Intergeneric Protoplast Fusion between *Rhizopus oryzae* and *Aspergillus oryzae*

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Conditions for the release and regeneration of protoplasts from *Rhizopus oryzae* and intergeneric protoplast fusion between *Rhizopus oryzae* and *Aspergillus oryzae* were studied. High yields of protoplasts from young germlings of *R. oryzae* were obtained by using lytic enzymes containing chitosanase (3 mg/ml), chitinase (3 mg/ml) and Novozym 234 (5 mg/ml). 0.5 M glucose was used as the osmotic stabilizer and optimum pH of buffer was determined to be pH 7.5~8.0. Under these conditions, protoplasts were formed after about 3~4 hrs incubation. Approximately, 1.0%~4.9% of these protoplasts regenerated on solid medium with a soft agar overlay. We have also carried out protoplast fusion between *R. oryzae* and *A. oryzae* and have succeeded in obtaining three types of intergeneric fusants. In these experiments, 35% PEG4000 and 10 mM $CaCl_2$ were used as fusogenic agents, and auxotrophic properties were used as a genetic marker to select fusants. Complementation frequency by protoplast fusion of *A. oryzae* and *R. oryzae* was 4.4×10^{-5}. The fusant strains of the first type were prototrophs showing an *Aspergillus* type morphology with dark-yellow sporulation, those of the second type were also *Apergillus* type morphology but showed no sporulation. And the strains of the third type stopped growing when fusion products grown on regeneration minimal medium were transferred to fresh minimal medium. The formation of fusion products was observed by fluorescent vital stains for complementary labelling of protoplasts from *R. oryzae* and *A. oryzae*. Rhodamine 6G and fluorescein diacetate were useful complementary vital stains of *Rhizopus* and *Aspergillus* protoplasts for visualization of frequency and type (dicell, multicell) of fusion.

KEY WORDS □ *Rhizopus oryzae*, *Aspergillus oryzae*, protoplast fusion

Fungal protoplasts have been used extensively in studies of cell wall regeneration (10) and, more recently, they have been used in the development of genetic manipulations which involve the uptake of nucleic acids. Fusion of protoplasts has allowed the selection of heterokaryons in some fungi, in most cases, is the only way of obtaining hybrids between two different species (4, 7).

Rhizopus oryzae is a very important organism in fermentation industry because it secretes a large amount of various enzymes extracellularly such as amylase and cellulase (15). The isolation, regeneration and fusion of *Rhizopus* protoplasts are essential steps in the use of genetic technique in *Rhizopus* strains. *Rhizopus oryzae* is classified in the Mucorales. The cell wall of Mucorales is different from other filamentous fungi in that it contains chitosan as one of the main components (9, 15). There are many reports concerning intra- and interspecific protoplast fusions of filamentous fungi including *Aspergillus* (8, 11), *Penicillium* (5, 9), *Trichoderma* (12), and *Cephalosporium* (7). However, only a few studies have been carried out in the protoplast fusion of Mucorales fungi (5, 14). In this work, we have standardized the necessary steps for isolation, regeneration, and fusion of *Rhizopus oryzae* protoplasts. Using these conditions investigated, we also carried out the protoplast fusion between *Rhizopus oryzae* and *Aspergillus oryzae* and studied the properties of the obtained fusants.

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MATERIALS AND METHODS

Strains and media

The strains used in this study were *R. oryzae* ATCC 22581-1(ino-), *R. oryzae* ATCC 22581 (wild type) and *A. oryzae* ATCC 16507 (lys-). *A. oryzae* ATCC 16507 and *R. oryzae* 22581 were obtained from ATCC and KTCC, respectively. Strain ATCC 22581-1(ino-) was derived from prototroph strain *R. oryzae* ATCC 22581 by NTG mutagenesis

(2) in this experiment.

Minimal medium (MM) and rich medium (CM) were used as described by Hamamoto *et al.* (5). In protoplast regeneration and fusion experiments, CM and MM were supplemented with osmotic stabilizer, 0.5 M glucose, and described RCM and RMM, respectively. In order to isolate single colonies after mutagenic treatment and fusion experiment, low pH agar media (pH 3.0) were used. The low pH was effective in assuring restricted growth of colonies which was essential for mutant isolation and detection of fusants. Agar in these media was autoclaved separately to avoid hydrolysis at the acidic pH.

Protoplast formation and regeneration

Sporangiospores of *Rhizopus* were inoculated into Erlenmyer flask (250 m*l*) containing 30 m*l* of complete broth medium and germinated at 30 °C with shaking for 7~8 hrs. Germlings (germinated spore) were collected by centrifugation at 5000 *g*, washed with 50 mM Tris-buffer (pH 7.5) containing 0.5 M glucose (protoplasting solution). The germlings were added to a lytic enzyme solution dissolved in the same buffer to give a final concentration of about 1×10^8/m*l* and incubate at 30°C for about 3~4 hrs with gentle shaking. Isolated spheroplasts were washed three times with stabilizer by centrifugation (at 1000 *g*). After this, they were added to melted (35~40°C) regeneration medium which was the same as the normal *Rhizopus* medium (CM or MM) except that the agar concentration was reduced to 0.6% (W/V) and 0.5 M glucose was present. Ten m*l* of this medium was poured onto plates already containing CM or MM plus 0.5 M glucose.

The preparation and regeneration of protoplasts derived from the mycelium of *A. oryzae* were performed according to the procedure of Lee *et al.* (8).

Fusion of protoplasts

Protoplast fusion was carried out basically by the methods of Anne and Peberdy (10) and Hamamoto *et al.* (5). Two protoplast suspensions derived from two strains, each having one different gene marker, were mixed (10^8/m*l* of each), and polyethylene glycol 4000 and $CaCl_2$ were added at final concentration of 35% (W/V) and 10 mM, respectively. After standing for 10 min at 30°C, the mixture was diluted by 5-fold by stepwise additions of liquid MM containing 0.5 M glucose and centrifuged (1000 *g*, 10 min). Protoplasts were washed twice with 8 m*l* portions of stabilizer. Serial dilutions were added to 10 m*l* hypertonic MM containing 0.6% agar, pre-incubated at 40°C, and overlayed on hypertonic MM containing 2% agar in 9 cm petri dishes. The fusion frequency was calculated from the ratio of colonies growing on RMM and RCM.

Fluorescent vital stains for complementary labelling of protoplasts

Stains used in this research were rhodamine 6G and fluorescein diacetate (6). Stock solutions of these stains were made as follows. Five milligrams of rhodamine 6G were dissolved in 1 m*l* of absolute ethanol. Seven milligrams of fluorescein diacetate were dissolved in 1 m*l* of dimethylsulfoxide.

For staining, the following quantities of stock solution were added to 1 m*l* aliquots of protoplasting solution, containing $1\times10^7-1\times10^8$ protoplasts, of rhodamine 6G (1× or 3×), 4 μ*l* and fluorescein diacetate, 4 μ*l*. And then, the mixture was incubated at room temperature for 10 min. Protoplasts were removed from the stain solution by centrifugation and resuspended in protoplasting solution, and this cycle was repeated. All observations were made using incident fluorescence microscopy. A Bausch and Lomb microscope equipped with FITC exciter filter (transmission between 350 and 490 nm), a dichroic filter with a transmission above 500 nm and a barrier filter transmitting below 550 nm were used.

Characterization of the fusants

Fusion products were picked up from the plate and inoculated on minimal agar slants. In order to test the genetic stability of hybrids obtained on protoplast fusion, an arbitrarily selected fusant colony was transferred onto the complete medium (CM) and cultivated for 14 days for 30°C for preparation of spore suspension. And then, their nutritional requirement was determined. The DNA contents of fusants were determined by the method of Stewart (13) and Burton (3) with calf thymus DNA (Sigma) as standard.

RESULTS AND DISCUSSION

Conditions for protoplast formation

In order to define optimal condition for enzymatic treatment of *Rhizopus*, a number of factors affecting protoplast formation were checked. First, the effects of sorbitol, mannitol, sucorse, glucose, $MgSO_4$, or KCl were examined as an osmotic stabilizer for protoplast formation. As shown in Table 1, solutions of sorbitol or glucose turned out to be more effective as osmotic stabilizers than salt solutions. $MgCl_2$ and KCl, which have been described as a stabilizer for isolating protoplasts in filamentous fungi (3, 8, 9), were not effective for *Rhizopus*. Protoplasts generated in salt solutions were very small or partially disrupted. While those prepared with the sugar alcohol solution showed a perfectly spherical form. And regeneration rates of protoplasts from potassium chloride solution were considerably lower than those from sugar or sugar alcohol solution (data not shown). We routinely

Table 1. *Influence of different osmotic stabilizers on the formation and stability of protoplasts of Rhizopus. oryzae.*

Osmotic stabilizer	Conversion rate(%)* 2 h	3 h
A. 0.6 M KCl	44.0	50.0
B. 0.5 M $MgSO_4$	15.4	46.2
C. 0.8 M Sorbitol	76.0	87.9
D. 0.5 M Mannitol	64.9	75.7
F. 25% Sucrose	50.0	72.5
G. 0.5 M Glucose	77.6	97.2

*The conversion rate of germlings into protoplasts was assayed by observation under a microscope.

Table 2. *Protoplast formation of Rhizopus oryzae using various lytic enzymes singly and together.*

Enzyme solusion	Protoplast formation (Incubation Time) 1 h	2 h	3 h	4 h
A. Novozym 234 (5 mg/ml)	−	−	−	−
B. β-glucuronidase (5 mg/ml)	−	−	−	−
C. Chitinase (3 mg/ml)	−	−	−	−
D. Chitosanase (3 mg/ml)	−	−	−	−
E. Novozym 234 (5 mg/ml) +Chitinase (3 mg/ml)	−	−	−	−
F. Novozym 234 (5 mg/ml) +Chitosanase (3 mg/ml)	−	−	±	±
G. Chitinase (3 mg/ml) +Chitosanase (3 mg/ml)	−	−	±	±
H. Chitosanase (3 mg/ml) + β-glucuronidasae (5 mg/ml)	−	−	−	±
I. Novozym 234 (5 mg/ml) +Chitinase (3 mg/ml) +Chitosanase (3 mg/ml)	±	+	+++	+++

Germlings (1.8×10^6 germlings/ml) were treated at 30 °C with each enzyme solution in 50 mM Tris-HCl buffer (pH 7.4) with 0.5 M glucose as an osmotic stabilizer. The conversion rate of germlings into protoplasts was assayed by observation under a microscope. Symbols −, ±, +, ++ and +++ indicate 0, < 30, 30~60, 60~90, > 90% conversion, respectively.

empolyed 0.5 M glucose solution as an osmotic stabilizer.

Novozym 234, chitinase, β-glucuronidase, and chitosanase were examined singly and in combinations as to their ability to release protoplasts (Table 2). The cell wall of Zygomycetes contains chitosan as main component, in contrast to most of other filamentous fungi which contain chitin and β-1.3-glucan as main cell wall component. There are several reports concerning the protoplast formation of Zygomycetes (5, 14),

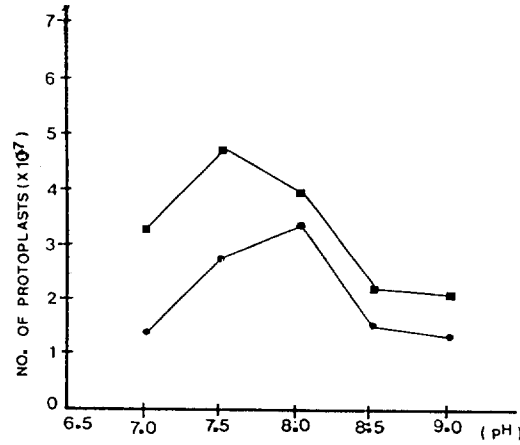

Fig. 1. *Influence of pH on the formation of protoplast of Rhizopus oryzae.*
Symbol (●) and (■) represent 3 hrs and 4 hrs incubations, respectively.

and chitosanase activity was required in each case. In this study, efficient method for protoplast formation of *Rhizopus* was established using three lytic enzymes, that is, Novozym 234, chitinase, and chitosanase combination (I). The germling could be completely converted into protoplasts by treatment with three lytic enzyme mixture (I) in 3~4 hrs at 30°C. No protoplast was obtained when these lytic enzymes were used alone (A, B, C, and D).

The optimum pH for protoplast formation on combined use of three lytic enzymes was determined using 50 mM Tris-HCl buffer containing 0.5 M glucose. The maximum yield of protoplasts was obtained at pH 7.5~8.0 (Fig. 1).

The culture age of the mycelium strongly affected the yield of protoplasts. Protoplasts were obtained most effectively from the germling 7~8 hrs old. Fig. 2(A) shows germlings at this stage. About 7~8 hr cultivation in YpG medium, the germling population showed some degree of asynchrony. In majority of germling, the germ tubes were short but a few longer ones could be observed. The germ tube was sensitive to enzymatic attack while the spore wall was not. And the efficiency of protoplast formation from longer germlings was lower. Photographs of intact germling 7~8 hrs old and of protoplasts prepared from them under the best conditions so far examined are shown in Fig. 2. Protoplast formation and regeneration of protoplasts derived from the mycelium of *A. oryzae* were performed according to the procedure of Lee *et al.* (8). Fig. 2(D) shows freshly isolated protoplasts from mycelia of *A. oryzae*.

Regeneration of protoplast

The regeneration frequency of *Rhizopus* and

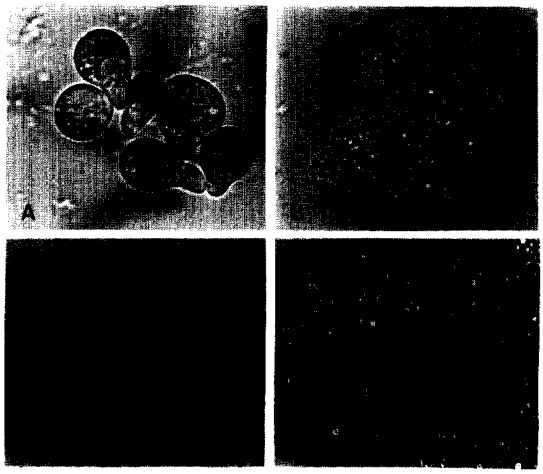

Fig. 2. *Photomicrography of Rhizopus oryzae and Aspergillus oryzae.*
A: *Rhizopus* germlings after 7~8 hrs growth in YpG medium. The majority of the germlings were of the shorter type. This size was the most convenient for obtaining protoplasts.
B: Protoplasts formed by the three lytic enzyme mixture (Table 2).
C: Mycelium of *A. oryzae* after 18 hrs growth in liquid malt extract medium.
D: Freshly isolated protoplasts from mycelia of *A. oryzae*.

Aspergillus protoplasts calculated from the number of colonies that appeared on the regeneration media and the total protoplast counts of the original suspension were shown in Table 3. The frequencies of regeneration of *Rhizopus* protoplasts were 1.0~4.9% depending on the regeneration media used. Regeneration frequency of *A. oryzae* protoplasts was 0.3~0.4%. Regeneration frequency of *Aspergillus* protoplasts was increased when 0.6 M KCl was used as an osmotic stabilizer (Table 3). Because 0.6 M KCl was not effective for *R. oryzae*, 0.5 M glucose was used as an osmotic stabilizer in the protoplast fusion between *Aspergillus* and *Rhizopus*.

Fusion of protoplasts

Intergeneric protoplast fusion between *Rhizopus* and *Aspergillus* was carried out using two auxotrophic strains combination (Table 4). Fusants that appeared on RMM were picked up and then cultivated on MM slant agar for subsequent analysis. As shown in Table 4, the fusion frequency was 4.4×10^{-5}. This frequency is 3~4 orders of magnitude higher than the reversion frequencies of the auxotrophic mutant strains.

The formation of fusion products was monitored by fluorescent vital stains for complementary labelling of protoplasts from *R. oryzae* and *A. oryzae*. Complementary vital staining of parental protoplasts prior to fusion is useful for several purposes. The formation of fusion products can be monitored and the frequency of formation of dicellular and multicellular heterofusants can be determined by direct microscopic observation. In this study, protoplasts stained with rhodamine 6G or fluorescein diacetate were fused in PEG solution (35%(w/v) PEG, 10 mM $CaCl_2$ in 50 mM Tris-HCl buffer) and observed (Fig. 3). Using these stains, heterofusants could be detected and observed. To determine whether protoplasts were injured by the stains used, viability of protoplasts stained with rhodamine 6G or fluorescein diacetate was tested with *R. oryzae* strain (Table 3). No deteriorative effects on protoplast viability were detected.

Therefore, rhodamine 6G and fluorescein diacetate are useful complementary vital stains of *R. oryzae* and *A. oryzae* protoplasts for visualization of frequency and type (dicell, multicell) of fusion.

We have carried out protoplast fusion between

Table 3. *Regeneration frequency of protoplasts from Aspergillus and Rhizopus.*

Strain	Regeneration frequency(%)			
	RCM	RMM	rhodamine 6G[a]	fluorescein[a] diacetate
R. oryzae ATCC 22581-1 (ino)	4.9	1.0	2.3	2.6
A. oryzae ATCC 16507 (lys)	0.4(6.4)[b]	0.4(13.0)	ND	ND

ND: Not determined.
RMM was supplemented with lysine (50 μg/ml) or inositol (50 μg/ml).
a : Stained protoplasts with rhodamine 6G or fluorescein diacetate were plated on the rich regeneration medium (RCM). Plates were incubated at 30°C for 3~7 days until the number of colonies could be counted.
b : Values in parentheses indicate the regeneration frequency of *A. oryzae* protoplasts when 0.6 M KCl was used as an osmotic stabilizer.

Table 4. *Frequency of intergeneric protoplast fusion between Aspergillus and Rhizopus.*

Protoplast pairs	Reversion frequency[a] (less than)	Fusion frequency[b]
R. oryzae ATCC 22581-1 (ino)	$<0.7\times10^{-9}$	
×		4.4×10^{-5}
A. oryzae ATCC 16507 (lys)	$<0.4\times10^{-8}$	

[a]: Reversion frequencies of two auxotrophic strains were defined as the ratio of the number of protoplasts generating on RMM and RCM. No colonies showed on RMM medium.
[b]: Protoplasts (1×10^{7} of each strain) were treated in 35% PEG 4000 solution containing 0.01 M $CaCl_2$ and 0.5 M glucose in 50 mM Tris-HCl buffer, pH 7.5. Regeneration was carried out on hypertonic MM and CM with overlaying and incubation was carried out for 3~4 days. Fusion frequency was shown as the ratio of colonies appearing on hypertonic MM and CM.

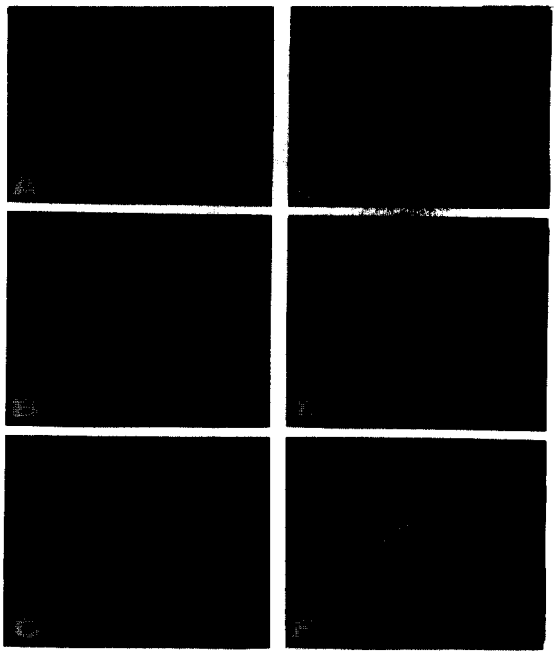

Fig. 3. *Complementary staining of protoplasts and demonstration of protoplast fusion of Rhizopus and Aspergillus using vital stains.*
A~F: Fusion between *R. oryzae* and *A. oryzae* protoplasts. Protoplasts of *Rhizopus* stained with rhodamin 6G (white) and protoplasts of *Aspergillus* stained with fluorescein diacetate (gray).

Rhizopus oryzae and *Aspergillus oryzae* and have succeeded in obtaining three types of intergeneric fusants. Fusant strains of the first type (F1) were prototrophs showing on *Aspergillus* type morphology with dark-yellow sporulation, those of the second type (F2) were also *Aspergillus* type morphology, but showed very poor growth and no sporulation. And the strains of the third type (F3) stopped growing when fusion products grown on RMM were transferred to fresh MM. Among them, the genetic stability of the first type of fusants was studied (Table 5). The second type and the third type could not be determined because of no sporulation and stopped growth, respectively. From the spore progeny of the first type of fusion colonies, prototroph (18~59%) could be isolated as well as double auxotrophic progeny (lys- ino-). We think that some of the progeny of F1 have become double auxotrophs during the cell division. But, we could not obtain the parental type auxotrophs. This result showed that the obtained progeny of fusants were recombinants. And the DNA contents of F1

Table 5. *Distribution of amino acid requirement of F1.*

Strain	Relative no. of colonies grown on minial medium supplimented with[a]			
	none	lys	ino	ino+lys
Fusants:				
F1-1	18	8	18	100(460)
F1-2	59	57	57	100(123)
F1-3	45	41	56	100(330)

[a]Number of colonies on each medium were expressed relative to the numbers on medium supplemented with two amino acids. Numbers in parentheses indicate the number of colonies grown on the medium with two amino acids.

Table 6. *Average DNA content of fusants.*

Strain	Total DNA (10^{-7} μg per spore)
Fusants:	
F1-1	1.7
F1-2	1.6
F1-3	1.1
Parents:	
Aspergillus oryzae	1.4
Rhizopus oryzae	3.2

strains were determined by method of Stewart (13) and Burton (3). The DNA contents of F1 strains were similar to those of *Aspergillus oryzae* as well as their morphorogy (Table 6). These results seggested that the first type of fusants was *Aspergillus* type recombinants. However, the mechanism of recombination remains unclear. Research is under way to determine the genetic character of these obtained fusants. These results will be soon published elsewhere.

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초 록: *Rhizopus oryzae*와 *Aspergillus oryzae*의 속간 원형질체융합

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*Rhizopus oryzae*의 원형질체 형성과 재생에 미치는 여러 요인의 영향을 조사하고 *Rhizopus oryzae*와 *Aspergillus oryzae*의 원형질체 융합 및 이들 융합체의 특성을 조사하였다. *Rhizopus*의 young germling으로부터 원형질체를 생성하는 데는 삼투안정제로 0.5 M glucose를 사용하고 lytic enzyme으로는 Novozym 234(5 mg/ml), chitinase(3 mg/ml), 그리고 chitosanase(3 mg/ml)를 함께 3~4시간 처리했을 때가 가장 효과적이었다. 완충용액의 pH는 7.5에서 8.0 사이가 최적이었으며 원형질체의 재생율은 1.0~4.0%이었다. *R. oryzae*와 *A. oryzae*의 원형질체 융합실험에는 PEG4000과 $CaCl_2$를 fusogenic agent로 사용하였고 융합체를 선별하기 위한 유전적 지표로는 아미노산 요구성을 사용하였다. *R. oryzae*와 *A. oryzae*의 융합율은 4.4×10^{-7}이었고 3가지 유형의 융합체를 얻었다. 첫번째 유형의 융합체들은 *Aspergillus* 형태로 황색의 포자를 형성했고 이들의 DNA 양은 *A. oryzae*와 비슷한 수준($1.1-1.7\times10^{-7}$ μg/spore)이었다. 두번째 유형은 *Aspergillus*의 형태를 나타냈으나 포자를 형성하지 못했으며, 세번째 유형의 융합체는 재생배지에서는 자라지만 이들을 새로운 최소배지로 옮겼을 때 성장을 멈추었다. Rhodamine 6G로 *R. oryzae*의 원형질체를 염색하고 fluorescine diacetate로 *A. oryzae*의 원형질체를 각각 염색한 후 다른 색으로 염색된 이들 원형질체를 융합시켜 융합되는 과정을 관찰하였다.