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Lytic Action of β(1-3)-Glucanase on Yeast Cells

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Candida utilis, *Saccharomyces cerevisiae*, *S. fragilis*, *Pichia polymorpha*, and *Hansenula anomala* yeast cells, harvested in the early logarithmic phase, were attacked with purified β(1-3)-glucanase from *Micromonospora chalcea*, which resulted in the liberation of protoplasts. The treated cells were observed under the electron microscope before the protoplasts were liberated. Differences in the cell walls of the enzyme-treated and untreated cells were observed. The action of the glucanase was also tested against isolated walls of *C. utilis*. The enzyme attacked the *S. cerevisiae* cell wall in a uniform manner. The attack on *S. fragilis* was located in certain zones of the cell wall, where breakage occurred and through which the protoplast emerged. On the other three yeasts, an intermediate attack was observed, not as definitely located as in *S. fragilis*, yet less uniformly than in *S. cerevisiae*.

A large amount of work has been done on the emergence of protoplasts from yeasts (11). Susceptibility of the yeast walls to selected enzymes was first tested by Eddy and Williamson (1), who found that the complex snail enzyme caused complete dissolution of cell wall. Tanaka and Phaff (10) have described a number of microorganisms from soil (*Bacillus* and *Streptomyces* species) which produce β(1-3)- and β(1-6)-glucanases, mannanases, and proteases. Although some alterations were caused by the action of the purified enzyme in the yeast cells, no protoplasts were formed. Recently Nagasaki et al. (7) obtained yeast protoplasts by means of the combined action of two agents isolated from *B. circulans*.

From the enzymes produced by *Micromonospora chalcea*, we have purified a β(1-3)-glucanase (Monreal and Villanueva, *in preparation*) that is active on young yeast cells and is capable of liberating protoplasts (12). This investigation is a study of the action of the purified enzyme on the cell walls of different yeasts.

MATERIALS AND METHODS

Organisms. Most of the experiments were carried out with strains of *Candida utilis* 1016 and *Saccharomyces fragilis* 1207. Differences in susceptibility to β(1-3)-glucanase were also investigated with strains of *S. cerevisiae* 1189, *Hansenula anomala* 1349, and *Pichia polymorpha* 1132. All cultures were from the Colección Española de Cultivos Tipo (CECT).

Media and conditions of growth. Flasks (300 ml) containing 100 ml of Hansen medium [30 g of sucrose; 10 g of peptone (Difco); 0.5 g of $MgSO_4 \cdot 7H_2O$; 0.5 g of K_2HPO_4; and 1,000 ml of distilled water] were inoculated with 1 ml of a 0.25 OD suspension of yeast cells. Cultures were incubated by shaking at 29 C, and after 12 hr cells were used.

Enzyme source. The endo-β(1-3)-glucanase was purified from culture filtrates of *M. chalcea* CECT 3195 as already described (Monreal and Villanueva, *in preparation*). The specific activity was 57 units/mg of protein (soluble laminarin from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England). The preparation was free of α-mannanase, β-glucosidase, β(1-6)-glucanase, β(1-4)-glucanase, and chitinase, but it showed traces of proteolytic activity.

Preparation of protoplasts. Cells harvested in the early logarithmic phase of growth were washed three times with distilled water and were suspended in 0.05 M citrate buffer (*p*H 5.6) containing 1 M $MgSO_4$ as stabilizer (3). A cell suspension sufficient to give an initial absorbance of about 0.5 was used. The enzyme preparation was added, and the mixture (1 ml = total volume) was shaken at 40 C. The extent of lysis and protoplast formation was measured in the Zeiss phase-contrast microscope.

For the most part, the protoplasts were free from cells of every kind of yeast described above after 2 hr of incubation, with the exception of *H. anomala* which needed 15 hr of enzymatic attack.

Preparation of the cell walls. Cell walls of *C. utilis* were prepared as described by Garcia-Mendoza and Villanueva (2). Only 30-hr cultures were used.

The cell walls were suspended in 1.0 ml of 0.05 M citrate buffer (*p*H 5.6), and the solution was divided into two equal fractions. The β (1-3)-glucanase solution was added to one of these fractions. Both fractions were then incubated at 40 C for 3 hr and centrifuged; samples of the sediment were examined in the phase-contrast and electron microscopes.

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Electron microscopy. Enzyme-treated and untreated cells were observed from samples taken after incubation. They were fixed with 5% $KMnO_4$ in 1 M $MgSO_4$ solution for 2 hr at room temperature, and then dehydrated through 25, 50, 75, and 100% acetone. During dehydration, the material was stained overnight in 2% uranyl acetate dissolved in 75% acetone, was embedded in Durcupan ACM (Fluka AG. chemische Fabrik, Bucks S6, Switzerland), was cut with an LKB Ultratome Microscope 4804 A Nife (Servicio de Microscopía Electrónica del C.I.B., Madrid) by use of glass knives, and was examined with a Zeiss EM 9 electron microscope. Pictures were taken at initial magnifications of 6,000.

The cell walls were fixed in 5% aqueous $KMnO_4$ for 2 hr at room temperature, and the dehydration and embedding were carried out as described above.

RESULTS

Figures 1, 3, 5, 7, and 9 show thin sections of the five yeasts before the attack of $\beta(1-3)$-glucanase. All are surrounded by a cell-wall which has an outside zone, thin and opaque, and an inside zone, thick and transparent to the electrons. This morphology is similar to that found by several investigators (8, 12). There is also possibly a third layer (4, 9). *C. utilis* has the thickest wall (1,400 A) followed by *S. cerevisiae* (1,200 A), *S. fragilis* (1,100 A), *H. anomala* (1,000 A), and *P. polymorpha* (800 A). Nuclei, mitochondria, vacuoles, and cytoplasmic components can be clearly observed.

Figures 2, 4, 6, 8, and 10 show the same yeasts after incubation with $\beta(1-3)$-glucanase. The plasmalemma has been detached from the cell wall, as also shown by Kobayashi, Friedman, and Kofroth (6). The cytoplasm was affected during the embedding process. This might be related to the fact that these cells lost a large share of their contents during dehydration and embedding, because there were no cell walls to protect the cytoplasm. This caused shrinkage and an increase in lipid content that may appear as large vacuoles with black points corresponding to polyphosphates, or as clear zones resulting from the loss of lipids during the preparation process. Frequently, cytoplasm was scattered by the breaking of the plasmalemma. Nuclei or mitochondria were rarely observed; when present, they were so shrunken that mitochondrial cristae were faintly visible. The appearance of the cell wall was different in each species of yeast. *S. cerevisiae* had a spongy and swollen wall evenly weakened around the cell. *P. polymorpha* and *C. utilis* showed similar, but less marked, appearance. The cell wall of *H. anomala* was most resistant and needed a longer incubation time for liberating the protoplasts. It still showed the two-layered structure with some degraded zones, and the cytoplasm was shrunken.

The cells of *S. fragilis* were the most variable. Even though they showed less degradation than the other cells, as was the case of *H. anomala*, they appeared broken in some zones as a conse-

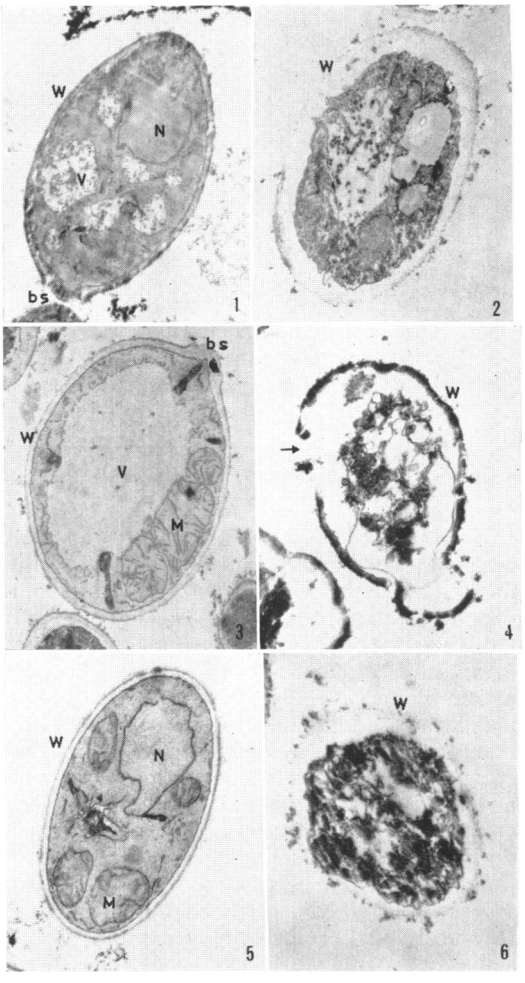

FIG. 1. *Thin section through a Saccharomyces cerevisiae untreated cell. N = nucleus; bs = bud scar; W = cell wall; and V = vacuole. × 10,200.*

FIG. 2. *Thin section through a Saccharomyces cerevisiae enzyme-treated cell. W = cell wall. × 16,800.*

FIG. 3. *Thin section through a Sacchromyces fragilis untreated cell. V = vacuole; M = mitochondria; and bs = bud scar. × 13,800.*

FIG. 4. *Thin section through a Saccharomyces fragilis enzyme-treated cell. W = cell wall. The arrow shows the degraded zones in the wall. × 15,000.*

FIG. 5. *Thin section through a Pichia polymorpha untreated cell. N = nucleus; M = mitochondria. × 19,000.*

FIG. 6. *Thin section through a Pichia polymorpha enzyme-treated cell. W = cell wall. × 18,600.*

quence of a preferential enzyme attack. Through these holes, protoplasts were liberated. This offers an explanation as to why we found intact cells, protoplasts on the way out, liberated protoplasts, and empty cell walls.

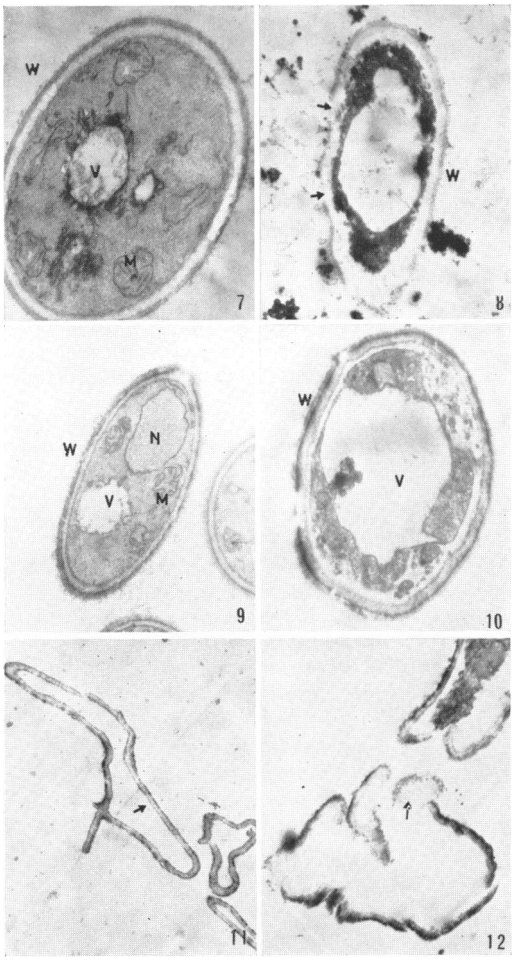

FIG. 7. *Thin section through a Candida utilis untreated cell. W = cell wall; M = mitochondria; and V = vacuole. × 18,000.*

FIG. 8. *Thin section through a Candida utilis enzyme-treated cell. W = cell wall. The arrows show the more degraded zones of the cell wall. × 23,200.*

FIG. 9. *Thin section through a Hansenula anomala untreated cell. N = nucleus; V = vacuole. × 15,600.*

FIG. 10. *Thin section through a Hansenula anomala enzyme-treated cell. W = cell wall; V = vacuole. × 19,800.*

FIG. 11. *Thin section through Candida utilis isolated and untreated walls. Note the third layer in the inner wall. × 12,000.*

FIG. 12. *Thin section through Candida utilis isolated and enzyme-treated walls. Note the degraded zones of the cell wall. × 15,000.*

Figures 11 and 12 show enzyme-treated and untreated cell walls of *C. utilis*. Isolated walls were similar to nonisolated ones, but they seemed to have another inner layer (see arrows). Thus, cells may have a three-layered wall; the third layer has not been detected by microscopic observation of the cell, probably because it is attached to the protoplasts.

DISCUSSION

Based on the photomicrographs, the attack of β(1-3)-glucanase on the cell walls of five yeasts varies greatly from *S. fragilis* to *S. cerevisiae*. The other three yeasts examined show results intermediate between those of these two yeasts.

The cell wall of *S. cerevisiae* is homogeneously degraded by the glucanase over the cell surface, leading us to suppose that glucan is evenly distributed upon it.

The localized attack on certain zones of the cell wall of *S. fragilis* suggests that glucan of *S. fragilis* accumulates in certain zones of the cell wall, namely, those that are attacked by the enzyme. On the other hand, it could be that, even if glucan is homogeneous over the cell surface, its structure differs such that it is easily attacked only on the above-mentioned zones. We must admit that, whereas cell walls consist of accumulated fibers, we do not know the orientation of the fibers on the cell-wall mesh. On the other hand, if the glucan is uniformly located around the cell wall, the differences in behavior could depend on localized mannan, the other polysaccharide of the cell wall (5).

Most analyses for mannan were from cells older than our culture of 12 hr. It is possible that the 12-hr cells have not yet produced mannan and the enzyme, not finding any mannan resistance, can homogeneously attack the *S. cerevisiae* cell walls.

There are no reports about the chemical composition of the *S. fragilis* cell walls. We can assume they have mannan which is not evenly distributed all around the cell surface, such that the enzyme finds mannan resistance only at the unattacked zones.

On the other hand, there must be a relation between yeast bud scars and attacked cell-wall zones. We know these scars have a different ultrastructure from the cell wall. For this reason, the *S. fragilis* cell walls are more readily degraded at the bud scars.

The other three yeasts show an intermediate behavior against β(1-3) glucanase. In this respect, we can order them as follows: *P. polymorpha*, *H. anomala*, and *C. utilis*.

P. polymorpha is similar to *S. cerevisiae*. *C. utilis* is more like *S. fragilis*, but in *C. utilis* the

differences between degraded and undegraded zones are not so great. On isolated walls, this degradation is greater, and this may result from an attack on the inner layer, with the possibility that glucan accumulates in it.

Of all yeasts tested, *H. anomala* is the least sensitive to enzyme attack. It needed an incubation period 7.5 times longer than the other yeasts to free the protoplasts. It showed the least cell-wall degradation of all the yeasts.

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