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Protoplast fusion of *Aspergillus oryzae**

Lee, Soo Youn, Joo Shil Lee and Yung Nok Lee
Department of Biology, Korea University, Seoul, Korea

*Aspergillus oryzae*의 원형질체 융합

이수연 · 이주실 · 이영록
고려대학교 이과대학 생물학과

ABSTRACT : As the bsic study about protoplast fusion of amylolytic fungus *Aspergillus oryzae* and nonamyloytic sugar fermenter, *Saccharomyces cerevsisae*, the intraspecific protoplast fusion of *A. oryzae* was carried out and the properties of the obtained fusants were investigated.

For protoplast fomation from mycellia of auxotrophs, Novozyme 234 as lytic enzyme was the most effective and optimal pH was determined to be pH 5.5-6.0. When the two types of protoplasts were treated with a fusogen including 30% PEG4000, they fused effectively and most of fusants were heterokaryons. Protoplasts aggregated with 30% PEG4000 after fusion treatment were observed by the microscope. Protoplast regeneration frequency was 1.46 to 13.8% and complementation frequency of fusion was 0.12 to 0.16. Fusant strains had a 1.5-fold DNA content compared to that of parent strain. And amylase activity was intermediate between those of parent strains.

KEY WORDS □ ***Aspergillus oryzae, fusion.***

Protoplast fusion technique, first described by Anné & Peberdy (1976) and Ferenczy *et al.* (1975b, 1976) in fungi, has proved to be a valuable method for breeding of new microorganisms or for investigation genetic problems (Peberdy, 1980). Recently, protoplast fusion has been carreid out with various filamentous fungi. In the genus *Aspergillus* intra-and interspesific protoplast fusion have been applied to obtain heterokaryons, heterodiploids and recombinants. Protoplast fusion has been usefully applied in breeding filamentous fungi including *Aspergillus, Penicillium* and *Trichoderma*(Anné and peberdy, 1976 ; Benitez *et. el.*, 1975 ; Ferenczy *et al.*, 1976 ; Hong et al., 1984).

In this experiment, as the basic study on the protoplast fusion of amyloytic fungus, *A. oryzae* and nonamylolytic sugar fermenter, *S. cerevisiae*, the intraspecific protoplast fusion of *A. oryzae* was carried out, and the properties of obtained fusants were investigated.

MATERIAL AND METHOD

Strians

A. oryzae ATCC 16507(lys⁻), *A. oryzae* ATCC 22788(wild type) and auxotrophic mutants(ATCC 22788-1 and ATCC 22788-2) induced(NTG) from *A. oryzae* ATCC 22788 were used. Auxotrophic mutants, ATCC22788-1(lys-) and ATCC 22788-2 (met-), were checked for their stability over several vegetative generation, and for their characteristic amylase activity.

Media

All strains were cultured on malt extract medium (CM) containing 20g of malt extract, lg of Bactopeptone and 20g of glucose per liter. Czapeck medium (3g of sodium nitrate, lg of potassium phosphate, 0.5g of magnesium sulfate, 0.01g of ferrous sulfate, and 30g of sucrose per liter) and SD medium(Sherman *et al.*, 1892) were used for the minimal medium(MM). In protoplast regeneration and fusion experiments CM and MM were supplemented with osmotic stabilizer, 0.6M KCl, and designated RCM, RMM, respectively.

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Proptplast formation and regeneration

The preparation and regeneration of protoplasts derived from the mycelium of *A. oryzae* were perfomed according to the modified procedure of Anné and Peberdy(1976). Conidiospores(1.0×10^7) of each strain were inoculated into Erlenmyer flask(250ml) containing 50ml of complete broth medium. Culture were then incubated on a rotary shaker at a speed of 180 rpm at 28℃ for 18~20 hrs, and mycelia were harvested by centrifugation and then washed twice with the 0.2M phosphate buffer containing 0.6M KCl. Pelleted mycelia (400mg/ml, wet weight) were treated with Novozyme 234(5mg/ml) in the stabilizer and then reacted for 3hrs at 30℃ with gentle shaking. Protoplasts were purified from the residual mycelial debris by filtration through the sintered glss filter and washed twice with stabilizer by centrifugation. The purified protoplast suspensions were aseptically added to 10ml RCM containing 0.5% agar, overlaied on the same RCM containing 2% agar in 9cm petri dishes, and incubated at 30℃ for 3~5 days. The regeneration frequency was shown as the ratio of the number of colonies fomed to the known number of protoplasts plated.

Protoplast fusion

The protoplasts from two parental strains were mixed(1.0×10^7 protoplasts, each) and sedimented by centrifugation. The pelleted protoplasts were suspened in 1ml of a prewarmed(3℃) solution of 30%(w/v) PEG 4000 in 0.01M $CaCl_2$ and 0.05M glycine, adjust to pH7.5 with 0.01M NaOH. After incubation for 10min at 30℃ the suspension was diluted with 6ml of liquid MM containing 0.6M KCl and centrifuged(700g, 5min). Protoplasts were washed twice with 8ml portions of stabilizer and finally resuspended in 1ml of stabilizer. Serial dilusions were added to 10ml of hypertonic MM containing of 0.5% agar, preincubated at 40℃, and overlaied on hypretonic MM containing of 2% agar in 9cm petri dishes. The fusion frequency was calculated from the ratio of colonies growing on RMM and RCM.

Stability of fusants

The colonies of fusants developed on MM were transfered to CM and incubated at 30℃ for 10~15 days. Conidiospores from the colonies that appeared after incubation were plated on CM, MM and MM supplimented with specific requirment to test auxotrophic requirment.

Measurement of DNA content

Dense conidial suspensions were centrifuged, resuspended and the spore number was estimated with a haemocytometer. The DNA fraction was prepared by the method of Stwart(1975). The washed conidia were incubated for 30min in 1.3ml of 10% perchloric acid at 0℃ to remove cold acid-soluble material.

The cold acid-insoluble material was collected by centrifugation. Nucleic acid was hydrolyzed by heating at 80℃ for 20min in 1.3ml of 10% perchloric acid. The hydrolysate was cooled to 0℃ and centrifuged, and then the supernatant was removed and retained. The DNA content was determined spectrophotometically by diphenylamine reagent(Burton, 1968). Calf thymus DNA(sigma) was used as standard.

Determination of amylase activity

Inoculum was prepared by germination the spores from the slants for 24hrs at 30℃ in 250ml flasks containing 50ml of minimal broth medium. For production of enzyme the fungal cells were grown in the above described medium except glucose was replaced with soluble potato starch, using 2% volume of inoculum of 24-germinated spores. The organism was cultured at 30℃ for 5days on a shaker. Mycelia wes removed by filtration and filtrate was used enzyme solution, Amylase activity was determined by procedure of Ogawa(1968) using 3′, 5′−dinitrosalycilic acid.

Results and Discussion

Formation and regeneration of protoplasts

Out of the various lytic enzymes, Novozyme234

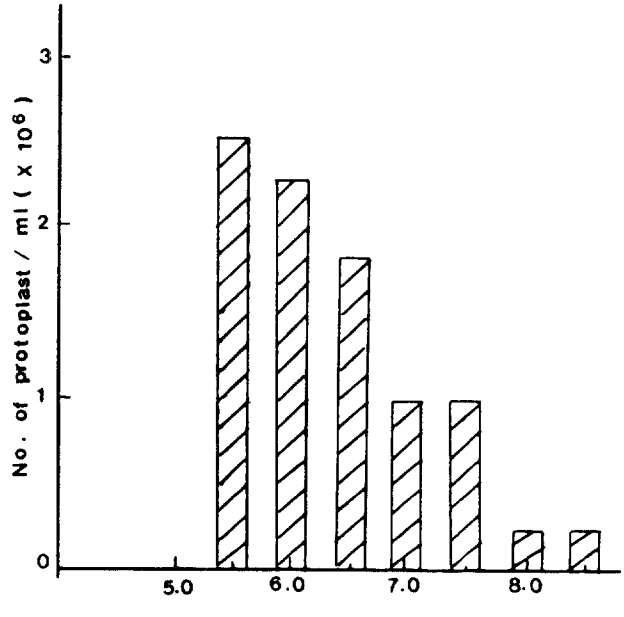

Fig. 1. *Effect of pH on the protoplast formation of A. oryzae ATCC 22788.*

Table 1. *Effect of various lytic enzymes on the protoplast formation of A. oryzae ATCC 22788.*

Expt No	Lytic enzyme	Final conc. of enzymes	No. of protoplasts/ml
1.	Novozyme 234	5mg/ml	7.5×10^{6}
2.	Novozyme 234	10mg/ml	2.1×10^{6}
3.	β-glucuronidase	6,000unit/ml	7.5×10^{6}
4.	β-glucuronidase	12,000unit/ml	7.5×10^{6}
5.	Novozyme 234 and Cellulase CP	both 5mg/ml	6.8×10^{6}
6.	Novozyme 234 and β-glucuronidase	5mg/ml and 6,000unit/ml respectively	1.3×10^{6}

and β-glucuronidase were the most effective for protoplast formation when the reaction was performed at 30℃ for 3hrs(Table 1). The optimal pH of protoplasting buffer was determined to be 5.5 to 6.0(Fig. 1). The regeneration frequency of protoplasts from various auxotrophic mutants were shown in Table 2. The frequency of regeneration varied depending on the strains and on the regeneration media used and were 1.46 to 13.0%. RSD medium using for regeneration of yeast protoplasts was more effective than RMM in the protoplasts regeneration of *A. oryzae*. In addition that, RSD medium was very effective for the restriction of colony size of *A. oryzae*.

Fusion of protoplasts

Fusion between protoplasts of nutritionally-complementing strains was detected by formation of colonies developing on RMM. After 48 to 72hrs, colonies appered from PEG-treated mixed protoplasts on both RMM and RCM. Unfused protoplasts, spores, and mycelia were unable to develop on these media. The frequency of fusion, based on the ratio of the number of colonies developing on RMM and RCM after fusion treatment, was 0.12−0.16, as determined from several experiments(Table 3). This value was more than 10^5 times higer than reversion rates of the used auxotrophs. This suggested that most of the colonies appearing on RMM after fusion were prototrophs through nutritional complementation. Protoplasts aggreated with 30% PEG 4 000 after fusion treatment were observed by the microscope (Fig. 2).

Table 2. *Regeneration frequency of protoplast from A. oryzae.*

Strain	Regeneration frequency(%)		
	RCM	RMM	RSD
A. oryzae ATCC 22788-1	2.12	1.46	ND
ATCC 22788-2	2.70	2.12	4.0
ATCC 16507	13.0	6.4	9.86

RMM and RSD were supplemented with specific amino acid(50μg/ml)
ND : Not determined

Table 3. *Complementation frequency by protoplast fusion of A. oryzae.*

protoplast pair	reversion+ frequency (less than)	fusion frequency
ATCC 22788-1 × ATCC 22788-2	1.7×10^{-6} / 1.3×10^{-6}	0.16
ATCC 22788-2 × ATCC 16507	1.3×10^{-6} / 1.4×10^{-6}	0.12

+ Reversion frequency was defined as the ratio of the number of protoplasts regenerating on hypertonic MM(minimal medium) and CM(complete medium).

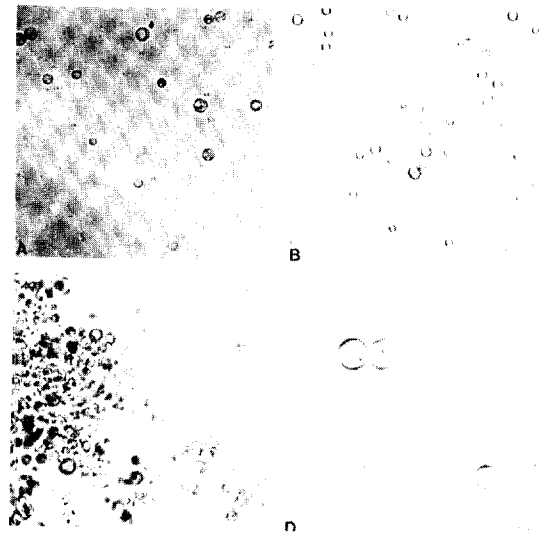

Fig. 2. *Photomicrography of Aspergillus oryzae.*
A. B : Freshly isolated protoplasts from mycellia of *A. oryzae* ATCC 22788-1 and *A. oryzae* ATCC 22788-2

C : Protoplasts aggreated with PEG4,000 after fusion treatment

D : Protoplast fusion process between *A oryzae* ATCC 22788-1 and *A. oryzae* ATCC 227882

Properties of fusants

The colonies of fusants that appeared on the RMM were transfered to MM agar slants, respectively. Most of colonies grown well on the slants. Most of fusants also segregated into two types of parental auxotrophs(Table 4). These results evidently showed

Table 4. *Segregation of heterokaryons after fusion and the appearance frequency of diploids.*

protoplast pair	segregation into auxotrophs(%)	appearance frequency of diploids
ATCC 22788-1	0.23	
×		3.7×10^{-4}
ATCC 22788-2	92.56	
ATCC 22788-2	69.3	
×		$7.2\times10^{-7}<$
ATCC 16507	27.7	

Appearance frequency of diploids was defined as the ratio of conidia regenerationg on CM and MM agar.

that the fusants were heterokaryons. And diploids frequency of heterokaryons were show also Table. 4. This result coincied with the protoplast fusion of other filamenteous fungi(Anné *at al.*, 1976 ; Ferenczy *at al.*, 1974 ; Ferenczy *at al.*, 1975). Apperance frequency of diploids was defined as the ratio of the number of conidia regeneration on CM and MM agar. The average DNA conlent per conidium of fusants was 16.9 to $23.7\times10^{-6}\mu g$. The DNA content of parental strains, *A. oryzae* ATTCC 22788-1 and *A. oryae* ATCC 22788-2, were 9.96 and 11. $5\times10^{-6}\mu g$, respectively. Fusant strins had a 1.5-fold DNA content compared to that of parental strains (Table 5).

Extracellular amylase activity of a wild strain,

Table 5. *Average DNA content per conidium for fusants and parental strains of Aspergillus oryzae.*

Strain	total DNA ($10^{-6}\mu g$ per conidium)
Fusants :	
Fus 1	18.7
Fus 2	17.0
Fus 3	23.7
Fus 4	16.9
Fus 5	18.3
Farents :	
ATCC 22788-1	9.96
ATCC 22788-2	11.5

Table 6. *Amylase activity for fusants and parents of A. oryzae auxotrophs compared to that of A. oryzae ATCC 22788.*

Strain	0.D	Relative activity(%)
ATCC 22788	0.780	100
ATCC 22788-1	1.40'	179.5
ATCC 22788-2	0.62	79.5
ATCC 16507	0.725	92.9
F1-1	0.635	81.4
F1-2	0.530	67.9
F1-3	0.760	97.4
F2-1	0.825	105.6
F2-2	1.18	151.3

The activity of wild type was set to control 100%, and the relative activity of auxotrophs and fusants were measured.

F1 : Fusants of ATCC 16507 and ATCC 22788-2

F2 : Fusants of ATCC 22788-1 and ATCC 22788-2

parental strain and the obtained fusants were tested (table 6). Amylase activity of the fusants was changed more of less according to the fusants. Among them, amyase activity of fuasnt F2-2 was 1.5times higher than that of the wild strain ATCC 227788.

적 요

Aamylase 활성이 높은 *Aspergillus oryzae*와 알콜발효능이 있는 *Saccharomyces cerevisiae*의 원형질체융합을 위한 기초연구로서, amylase 활성이있는 *A. oryzae*의 종내원형질체를 융합시켜 이들융합체의 특성을 조사하였다.

영양요구성 돌연변이 균주의 mycellia로 부터 원형질체를 생성하기 위해서는 lytic enzyme으로 Novozyme 234가 효과적이었고 완충용액의 pH는 5.5에서 6.0사이가 최적이었다. Fusogen으로 30% PEG4,000를 사용하였을 때 효과적으로 원형질체의 융합이 이루어졌으며 이들 융합체의 대부부은 heterokaryons이었다. 원형질체의 형태와 PEG처리후 융합되는 과정을 광학현미경으로 관찰하였다. 원형질체의 재생율은 재생배지와 균주에 따라 1.46~14.%이었고, *A. oryzae* 종내융합율은 0.12~0.16이었다. 융합체의 DNA함량을 조사한 결과 모균주보다 약 1.5배정도 증가됨을 보였고, 융합체들의 amylase 활성은 융합체에 따라 다소 차이를 나타내었다. 가장 높은 amylase 활성을 나타낸 융합체 F2-2에 있어서는 야생균주 ATCC 22788의 그것보다 amylase 활성이 약 1.5배 가량 높았다.

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