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Use of a Chitinase Complex and β-(1,3)-Glucanase for Spheroplast Production from *Candida albicans*[1]

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The digestive juice of the snail *Helix pomatia*, composed of thirty or more enzymes (H. J. Phaff, Ann. Rev. Microbiol. **17**:15, 1963), has been used for cell wall lysis and spheroplast production from *Candida albicans* (S. Sugawara, Nature **212**:92, 1966; G. S. Kobayashi, L. Friedman, and J. F. Kofroth, J. Bacteriol. **88**:795, 1964). In this study, we investigated the use of specific enzymes for this purpose.

Since more than one enzyme is needed for fungal cell wall lysis (K. Horikoshi and S. Iida, Nature **183**:186, 1959; J. J. Skujins, H. J. Potgieter, and M. Alexander, Arch. Biochem. Biophys. **111**:358, 1965), a chitinase complex and β-(1,3)-glucanase were selected for these experiments. Their substrates, chitin and glucan, are major components of the cell wall of *C. albicans* (W. J. Nickerson, Bacteriol. Rev. **27**:305, 1963; H. J. Phaff, Ann. Rev. Microbiol. **17**:15, 1963).

The exocellular chitinase complex was isolated according to the method of D. M. Reynolds (J. Gen. Microbiol. **11**:150, 1954), with a culture of *Streptomyces griseus* C-10-X obtained from Dr. Reynolds. The complex was concentrated to one-tenth its original volume on a flash evaporator and was purified by dialysis against distilled water. No procedure was used to separate chitinase and chitobiase, the enzymes of this lytic complex (L. R. Berger and D. M. Reynolds, Biochim. Biophys. Acta **29**:522, 1958).

The exocellular enzyme β-(1,3)-glucanase, obtained from Myrna Thomas, Biology Department, Temple University, was produced by fungal growth on an inorganic salt medium with laminaran as the sole source of carbon. The methods of J. J. Skujins et al. (Arch. Biochem. Biophys. **111**:358, 1965) were used for the purification of this enzyme and for the assay and determination of the unit of both β-(1,3)-glucanase and the chitinase complex. Both enzyme preparations were free from proteolytic activity when tested by the method of Anson (J. Gen. Physiol. **22**:79, 1938).

For these experiments, the centrifuged sediment of a culture of *C. albicans* (stock culture # 15), grown for 6 hr in Sabouraud's broth at room temperature on a rotary shaker, was suspended in 2 ml of physiological saline and was divided into two equal portions. One portion was treated with 5 ml of a solution containing 0.15 M mercaptoethanol, a reducing agent, and 0.04 M ethylenediaminetetraacetic acid (EDTA), a chelating agent, as proposed by E. A. Duell et al. (J. Bacteriol. **88**:1762, 1964). The other portion was suspended in physiological saline. After incubation at room temperature for 30 to 45 min, the centrifuged sediments were washed twice with distilled water and twice with physiological saline; each portion was suspended in 2 ml of saline and was divided into two 1-ml portions.

One of the samples from each of the original suspensions was mixed with 10 ml of a solution containing 1.2 M mannitol, 0.03 M phosphate-citrate buffer (*p*H 5.8), 100 units of penicillin per ml, 41 milliunits of β-(1,3)-glucanase, and 3 milliunits of the chitinase complex per ml. The two remaining portions were each treated with 10 ml of the same solution omitting the enzymes.

After incubation on a rotary shaker at room temperature, one drop of each sample was taken at intervals varying from 10 to 90 min and 2 to 16 hr, examined under the oil immersion lens of a light microscope, and photographed with a camera microscope Ultraphot II (Carl Zeiss, Inc., New York, N.Y.), by use of the phase-contrast microscope.

The appearance of normal *C. albicans* cells is shown in Fig. 1. Spheroplasts were produced only in the presence of all reagents and within the 1-hr incubation period. At first, the cell surface appeared wrinkled; 5 min later, we observed a spherical body with cellular debris which remained attached for several minutes (Fig. 2). The spheroplasts were stable in 1.2 M mannitol solution and burst when suspended in distilled water.

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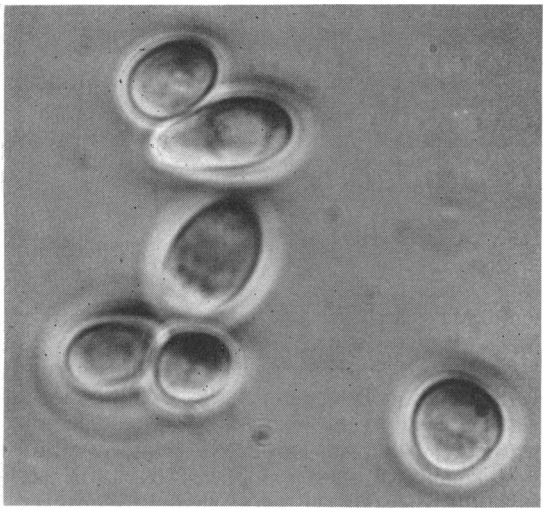

FIG. 1. *Phase micrograph of untreated Candida albicans cells suspended in a hypertonic buffer solution.* × *3,600.*

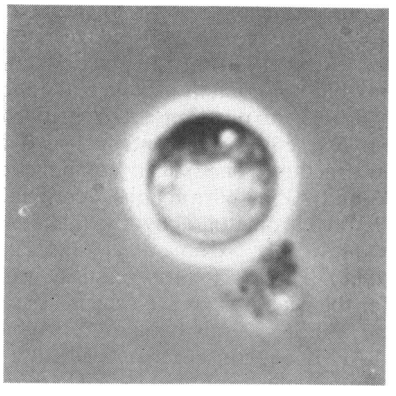

FIG. 2. *Phase micrograph of a Candida albicans spheroplast with residual cellular material attached.* × *2,800.*

Table 1 shows that the amount (μg per ml) of *N*-acetyl-glucosamine released in 5 hr by the chitinase complex alone was increased almost four times when β-(1,3)-glucanase was added to the system. This suggested that a glucan layer may be combined with or may cover the chitin in the cell wall and that the action of the chitinolytic enzymes is blocked until the glucan is hydrolyzed by its specific enzyme, β-(1,3)-glucanase. Similar observations regarding the location of these cell wall constituents have been made for the hyphal fungi *Fusarium solani* and *Aspergillus oryzae* and for the yeast *Saccharomyces cerevisiae* (H. J. Potgieter and M. Alexander, J. Bacteriol. **91**:1526, 1966; J. J. Skujins et al., Arch. Biochem. Biophys. **111**:358, 1965; K. Horikoshi and S. Iida, Nature **183**:186, 1959; J. S. D. Bacon et al., Biochem. J. **101**:36C, 1966).

This procedure for spheroplast production from *C. albicans* offers a method for the isolation of fungal organelles in a solution free from proteolytic activity.

TABLE 1. *Decrease in absorbance and release of N-acetylglucosamine from Candida albicans cell suspensions after treatment with mercaptoethanol and EDTA and incubation in hypotonic buffered solutions of the chitinase complex alone and in combination with β-(1,3)-glucanase for 5 hr*[a]

Time (hr)	Decrease in absorbance[b]		Release of *N*-acetylglucosamine[c]	
	Chitinase complex	Chitinase complex + β-(1,3)-glucanase	Chitinase complex	Chitinase complex + β-(1,3)-glucanase
			μg/ml	μg/ml
0	0.425	0.427	0.0	0.0
1	0.412	0.361	8.0	26.3
2	0.403	0.300	12.5	49.3
3	0.395	0.255	16.2	67.8
4	0.378	0.225	20.0	78.2
5	0.367	0.216	22.7	85.0

[a] Light absorption was measured in a Spectronic-20 colorimeter. *N*-acetylglucosamine was determined by the method of Reissig et al. (J. Biol. Chem. **217**:959, 1955). The hypotonic buffered solution was composed of 0.03 M phosphate-citrate buffer, *p*H 5.8.

[b] Decrease in absorbancy in the presence of β-(1,3)-glucanase alone was from 0.425 at zero-hour to 0.410 in 5 hr.

[c] Measurement of release of *N*-acetylglucosamine in the presence of β-(1,3)-glucanase alone was from 0 at zero-hour to 6 μg/ml in 5 hr.

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