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Effects of 2-Mercaptoethanol on the Protoplast Formation and Osmotic Stabilizers on the Protoplast Reversion of *Pyricularia oryzae* Cavara

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벼 稻熱病菌의 原形質體 生成에 미치는 2-Mercaptoethanol과 復歸에 미치는 滲透壓 安定劑의 影響

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ABSTRACT: The mycelia of *Pyricularia oryzae* were treated with enzyme solution mixture consisting of Driselase, β-Glucuronidase, Cellulase, and Macerozyme R-10 for the production of protoplasts. More protoplasts were formed from mycelia of race KJ 101 of *P. oryzae* than that of race KI 315a in the enzyme mixtures. The number of protoplasts was decreased in the untreated control three hrs after the enzyme treatment, whereas the number was increased in the treatments with 10, 50 and 100 mM 2-mercaptoethanol, respectively. The number of protoplasts increased to reach maximum at five hrs after treatment of 10 mM 2-mercaptoethanol, but the least was found in 200 mM.

The protoplasts of *P. oryzae* in a liquid medium containing 2.5% yeast extract, and 2% dextrose reverted to the mycelia after five hrs shaking incubation at 27°C. Some protoplasts produced yeast like buds and the buds were developed to irregularly shaped chains of cell protruded a germ tube like hypha from the distal cell. Once in a while a germ tube like hypha protruded directly from the protoplasts. Except in the first type of reversion, other protoplasts reverted to the normal mycelia. The reversion frequency was highest on PDA with stabilizer of 0.6 M KCl. No reversion of protoplasts occurred on water agar regaardless oftreaatments.

KEYWORDS: ***Pyricularia oryzae*, Protoplast formation and regeneration, Stabilizer, Rice blast fungus**

Rice blast, which is caused by *Pyricularia oryzae* Cavara is one of the most important limiting factors in the production of rice throughout the world. Breeding of resistant cultivars and chemical spray have been major methods for controlling rice blast disease in most rice-growing areas. Although these methods have contributed to the increase of rice yield, the break down of blast resistance of rice cultivars because of shifting race populations of *P. oryzae* (Chung, 1979) and occurrence of fungicide tolerance became serious problems in the stable production of rice (Chung *etal.*, 1988). Therefore the studies of the genetic variation of the pathogen are needed prior to the breeding of blast resistant cultivars, but little information is available on the genetic aspect in *P. oryzae*.

In recent years, there has been an increase in the use of protoplasts for physiological, biochemical, and genetic studies of microorganisms (Harris, 1982). Thus, improved methods are needed to develop for efficient formation and reversion of protoplasts. Although the principles of the techniques are known to be similar, there are differences in details of the techniques employed because of the complexities in cell wall structure and com-

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position of each species. The efficient techniques of protoplast formation and reversion in *P. oryzae* must be developed in order to carry out genetic studies of the fungus.

Protoplast formation in filamentous fungi was observed for the first time in *Neurospora crassa* (Bachmann, 1981) using snail digestive juice. Protoplasts fusion of *Gibberella fujikuroi* causing the bakanae disease of rice (Harris, 1982) was used to obtain the sexual stage of the pathogen and to find out the genetic control of gibberellin biosynthesis. The formation of protoplasts was also described in *Aspergillus nidulans* (Bos and Slakhorst, 1981), *Pythium* PRL (Sietsma and Boer, 1973), *Rhizoctonia solani* (Hashiba and Yamada, 1982) and *Trichoderma koningii* (Cho *et al.*, 1981). In the protoplast formation of *P. oryzae*, 0.6 M KCl was the best osmotic stabilizer at pH 7.0 (Lee *et al.*, 1985). The same stabilizer was also reported to be the best one, but pH of the buffer was at 5.2 (Han *et al.*, 1987). To improve the number of protoplasts released from fungi, the SH-compound, known as reducing disulphate linkage of the protein, has been frequently recommended. The pretreatment with SH-compound had a stimulatory effect on the protoplast formation from young mycelium of *Geotrichum candidum* (Dooijewaard-kloosterziel *et al.*, 1973; Sietsma *et al.*, 1973). It is thought that numbers of released protoplasts of *P. oryzae* can be improved by the SH-compound such as 2-Mercaptoethanol.

When protoplasts are suspended in an osmotically stabilized medium, part of the protoplasts starts to synthesize a new cell wall and eventually return to normal hyphal form. The reversion of protoplasts was investigated in the various filamentous fungi, *Geotrichum candidum* (Fukui *et al.*, 1969), *Pythium* sp. (Sietsma and Boer, 1973) *Trichoderma koningii* (Cho *et al.*, 1981) and *Pleurotus ostreatus* (Yoo *et al.*, 1985). Osmotic stabilizer is known to be the major factor affecting the protoplast reversion. Inorganic salts including sodium chloride, magnesium sulfate, and potassium chloride inhibited the protoplast reversion of *Pythium* sp., whereas organic compounds such as mannitol, sorbitol and sorbose increased its efficiency (Sietsma and Boer, 1973). But more protoplast reversion of *Pleurotus ostreatus* was observed on the reversion medium containing 0.6 M KCl than mannitol or sorbitol (Yoo *et al.*, 1985). A suitable osmotic stabilizer is required for the reversion of protoplasts in *Cephalosporium acremonium* (Hamlya, 1982).

The purpose of this study was to determine the effects of 2-mercaptoethanol and osmotic stabilizers on the protoplast formation and the protoplast reversion of *P. oryzae*.

Materials and Methods

Pathogen used

Two races of *Pyricularia oryzae* Cavara, KJ 101 and KI 315a, kept in this laboratory previously were used for the formation and reversion of protoplasts. These were cultured seven days on potato dextrose agar at 27°C before used.

Protoplast Formation and pretreatment of 2-Mercaptoethanol

Protoplasts of *P. oryzae* were produced by modifying Lee's method (Lee and Chung, 1985). Fungal mycelia cultured on potato dextrose agar for seven days were removed from the medium using five m*l* of the sterile distilled water. The mycelial suspension was then inoculated into 50 m*l* of potato dextrose broth in a 250 m*l* Erlenmeyer flask and incubated 48 hrs on a rotary shaker at 27°C. Mycelia in the flask were washed twice with sterile distilled water by centrifuging 40 min at 4500g. The mycelia were suspended in the enzyme solution at 27°C for releasing protoplasts, and then the number of released protoplasts was counted with hemocytometer under the light microscope. The enzyme solution was prepared as follows: 2% Cellulase ONOZUKA-RS, 1% Macerozyme R-10, 1% Dricelase and 0.01% β-Glucuronidase Type H-2 were mixed in 0.02 M phosphate buffer containing 0.6 M KCl, and it's pH was adjusted to 7.0. The enzyme solution was centrifuged 40 min at 4500g and sterilized with a membrane filter (pore size: 0.22 um, Gelman Sci. Inc.). The pretreatment of mycelia of *P. oryzae* with 2-mercaptoethanol was done at concentrations of 10, 50, 100 and 200 mM.

Reversion of Protoplast

The crude protoplast suspension was passed through a sintered glass filter (pore size: 20-30 μm) to remove mycelial debris after the treatment of enzyme solution. The filtrate was washed twice with 6 m*l* of sterile osmotic stabilizer, and the pu-

rified protoplasts were suspended in a reversion liquid medium containing 2.5% yeast extract, 2% dextrose with 0.6 M KCl and incubated at 27°C on a rotary shaker with gentle shaking. The reversion types of protoplasts were observed periodically under the phase contrast microscope. To determine the efficiency of protoplasts reversion, the known number of protoplasts was poured evenly over potato dextrose agar containing osmotic stabilizer and the media were incubated five days at 27°C. The frequency of protoplast reversion was determined by the number of visible colonies.

$$\text{Reversion frequency(\%)} = \frac{\text{Number of colonies on supplemented reversion media}}{\text{Number of protoplasts inoculated}} \times 100$$

Results

The formation of protoplast was evaluated by counting the number of protoplasts at various intervals after enzyme treatment. The first change of the mycelium in the lytic enzyme solution was swelling of hyphal tips followed by the release of small nonvacuolate protoplasts 30 min after incubation. Continuous incubation showed that the swelling of subapical region of the hyphae and the vacuolation of protoplasts were induced during continuous incubation. Throughout the treatment period, more protoplasts were produced in the race KJ 101 than those of the race KI 315a, but the trend of protoplast formation in the both races was similar (Fig.1). With the incubation time in the enzyme solution, the number of protoplast sincreased gradually and reached maximum at three hrs, then decreased there after.

The effect of 2-mercaptoethanol on formation of protoplasts was observed with incubation time (Fig.2). After one hr of enzyme solution treatment the highest number of released protoplasts from the mycelia was obtained in 50 mM. With incubation time, untreated control increased the protoplast formation as compared to the treatments of two and three hrs. After that, the number of protoplasts from the untreated control decreased rapidly, whereas 10 mM increased the number continuously up to 2.5×10^8/g five hrs after the treatment. In 200 mM 2-mercaptoethanol treatment, the yield of protoplasts was the lowest throughout the incubation period.

Three basic patterns of protoplast reversion were observed. In the first type, protoplasts produced yeast like buds and they developed into irregularly shaped chains of cells five hrs after treatment (Fig.3a). The cytoplasm occasionally was cleaved into two parts locating at opposite

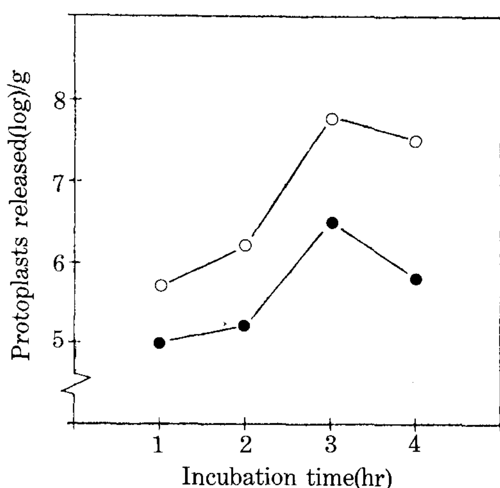

Fig.1. Comparison of protoplast yields of two races, KJ101(○) and KI315a(●), included by *Pyricularia oryzae* in the enzyme solution with 0.6 M KCl at pH 7.0.

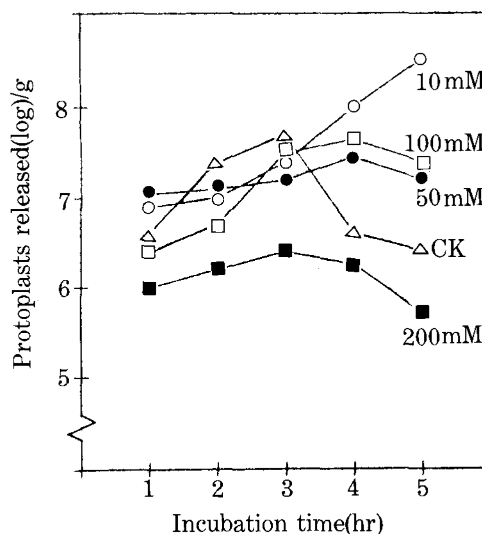

Fig.2. Comparison of protoplast yields of *Pyricularia oryzae* in the enzyme solution with 0.6 M KCl at pH 7.0 after the pretreatments with 2-mercaptoethanol of ■: 200 mM, □: 100 mM, ●: 50 mM, ○: 10 mM and △: untreated control.

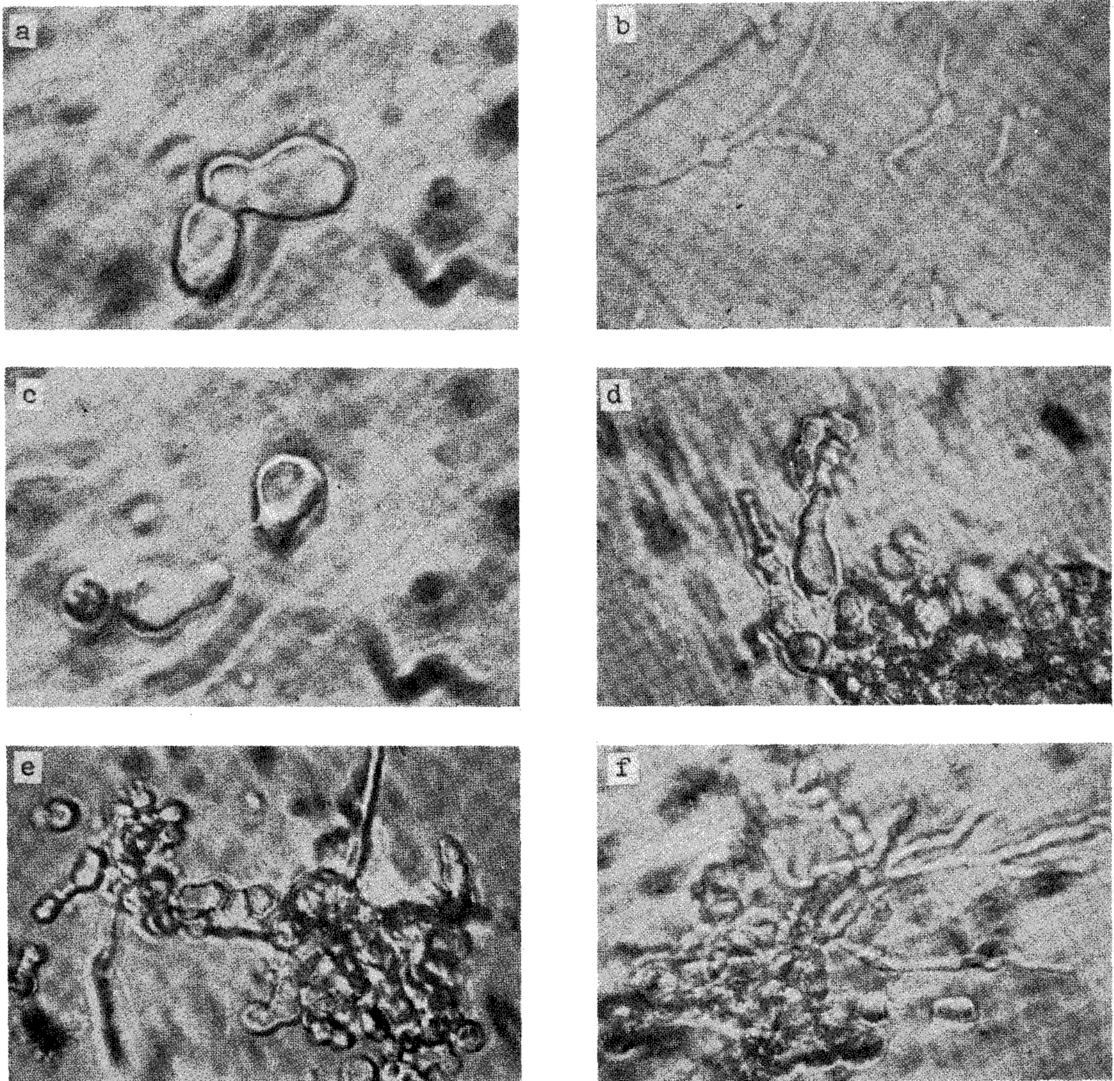

Fig.3. Reversion process of protoplasts released from mycelia of *Pyricularia oryzae* whith phase contrast microscope.
a) Irregularly formed cells emerging from the original protoplast after 5 hr incubation (×1000)
b) The germ tube like hyphae from the protoplast after 5 hr incubation (×400)
c) The cleaved cytoplasm at opposite ends of the chain after 8 hr incubation (×1000)
d), e) The germ tube like hyphae protruded from the distal cell at the chain of irregularly formed cells (×400)
f) Protoplasts developed into normal hyphal structure (×400)

ends of the chains eight hrs after treatment (Fig.3c). Finally cells autolyzed without hyphal development. The second type was similar to the first type. After development of an irregularly shaped chain of cells, a germ tube like hypha protruded from the distal cell and a normal apical type of hyphal growth was restored(Fig.3d, e). A germ tube like hypha protruded directly from the protoplastand developed into normal hyphal structure in the third type (Fig.3b).

The frequency of protoplast reversion on potato dextrose agar with various osmotic stabilizers

Table 1. Reversion percentage* of *Pyricularia oryzae* protoplasts on the potato dextrose agar and water agar with osmotic stabilizer** at pH 7.0.

Osmotic Stabilizer	Reversion(%)	
	PDA	WA
Mannitol	6.5	0
Sorbitol	6.1	0
KCL	33.4	0
$MgSO_4$	11.3	0

*: Reversion(%) = Number of colonies × 100
Number of protoplasts
**: Concentration of osmotic stabilizer; 0.6 M

was shown in Table 1. The highest reversion frequency was obtained five days incubation on potato dextrose agar containing 0.6 M KCl with its value of 33.4%. The percentages of protoplast reversion induced by mannitol and sorbitol were 6.1 and 6.5%, respectively. Potassium chloride was the best osmotic stabilizer among those tested for protoplast reversion. However, no protoplast reversion was observed on water agar regardless of osmotic stabilizers.

Discussion

The thiol compounds have been known to increase the protoplast fromation in several filamentous fungi. The protoplast formation of *P. oryzae* increased continuously in the 10 mM 2-mercaptoethanol up to five hrs after enzyme treatment. This suggested that S-S bond of protein on the cell wall of *P. oryzae* was attacked by 2-mercaptoethanol and enzyme activity increased to produce more protoplasts. Nakajima *et al.* (1970) found that cell wall of *P. oryzae* was comprised of 4.6% protein. Similarly, protoplast formation of some other filamentous fungi such as *Geotrichum candidum* and *Pythiym* sp. consisting of an outer protein layer was stimulated by SH-compound (Dooijewaard-kloosterziel *et al.*, 1973; Sietsma and Boer, 1973). The decrease of protoplast formation in 200 mM of 2-mercaptoethanol comparing to the untreated control may be due to the inactivation of enzymes treated on the mycelia of *P. oryzae* by 2-mercaptoethanol.

Han *et al.* (1987) observed only one type of protoplast reversion in *P. oryzae*. Like the second reversion type in the present study, a germ tube like hypha protruded from the distal cell after development of an irregularly shaped chain of cells. Similar three types of protoplast reversion of *P. oryzae* reported (Tanaka *et al.*, 1981) were also obtained in the present study. The reason for the failure of the yeast like buds to revert to normal mycelia has not been found, but the absence of a nucleus may be one of those reasons as reported by Hamlya (1982). In the filamentous fungi, another significant factor affecting reversion of protoplasts is the origin of protoplasts in relation to hyphal organization; protoplasts from distal region of hyphae may be lacking in the capacity of reversion (Peberdy, 1979).

There are conflicting results on the effects of osmotic stabilizers on the protoplast reversion in other fungi. Ann'e (1977) found that the nature of the osmotic stabilizer had little influence on the percentage of protoplast reversion in *Penicillium chrysogenum*, whereas the reversion of *Pythium* and *Geotrichum* protoplasts was completely inhibited by inorganic salts. The frequency of protoplast reversion of *P. oryzae* was highest on PDA incorporated with 0.6 M KCl (Han *et al.*, 1987). In the present study, potassium chloride was also the best as an osmotic stabilizer for the protoplast reversion of *P. oryzae*. Although protoplasts of *Rhizoctonia solani* (Hashiba and Yamada, 1982) reverted to normalmycelia on water agar, the protoplast reversion of *P. oryzae* did not occur in the present study. Hamlya (1982) reported that the choiceof reversion medium did not appear to affect the reversion frequency. In thepresent study, however, the choice of reversion medium with suitablenutrients was required essential for the high frequency of protoplastreversion in *P. oryzae*.

The technique of protoplast formation and reversion in the present study might be useful in studying the mechanism of pathogenicity and genetics of *P. oryzae* and the other pathogenic fungi.

摘　要

벼 稻熱病菌(*Pyricularia oryzae*)을 감자 液體培地에서 27℃, 48時間 培養한 後, 菌絲體에 Driselase,

β-Glucuronidase, Cellulase, Macerozyme R-10의 混合 酵素 液을 處理한 30분後부터 原形質體가 形成되었다. Race KJ 101이 KI 315a보다 더 많은 原形質體가 形成되었다. 2-Mercaptoethanol을 混合 酵素液 處理前, 菌絲體에 前處理 함으로써 3時間 후부터 줄어들던 對照區보다 原形質體 形成量을 增加시킬 수 있었다. 특히 2-Mercaptoethanol 10 mM 處理에서는 酵素液處理 5時間 後에 最大의 原形質體 形成量을 보였으나 200-mM 處理區에서는 오히려 原形質體 形成을 抑制하였다. 菌絲體로부터 形成된 原形質體를 27℃의 液體培地(2.5% yeast extract, 2% dextrose)에서 진탕 培養하면 5時間 後부터 크게 세가지 形態로 再生, 復歸되었다. Yeast와 같은 連鎖 사슬形態로 되거나, 連鎖사슬의 선단부에서 發芽管과 類似한 菌絲體가 形成되거나, 혹은 처음부터 發芽管과 같은 菌絲가 形成되었다. 滲透壓 安定劑를 添加한 固體 培地에 稻熱病菌의 原形質體를 接種하여 27℃에서 5일간 培養하면 正常的인 菌絲體로 復歸되어 菌叢을 形成하였다. 이때 使用한 Mannitol, Sorbitol, KCl, $MgSO_4$ 등의 滲透壓 安定劑中에서 0.6 M KCl을 감자寒天培地에 添加했을 때 33.4%의 가장 높은 復歸率을 보였으나, 물 寒天培地에서는 滲透壓 安定劑의 種類와는 關係없이 原形質體가 正常的인 菌絲體로 復歸하지 못하였다.

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