td>positive</td>
<td>0.61</td>
</tr>
<tr>
<td>1671</td>
<td>sputum</td>
<td>positive</td>
<td>0.39</td>
</tr>
<tr>
<td>1712</td>
<td>sputum</td>
<td>positive</td>
<td>0.50</td>
</tr>
<tr>
<td>3375</td>
<td>sputum</td>
<td>positive</td>
<td>0.50</td>
</tr>
<tr>
<td>2847</td>
<td>neck wound</td>
<td>positive</td>
<td>0.49</td>
</tr>
<tr>
<td>Apos</td>
<td></td>
<td>positive</td>
<td>0.43</td>
</tr>
<tr>
<td>Aneg</td>
<td></td>
<td>negative</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Note: Apos is the culture previously determined to be positive by Price, et al., for phospholipase production. Aneg was previously determined to be negative by Price, et al.
Hyphal Phase Experiments

In order to compare the phospholipase production between the two phases, four different experimental protocols were used in an attempt to demonstrate phospholipase production by *C. albicans* on a solid medium.

1) Cultures were shifted to the hyphal phase using albumin-enriched Lee's medium, spread onto the egg yolk-enriched SDA plates with a sterile loop, and incubated at 37°C for approximately 72 hours. However, at the end of the incubation period simple stains with crystal violet showed that the cells had shifted back to blastospores.

2) A second method involved adjusting the pH of SDA plates. This was accomplished by raising the pH of Sabouraud Dextrose broth (pH 5.7) to 7.5 with the addition of NaOH. Then 1.8% Bacto-Agar was added to the medium. The mixture was autoclaved for 15 minutes, cooled to 50°C in a rotary water bath, combined with 8% sterile egg yolk (whose temperature had been raised to 50°C in the same water bath), and poured into plates. When the egg yolk was added, some precipitation occurred. Cultures were shifted to the hyphal form in Lee's medium by the method described earlier and streaked onto these plates. Plates were incubated at 37°C for approximately 72 hours. At the end of that period simple stains with crystal violet showed that the cells had shifted back to blastospores.

3) Since methionine has been used to induce hyphal growth in liquid media, it was used here in an attempt to grow hyphae on egg yolk-enriched SDA plates. After pouring the plates and
allowing them to solidify, 0.25 mg of methionine (from a stock solution of 2.5 mg/ml) were placed on each plate, spread over the surface of the plate with a sterile bent glass rod, and allowed to dry. Cultures were shifted to hyphae in Lee’s medium, spread onto the plates, and incubated for 72 hours at 37°C. After the growth period simple stains with crystal violet showed the cultures had shifted back to blastospores.

4) A special agar containing Quaker Oat’s Cream of Wheat was formulated. Ten grams of Cream of Wheat and ten grams of SDA were put in one liter of deionized H₂O and autoclaved. The mixture was poured into plates and left to solidify. However, the agar did not solidify completely, and the plates were discarded. The method was then attempted using a higher ratio of agar and Cream of Wheat to water. This time 10 g of Cream of Wheat were added to 150 ml of deionized H₂O. The pH of the mixture was 6.6. Then 10 g of SDA were added, and the mixture was autoclaved and poured into plates. This agar was opaque but solid. Cultures were shifted to hyphae in Lee’s medium and streaked onto two plates. The plates were incubated at 37°C for approximately 72 hours. No hyphal growth was detected on one plate (through the use of a simple stain with crystal violet) but on the second a small number of cells had germ tubes.
DISCUSSION

*C. albicans* isolates grow in the yeast phase on SDA. Shifting to the hyphal form in liquid media is mediated by addition of albumin and an increase in the growth temperature. Methionine can be used instead of albumin, but albumin tends to give a more reliable shift.

Unlike the results obtained by the designers of the egg yolk-enriched SDA, the data collected here showed no phospholipase negative strains except for the negative strain obtained from the Baylor University group. Furthermore, the Pz values were generally lower than those published. Interestingly, our plates required an average of 72 hours incubation before one could see phospholipase production while the Baylor group needed only 48 hours.

One notable observation from the research is that the only phospholipase negative culture was also the only culture that was not found to be dimorphic. Since only one strain is involved, no conclusions can be drawn, but further investigation might show a correlation.

Although the purpose of this study was to correlate amount of phospholipase production with morphological phase of *C. albicans*, that end has not yet been reached. Before this can be done, a solid medium which would allow hyphal growth must be developed. Then egg yolk would be incorporated into that medium in order to demonstrate phospholipase production. This has been attempted by incubating hyphal cells at 37°C on (1) SDA with egg yolk, (2) SDA with pH elevated to 7.5, (3) SDA whose surface was covered with
methionine, and (4) SDA containing Quaker Oat's Cream of Wheat. Only the cells grown on SDA with Cream of Wheat showed any hyphal growth, but the amount was insignificant. The next step in this attempt would probably be to use the Cream of Wheat agar with its pH adjusted to different levels before addition of SDA. Perhaps plain agar should be used instead of SDA. The amount of Cream of Wheat mixed with the agar should probably be reduced also in order to make the plates more translucent. If changing the pH proves unsuccessful, other media would need to be tried at various pH values.

Since hyphal growth can be induced in liquid media by manipulating certain environmental parameters, it might be possible to obtain hyphal growth on solid media by manipulating these same factors.
REFERENCES


