An Examination of the Production of Hydrolytic Enzymes and Toxins by Pathogenic Strains of *Candida albicans*

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**SUMMARY**

Attempts to demonstrate the production of toxin by pathogenic strains of *Candida albicans* have proved negative; a variety of growth conditions and methods of organism extraction were used, several of which were previously described by other workers as yielding toxins. Toxin production appears to be a strain characteristic and not a property of all pathogenic strains. Acid phosphatases with pH optima at 3-6 and 5-6, a peptidase with optimum pH 6-6 and β-glucosidase have been isolated from both blastospore and mycelial forms of *C. albicans* and their properties examined. The occurrence in organism extracts of alkaline phosphatase and peptidase (optimum pH 3-6) has been confirmed; no activity of mucopolysaccharase, neuraminidase, β-glucuronidase or phospholipase A and B could be demonstrated. The possible role of hydrolytic enzymes in pathogenicity is discussed.

**INTRODUCTION**

The importance of *Candida albicans* as a pathogen is being increasingly appreciated. Patients suffering from endocrine disorders, including diabetes mellitus and hypoparathyroidism, are particularly susceptible to infection, as are pregnant women and those receiving oral contraceptives. There is also an increased incidence of infection amongst patients receiving prolonged antibiotic therapy or corticosteroids. The mechanisms by which it, the pathogen, invades susceptible tissues are, however, not well defined. The probable formation of toxins was indicated by a variety of deleterious effects induced in mice, rabbits, dogs and humans by extracts of organisms or culture filtrates, and earlier literature has been reviewed by Seelig (1966). In addition, Isenberg, Allerhand & Berkman (1963) and Kobayashi & Friedman (1964) have found phenolic extracts of Candida organisms to contain an endotoxin-like material, but the latter authors indicated that their material differed in a number of properties from bacterial endotoxins. None of the foregoing materials was extensively purified, but Mankowski (1968) has isolated a glycoprotein from culture filtrates which retarded the growth of newborn mice on repeated subcutaneous injection. This material has very similar properties to that isolated by Masler, Šikl, Bauer & Sandula (1966) from culture filtrates of strains of *C. albicans*, with widely varying pathogenicity, so that its significance as a pathogenic agent must be in doubt. The most detailed claim for the production of a toxin is that by Iwata, Uchida & Endo (1967), who isolated a material termed canditoxin from a strain of *C. albicans* obtained from a case of meningitis. Subsequent work (Iwata & Uchida, 1967a, b, 1968) showed the purified material...
to be lethal to mice at a dose of 18 \(\mu\)g. and to be a prophosphatase; the enzyme activity was induced by heat and shown to be an alkaline phosphatase, such activation resulting in a loss of toxic properties.

Toxic activity may be associated with the activity of hydrolytic enzymes, and the occurrence of an alkaline phosphatase has been shown histochemically by Kurup (1963), and in cell extracts by Bartels & Christopherson (1970), a leucine aminopeptidase in cell extracts by Kim, Adachi & Chow (1962), a plasma coagulase by Zaikina & Elinov (1968), and an excreted peptidase by Staib (1965). The last was isolated from culture filtrates (Remold, Fasold & Staib, 1968) grown on a serum albumin containing medium at pH 4.0.

There have been many suggestions that tissue invasion is associated with development of the mycelial form of the organism. However, the conditions of growth for the preparation of the toxic materials mentioned above would give rise to the blastospore form and none of the authors appear to have examined toxin production by mycelium.

The present paper describes attempts to repeat the preparation of toxin-like materials under the conditions used by Mourad & Friedman (1961), Kobayashi & Friedman (1964), Louria, Brayton & Finkel (1963) and Iwata et al. (1967), and attempts to obtain toxin-like activity from both morphological forms grown on medium C of Chattaway, Holmes & Barlow (1968) using strains of Candida albicans isolated from cases of candidiasis. A number of these strains have been screened for the presence of hydrolytic enzyme activity in culture filtrates and cell extracts from both morphological forms and the partial purification of phosphatases and peptidases is described.

**METHODS**

**Organisms.** All strains of Candida albicans (Robin) Berkhout used were primary isolations from human sources either at the Royal Infirmary, Huddersfield, or with the co-operation of Dr E. G. V. Evans at the Leeds General Infirmary. The strains used for preparation of toxin and most of the enzyme work were designated c-a-1, c-a-2, c-a-5, c-a-6, the first of these being the strain used in previous work (Chattaway et al. 1968). In addition, 33 other strains were examined for the presence of peptidase activity at pH 3.2. Organisms from the first subculture (18 h. at 30°) on Sabouraud maltose agar with added chloramphenicol were resuspended in malt extract broth (Oxoid) and grown for 18 h. at 37° with shaking, and the centrifuged growth resuspended in preserving medium, lyophilized and stored in sterile ampoules at room temperature. Cultures were reconstituted by incubation in malt extract broth for 18 h. at 30° and then subcultured on to Sabouraud maltose agar.

**Media.** The principal medium used was medium C of Chattaway et al. (1968) which yields blastospores on incubation at 30° and the mycelial form at 40°; on occasion this was substituted by a synthetic medium modified from that of Kobayashi, Friedman & Kofroth (1964), which gave the two morphological forms under the same conditions, although growth was poorer and the proportion of mycelium produced was lower. Percentage composition of this medium was: glucose, 6 g.; MgSO\(_4\)·7H\(_2\)O, 10 mg.; (NH\(_4\))\(_2\)HPO\(_4\), 25 mg.; KH\(_2\)PO\(_4\), 20 mg.; biotin, 1 ng.; NaHCO\(_3\), 145 mg.; pH 7.5. In some experiments the medium of De Palma (1966) was used which gives blastospores at 37° and the mycelial form at the same temperature when sodium bicarbonate is added to give pH 7.5. For preparation of the extracellular peptidase the medium of Remold et al. (1968) was used.

**Inoculation, growth and preparation of organism extracts.** Organisms for inoculation were grown on Sabouraud maltose agar using the second subculture after reconstitution of
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lyophilized cells; a standard inoculum of $2 \times 10^6$ organisms/ml. was used. Cultures were shaken in a New Brunswick environmental incubator shaker (100 rev./min.). Blastosporas and mycelium were harvested as described by Chattaway et al. (1968); supernatants from extracts were obtained by disruption of organisms in a French press (and, where indicated, by ultrasonication) and centrifugation of the suspension at 10,000 g. The supernatants, and sometimes residues, were examined for toxin or enzyme activity.

Toxic activity. *Candida albicans* strains were tested for pathogenicity as recommended by Sandula, Kocková-Kratochvílová & Zamecnikova (1963) by injection of $10^6$ organisms suspended in 0.5 ml. 0.9 % saline into the tail vein of female Swiss albino mice. Preparations from culture filtrates or extracts were tested for toxicity to mice by intravenous injection of 0.5 ml. volumes into groups of five mice together with the necessary control groups.

Enzyme assays. Peptidase was determined by the method of Remold et al. (1968) using 1 % bovine serum albumin (fraction V, Sigma) and reading extinction of the supernatant at 280 nm. Phosphatases were measured as described by Linhardt & Walter (1963), mucopoly saccharase by the method of Mathews (1966), neuraminidase according to Mahadevan, Nduaguba & Tappel (1967), $\beta$-glucosidase by the method of Dueksen & Halvorson (1958), $\beta$-glucuronidase according to Fishman (1963), phospholipase A according to Hanahan (1952) using ethereal egg lecithin as substrate and estimating the decrease in substrate concentration as in Augustyn & Elliott (1969) and phospholipase B according to Dawson (1956).

Chemical estimations. Orthophosphate was measured according to Fiske & Subbarow (1925) and protein according to Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Toxin production. The growth conditions and methods of treatment used by four groups of workers claiming the preparation of toxins from *Candida albicans* have been repeated and also extracts prepared from both morphological forms of organisms grown on neopeptone-starch-yeast extract medium have been examined. All products were tested in mice, all workers having used the same test for toxin activity. The results are shown in Table 1, together with the variety of media and growth times employed. The only preparation which showed any toxic activity was a phenol extract prepared from organisms grown on the medium of Kobayashi & Friedman (1964) when all five mice died within 6 h. Two further attempts to repeat this result were negative. It appeared that the development of toxin activity for mice is dependent upon strain characteristics, rather than cultural conditions.

Detection and separation of hydrolytic enzyme activity. The above result suggests that pathogenicity may be more related to the local activity on host tissue components of enzymes elaborated by the micro-organism during its growth. Accordingly, a survey has been made for hydrolytic enzymes present in culture filtrates and organism extracts. Strains were grown for 4 h. on the neopeptone-starch broth so that enzyme activities in both growth forms could be compared, this growth period having been shown to give the highest yield of mycelium at $40^\circ$ (unpublished results). Culture filtrates were concentrated to low volume, but no enzyme activities have been detected in these preparations. Press supernatants contained phosphatases, peptidases and $\beta$-glucosidase (Table 2) while tests for mucopolysaccharases, neuraminidase, $\beta$-glucuronidase and phospholipases A and B were uniformly negative. The phosphatase and peptidase activities were separated by passage through DEAE-cellulose (Fig. 1) the same elution profile being obtained with extracts from blastospores or mycelium. Three phosphatase fractions were obtained with optimum
pH 3.6, 5.6 and 8.6; protease activity with optimum pH 6.6 was found only in tubes nos. 70 to 90.

**Phosphatases.** The specific activity of the three phosphatase fractions described above reveal that the acid (pH 5.6) phosphatase is a relatively minor component of the supernatants (Fig. 2), but the significance of this enzyme within the organism may be greater than is

Table 1. *Attempted preparation of toxin from Candida albicans*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time (h.)</th>
<th>Temp.</th>
<th>Ref.</th>
<th>No.</th>
<th>Morphol. form</th>
<th>Type of extract injected</th>
<th>No. mice used</th>
<th>No. dead in 24 h.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casamino acids</td>
<td>24</td>
<td>37°</td>
<td>Mourad &amp; Friedman (1961)</td>
<td>1</td>
<td>Blastospores</td>
<td>Supernatant from ultrasonication</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Glucose-salts biotin</td>
<td>72</td>
<td>?</td>
<td>Kobayashi &amp; Friedman (1964)</td>
<td>1</td>
<td>Blastospores</td>
<td>Phenol</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Sabouraud broth</td>
<td>504</td>
<td>37°</td>
<td>Louria <em>et al.</em> (1963)</td>
<td>1</td>
<td>Blastospores</td>
<td>Culture filtrate</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>10</td>
<td>37°</td>
<td>Iwata <em>et al.</em> (1967)</td>
<td>4</td>
<td>Blastospores</td>
<td>French press supernatant</td>
<td>20†</td>
<td>0</td>
</tr>
<tr>
<td>Neopeptone–starch yeast extract</td>
<td>18</td>
<td>30°</td>
<td>Chattaway <em>et al.</em> (1968)</td>
<td>4</td>
<td>Blastospores</td>
<td>French press supernatant</td>
<td>20†</td>
<td>0</td>
</tr>
<tr>
<td>Neopeptone–starch yeast extract</td>
<td>18</td>
<td>40°</td>
<td>Chattaway <em>et al.</em> (1968)</td>
<td>4</td>
<td>Mycelium</td>
<td>French press supernatant</td>
<td>20†</td>
<td>0</td>
</tr>
</tbody>
</table>

* Strain c-a-1 was used for all preparations and additionally strains c-a-2, c-a-5 and c-a-6 for canditoxin production (Iwata *et al.* 1967) and for comparison of the two morphological forms.
† Five mice for extract from each strain.

Table 2. *Hydroltyic enzyme activities in Candida albicans extracts*

Specific activity of peptidases in French press supernatants expressed as extinction at 280 nm./h./mg. protein $\times 10^3$, of phosphatases as nm phosphate released/min./mg. protein and $\beta$-glucosidase as nm $\beta$-nitrophenol/min./mg. protein.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH of test</th>
<th>c-a-1</th>
<th>c-a-2</th>
<th>c-a-5</th>
<th>c-a-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidase</td>
<td>3</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>21</td>
<td>15</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>15</td>
<td>25</td>
<td>15</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
<td>17</td>
<td>7</td>
<td>34</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>5</td>
<td>36</td>
<td>55</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>$\beta$-glucosidase</td>
<td>6-8</td>
<td>10</td>
<td>16</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

B = blastospores. M = mycelium.

suggested by this comparison since it will be acting near to its optimum pH. The acid (pH 3.6) phosphatase has appreciably higher specific activity in mycelium than in blastospores. Examination of residues of the extracts used to prepare the above fractions showed only acid phosphatase activity, principally with optimum pH 4. A similar activity is shown by whole cells (Fig. 3) but here it is seen that blastospores show an appreciably greater activity than mycelium.

The results for phosphatase activity were all recorded using $\beta$-nitrophenyl phosphate as substrate; the substrate specificity of these enzymes has been examined against a number
of sugar phosphates and nucleotides (Table 3). The highest activities were found with fructose-1,6-diphosphate as substrate for the acid (pH 3.6) and the alkaline phosphatase; the acid (pH 5.6) phosphatase showed no 5'-ribonucleotide phosphohydrolase activity. There were no significant differences in specificity between the enzymes isolated from blastospores or mycelium and none of them showed any activity against cyclic-3',5'-adenosine monophosphate or bis-p-nitrophenylphosphate.

![Graph](image)

**Fig. 1.** Chromatography of Candida albicans extract. DEAE-cellulose was used in a column (20 x 1.5 cm.); the sample of French press supernatant (120 mg. protein) was applied in 0.05 M tris buffer, pH 7.5, and initial elution was with this buffer, then a linear salt gradient in the buffer was applied up to 1 M NaCl (--). Elution was monitored by extinction readings at 280 nm (-----) on the 9 ml. fractions collected. Phosphatase, pH 3.6 (○), pH 5.6 (●), pH 8.6 (□).

**Table 3. Relative substrate specificities of phosphatases of Candida albicans strain C-a-2**

Activities were determined on French press supernatants fractionated as described in Fig. 1 and incubated with substrates (5 mM) at 30° for 1 h. The results are recorded as percentages of the activity of each mycelial fraction against p-nitrophenyl phosphate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acid (pH 3.6)</th>
<th>Acid (pH 5.6)</th>
<th>Alkaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>M</td>
<td>B</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>62</td>
<td>64</td>
<td>56</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>47</td>
<td>46</td>
<td>50</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphate</td>
<td>112</td>
<td>122</td>
<td>101</td>
</tr>
<tr>
<td>Guanosine-3'-monophosphate</td>
<td>49</td>
<td>50</td>
<td>120</td>
</tr>
<tr>
<td>Adenosine-5'-triphosphate</td>
<td>54</td>
<td>66</td>
<td>21</td>
</tr>
<tr>
<td>Cytosine-5'-triphosphate</td>
<td>23</td>
<td>46</td>
<td>30</td>
</tr>
<tr>
<td>Guanosine-5'-triphosphate</td>
<td>44</td>
<td>61</td>
<td>38</td>
</tr>
<tr>
<td>Uridine-5'-triphosphate</td>
<td>28</td>
<td>46</td>
<td>69</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>99</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>61</td>
<td>59</td>
<td>86</td>
</tr>
</tbody>
</table>

B = blastospores. M = mycelium.
Michaelis constants have been determined for the partially purified enzymes prepared from the two growth forms and for the acid (pH 4.0) phosphatase using whole cells as source of enzyme (Table 4).

The alkaline phosphatase has been shown to require $3 \times 10^{-4}$ M-Mg$^{2+}$ for optimum activity, no magnesium requirement was found for the other two enzymes.

Fig. 2. Variation of phosphatase activity with pH in morphological forms of Candida albicans. Enzyme activity was determined on French press supernatants fractionated as described in Fig. 1 and incubated with p-nitrophenyl phosphate (5 mM) at 30° for 1 h. Results are given for acid phosphatase (pH 3.6, △), acid phosphatase (pH 5.6, □) and alkaline phosphatase (○), for blastospore (open symbols) and mycelial (solid symbols) extracts. 0.1 M-Citric acid-NaOH buffers were used for pH 3.0 to 7.0 and 0.1 M-tris-HCl buffers for pH 6.6 to 9.3.

Fig. 3. Variation of phosphatase activity with pH in whole organisms and residues of extracts of Candida albicans. Assay conditions as in Fig. 2. Activity shown for blastospores (whole cells, □; residue, ○) and mycelium (whole cells, ■; residue, ●).

Peptidases

(a) Optimum pH 6.6. The specific activity of this enzyme prepared from mycelium is approximately twice that obtained from blastospores (1.41 ± 0.04 o.d. 280 nm./min./mg. protein from mycelium; 0.79 ± 0.20 o.d. 280 nm./min./mg. protein from blastospores—mean of four experiments). It is an unstable enzyme, appreciable losses occurring during purification
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and all activity being lost in 48 h. at 0°C after the described separation; thus the significance of the different activities in the two forms and the role of the enzyme in the life of the organism remain to be determined.

(b) Extracellular peptidase, optimum pH 3.2. This enzyme was described by Staib (1965) as being released from 75/100 strains of Candida albicans when grown on acid-serum-agar. Its formation under the conditions described by Staib has been confirmed in 36/37 strains tested. The one strain (c-a-I) which did not produce it was isolated from a child with widespread candidiasis of several years duration and was highly lethal to mice; Staib (1965) describes a strain of similar origin, but this and other strains which did not produce the enzyme were found by Remold et al. (1968) not to cause widespread infection in mice.

Table 4. Michaelis constants for phosphatases from Candida albicans

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Optimum pH</th>
<th>Growth form</th>
<th>Michaelis constant (x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partially purified</td>
<td>3.6</td>
<td>Blastospore</td>
<td>0.715 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mycelium</td>
<td>0.673 ± 0.03</td>
</tr>
<tr>
<td>French press supernatant</td>
<td>8.6</td>
<td>Blastospore</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>Blastospore</td>
<td>3.71 ± 0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mycelium</td>
<td>4.38 ± 0.33</td>
</tr>
<tr>
<td>Whole organisms</td>
<td>4.0</td>
<td>Blastospores</td>
<td>0.637 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mycelium</td>
<td>0.650 ± 0.034</td>
</tr>
</tbody>
</table>

DISCUSSION

Several groups of workers have reported the production of toxin by single strains of Candida albicans, using a variety of growth conditions. The attempt to apply these conditions to toxin production by a small number of strains has underlined the fact that the production of toxin appears to be a strain characteristic since none of the strains used in this work produced an active toxin. These strains were primary isolations from human cases of candidiasis and of proven lethality to mice following intravenous injection; it would seem that the production of toxin tested in this way bears little relationship to the pathogenicity of this organism.

Enzyme activities implicated in the pathogenic action of many bacteria, such as mucopolysaccharases, neuraminidases and phospholipases, have not been found in Candida albicans. The presence of an alkaline phosphatase has been confirmed and in addition the presence demonstrated in cell extracts of two acid phosphatases with pH optima at 3.6 and 5.6. The pathogenic significance of these enzymes will depend in part on whether or not they are released from the organism or are associated with its surface and so available to attack substrates from the host.

Washed whole organisms serve as a source of the acid phosphatase (pH 4.0) but not of the others and some activity due to this enzyme is associated with the residue after disruption, suggesting that it may, in part, be associated with, or close to, the cell surface. The close similarity of the pH optima of whole organism, residue and supernatant acid phosphatases (4.0, 4.0 and 3.6 respectively) and the near identity of Michaelis constants for the whole organism and supernatant enzymes (Table 4) strongly suggests that the two activities may in fact be due to one enzyme. The acid phosphatase (pH 5.6) may be acting nearer to its optimum in the in vivo situation but has been shown to be of lower activity and narrower specificity than the other phosphatases and there is no evidence at present to indicate its association with the cell surface or its ready release from the organism.
Repetition of the purification of canditoxin by Iwata et al. (1967b) on DEAE-cellulose gave identical profiles for the elution of protein fractions, but showed that the first fraction eluted from the column was the acid phosphatase (pH 3.6) while the corresponding fraction from the Japanese strain was canditoxin which on heating had alkaline phosphatase activity, indicating that these enzymes from different strains may show markedly different physical properties.

Proteolytic enzymes are clearly potential agents of pathogenicity and the occurrence of an extracellular peptidase of optimum pH 3.2 has been confirmed, but its significance in this respect must be in doubt since it had no activity above pH 5.0 against serum albumin. The instability of the peptidase with optimum pH 6.6 demonstrated in press supernatants has so far prevented any detailed study of its properties.

The association of the presence of mycelium with the invasion of tissue could be due to greater invasive power of the mycelial form or to the more rapid spread of the mycelial form once invasion has occurred as suggested by Hurley & Stanley (1969), who found no quantitative difference between the two forms in their ability to attack mouse epithelial cells in tissue culture. The present results indicate greater specific activity for the acid phosphatase (pH 3.6) and the peptidase (pH 6.6) in mycelial extracts. However, the assay of this acid phosphatase using whole organisms indicates greater specific activity for the blastospore form and it may be that the different wall structure in the two forms may affect the association of activity with the cell surface and this may be a more important factor in pathogenicity than the activity in extracts.

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