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Ultrastructural Process of Protoplast Fusion Between *Lentinula edodes* and *Coriolus versicolor*

Chaekyun Kim* **and Byong Kak Kim**

Department of Microbial Chemistry, College of Pharmacy, Seoul National University, Seoul 151-742, Korea

Protoplast fusion is a useful technique for establishing fungal hybrids to overcome the natural barriers. The ultrastructure of protoplast and its fusion process were observed using a scanning electron microscopy (SEM) and a transmission electron microscopy (TEM). The protoplasts were variable in size from 0.5~15 μm in diameter, and the mean diameter was about 3~5 μm. It was impossible to discriminate protoplasts of *Lentinula edodes* from protoplasts of *Coriolus versicolor* by size and surface structure. Big aggregates of the dehydrated protoplasts were observed, after polyethylene glycol 4000 treatment. Nucleus, mitochondria, lipid granules and various vesicles having granules were scattered in the cytoplasm. The vesicles were heterogeneous in size and vary from one protoplast to another. The fused membrane layer of the two protoplasts was observed. True protoplast membrane contact and reorganization of membrane components were essential condition for protoplast fusion. Transmission electron micrograph showed fused protoplasts and flattening of the cells in the area of the membrane contact. We hope that our electron microscopic observations provide some insights into the understanding of the fusion process of protoplast in fungi.

KEYWORDS: Protoplast fusion, *Lentinula edodes*, *Coriouls vesicolor*, SEM, TEM

The development of fusion techniques using protoplasts has opened up many possibilities for generating hybrids and understanding the genetic mechanisms of fungi. Protoplast fusion is a useful technique for establishing fungal hybrids to overcome the natural barriers to gene exchange by conventional breeding systems (Ogawa, 1993).

Lentinula edodes and *Coriolus versicolor* have been claimed to possess several pharmacological effects. *L. edodes* inhibits the growth of tumor cells, the replication of herpes simplex virus, HIV infection, and blood cholesterol level (Fujii *et al.*, 1978; Hamuro *et al.*, 1980; Sakar *et al.*, 1993; Suzuki *et al.*, 1989; Kabir *et al.*, 1987). *C. versicolor* inhibits tumor growth, the interaction between HIV-1 gp120 and CD4 receptor, and HIV reverse transcriptase activity (Ohno *et al.*, 1975; Dong *et al.*, 1996; Tochikura *et al.*, 1987; Collin and Ng, 1997). To investigate the possibility of chimeric mushroom between different orders (Agaricales: *L. edodes* and Aphyllophorales: *C. versicolor*) and to generate the strains having combined or synergistic pharmacological effects, we performed protoplast fusion between *L. edodes* and *C. versicolor* (Kim *et al.*, 1997b, c; 2000). Our success in the production of hybrids between *L. edodes* and *C. versicolor* opened up possibilities for generating hybrids between genetically unrelated orders as well as understanding the genetic mechanism of fungi.

Ultrastructure of fungi has been studied since Braker (1967) published "Ultrastructure of Fungi". However the structure of protoplast and its fusion process in fungi is hardly studied. In the present study, we report the ultrastructual process of protoplast isolation and their fusion between *L. edodes* and *C. versicolor.* This study helps us understand the detail structure of protoplast and their fusion process.

*Corresponding author <E-mail: chaekyun@hotmail.com>

Materials and Methods

Protoplast isolation and fusion. Protoplasts were prepared from *L. edodes* and *C. versicolor* as previously described by Kim *et al.* (Kim and Kim, 1997a; Kim *et al.*, 1997c, 2000). Briefly, the mycelia were treated with 10 mg/m*l* Novozyme 234 (Novo, Bagsvaerd, Denmark) and 10 mg/m*l* Cellulase Onozuka (Yakult, Japan) at 30°C. Hyphal debris was removed by filtration followed by centrifugation for 5 min at 1,000 rpm. After removing enzyme solution by centrifugation, protoplasts were washed with 0.6 M sucrose. Protoplast fusion was started by mixing same numbers of protoplasts (1×10^7/m*l*) from both stains. The protoplast mixture was centrifuged and the pellet was reconstituted in 1 m*l* of 30% polyethylene glycol (PEG 4000) in 10 mM $CaCl_2$-glycine solution (pH 8.0). The fusion mixture was incubated for 15 min at 30°C with gentle shaking, and suspended in 10 m*l* of 0.6 M sucrose. After centrifugation for 7 min at 2,500 rpm, the pellet was suspended in 0.6 M sucrose.

Scanning electron microscopy. For the electron microscopic observations, samples from each step were prefixed in 3% (v/v) glutaraldehyde in 0.6 M sucrose-phosphate buffered saline (S-PBS; pH 7.6) for 5 h at 4°C, and were washed with 0.6 M sucrose. Samples were dehydrated in 30, 50, 60, 70, 80, 90, 95% ethanol for 20 min

and twice in 100% ethanol. Dehydration was completed by standing in acetone for 30 min. Samples were placed on cover glasses and freeze dried. After coating sample slides with fine gold particles, samples were scanned using SEM (JSM-T200, Jeol, Japan).

Transmission electron microscopy. Samples for the TEM observation were prepared by post fixations in 1.0% osmium tetroxide in S-PBS for 2 h at 4°C. The samples were washed with 0.6 M sucrose, dehydrated in ethanol series (30, 50, 70, 80, 90, 100%) and propylene oxide. After dehydration, the samples were embedded and polymerized in epon (nadic methyl anhydride 11.3 m*l*, dodecenyl succinic anhydride 9.9 m*l*, EM bed-812 18.8 m*l*, and 2,4,6-tri(dimethylaminoethyl)phenol 0.6 m*l*; Electron Microscopy Science, PA) for 24 h at 44°C, 50°C, and then 60°C. Ultrathin sections (50~70 nm) were prepared using ultramicrotome (Super Nova, Austria), and were stained with uranyl acetate and lead citrate, and analyzed by TEM (JEM-1200 EXII, Jeol).

Results and Discussion

Observation of protoplast by SEM. The protoplasts were variable in size from 0.5~15 μm, and the mean diameter was about 3~5 μm (Fig. 1A). Yoon (1985) reported that the mean diameter of *L. edodes* was 4 μm, and it is consistent with our data. It was impossible to discriminate protoplasts of *L. edodes* from protoplasts of *C. versicolor* by size and surface structure. The protoplasts were round and smooth (Fig. 1A). After PEG 4000 treatment, big aggregates of the dehydrated protoplasts were observed.

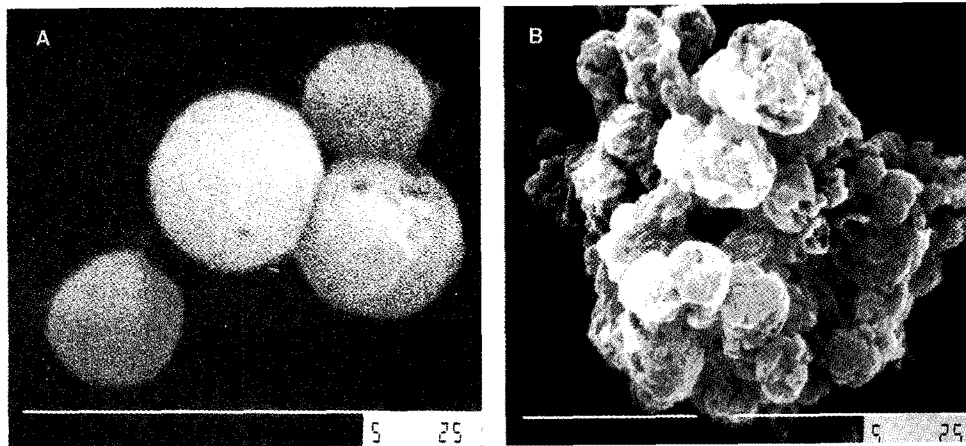

Fig. 1. Scanning electron microscopic structure of the protplasts of *Coriolus versicolar*. Protoplasts (×13,000; A) and aggregates after PEG 4000 treatment were observed (×6,000; B). Scale bar represents 5.0 μm.

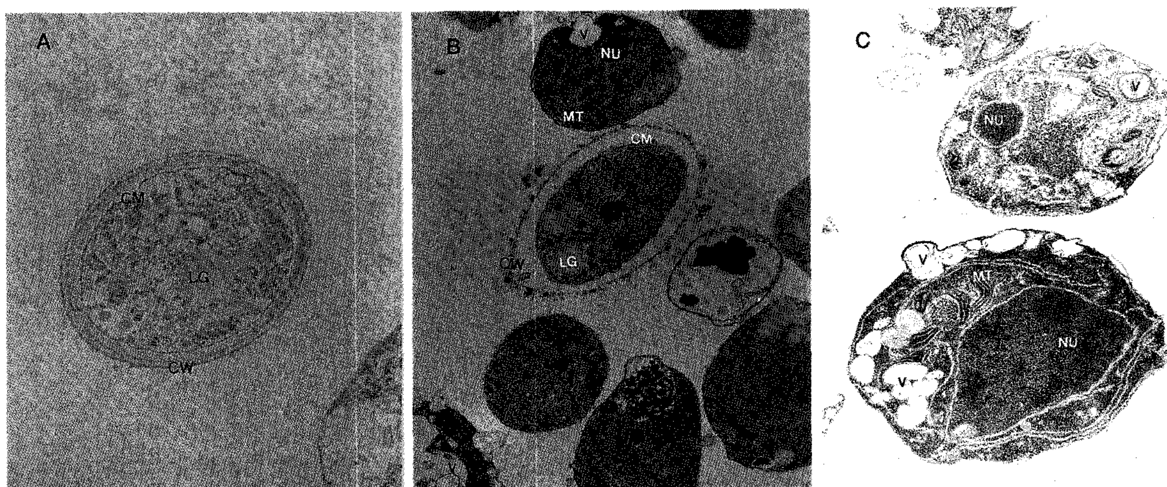

Fig. 2. Transmission electron microscopic observation of protoplasts prepared by standard doublefixation using glutaraldehyde and osmium tetroxide. Various organelles, nucleus (NU), lipid granule (LG), mitochondria (MT), and vacuoles (V) are well developed. (A) Structure of fungal hyphae (×20,000), which shows intact cell wall (CW) and cell membrane (CM). (B) Cell wall starts to separate from cell membrane after lysis enzyme treatment (×12,000). (C) The purified protoplasts, which contain nucleus, mitochondria, lipid granule, and vacuoles (×20,000).

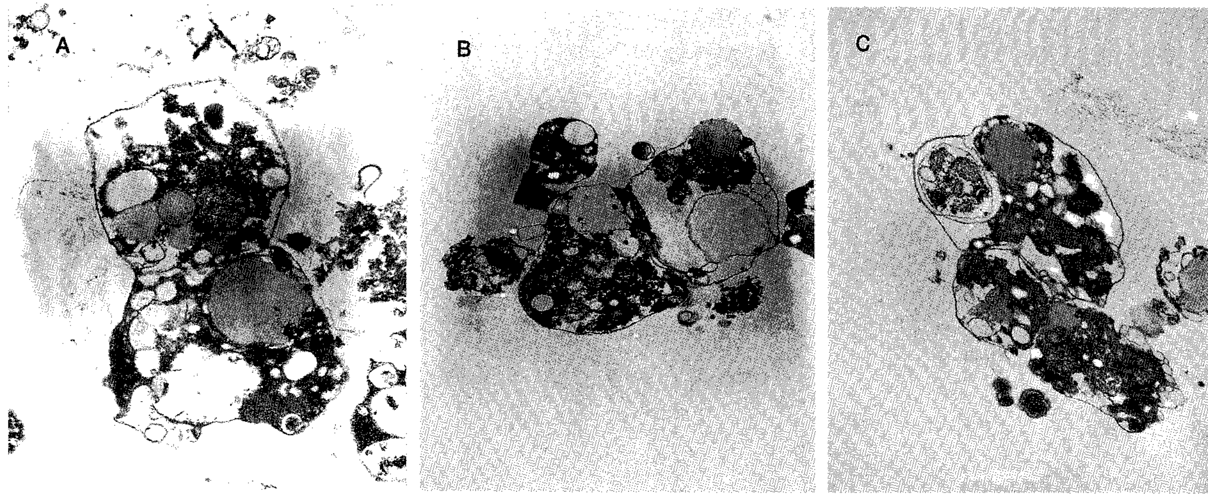

Fig. 3. Transmission electron microscopic observation of protoplast fusion between *Lentinula edodes* and *Coriolus versicolor.* (A) Flattening of cells in the area of the membrane contact was observed (×20,000). (B) Big vacuole is important for protoplast fusion (×15,000). (C) Protoplast including small protoplast in vacuole was observed (×15,000).

The rough surfaces which were made by empty holes and collapsed vesicles on the surface of protoplasts were found (Fig. 1B). It caused by the small vesicles inside the protoplast, which move to the surface and erupted (Cohen *et al.*, 1982). The membrane alteration and other morphological features associated with PEG treatment are well described by Robinson *et al.* (1979). Once cell wall lysis enzyme started to partially destroy cell wall, the gap between cell wall and cell membrane was made. And then protoplast was released by the pushing out force.

Observation of protoplast and its fusion process by TEM. With the aid of osmium tetroxide, protoplast at various stages of fusion process has been fixed for TEM. TEM enabled us to visualize the protoplast and the sequence of events and more in the protoplast fusion in the presence of polyethylene glycol. Figure 2A shows horizontal section of hyphae, which cell wall and membrane were intact, and various organelles were well developed. The gap between cell wall and cell membrane were made after PEG 4000 treatment (Fig. 2B). Figure 2C shows isolated protoplasts after 6 h treatment with cell wall lysis enzymes. Nucleus, mitochondria, lipid granules and various vesicles having granules were scattered in the cytoplasm. Figure 3 shows protoplast and various fusion processes of the two or more protoplasts. Vacuoles are very important for protoplast fusion and regeneration (Peberdy, 1979). Big and small protrusions were also observed on the protoplast surface, and these subprotoplasts do not have cell contents. The uptake of water after incubation with PEG 4000 was accompanied by conspicuous structural changes on protoplast and its layer. The fused membrane layer of the two protoplasts was observed. Membrane contact and reorganization of membrane components were required for the membrane fusion. Flattening of the cells in the area of the membrane contact was observed. The protoplast which including small protoplast inside the vacuole was also observed. In this case, the included protoplast membrane was still remained intact.

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