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Studies on Protoplast Formation and Regeneration of *Ganoderma lucidum*

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Abstract□To obtain a new strain of *Ganoderma lucidum* by protoplast fusion technique, its protoplast formation and regeneration were studied. Several factors affecting the protoplast formation and regeneration were investigated to find their optimum conditions. The mycelium was grown for four days on the cellophane membrane placed on *G. lucidum* complete medium (GCM). When various commercial lytic enzymes were examined for protoplast isolation, the combination of Novozym 234 and β-glucuronidase was found to be effective. An osmotic stabilizer, 0.6 M sucrose in 20 mM phosphate buffer pH 5.8, gave the highest yield of protoplasts. Three-hour incubation in shaking incubator was most suitable for releasing protoplasts. To increase the protoplast yield, pretreatment with 2-mercaptoethanol was carried out. The regeneration frequency in GCM containing 0.6 M $MgSO_4 \cdot 7H_2O$ was shown to be 0.66%.

Keywords□ *Ganoderma lucidum, Polyporaceae,* Protoplast, Lytic enzyme, Osmotic stabilizer, Regeneration frequency.

The carpophores of *Ganoderma lucidum* (Leyss. ex Fr.) Karsten (family *Polyporaceae*) have been used to treat hepatopathy, chronic hepatitis, nephritis, gastro ulcer, hypertension, arthritis, neurasthenia, insomnia, bronchitis, asthma, and allergy. The reports on protoplast isolation from yeasts[1] and filamentous fungi[2] began to appear in 1957. Consequently, protoplasts derived from *Saccaromyces cerevisiae* have proved of great value in studies on organelles and in biosynthetic studies[3]. More recently interest in protoplasts has again moved to their usefulness as tools for particular relation to certain new approaches to the genetic modification of bacteria, fungi and plants. Removing the wall and exposing the protoplast membrane allow manipulation involving fusion or uptake of nucleic acids, processes that are less achievable with intact cells.

The first protoplast fusion was carried out in *Geotrium candidium*[4]. The fusion frequency was enhanced by using PEG-Ca^{+2} [5]. Furthermore, current research into electro-fusion method was able to avoid the most of the disadvantages of the chemically induced fusion procedures. In higher fungi, protoplasts were isolated first in *Polystictus versicolor*[6], and then in *Schizophyllum commune*[7], *Coprinus cicereus*[8], *Lentinus edodes*[9], *Tricholoma matsutake*[10], *Flammulina velutipes*[11] and *Peleurotus ostreatus*[12].

Recently, *G. lucidum* was found to exhibit activity of improving blood circulation, inhibition of degranulation in the mast cell, efficacies in hypertension, thrombosis, hyperglycemia, and types I-IV of allergy. However, anastomosis or antagonist line between the mycelia of different strains which have different activities did not occur when they were cultured in the same medium. Therefore, protoplast fusion between the different strains may produce a new strain which will show several activities.

In this investigation, attempts were made to determine optimal factors for protoplast formation and regeneration of *G. lucidum*.

EXPERIMENTAL METHODS

Materials

Ganoderma lucidum (Fr.) Karsten was used. This organism was kindly provided by the Institute of Agricultural Sciences, Suwon, Korea. *Ganoder-*

Table I. The composition of *Ganoderma lucidum* complete medium (GCM)

Glucose	30
Sucrose	20
$MgSO_4 \cdot 7H_2O$	0.5
K_2HPO_4	1.0
KH_2PO_4	0.46
Yeast extract	10
Bacto peptone	4
Casamino acid	5
Agar	20 (g/l)
Added distilled water to 1 L	

ma lucidum complete medium (GCM) and potato dextrose agar medium (PDA, Difco Lab., U.S.A., 39 g/l) were used (Table I). Regeneration media were prepared by adding each osmotic stabilizer to GCM.

Protoplast Formation

1) Enzyme Preparation

Novozym 234 (Novo Industri, Denmark), β-glucuronidase (Sigma Chem. Co.) and β-D-glucanase (BDH Chem., Ltd., U.K.) were used as lytic enzymes. The enzyme solution containing the osmotic stabilizer was filtered through the 0.2μm membrane filter (Gelman Sci. Co.). For the most effective release of protoplasts, these commercial enzymes were examined and their appropriate concentrations were determined.

2) Osmotic Stabilizers

Several inorganic salts, sugars and sugar alcohols were used. Among them, for the optimal concentration of the selected osmotic stabilizer, various concentrations of sucrose were examined. All the osmotic stabilizers tested were dissolved in 20 mM phosphate buffer (pH 5.8).

3) Isolation of Protoplast

The mycelia of the fungus were grown for four days prior to inoculation onto cellophane membrane in GCM Petri-dish. The cellophane sheets were removed together with the mycelia from the media after four days and placed in empty Petri-dish. And then they were added with the lytic enzyme solution containing 0.6 M sucrose and 20 mM phosphate buffer (pH 5.8). Consequently, they were incubated in shaking incubator with 120 strokes/min at 30°C for three hours for the protoplast releasing, and the generated protoplasts were counted with a hemacytometer.

Protoplast Regeneration

After the released protoplasts were filtered through sintered glass filter (porosity 1) to remove the mycelial debris, their filtrates were centrifugated for 15 minutes at 1000 rpm and then washed twice with 0.6 M sucrose solution. The prepared protoplast suspension was diluted serially to 10^3, 10^4 10^5 and 10^6/ml by adding the osmotic stabilizer, 0.6 M sucrose. Regeneration medium containing a suitable osmotic substance was prepared prior to plating serially diluted protoplast suspension. Five ml of soft agar (0.75%, 42°C) was mixed with 0.5 ml of serially diluted protoplast suspension, immediately overlayed and spread uniformly on the regeneration medium. In order to calculate the regeneration frequency, the number of regenerated colonies which were 100-200 per Petri-dish was counted, and its ratio was calculated.

RESULTS AND DISCUSSION

Formation of Protoplasts

1) Effects of Lytic Enzymes

The lytic enzyme was one of the major factors for protoplast formation. The effectiveness of dif-

Table II. Comparison of commercial enzymes for the release of protoplast in *Ganoderma lucidum*

Exp. I

Enzyme (mg/ml)	No. of protoplast (X 10^6/ml)
Novozym 234 (10)	1.57 ± 0.73*
Novozym 234 (15)	1.20 ± 0.41
Novozym 234 (20)	1.04 ± 0.56

Exp. II

Enzyme (mg/ml)*	No. of protoplast (X 10^6/ml)
Novozym 234 (10)	0.81 ± 0.04**
Novozym 234 (10) + β-Glucuronidase (10)	5.96 ± 0.56
Novozym 234 (10) + β-Glucuronidase (10) + β-D-Glucanase	3.09 ± 0.04

* Buffer: 20 mM phosphate buffer
Incubation time: 3 hours

** Mean ± standard deviation of triplet

ferent commercial enzymes in releasing protoplasts from *G. lucidum* was shown in Table II. Novozym 234 was the most effective enzyme and its suitable concentration was 10 mg/ml.

The enzymes varied in their ability to produce protoplasts from the different fungi. The combinations of enzymes were normally required to obtain high yields of protoplasts. A combination of the enzymes, Novozym 234 and cellulase CP gave the best results with *Aspergillus nidulans*[13]. Both Novozym 234 and β-D-glucuronidase readily converted cells of *Saccharomyces cerevisiae* to protoplasts. Novozym 234 was the most effective enzyme with *Penicilium chrysogenum* and *Volvariella volvacea*. Generally, Novozym 234 was effective in most of the fungi[14]. In *G. lucidum*, a combination of Novozym 234 and β-glucuronidase was most effective.

Eddy and Williamson introduced the use of *Helix pomatia* digestive juice as a lytic enzyme[1]. Some enzymes have been used by many other workers with several different yeasts and are available as helicase, sulfatase and glusulase[15]. A second enzyme, zymolase, derived from *Arthrobacter luteus*, has also been used for the isolation of protoplast from *S. cerevisiae* and other yeasts. Various Actinomycetes produce enzymes that are lytic against yeasts but these are not commercially available[16-17].

2) *Effects of Osmotic Stabilizers*

Osmotic stabilizers are clearly essential to provide osmotic support to the protoplast following the removal of the cell wall. This wide ranges of inorganic salts, sugars and sugar alcohols have proved more effective for filamentous fungi, and sugars or sugar alcohols were more effective for yeasts[21]. However, the virtues of particular stabilizers were understood only in an empirical sense, and differences in effectiveness must relate to as yet unknown factors in the uptake and utilization of the particular compounds.

In *Fusarium culmorum*, ammonium chloride gave the best result. In *A. nidulans* and *A. flavum* ammonium chloride and potassium chloride were good stabilizers, and in *P. chrysogenum* potassium chloride was effective. In *C. acremonium* 1.6 M sodium chloride was more effective than sucrose, mannitol and magnesium sulfate[14]. In *V. volvaceae* 0.6 M potassium chloride was effective. In *G. lucidum* 0.6 M sucrose was more effective than glucose and mannitol (Table III). One of the more interesting observation of osmotic stabilizers was made by De Vries and Wessels who identified a distinct property exhibited by 0.6 M magnesium sulfate when used for filamentous fungi. Two effects were observed; the mycelium became extensively fragmented in the early period of lytic digestion, and then they found that a large proportion of the protoplast released had large vacuoles[22].

Table III. Effects of osmotic stabilizer on the protoplast release in *Ganoderma lucidum*

Osmotic stabilizer*	No. of protoplast (X 10^5/ml)
0.6 M $MgSO_4 \cdot 7H_2O$	0.50 ± 0.07**
0.6 M NH_4Cl	0.25 ± 0.03
0.6 M Sucrose	8.31 ± 0.59
0.6 M Glucose	2.00 ± 0.41
0.6 M Mannitol	0.75 ± 0.05

* Enzyme: 10 mg/ml Novozym 234 in 20 mM phosphate buffer (pH 5.8)
Incubation time: 3 hours
No pretreatment was done
** Mean ± standard deviation of four plates

3) *Buffer System and pH*

Buffer system and its pH influenced the lytic enzyme activity. Phosphate buffer was more effective than Na-malate buffer (Table IV). The optimum pH for protoplast formation was between pH 5.8 and 6.0 (Figure 2). It was likely that the effectiveness of these enzyme could be increased by determining optimum conditions.

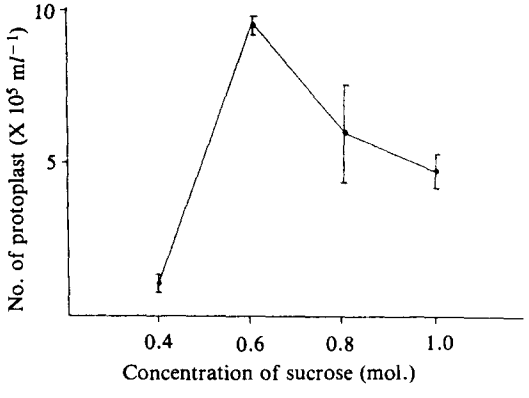

Fig. 1. Effect of osmotic stabilizer sucrose concentration on the protoplast releasing from *G. lucidum*.
Enzyme: 10 mg/ml Novozym 234
Buffer: 20 mM phosphate buffer (pH 5.8)
Incubation time: 3 hours
No pretreatment was done
Each bar: standard deviation of three plates

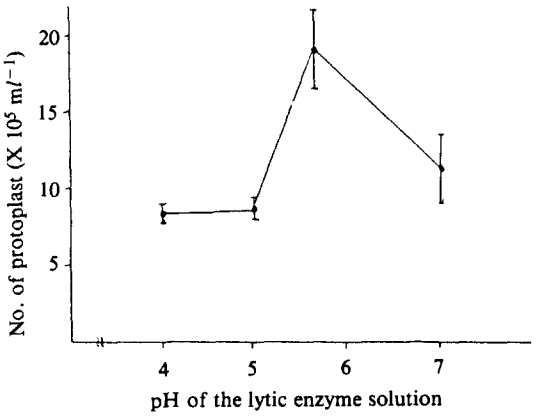

Fig. 2. Effect of pH of the lytic enzyme solution on the protoplast releasing from *G. lucidum*.
Osmotic stabilizer: 0.6 M sucrose in 20 mM phosphate buffer
Enzyme: 10 mg/ml Novozym 234
Incubation time and temperature: 3 hours and 28 °C
No pretreatment was done
Each bar: standard deviation of three plates

4) Influence of Incubating Time

After the treatment of the enzyme solutions, the protoplasts began to release. The yield of the protoplasts reached the maximum after two and four hours, and then decreased gradually. No protoplast was observed after twenty-four hours. Thus the gradual decrease in the number of the protoplast was mainly due to the lysis of the protoplast by the enzyme. The results showed that incubating time was not proportional to releasing of the protoplasts and the optimum incubating time was from two to four hours after the release of the protoplasts began (Figure 3).

Table IV. Comparison of different buffer solution on the release of protoplast in *Ganoderma lucidum*

Buffer*	No. of protoplast (X 10^6/ml)
20 mM Phosphate buffer (pH 5.8)	1.96 ± 0.26**
50 mM Na-malate buffer (pH 5.8)	0.81 ± 0.01
Control (distilled water)	1.31 ± 0.61

* Enzyme: 10 mg/ml Novozym 234
Protoplast stabilizer: 0.6 M sucrose solution in 20 mM phosphate buffer
Incubation time: 3 hours
No pretreatment was done
** Mean ± standard deviation of three plates

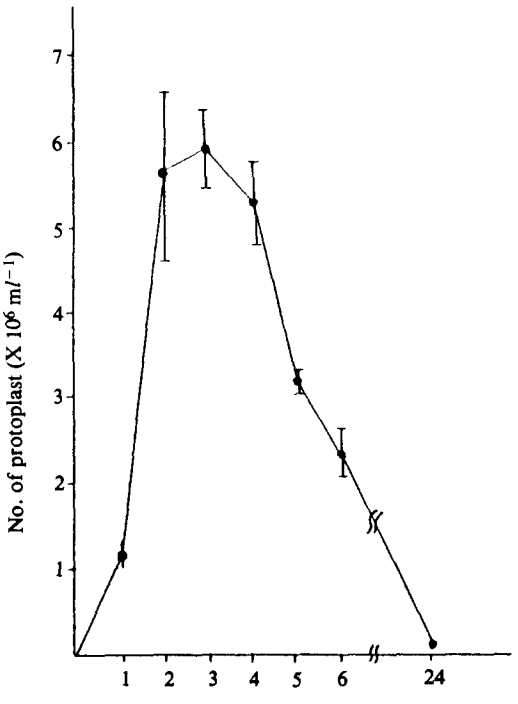

Fig. 3. Influence of the incubating time on the protoplast releasing from *G. lucidum*.
Osmotic stabilizer: 0.6 M sucrose in 20 mM phosphate buffer (pH 5.8)
Enzyme: 10 mg/ml Novozym 234
Incubation temperature: 28 °C
No pretreatment was done
Each bar: standard deviation of three plates

5) Influence of Mycelial Amount

Influence of the mycelial concentration on the release of protoplasts was tested (Figure 4). Four ml of the enzyme mixture, Novozym 234 and β-glucuronidase, 10 mg/ml each, was used for protoplast release and incubated for four hours. When the mycelia grew up to six colonies with an area of 35.5 cm^2, eight with that of 42.4, 10 with that of 56.6 and 12 with that of 61.0, they were used in the subsequent procedures. One ml of the mixture solution was suitable for two colonies and the protoplast yield was not proportional to the amount of the mycelia.

6) Age of Mycelium

The mycelia from the cellophane cultures of different ages were tested for protoplast production (Figure 5). The appropriate age of the mycelium was four-day. At the population level, the physiological status of the culture was a major factor in

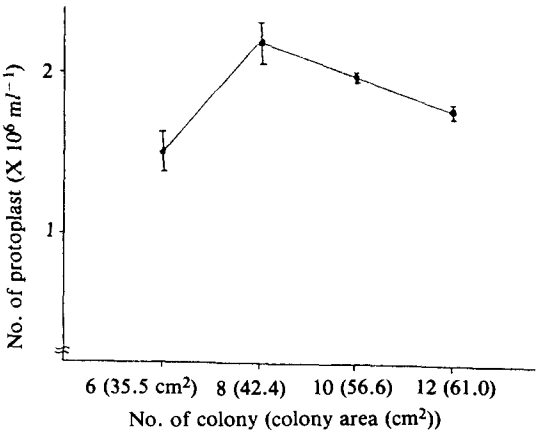

Fig. 4. Influence of the mycelial amounts on the protoplast release of *G. lucidum*.
Osmotic stabilizer: 0.6 M sucrose in 20 mM phosphate buffer (pH 5.8)
Enzyme: 10 mg/ml Novozym 234
Incubation time and temperature: 3 hours and 28 °C
No pretreatment was done
Each bar: standard deviation of three plates

determining protoplast yield. The isolated cell walls of *G. candidicum* obtained from cells at different culture ages varied in their susceptibility to lysis with snail digestive juice[15]. The walls from the cultures in the early and mid-exponential phases of growth were more susceptible than the walls derived from the older cultures of the fungus. Peberdy *et al.* found that the protoplast yields from *A. flavus* were the highest when the cultures in the exponential phase of growth were used. The young cells from the exponential cultures were readily converted to protoplasts, but the cells from the stationary state cultures were resistant to lysis. In addition, the occurrence of a duplication cycle in the terminal segment of the hyphae also had significant and interesting implication in relation to protoplast release. The signification of mycelial age for induction of protoplast was unknown clearly; however, it is most probably associated with changes in the hyphal wall, *e.g.*, in *A. nidulans* the deposition of α-1, 3-glucan as an outer wall layer in the older hyphae may be a factor.

7) Pretreatment of the Mycelia

To increase the protoplast yield, the pretreatment of thiol compounds and chelating agents was carried out. For pretreatment conditions, fungal mycelia were incubated with the above reagents for 30 minutes at 30 °C and washed twice with 0.6 M sucrose. In *G. lucidum* the pretreatment of the mycelia with a disulfide reducing agent such as 2-mercaptoethanol was necessary for the efficient production of the protoplasts.

Certain modification or change in cell wall might be involved in altering its susceptibility to lysis. This also seems likely in the several situation where the cells have been given some pretreatment that improves protoplasts yields. Thiol compounds have been used extensively with yeasts 18) and some filamentous fungi, including *Cephalosporium acremonium*[19] and *Histoplasma capsulatum*. Another treatment was shown to improve protoplast release involved the use of Triton X-100 with *Pythium*[20]. The enhancing effect was presumed to result from

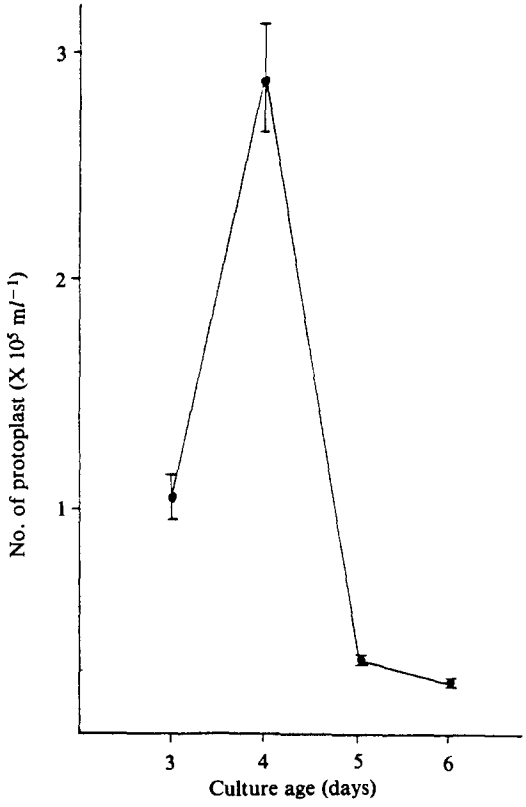

Fig. 5. Effect of the culture age in the yields of the protoplast per unit area of the mycelial colony of *G. lucidum*.
Osmotic stabilizer: 0.6 M sucrose in 20 mM phosphate buffer (pH 5.8)
Enzyme: 10 mg/ml Novozym 234
Incubation time and temperature: 3 hours and 28 °C
No pretreatment was done
Each bar: standard deviation of three plates

the removal of a lipid layer, and this idea was further supported by experiments in which lipase was found to be an adjuvant in protoplast isolation from this fungus.

Influences of the pretreatment on the *G. lucidum* protoplast release were shown in Table V. These results suggest that the hyphal wall of the disulfide reducing agents could enhance the enzymatic activity.

Regeneration of Protoplasts

The process of wall regeneration and subsequent reversion has been extensively studied. It has been investigated with respect to the mechanism of wall polymer biogenesis and deposition in an attempt to gain an understanding of the process in the intact cell. The developmental aspects of protoplast reversion are also of interest, providing a model system for investigation into the basis for changes in shape and the possible role of the cell wall.

1) Effects of Osmotic Stabilizers on Regeneration

The protoplast regeneration frequency was affected by various osmotic substances which were added into the regeneration media for protection against the osmotic pressure of the protoplast during regeneration. As shown in Table VI, among the osmotic stabilizers tested, 0.6 M sucrose and 0.6 M magnesium sulfate showed 0.46 and 0.66% regeneration frequencies. However, in case of magnesium sulfate, the medium did not harden easily because of its acidity. Therefore, 0.6 M sucrose was used as osmotic stabilizer for the subsequent experiments.

Table V. Influences of pretreatment mycelium on the protoplast release in *Ganoderma lucidum*

Pretreatment agent*	No. of protoplast (X 10^6/ml)
5 mM Na-EDTA	1.10±0.15**
50 mM Na-EDTA	0.53±0.08
5 mM 2-Mercaptoethanol	1.09±0.32
50 mM 2-Mercaptoethanol	1.12±0.26
1.2 M Sucrose	0.33±0.11
1.2 M $MgSO_4 \cdot 7H_2O$	0.15±0.03
Control (without treatment)	0.39±0.09

* Enzyme: 10 mg/ml Novozym 234
Osmotic stabilizer: 0.6 M sucrose solution in 20 mM phosphate buffer (pH 5.8)
Pretreatment time and temperature: 30 min and 30°C
Incubation time: 3 hours
** Mean ± standard deviation of triplet

Table VI. Regeneration frequency of *Ganoderma lucidum* protoplast on the several regeneration media

Osmotic stabilizer*	Regeneration frequency (%)
0.6 M Sucrose	0.46±0.07**
0.6 M $MgSO_4 \cdot 7H_2O$	0.66±0.24
0.6 M Glucose	0.10±0.06
0.6 M Mannitol	0.12±0.03

* Buffer: 20 mM phosphate buffer (pH 5.8)
Top agar: 0.75% soft agar of 42°C
** Mean ± standard deviation of four plates

2) Regeneration Frequency

The regeneration frequency was obtained by viable count determination following plating of protoplasts in osmotically stabilized agar media. The regeneration ratio calculated from the following equation:

$$\text{Regeneration ratio} = \frac{\text{No. of colonies}}{\text{No. of protoplast}} \times 100$$

The regeneration ratios of *Schizophillum commune* and *Coprinus cinereus* were 50% and 10%, respectively. However, *V. volvaceae* showed a lower rate, only 1-3% because of the absence of nuclei in protoplast.

The absence of a nucleus was one factor of these results and this can be a feature of a large proportion of a protoplast preparation. In the filamentous fungi, another significant factor may be the origin of protoplasts in relation to hyphal organization; protoplasts from distal regions of hyphae may be lacking the capacity for reversion. Refinements in techniques for protoplast isolation and possible fraction of protoplast may aid in the understanding of this fundamental question.

Two basic patterns of regeneration were observed. In the first, the spherical protoplast directly produced one or more germ tubes. In the second, the protoplasts developed into bud-like structures and finally produced germ tubes.

CONCLUSION

The mycelium of *Ganoderma lucidum* was grown on the cellophane membrane placed on GCM for four days. The most effective concentrations of both Novozym 234 and β-glucuronidase were 10 mg/ml. An osmotic stabilizer, 0.6 M sucrose in 20 mM phosphate buffer (pH 5.8), was found to be adequate. Three-hour shaking incubation was the most suitable condition for releasing

protoplasts. The regeneration frequency in GCM containing 0.6 M magnesium sulfate was shown to be 0.66%. Two patterns of regeneration were observed. The protoplast directly produced one or more germ tubes and the bud-like structures.

ACKNOWLEDGMENT

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