Phosphatase Activity Among Candida Species and Other Yeasts Isolated from Clinical Material

RODNEY F. SMITH, DIANNA BLASI, AND SANDRA L. DAYTON
Division of Microbiology, Shriners Burns Institute, University of Texas Medical Branch, Galveston, Texas 77550

Received for publication 26 April 1973

A group of 277 yeasts isolated from burned children and 14 reference strains were tested for phosphatase activity by using phenolphthalein phosphate substrates. Phosphatase activity was widely distributed among various species and strains representing seven genera. Candida albicans, which was the most common yeast isolated from clinical material, was notably absent in producing the enzyme, whereas Candida tropicalis was the most consistent, strong, and rapidly active phosphatase-producing organism. The characteristic enzyme activity of a selected isolate of C. tropicalis was demonstrated in the presence of concentrations of inorganic phosphate which inhibited enzyme activity of other species. The greater enzyme activity of C. tropicalis was not related to more rapid or greater cell growth or decrease in the pH of culture media. Extracellular constitutive heat-labile acid phosphatase was found in broth filtrates of C. tropicalis, C. krusei, and a strain of Staphylococcus aureus.

Acid and alkaline phosphatases have been demonstrated cytologically in a variety of bacteria and yeasts (3). Phosphatase production has also been useful in the identification or biotyping of members of the family Micrococaceae (1). Acid phosphatase activity has been correlated with the virulence of Staphylococcus aureus (2) and has also been studied in connection with virulence of Candida albicans (7). Very little is known about phosphatase activity of other Candida species and related yeasts isolated from human clinical specimens.

In this hospital, studies in progress deal with the ecology and epidemiology of yeasts in a population of burned children. During routine procedures to identify these organisms, studies were also conducted to determine the phosphatase activities of C. albicans and other species of yeasts.

MATERIALS AND METHODS

Isolation. Yeasts were primarily isolated from burned children using various swab collection methods previously reported (9, 10). Snyder agar and Littman Oxgall agar (BBL) were used to isolate yeasts. These media were incubated aerobically at 35 C for 3 days. Representative colonies were transferred to Sabouraud 4% dextrose (SD) broth (BBL) and further streaked on the SD agar plates, if necessary, to purify the cultures. The cultures were maintained on SD agar slants.

Identification. Clinical isolates were identified according to the procedures of Dolan and Ihrke (6) and Webb et al. (12). One culture each of C. albicans, C. parapsilosis, and C. tropicalis was obtained from the Department of Dermatology, University of Texas Medical Branch. One culture each of C. albicans, Saccharomyces ellipsoideus, Schizosaccharomyces octosporus, and Geotrichum candidum was obtained from the midwest Culture Service, Terre Haute, Ind. One culture each Candida stellatoidea, C. tropicalis, C. pseudotropicalis, C. krusei, C. parapsilosis, and C. guilliermondii was obtained from the Texas State Health Department, Austin (courtesy of Carl D. Heather).

Phosphatase activity. Screening tests to detect enzyme activity were conducted in SD broth. One-per-cent solutions of phenolphthalein diphosphate or phenolphthalein monophosphate (PMP), obtained from Sigma Chemical Co., St. Louis, Mo., were prepared in distilled water and sterilized by filtration with 0.45-µm disposable filter units (BBL). The substrates were added to 5-ml quantities of SD broth to give a final concentration of 0.02% phenolphthalein diphosphate or PMP. Yeast strains were inoculated into the broths with a loop from 24-h SD broth cultures of each organism. The substrate containing broths were incubated at 35 C. At 24 and 48 h, the tubes were mixed on a vortex, and 1 ml was removed and tested for phosphatase activity by adding one drop of ammonium hydroxide to 1 ml of broth (5). The color intensity of the reaction was scored at 1-4, which ranged from a pale pink hue to a deep red. Enzyme activity, using the PMP substrate, was also compared in Mycophil broth at pH 7.0 (BBL), in Mycophil broth at pH 4.7, in Sabouraud 4% maltose
broth (BBL), and in SD broth. Enzyme activity was
detected with ammonium hydroxide at 24-, 48-, and
72-h intervals. Phosphatase activity was examined in
Sabouraud maltose broth with PMP substrate in the
presence of gradient concentrations of KH₂PO₄, (11).

Comparative quantitative phosphatase activity
was measured in conjunction with viable cell counts
and changes in medium pH as a function of growth by
using SD broth. Two broths with 0.01% PMP and two
SD broths without substrate were inoculated with 10⁴
viable cells per ml (colony-forming units) of test
cultures. One set of broths with and without PMP
was incubated at 30 C, and the other set was
incubated at 37 C. All tubes were removed from the
incubator at 24 and 48 h. Colony-forming units per
milliliter of cultures in one tube incubated at 30 and
37 C was determined by spreading samples diluted in
saline on SD agar plates. The pH of the broths was
taken potentiometrically. The remainder of the broths
containing PMP were centrifuged at 5,000 rpm for 10
min. One milliliter of broth supernatant was removed
and mixed with 5 ml of 0.1 N NaOH. The quantity of
free phenolphthalein released from the substrate was
measured at 550 nm in 0.5-inch (approximately 1.27
cm) standardized tubes using the Bausch and Lomb
Spectronic 50 colorimeter. The results were compared
with a standard concentration gradient of phenol-
phthalein. Constitutive phosphatase production was
detected in culture filtrates of SD broth without
PMP. Broth filtrate (0.5 ml) was added to a sterile
tube containing 0.5 ml of SD broth containing 0.02%
PMP to give of final substrate concentration of 0.01%
per ml (pH 5.6). One sample of each filtrate was
boiled for 15 min as a control. All tubes were placed in
a 37 C waterbath for 60 min at which time 5 ml of 0.1
N NaOH was added to each tube. Enzyme activity
was again determined by the quantity of phenol-
phthalein released. A standard curve was also pre-
pared to measure acid phosphatase by preparing
gradient concentrations of acid phosphatase from 1
U/ml to 0.06 U/ml (Sigma) in SD broth. In these latter
experiments, an endemic strain of S. aureus, phage
type 54/85 (9) was tested with the yeasts. Trypticase
soy glucose broth (BBL) was used in place of SD broth
for testing the bacterium.

RESULTS AND DISCUSSION

A group of 277 yeasts isolated from burned
children together with 14 named reference
strains were screened for phosphatase activity
(Table 1). Species of seven generic groups were
represented, including seven species of the
genus Candida. Phosphatase activity was ob-
served in a variety of the strains but each of 200
C. albicans strains was negative after 24 h of
incubation. All of the C. tropicalis strains were
positive at 24 or 48 h and produced intense red
(3+ to 4+) reactions. All of the other yeasts
which were phosphatase positive at 24 h pro-
duced weaker positive reactions judged to be 1+
to 2+.

Fifty-two strains of candida were further
tested for phosphatase activity in four broth
media (Table 2). Some variations occurred
among some species with regard to phosphatase
activity detected in each medium, but C.
tropicalis produced 3+ to 4+ reactions in all of
the media after 24 h of incubation.

Further studies with selected strains of yeast
species showed that the addition of gradient
amounts of KH₂PO₄ to phosphatase test broth
was the least inhibitory to C. tropicalis enzyme
activity (Table 3). With this organism, phos-
atase activity occurred in the presence of
KH₂PO₄ concentrations 50 times greater than
those which completely inhibited the enzyme
activity of other candida species.

A comparative quantitative assay of phos-
atase activity with six yeasts and one strain of
S. aureus (Table 4) revealed that C. tropicalis
was the most potent phosphatase active organ-
ism tested at 30 and 37 C. The greater
enzyme activity of this organism was not related
to greater growth than the other strains or a
more rapid and lower decrease in the pH of the
culture media at either 30 or 37 C.

Phosphatase activity was detected in the
substrate-free broth filtrate of C. tropicalis, C.
krusei, and S. aureus (Table 5) with the C.
tropicalis broth filtrate having the most enzyme
activity. Among the three organisms with activity,
the amounts of phosphatase present were
less than 0.05 U/ml of broth based on an acid
phosphatase standard. All enzyme activity was
destroyed in these broth filtrates by boiling.

Chattaway, Odds, and Barlow (4) demon-
strated alkaline and acid phosphatases in C.
albicans and indicated that since washed whole
cells served as a source of acid phosphatase, the
enzyme was associated with or near the cell

| Organism tested | No. of strains tested | No. of strains positive |
|-----------------|-----------------------|-------------------------|
|                 | 24 h                  | 48 h                    |
| Candida species |                       |                         |
| C. albicans     | 200 0                 | 5                       |
| C. stellatoidea | 7 0                   | 1                       |
| C. parapsilosis | 12 3                  | 10                      |
| C. tropicalis   | 32 1                  | 32                      |
| C. pseudotropicalis | 6 2               | 3                       |
| C. krusei      | 7 2                   | 4                       |
| C. quillermondii| 6 1                   | 3                       |
| Torulopsis glabrata | 4 1             | 3                       |
| Trichosporon cutaneum | 9 2        | 4                       |
| Geotrichum candidum | 2 0            | 0                       |
| Saccharomyces cerevisiae | 3 2     | 3                       |
| Saccharomyces ellipsoideus | 1 0  | 1                       |
| Saccharomyces octosporus | 1 0  | 1                       |
| Rhodotorula species | 1 0               | 1                       |

*Sabouraud dextrose broth containing 0.02% phenol-
phthalein diphasate incubated at 35 C.
Table 2. Comparative phosphatase activity of Candida species in various broth media

| Species tested | Strains tested | Sabouraud broth | Mycofil broth |
|----------------|----------------|-----------------|---------------|
|                |                | Dextrose | Maltose | pH 7.0 | pH 4.7 | Dextrose | Maltose | pH 7.0 | pH 4.7 | Dextrose | Maltose | pH 7.0 | pH 4.7 |
|                |                | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| C. albicans    | 16             | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 7    |
| C. stellatoidea| 3              | 0    | 0    | 0    | 2    | 0    | 0    | 0    | 3    | 0    | 3    | 0    | 3    |
| C. tropicalis  | 18             | 18   | 18   | -    | 18   | -    | 18   | -    | 18   | -    | 18   | -    | -    |
| C. pseudotropicalis | 3       | 3    | 1    | 1    | 2    | 0    | 2    | 2    | 1    | 1    | 1    | 1    | 3    |
| C. parapsilosis| 6              | 1    | 1    | 4    | 0    | 1    | 5    | 0    | 2    | 6    | 0    | 4    | 6    |
| C. guillermondii| 3            | 0    | 1    | 2    | 0    | 2    | 2    | 0    | 2    | 2    | 0    | 2    | 2    |
| C. krusei     | 3              | 1    | 2    | 3    | 0    | 3    | 4    | 1    | 2    | 3    | 1    | 2    | 3    |

* Broths contained 0.02% phenolphthalein monophosphate incubated at 35°C.
* Number of strains positive at 24, 48, or 72 h of incubation.
* No further tests were done.

Table 3. Comparative phosphatase activity of selected Candida species in the presence of monobasic potassium phosphate

| Species and strain | KH₂PO₄ (µM/ml) |
|-------------------|---------------|
|                   | 0 | 1 | 5 | 10 | 50 |
|                   | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| C. albicans TSHD  | 0³ | 0 | 3+ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C. tropicalis TSHD| 3+ | 4+ | 4+ | 2+ | 4+ | 4+ | 2+ | 4+ | 4+ | 1+ | 4+ | 4+ |
| C. parapsilosis UTMB | 0 | 2+ | 3+ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C. pseudotropicalis TSHD | 1+ | 3+ | 4+ | 0 | 2+ | 3+ | 0 | 1+ | 2+ | 0 | 0 | 0 |
| C. krusei TSDH    | 2+ | 4+ | 4+ | 1+ | 2+ | 4+ | 0 | 1+ | 0 | 0 | 0 | 0 |

*Sabouraud dextrose broth containing 0.02% phenolphthalein monophosphate was incubated at 35°C and tested for activity at 24, 48, and 72 h. The initial pH of broth without added KH₂PO₄ was 5.6 and ranged from pH 5.6 to pH 5.2 with the addition of 50 µM KH₂PO₄ per ml.
* Intensity of color reaction 0, negative; 1+ to 4+, positive.

Table 4. Relationships between growth and phosphatase activity of yeasts and Staphylococcus aureus

| Strain tested | 37°C | 30°C |
|---------------|------|------|
|               | 24 h | 48 h | 24 h | 48 h |
| C. tropicalis TSDH | 6.68 | 5.1 | 0.0 | 6.90 | 4.7 | 66.0 | 7.04 | 4.7 | 48.0 | 7.41 | 4.5 | 57.0 |
| C. pseudotropicalis | 7.00 | 4.2 | 18.0 | 7.20 | 4.0 | 19.0 | 7.25 | 4.1 | 23.0 | 7.63 | 3.8 | 26.0 |
| C. krusei TSDH    | 7.25 | 4.8 | 28.0 | 7.41 | 4.3 | 29.0 | 7.84 | 5.0 | 31.0 | 7.94 | 4.3 | 31.0 |
| C. albicans UTMB  | 7.10 | 5.0 | 0.0 | 6.70 | 4.7 | 2.0 | 7.17 | 5.0 | 0.0 | 7.38 | 4.4 | 0.0 |
| C. parapsilosis TSHD | 6.77 | 5.3 | 2.0 | 7.25 | 4.9 | 3.8 | 7.44 | 5.3 | 2.3 | 7.67 | 4.8 | 10.0 |
| S. cerevisiae MCS³ | 6.83 | 5.7 | 28.0 | 9.52 | 5.6 | 29.0 | 9.20 | 6.1 | 25.0 | 9.14 | 5.4 | 23.0 |

* Yeasts were grown in Sabouraud dextrose broth with initial pH of 5.7. S. aureus was grown in Trypticase soy glucose broth (initial pH 7.2). Enzyme activity was expressed as percentage of phenolphthalein released from 0.01% PMP substrate.
* CFU, colony-forming units.
* This strain grew poorly at 37°C and tests were not conducted at this temperature.
Table 5. Constitutive phosphatase activity in broth filtrates of yeasts and Staphylococcus aureus

| Strains tested          | Enzyme activity* |
|-------------------------|------------------|
|                         | 37°C  | 48h      | 24h  | 30°C  | 48h  |
| Candida tropicalis      | 3.9   | 9.9      | 3.2  | 7.9   |      |
| C. pseudotropicalis     | 0.0   | 0.0      | 0.0  | 2.0   |      |
| C. krusei               | 1.4   | 5.0      | 1.4  | 3.2   |      |
| C. albicans             | 0.0   | 0.0      | 0.0  | 0.0   |      |
| C. parapsilosis         | 0.0   | 0.0      | 0.0  | 0.0   |      |
| Saccharomyces cerevisiae| *     | —        | 0.0  | 0.0   |      |
| Staphylococcus aureus   | 0.0   | 2.0      | 1.0  | 1.4   |      |

*Expressed as percent of phenolphthalein released from 0.01% PMP.

wall. The acid phosphatases of C. tropicalis, or other yeasts for that matter, appear to be fundamentally different from that of C. albicans with respect to the extra-cellular activity of the enzyme in actively growing cultures. Phenolphthalein phosphate substrates were used in this study because of their potential colorimetric diagnostic applications, but additional work in progress indicates that the acid phosphatase enzyme of C. tropicalis acts upon p-nitrophenyl phosphate substrate (unpublished data).

In view of the well established methods which are available and used successfully for the identification of clinically isolated yeasts, the phosphatase test is not proposed here as a routinely useful diagnostic test but could be a simple and rapid optional method for the separation of C. albicans from other closely related species or for the identification of C. tropicalis.

Since candida species other than C. albicans may cause infection, particularly in the compromised host (8), the demonstration of acid phosphatases in several yeasts which are not commonly pathogenic in humans may also indicate that such enzymes can not be associated with virulence as in the case of C. albicans (4, 7) but could be useful to study in comparison to C. albicans.

ACKNOWLEDGMENT

This work was supported by funds from the Shriners of North America.

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