

The Conidial Protoplast Fusion of Cellulolytic Fungus, *Trichoderma koningii*

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섬유소 분해균인 *Trichoderma koningii* 의 분생자 원형질체 융합에 관하여

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ABSTRACT

Improved methods for the isolation and purification of conidial protoplast were investigated and conidial protoplast from auxotrophic mutants were fused. The reaction time for isolation of protoplasts from the swollen conidiospores preincubated in liquid minimal medium supplemented with 2-deoxy-D-glucose was shortened by reaction with mixture of 2% driselase and 2% β -glucuronidase (1 : 1). The conidial protoplast could be highly purified by using 5% Ficoll 400 as a centrifugation medium. Nucleus of the conidial protoplast was stained with Giemsa stain and the conidial protoplast had one nucleus. It was also confirmed that the conidial protoplast was true protoplast with no cell wall remnant at the outside of plasma membrane. Fusion frequencies of the conidial protoplast from auxotrophic mutants ranged from 3.4×10^{-1} to 4.9×10^{-1} . These values were higher than those of mycelial protoplast by a factor of 5 to 28.

INTRODUCTION

Induced fusion of microbial protoplast is widely used in both basic and applied areas. A short review of the whole area of induced fusion in microbial protoplast was given by Ferenczy (1981).

Cellulolytic fungus *Trichoderma koningii* has already been used in numerous studies on cellulose degradation (Halliwell and Riaz, 1970; Wood and McCrae, 1972; Halliwell and Griffin, 1973). However, the genetic study of these organisms has not been done because of its asexual nature. As one of approaches towards genetic study and strain improvement in industrially important strain, genus *Tricho-*

derma, protoplast fusion technology has been recently applied. Only a few studies, however, reported on the isolation (Benitez *et al.*, 1975; Picataggio *et al.*, 1983; Cho *et al.*, 1981a, b; Lim *et al.*, 1983) and fusion of protoplast from mycelium of *Trichoderma* spp. (Hong *et al.*, 1984a, b; Park *et al.*, 1984 a, b, c).

Most of fusion experiments in filamentous fungi have been done with protoplast from mycelium (Ferenczy *et al.*, 1975; Anné and Peberdy, 1976; Ohnuki *et al.*, 1982). Protoplasts from mycelium vary in size, in number of nucleus, and in organelle constitution. Such heterogenous protoplast suspension from the filamentous fungi are less suitable for high frequency heterokaryon formation, recombination, and regeneration experiments (Bos and

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Slakhorst, 1981). These difficulties might be overcome by the isolation of protoplast from conidiospores. Recent reports concerned with the isolation of protoplast from conidiospores in filamentous fungi are the cases; *Aspergillus flavus* (Moore and Peberdy, 1976), *Aspergillus nidulans* (Bos and Slakhorst, 1981), *Trichoderma reesei* (Toyama *et al.*, 1983), and *Trichoderma koningii* (Park *et al.*, 1983, 1984c). There are only few reports on fusion of protoplast from conidiospore (Toyama *et al.*, 1984), and there is no reports on the fusion of conidial protoplast in *Trichoderma koningii*.

We report here modifications of previous methods (Park *et al.*, 1983) for isolation and purification of conidial protoplast and the fusion frequency of conidial protoplast in cellulolytic fungus, *Trichoderma koningii*.

MATERIALS AND METHODS

Organisms

Trichoderma koningii ATCC 26113 and its auxotrophic mutants obtained by the methods described below were used in this experiment.

Media

Malt extract medium and modified Mandel's medium were used as the complete (CM) and minimal medium (MM), respectively (Hong *et al.*, 1984a, b). For the regeneration complete (RCM) and regeneration minimal medium (RMM), the CM supplemented with 0.6 M $MgSO_4$ and the MM supplemented with yeast nitrogen base without amino acid (0.65%, Difco) and 0.6 M $MgSO_4$ were used. For preparing regeneration agar medium, 2% agar was autoclaved separately to avoid acid hydrolysis, and Triton X-100 (0.05%, v/v) was added to the solid medium for the restriction of colony size (Park *et al.*, 1984a). For the preincubation of conidiospore, liquid minimal medium supplemented with 2-deoxy-D-glucose (25 $\mu g/ml$, w/v) was used. If required, vita-

mine free casamino acid (2 mg/ml) and adenine (5 $\mu g/ml$) were added to the preincubation medium for swelling of conidiospores from auxotrophic mutants.

Mutagenesis

Auxotrophic mutants were derived by mutagenesis with ultraviolet light (UV) and N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The conditions of mutagenesis with UV has been described previously (Hong *et al.*, 1984b). The conditions of mutagenesis with NTG is as follows: Conidiospore suspension of parental strains ($1.0 \times 10^7/ml$) in 0.01 M phosphate buffer (pH 5.8) containing 0.01% tween 80 was treated with NTG (0.2 mg/ml) for 50 min at 30°C to kill 99% of the population. Auxotrophic mutants were enriched by the filtration methods (Hong *et al.*, 1984b). The lineage of auxotrophic mutants is shown in Fig 1.

Protoplast preparation

Protoplasts were prepared from the conidiospores of *T. koningii* according to the methods described before (Park *et al.*, 1983) with minor modifications. The conidiospores preincubated for 8.5 hrs in liquid minimal medium containing 2-deoxy-D-glucose (2-DG) were harvested by centrifugation ($750 \times g$, for 15 min), and washed twice with osmotic stabi-

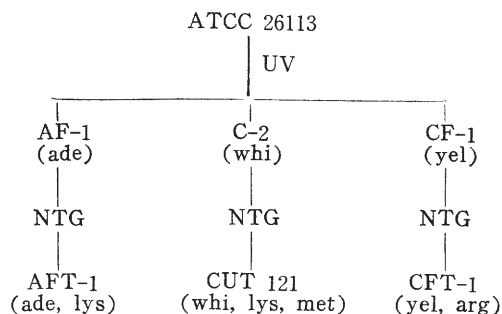


Fig. 1. Lineage of mutants derived by mutagenesis with UV and NTG treatment. Abbreviations: yel: yellow color conidia; whi: white color conidia; ade: adenine requiring; arg: arginine requiring; lys: lysine requiring; met: methionine requiring.

lizer (0.6 M MgSO_4 in 0.01 M phosphate buffer pH 5.8). The washed swollen conidiospores were treated with combinations of driselase and β -glucuronidase. To test the effect of cell wall lytic enzymes, combined solutions of driselase and β -glucuronidase (1% driselase, 2% driselase, 1% β -glucuronidase, 2% β -glucuronidase, and one to one volume mixture of 2% driselase and 2% β -glucuronidase) were added to the pelleted conidiospores and reacted at 28°C. The reaction mixture was sampled periodically and number of protoplasts was determined with the aid of haemocytometer.

Purification of protoplasts

One or two milliliter of reaction mixture was overlayed onto 5 ml of Ficoll solution (5%, w/v) dissolved in osmotic stabilizer in 10 ml centrifuge tube. After centrifugation with horizontal rotor (400×g, for 30 min with slow accelerator), the upper layer was removed into a new centrifuge tube with sterilized Pasteur-pipette. The purified protoplasts were washed twice with osmotic stabilizer by centrifugation.

Staining of protoplast

The purified protoplasts were fixed with 3% glutaraldehyde in osmotic stabilizer for 4~6 hrs at 4°C, and washed with distilled water. The fixed and washed protoplasts were allowed to stand in 5 N HCl at 60°C for 10 min, and washed with and resuspended in distilled water. One loopful of protoplast suspension was smeared on a slide glass, and freeze-dried in freeze-dryer (Labconco). The freeze-dried specimen was soaked in various staining solutions for 1 hr at room temperature. Thereafter, excessive stain was decolorized with 95% ethanol solution. The specimen was examined with a multipurpose light microscope and photographed (Nikon Nippon Kogaku K.K., Japan).

Preparation of specimen for electron microscopy:

Protoplasts were fixed with 3% glutaraldehyde in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.6 M MgSO_4 . The mixture was then gently stirred, allowed to stand overnight at 4°C, washed twice with buffer, and pelleted by centrifugation. Afterwards, these pellets were resuspended in osmium tetroxide (1%), allowed to stand for 2 hrs and rinsed with buffer. For transmission electron microscopy (TEM), the post-fixed sample was pelleted and solidified with pre-warmed agar (2% in buffer). Agar block was cut on a slide glass into pieces approximately by 1 mm². These blocks were suspended in 0.5% uranyl acetate, and dehydrated using a series of ethanol solution of increasing concentration (60, 70, 80, 90, 95%) and absolute ethanol twice for 20 min. The specimen was then embedded in an epoxy resin, Epon. After polymerization by incubating for 3 days at 65°C, the section was cut on a microtome and Sorvall MT-2 ultramicrotome with glass knives, stained with uranyl acetate, and examined with an electron microscope (Hitachi H-500, Japan). In preparing the specimen for scanning electron microscopy (SEM), the sample post-fixed with osmium tetroxide was filtered through a membrane filter (0.45 μm) and dehydrated using a series of the ethanol solution. These samples on the membrane filter were treated with isoamylacetate for 30 min and dried with a critical point dryer (Hitachi HCP-2) using liquid CO_2 . The dried sample was then coated with gold on aluminium stub and examined with a scanning electron microscope (Hitachi S-450, Japan).

Fusion of protoplast

Protoplast fusion was carried out by the methods described previously (Hong *et al.*, 1984b). Protoplasts from two parental strains were mixed (5.0×10^6 each) and sedimented

by centrifugation. The pelleted protoplasts were resuspended in the minute volume of the remaining stabilizer by gentle mixing. The protoplasts were treated with 1 ml of pre-warmed 30% polyethylene glycol 6,000 (containing 10 mM CaCl_2) in 0.05 M glycine (pH 5.5) and incubated for 10 min at 28°C. After incubation, PEG-treated protoplasts were serially diluted and plated on the RCM and RMM, and incubated at 28°C. Fusion frequency was calculated by dividing the number of colonies per milliliter appeared on the RMM by the number of colonies per milliliter on the RCM.

RESULTS AND DISCUSSION

Effect of cell wall lytic enzymes

According to the results shown in Fig. 2 and reported previously (Park *et al.*, 1983), the yield of protoplast production with driselase (1%, 2%) was slightly higher than that of β -glucuronidase (1%, 2%). However, the yield of protoplast production with mixture of 2% driselase and 2% β -glucuronidase solution (1:1) was almost the same as that with 2% driselase and protoplast production reached maximum value within 2 hrs reaction, differently from the case of 2% driselase. In general, regeneration frequency of protoplast was declined with prolonged incubation with cell wall lytic enzyme, probably because small amount of contaminated proteolytic enzymes in lytic enzyme preparation might cause lethal damage to protein or lipoprotein components of plasma membrane (Gaupy and Fevre, 1982). The protoplast was highly vacuolated after 3hrs incubation and it could hardly pelleted in washing step. Consequently, it would be better to be able to shorten the reaction time with cell lytic enzymes. For fusion experiment, the mixture of driselase and β -glucuronidase was used as a protoplasting system.

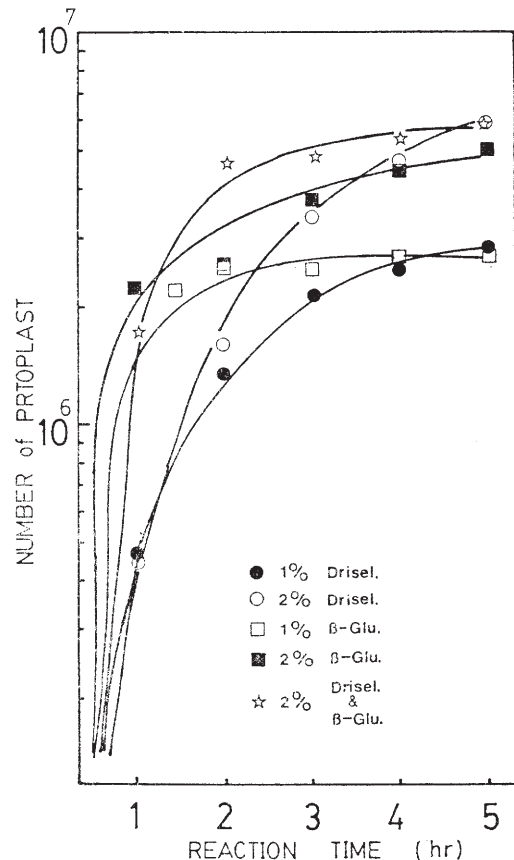


Fig. 2. Yield of protoplast production treated with combinations of enzymes. Conidiospores preincubated in liquid minimal broth for 8.5 hrs were reacted with various combinations of cell wall lytic enzymes. The yield of protoplast production were determined periodically with the aid of haemocytometer.

Purification of protoplast

Sucrose (30% in stabilizer) used to separate the protoplasts from conidial cell wall debris and undigested conidiospores (Bos and Slakhorst, 1981; Park *et al.*, 1983) was not good enough to obtain highly purified protoplast preparation. To enhance purity of the protoplast, we used Ficoll 400 (m.w. 400,000, Pharmacia Fine Chemical) as a centrifugation medium. A copolymer of sucrose and epichlorohydrine, Ficoll, is particularly suitable for density gradient centrifugation of membrane-bound particles. High density solution with low osmotic pressure can be prepared with it.

Thus Ficoll has been used to purify protoplast and membrane-bound vesicles. Varied concentration of Ficoll solution in osmotic stabilizer (5, 10, 20%) was examined for purification of protoplasts. The protoplasts were successfully separated from the conidial cell wall debris and undigested conidiospores using 5% Ficoll as the centrifugation medium.

Nucleus staining of protoplast

Nucleus of the purified protoplast was stained with several staining solution. The purified protoplasts were fixed with glutaraldehyde and allowed to stand in 5 N HCl at 60°C for 10 min. Thereafter, nucleus of the protoplast was stained. Treatment of the fixed protoplast with 5 N HCl was essential step for good staining. For the attachment of protoplast on slide glass, protoplast suspension was smeared on slide glass and lyophilized. This procedure was more convenient and effective than egg albumin method. Several stains were tested for nuclear staining. The nucleus was stained with Giemsa, lacto-orcein, and acetocarmine, however, best result was obtained with Giemsa staining. The nucleus of the protoplast was easily observed when the glutaraldehyde-fixed protoplasts were allowed to soak in 5 N HCl for 10 min at 60°C and stained with Giemsa solution for 1 hr, and washed with 95% ethanol (Fig. 3). Because most of the stained protoplasts have one nucleus, it is possible that regeneration frequency of the conidial protoplast was higher than that of heterogeneous mycelial protoplast (Cho *et al.*, 1981a; Lim *et al.*, 1983).

Electron microscopic observation of conidial protoplast formation

After cell wall lytic enzyme treatment to the swollen conidiospores preincubated in liquid minimal medium supplemented with 2-DG, process of protoplasting from the conidiospores was observed with scanning and

transmission electron microscopy. In SEM observation, protoplast in the releasing process and protoplast released were observed (Fig. 4). In TEM observation, various structure such as partially broken conidial cell wall, peripheral vesicles, vacuoles with electron dense material, protruding vacuoles, protoplast in the releasing process, protoplast without cell wall material, and ghost of the conidial cell wall were observed (Fig. 5). These results indicated that the protoplast was not produced by complete digestion of conidial cell wall but released by protrusion through pore of conidiospore (Park *et al.*, 1983, 1984c). It was also confirmed that the conidial protoplast was not the sphaeroplast with cell wall remnant but true protoplast with no cell wall remnant at the outside of plasma membrane.

Fusion of conidial protoplast

Conidial protoplast fusion between auxotrophic mutants was carried out according to the conditions for mycelial protoplast fusion (Hong *et al.*, 1984b). As shown in Table 1, fusion frequencies for complementing heterokaryons of the cross CFT-1(arg) × AF-1(ade), CUT 121(lys, met) × AF-1(ade), and CUT 121(lys, met) × AFT-1(ade, lys) were 4.8×10^{-1} , 3.4×10^{-1} and 4.9×10^{-1} , respectively. As expected, these values of fusion frequency were

Table 1. Fusion frequency for complementing heterokaryon formation between protoplast from auxotrophic mutants

Cross (genotype) ^a	Fusion frequency	
	Conidial	Mycelial ^b
CFT-1 × AF-1 (arg) (ade)	4.8×10^{-1}	1.7×10^{-2}
CUT 121 × AF-1 (lys, met) (ade)	3.4×10^{-1}	7.4×10^{-1}
CUT 121 × AFT-1 (lys, met) (ade, lys)	4.9×10^{-1}	ND ^c

a) All the abbreviations are given in the legend to Fig. 1

b) Park *et al.*, 1984b

c) Not determined.

higher than those of mycelial protoplast by a factor of 5 to 28.

In cross between CUT 121(lys, met) × AFT-1 (ade, lys), prototrophic colonies were detected on RMM. This result suggests that the mutation site of both strains on the gene concerned with lysine biosynthesis may be different and the defect on lysine gene in both strains may

be overcome by complementing heterokaryon formation through protoplast fusion.

The results reported here indicate that high frequency heterokaryon formation can be achieved by conidial protoplast fusion, and determination of complementation group among auxotrophic mutants can be done with protoplast fusion technique.

적 요

2-deoxy-D-glucose가 첨가된(25 µg/ml) 최소액체 배지에 8.5시간 전 배양하여 swelling 시킨 conidiospore에 2% driselase와 2% β-glucuronidase를 동량 혼합한 효소용액을 처리하여 반응시킨 결과 protoplast 형성 시간을 2시간 이내로 단축시킬 수 있었다. 5% Ficoll(m.w. 400,000)용액을 사용하여 conidial protoplast를 보다 순수하게 분리 정제할 수 있었으며, protoplast를 Giemsa로 핵 염색한 결과, 대부분의 protoplast는 하나의 핵을 갖고 있었다. 또한 전자현미경으로 관찰한 결과 conidiospore에서 유래된 protoplast는 세포벽 성분이 남아 있지 아니한 완전한 protoplast인 것으로 판명되었다. 영양요구성 돌연변이주에서 유래된 conidial protoplast의 융합률은 3.4×10^{-1} 에서 4.9×10^{-1} 수준으로 이는 mycelial protoplast의 경우보다 5~28배 가량 높은 수준의 것이었다.

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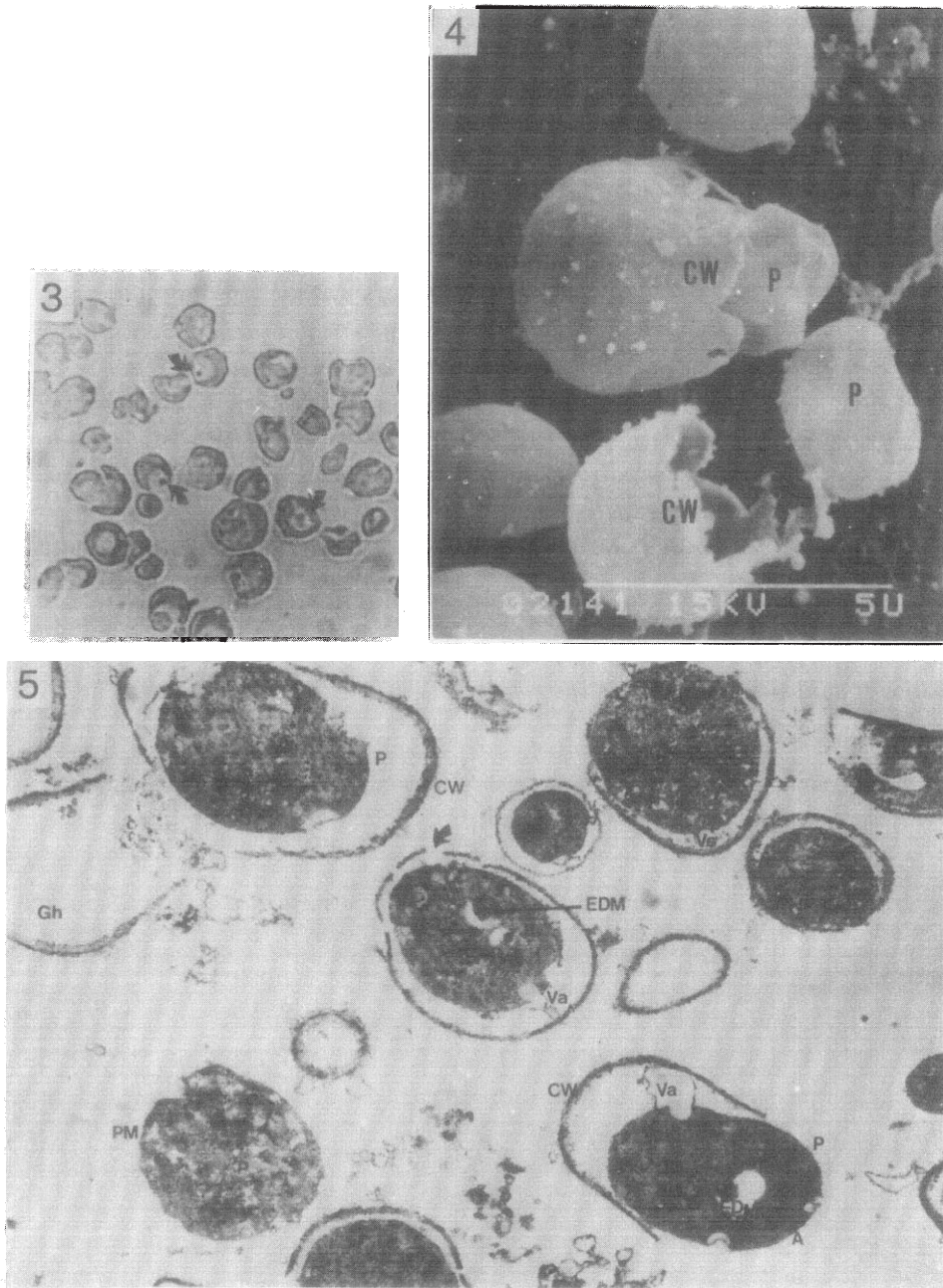


Fig. 3. Nucleus staining of conidial protoplast. After treatment of glutaraldehyde-fixed protoplasts in 5N HCl for 10 min at 60°C, protoplasts were stained with Giemsa for 1 hr. Nucleus is indicated with arrow.

Fig. 4. Scanning electron microscopic observation of protoplasting from swollen conidiospore. Protoplast in the releasing process and released protoplast are shown. W: cell wall; P: protoplast.

Fig. 5. Transmission electron microscopic observation of protoplasting from conidiospores. Various structures such as partially broken conidial cell wall (arrow), peripheral vesicles (Ve), vacuole with electron dense material (EDM), protruding vacuoles (Va), protoplast in releasing process (A), protoplast without cell wall remnant (P), and ghost of the conidial cell wall (Gh) are observed ($\times 3,500$).