Note

Degradation of Cell Wall of *Mucor mucedo* by Chitosanase from *Bacillus R-4*

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Chitosanase is an enzyme which hydrolyzes chitosan, β -1,4-linked glucosamine polymer. There have been only a few reports published concerning chitosanase.¹⁻⁴⁾ We have already purified the chitosanase produced by *Bacillus R-4*⁵⁾ and confirmed that the enzyme plays an important role in the lysis of *Rhizopus* cell walls which are mainly composed of chitosan and chitin.⁶⁾ In this report, we will describe the lysis of cell wall and the preparation of spheroplast of *Mucor mucedo* which belongs to the same *Mucorales* as *Rhizopus* by the purified chitosanase of *Bacillus R-4*.

The chitosanase was purified to an ultracentrifugally and electrophoretically homogeneous state from the culture broth of *Bacillus R-4* by SP-Sephadex, Sephadex G-100 and G-75 column chromatographies as described previously.⁵⁾ The cell wall of *Mucor mucedo* was prepared by the procedures described previously.⁸⁾ Hexosamine content of the cell wall was

determined by the method of Randle and Morgan after hydrolysis with 4 N HCl in a sealed evacuated tube at 110°C for 6 hr. The cell wall of *Mucor mucedo* used in this experiments contained 414 μ g of hexosamine (as glucosamine) per mg.

The lysis of *Mucor* cell wall by the purified chitosanase was examined by measuring the decrease in turbidity of the reaction mixture and the increase in the amount of glucosamine liberated. Five milligrams of the cell wall were suspended in 4 ml of 0.2 M acetate buffer (pH 5.6) and homogenized with an ultrasonic oscillator (20 kHz, 3 min). One milliliter of the enzyme solution (10 μ g of enzyme protein) was added to the cell wall suspension, and the reaction mixture was incubated with occasional shaking at 40°C. At various intervals, the decrease in the optical density at

FIG. 1. Lysis of *Mucor* Cell Wall by Chitosanase. The experimental details are described in the text. ●—●, Turbidity; ○—○, Amount of glucosamine liberated.

FIG. 2. Electron Micrographs of *Mucor* Cell Wall Degraded by Chitosanase.

One milliliter of the enzyme solution (10 μ g) was added to 5 mg of the cell wall suspended in 4 ml of 0.2 M acetate buffer (pH 5.6) and was incubated with occasional shaking at 40°C. After 2-hr incubation, the debris of cell wall was collected by centrifugation (15,000 rpm, 20 min) and thoroughly washed with deionized water. The cell wall degraded by the chitosanase was shadowed with gold and was examined with a scanning electron microscope (Japan Optics Laboratory Co., Ltd., Type JMS-2). The control was treated by the same procedures as those described above except for the addition of 1 ml of deionized water instead of the enzyme solution.

660 nm of the reaction mixture was measured with a Hitachi Photoelectric Photometer, and the amount of hexosamine liberated was determined by the method of Randle and Morgan.⁷⁾ As shown in Fig. 1, a rapid decrease in turbidity was observed in an early stage of the reaction, and the optical density was reduced to about 60% after 2-hr incubation. The amount of glucosamine liberated from the cell wall gradually increased and, after 5-hr incubation, reached about 30% of total hexosamine in the cell wall. Electron micrographs of the cell wall degraded by the action of chitosanase are shown in Fig. 2.

Mucor mucedo used in the preparation of spheroplast was grown aerobically at 28°C for 24 hr in a medium containing 3% of malt extract, 2% of glucose, 0.5% of peptone, 0.1% of yeast extract, 0.2% of KH_2PO_4 , 0.2% of $(\text{NH}_4)_2\text{PO}_4$ and 0.1% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, (pH 6.0). The cells were harvested by centrifugation (10,000 rpm, 10 min) and thoroughly washed with cold water containing 0.8% of NaCl. The washed cells

were suspended in 0.2 M acetate buffer (pH 5.6) containing 50% of sucrose to give an optical density of 2.0 at 660 nm. Three milliliters of the cell suspension were incubated with 3 ml of the enzyme solution (30 μg of enzyme protein) with occasional shaking at 40°C. After 1-hr incubation, the reaction mixture was divided into three portions. Two of them were diluted with deionized water, and their concentrations of sucrose were reduced to 12.5% and 6.25%, respectively. The microphotographs of the cells of *Mucor mucedo* at each concentration of sucrose are shown in Fig. 3. The cells gradually swelled, changed into spherical shapes as the concentration of sucrose was lowered, and finally burst. Ghosts of the cells were observed at the concentration of 6.25%. After the cells were removed from each suspension by centrifugation (15,000 rpm, 30 min), the ultraviolet absorption spectra of the supernatant solution were examined with a spectrophotometer. The optical density at 260 nm of the supernatant solution increased as

FIG. 3. Spheroplast Formation of *Mucor mucedo* by Chitosanase. The experimental details are described in the text. (A) Intact cells. (B) Cells incubated with the chitosanase in 25% sucrose solution. (C) Cells suspended in sucrose solution of which the concentration was reduced to 12.5% by dilution. (D) Cells suspended in sucrose of which the concentration was reduced to 6.25% by dilution.

the concentration of sucrose was lowered. These results indicate that the cells of *Mucor mucedo* were converted into osmotically sensitive forms, spheroplasts, by the chitosanase of *Bacillus* R-4.

From the result that the cell wall was degraded by the purified chitosanase, it was confirmed that the chitosan was present in the cell wall of *Mucor*, as well as in that of *Rhizopus* which belongs to the same order, playing an important role in maintaining the structure of the cell wall. Furthermore, since the spheroplast of *Mucor mucedo* was formed by the chitosanase, it was proved that the chitosanase was able to react on intact fungal cell walls. The chitosanase produced by *Bacillus* R-4 may provide an effective tool for studying the structure and function of fungal cell walls which still remain obscure.

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