Formation of Protoplasts from *Pyricularia oryzae*

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벼 稻熱病菌, *Pyricularia oryzae*의 原形質体 形成

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The optimum conditions of protoplasts formation from *Pyricularia oryzae* were investigated with lytic enzymes and osmotic stabilizers. The mycelia were begun to release the protoplasts in response to the complex enzyme solution after 30-60 minutes and reached to maximum after 2-3 hrs. Among the lytic enzymes tested, the mixture solution containing β-Glucuronidase (0.01 ml/ml), Cellulase ONOZUKA-RS (20 mg/ml), Driselase (10 mg/ml), and Macerozyme R-10 (10 mg/ml) resulted in the highest rate of protoplasts releasing of *Pyricularia oryzae*. The best stabilizer was 0.6M KCl at pH 7.0. When the mycelia were digested with enzyme mixture, the stationary culture was better than shaking culture for higher protoplast formation.

Rice blast disease, caused by *Pyricularia oryzae* Cav., has been one of the major limiting factors for stable rice production in Korea. Although the breeding of blast resistant cultivars has been emphasized, break-down of the disease resistance has occurred because of shifting of race populations of *Pyricularia oryzae* in Korea (Chung, 1979). Therefore mechanisms of the genetic variations of the pathogen have to be studied prior to the breeding of disease resistant cultivars, however little informations are available on this aspect in *Pyricularia oryzae*.

In recent years, there has been tremendous increase in the use of protoplast for physiological, biochemical, and genetic studies of microorganisms (Peberdy, 1979; Harris, 1982). As a results, the improved methods are continuously being developed for their efficient production from a wide variety of bacterial, fungal, and plant cells (Evans et al., 1972; Finkelman et al., 1980; Peberdy, 1980), but there is only a few reports on the formation of protoplasts from plant pathogenic fungi. Protoplast formation in plant pathogenic fungi was first observed in *Fusarium culmorum* (Rodriquz et al., 1964) and followed by *Rhizoctonia solani* (Hashiba and Yamada, 1982), *Gibberella fujikuroi* (Harris, 1982), *Pyricularia oryzae* (Tanaka et al., 1981), recently. The formation of cell wall-free state of pathogen is important to carry out genetic studies of plant pathogens.

The objectives of this study were to obtain basic data for genetic ifnormations of *Pyricularia oryzae* for breeding blast resistant cultivars, to apply protoplast fusion in future, and to pursue physiology ad pathogenecity of plant pathogens.

MATERIALS AND METHODS

Fungal isolate used

The isolate of *Pyricularia oryzae* Cav. (KJ-101)

used in this study was originally obtained from the Institute of Agricultural Science, Office of Rural Development, Suweon, Korea. The stock cultures were maintained on potato sucrose agar (PSA).

Growth of mycelium for protoplast formation

Preliminary experiments were conducted to obtain high yield of protoplasts from the mycelia of *Pyricularia oryzae*. To obtain young mycelia, 7-day-old mycelia from a stock culture were inoculated into an agar slant. After 3 days of culturing at 28°C, fungal growth was completely removed from an agar slant and the mycelial suspension was homogenized at 50 stroke for 30 minutes by Homogenizer (Virtis). Mycelial suspension was inoculated into 50ml of potato sucrose broth in 250ml Erlenmeyer flask. The cultures were incubated on rotary shaker at 28°C for 18 hours.

Osmotic stabilizers

$MgSo_4$, KCl and mannitol were tested as an osmotic stabilizer for their ability to support the release of protoplasts from mycelia of *Pyricularia oryzae*. The concentrations of osmotic stabilizers were adjusted to 0.4, 0.6, 0.8M in 0.02M phosphate buffer with pH 7.0, respectively. The pH of 0.6M KCl was adjusted to 4.0, 5.8, 7.0 8.0 with 0.02M phosphate or citrate phosphate buffer, respectively.

Lytic enzymes

Cellulase ONOZUKA-RS from *Trichoderma viride* and Macerozyme R-10 from *Rhizopus* sp. were obtained from Yakult Phamaceutical Industry Co., Ltd. (Nishinomiya, Japan) and Driselase from basidiomycetes was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). β-Glucuronidase Type H-2 from *Helix pomatia was* obtained from Sigma Chemical Co. The enzymes were tested singly or in combinations for their lytic activity. They were dissolved in 0.02M phosphate buffer containing osmotic stabilizer. After 24 hours at 4°C with agitation occasionally, the enzyme solutions were centrifuged at 4,500g for 40 minutes and were sterilized by passing them through a membrane filter (pore size: 0.22μm, Type H4). The filtrates were used as lytic enzyme solutions.

Protoplast formation

Mycelia were harvested by centrigugation (4,500g/30 minutes) and washed twice with osmotic stabilizer buffered with 0.02M phosphate or citrate-phosphate buffer. Mycelia were suspended in 5ml enzyme solutions. The suspensions were incubated at 28°C with stationary state or gentle shaking for up to 3-4 hours. Protoplasts were identified microscopically as spherical, osmotically sensitive bodies and the yield obtained were determined by haemacytometer counters.

Purification of protoplasts

The lytic suspension was filtered through a sintered glass filter (pore size: 20-30 um) to remove mycelial fragments and the filtrate was centrifuged at 2,000g for 10 minutes. The pellet was washed by centrifugation (2,000g/10 minutes) with osmotic stabilizer to remove lytic enzymes. The pellet was resuspended in osmotic stabilizer.

RESULTS

After 30 minutes incubation in the lytic suspension, some hyphal tips became swollen and protoplasts subsequently emerged from an extrusion of cytoplasm at the digested tips or the subapical region. (Plate 1A and B) The rate of protoplast releasing was different in accordance with enzyme solutions, but the number of protoplast was reached the maximum in 2-3 hours. It was shown that

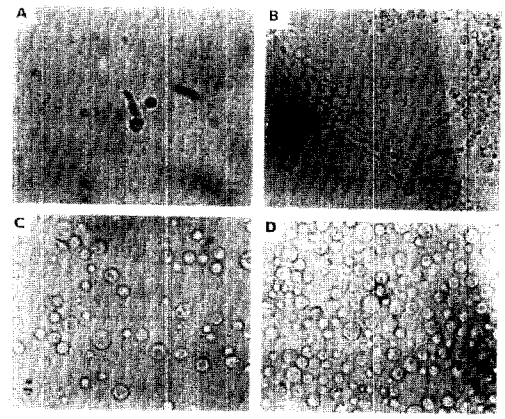

Plate 1. *Formation and purification of protoplasts from mycelia of Pyricularia oryzae.*
A) protoplast emerging from hyphal tip.
B) protoplasts released from subapical hyphae
C) protoplasts released after 3 hrs digestion
D) protoplasts purified through sintered glass Filter, and by centrifugation.

there were dissimilarities in shape and size of protoplasts released according to the increasing of the time. Protoplasts released after 1 hr. in the lytic enzyme solutions were dense and non-vacuolated, and vacuoles were observed after 2hr. incubation. The size of protoplasts and vacuoles was increased as increasing the time, and the detercted protoplasts were ranged 2-10 um. (Plate 1C, and D).

Comparison of lytic enzymes

Commercially available enzyme preparations (Cellulase ONOZUKA-RS, Driselase, Macerozyme R-10, β-Glucuronidase Type H-2) were screened singly or in combinations for their lytic activities. Cellulase ONOZUKA-RS and Driselase were active, but Macerozyme R-10 was inactive for cell wall digestion. The yield of portoplasts obtained after 2 hrs digestion in the presence of Macerozyme was only 0.24×10^4/g.

The effectiveness of enzyme combinations was compared as shown in Table 1. The yield of protoplasts was higher in enzyme combinations than single enzyme treatment. Enzyme mixture A and B contained four kinds of enzyme equally, and the concentration of enzyme mixture A was two times of enzyme mixture B. It was apparenpt that the number of protoplasts increased significantly at higher enzyme concentration. The enzyme mixture A containing Cellulase Onozuka-rs 2%, Macerozyme R-10 1%, Driselase /%, B-glucuronidase Type H-2 0.1% was the most active and yield 4.1×10^7/g after 3 hrs. incubation.

Table 1. *Comparison of different enzymes on releasing of protoplasts from* ***Pyricularia oryzae*** *mycelia*

Enzymes	Yield (protoplasts/g[a])	
Cellulase ONOZUKA-RS	2%	3.1×10^6
Macerozyme R-10	1%	0.25×10^4
Driselase	1%	5.4×10^6
Enzyme mixture A[b]		4.1×10^7
Enzyme mixture B[c]		3.0×10^7

a/ mycelial fresh weight
b/ mixture solution containing Cellulase ONOZUKA-RS 2%, Driselase 1%, Macerozyme R-10 1%, β-Glucuronidase Type H-2 0.1%
c/ mixture solution containing Cellulase ONOZUKA-RS 1%, Driselase 0.5%, Macerozyme R-10 0.5%, β-Glucuronidase Type H-2 0.05%

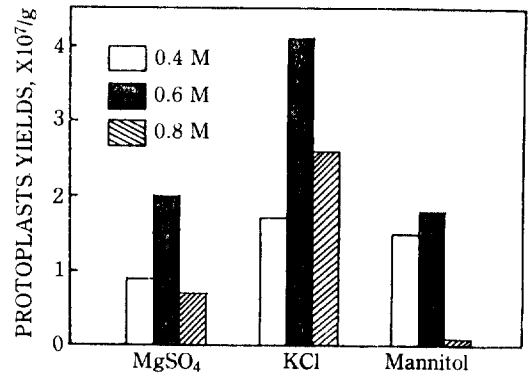

Fig. 1. *Comparison of protoplasts yields of Pyricularia oryzae using different osmotic stabilizers and altered concentrations.*
(Lytic enzyme solution contains Driselase 1%, Macerozyme R-10 1%, B-glucuronidase 0.1%, (ellulase onozuka-RS 2%)

Comparison of osmotic stabilizers

The effect of osmotic potential on protoplast formation was examined with different concentrations of $MgSO_4$, KCl, and mannitol. (Fig. 1) Smaller amounts of protoplasts were released and supported in $MgSO_4$ and mannitol than in KCl. The maximum yields of protoplasts in all conditions were obtained afte 3 hrs incubation. The yield of protoplasts with 0.6M KCl was the highest among osmotic stabilizers tested and was two times than

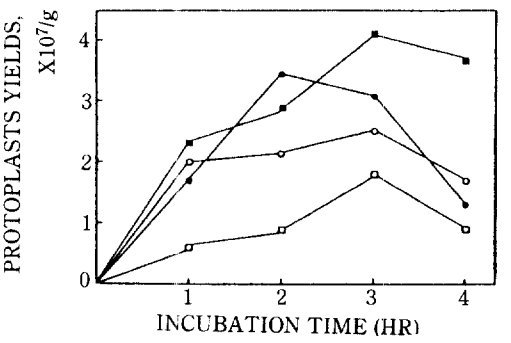

Fig. 2. *Effect of altered pH on protoplast yield of Pyricularia oryzae.*
(Lytic enzyme solution contains Driselase 1%, Macerozyme R-10 1%, β-Glucurouidase 0.1%, Cellulase ONOZUKA-RS 2%, and 0.6M KCl as an osmotic stabilizer)
(• pH 5.0, o: pH 5.8, □: pH 7.0, □:8.0)

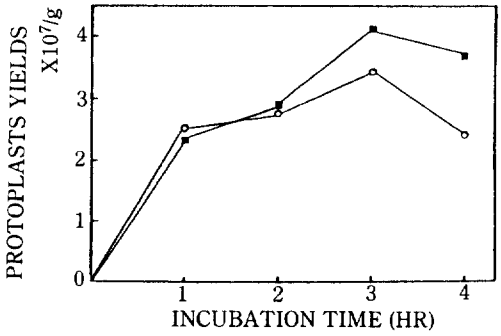

Fig. 3. *Comparison of digestion methods on protoplast formation of Pyricularia oryzae.*
(Lytic enzyme solution contains Driselase 1%, Macerozyme R-10 1%, β-Glucuronidase 0.1%, Cellulase ONOZUKA-RS 2%, and 0.6M KCl as an osmotic stabilizer)
(o: Shaking, □: Stationary)

that with $MgSO_4$ or mannitol.

The effect of altered pH

The effect of pH was investigated using active enzyme preparation consisted of 2% Cellulase, 1% Driselase, 1% Macerozyme, 0.1% β-Glucuronidase with 0.6M KCl as a osmotic stablilizer. (Fig. 2) The osmotic stabilizer was buffered with 0.02M citrate phosphate buffer for pH 5.0 and with 0.02M phosphate buffer for pH 5.8, 7.0, and 8.0. The number of protoplasts at pH 7.0 was the maximum after 3 hrs incubation, but the number was decreased at all pH levels after 4 hrs except at pH 5.0. With this observation, the lytic systems were routinely adjusted to pH 7.0.

Comparison of digestion methods

When the mycelia were digested in the lytic enzyme solution, comparisons were made with stationary culture vs. shaking culture for protoplast releasing of *Pyricularia oryzae* (Fig. 3). The rate of protoplast release in shaking and stationary was similar for the first 2 hr incubation. With the both systems, however, the number of protoplast reached the maximum after 3 hr incubation, then decreased after 4 hrs. The number with shaking culture was decreased more sharply than stationary culture. There was a great difference in the releasing protoplasts between two systems after 4 hrs incubation.

DISCUSSIONS

Formation of protoplasts from *Pyricularia oryzae* may be interest to plant pathologists, because fungal protoplasts can be used in variety of experiments involving genetic manipulation that may aid in understanding host-parasite interactions. Optimum conditions for releasing protoplasts are very important because the successful applications of these protoplasts may lead to valuable researches. Although an active enzyme complex could be prepared in the laboratory, this approach was considered to be laborious for routine use. To find a suitable enzyme system, commercially available enzymes were tested for lytic activity against *Pyricularia oryzae* mycelia.

The yield of protoplasts obtained in the present study with each enzyme was greatly differed from *Rhizoctonia solani* (Hashiba and Yamada, 1982), *Trichoderma koningii* (Cho et al., 1981 Akamatsu et al., 1983; Ishizaki et al., 1983). It seemed that the phenomena were resulted from the difference of the organisms, the cell wall compositions and physiological states of organisms tested. And the combinations of these enzymes were significantly more active than each enzyme singly tested. It was probable that combinations of these enzyme mixtures were resulted in synergistic action of the major components, because β-Glucuronidase. Macerozyme R-10, Driselase were undefined mixtures containing several enzymes. Synergistic action of lytic enzymes have been studied (Torres-Bauza and Riggsby, 1980; Bradshaw, 1983) and Bartnicki-Garcia (1968) reported that the phenomena of synegism arised from the complexity of fungal cell wall components of *Pyricularia oryzae* were mainly consisted of B-D-1,3 glucan, heteroglycan and chitin-like substance.

The formation of protoplast was shown to be influenced by a variety of factors other than the lytic enzymes. Osmotic stabilizers are essential to provide osmotic support after removal cell wall. A wide variety of inorganic salts, sugar alcohols, and other organic compounds have been investigated (Villanueva and Acha, 1971). According to Sietsma et al. (1973), inorganic salt were more favorable than organic compound to *Pythium* PRL 2142. Tanaka et al. (1981) reported that protoplast of *Pyricularia oryzae* formed with mannitol gradually disinter-

grated probably as a result of metabolic lysis. In this experiment, KCl was proved to be more effective for the stabilizing of protoplasts than mannitol or $MgSO_4$. Although the numbers of protoplasts were reached the maximum during 3 hrs incubation in the three kinds of osmotic stabilizers tested, the numbers were decreased after 3 hrs incubation. The reason can not be explained clearly in this experiment but should be solved, because the stability of protoplasts is very important for further study.

When digestion methods were compared for cell wall digestion, intial rate of protoplast releasing in gentle shaking was higher than stationary culture. It seemed that enzyme activities have been improved with shaking in initial digestion. The age and physiological state of mycelia used for preparation of protoplasts can also influenced protoplast releasing. Buckly (1979) reported that mycelia from older culture at the latter stage of the linear growth phase gave much fewer protoplasts. In this study, only 18 hrs old mycelia were taken as a young mycelia throughout the experiment. Not only the age and physiological state but strains or races of the organisms must be involved to improve protoplast formation in future.

적 요

벼 稻熱病菌 *Pyricularia oryzae*의 原形質体를 形成하는 最適條件을 分解酵素와 滲透圧調整劑를 処理해서 究明하였다. 菌糸体는 酵素混合液과 30~60分 反応하여 原形質体를 形成하기 시작해서 2~3時間에 最高에 이르렀다. 酵素液 組合 중에서 β-glucosidase (0.01mg/ml), Cellulase ONOZUKA-RS (20mg/ml), Dricelase (10mg/ml), Macerozyme R-10 (10mg/ml)을 含有한 것이 가장 좋은 結果를 가져왔다. 最適調整劑는 pH 7.0에서 0.6M KCl이었다. 그리고 振湯培養하는 것 보다 靜置培養하는 것에서 더 많은 原形質体가 形成되었다.

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