Giant Protoplasts of *Pyricularia oryzae* Cavara*

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Abstract

Protoplasts of *Pyricularia oryzae*, which were isolated from mycelium or germ-tube by 1% Cellulase “Onozuka” R-10 and 1% Driselase, kept spherical shape and sensitivity to osmotic change, and became larger in size when incubated in potato sucrose broth or the synthetic medium containing 0.6 M sucrose and also the enzymes. Twenty-four hr after incubation, more than half of the protoplasts became double or more in size. The formation of the giant protoplasts was inhibited by adding IBP or kasugamycin, but not by adding polyoxin D. The incubated protoplasts were fractionated to the giant-rich fraction and the original-size fraction by the two phase system with 0.6 M mannitol and 0.6 M sucrose. The giant protoplasts kept their viability as well as the original-size did, and had higher metabolic activity in all biosynthesis of protein, DNA, RNA, lipid and cell wall than the original-size had. These results suggest that this protoplast incubation system with the enzymes may be not so poisonous that the protoplasts could keep viability and grow without cell wall, and consequently become giant.

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Key words: giant protoplasts, *Pyricularia oryzae*, viability, metabolic activity.

INTRODUCTION

Fungal protoplasts have been isolated from various fungi with commercially available enzymes, and applied as tools for physiological, biochemical and genetic studies. But often fungal protoplasts have not so high viability and not so easily regenerate the cell wall and reverse to mycelium. Probably various factors in protoplast isolation and incubation, such as the enzyme system for the isolation, the incubation system including osmoticum and the frequency of isolating protoplasts without nucleus, may affect the viability of protoplasts and/or the ability to regenerate and reverse. Generally, fungal protoplasts are smaller than plant protoplasts. Therefore the manipulations in fungal protoplasts, such as injection of foreign DNA into protoplast or protoplast fusion, are often difficult.

In *Pyricularia oryzae*, the protoplasts were firstly isolated by Hosokawa and Kozaka3). And then the enzyme composition for the isolation was modified by Yaegashi *et al.*9) and later reported by Asai *et al.*1) As unexpected, we had observed abnormally giant protoplasts with spherical shape when incubated in potato sucrose broth containing 0.6 M sucrose and also enzymes for the protoplast isolation, which was composed of 1% Cellulase R-10 and 1% Driselase according to the modified method of Hosokawa and Kozaka9). We expected that the protoplasts could keep viability and grow larger without cell wall, and that the occurrence of the giant protoplast was not caused by protoplast fusion. So, we will report in this paper that the giant protoplast keeps viability and has higher metabolic activity than the original-size have and probably results from its growing without cell wall, and discuss its application for some fields.

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MATERIALS AND METHODS

Chemicals. Enzymes for protoplasts isolation, Cellulase “Onozuka” R-10 and Driselase, were purchased from Yakult Biochemical Co. Ltd. and Kyowa Hakko Kogyo Co. Ltd., respectively. All radioactive precursors were purchased: [U-14C]amino acid mixture (57 mCi/milliatom) from New England Nuclear Co. Ltd.; and [2-14C]uridine (52 mCi/mmol), [2-14C] thymidine (58 mCi/mmol), [U-14C] sodium acetate (58 mCi/mmol), [1-3H]N-acetylglucosamine (3.4 Ci/mmol) and [U-14C] glucose (270 mCi/mmol) from Amersham Co. Ltd. IBP (S-benzyl-O,O'-diisopropylphosphorothiolate, Kitazin P; more than 96.27% purity), kasugamycin (sulfate salt; 68.6% purity) and polyoxin D (98.9% purity) were provided by Kumiai Chemical Industry Co.Ltd., Meiji Seika Co. Ltd., and Kaken Pharmaceutical Co. Ltd., respectively. Calcofluor White to stain glucans and fluorescent diacetate (FDA) for vital staining were available from Sigma Biochemical Co. Ltd.

Organism. Isolate P-2 of Pyricularia oryzae was mainly used in this study. Likewise, a field isolate A4-1, H13-6 and G4-5 were used as a sensitive and resistant isolate to IBP, and a resistant isolate to kasugamycin, respectively. And a mutant RM P-2 resistant to polyoxin D, which is one of the resistant mutants reversed from protoplasts of P-2, was also used. All isolates were grown on potato sucrose agar for about 2 weeks at 28°C and then stocked at 20°C.

Protoplast isolation. Protoplasts of P. oryzae were isolated by the enzymatic procedure as described previously7). Mycelia cultured in potato sucrose broth (PSB) for 3 or 4 days at 28°C, or spores germinated in PSB for 1 days at 28°C were collected by centrifugation (550 g, 5min) and immersed for 3 to 4 hr in an enzyme solution, which was composed of 1% Cellulase “Onozuka” R-10 and 1% Driselase in the synthetic medium7) containing 0.6M KCl (SMK), and filtered through a Millipore filter (0.22 μm, pore size). The undigested mycelia or spores were removed through nylon sieves (82 and 25 μm). The isolated protoplasts were washed 2 or 3 times with SMK to remove enzymes. The number of protoplasts was counted with hemacytometer. Viability of protoplasts was determined by vital staining with FDA and/or neutral red.

Incubation systems for protoplasts. The isolated protoplasts were finally suspended in the synthetic medium containing 0.6 M sucrose (SMS) or PSB containing 0.6 M sucrose (PS) with the above enzymes for protoplast isolation, and incubated at 28°C. For protoplast regeneration and reversion, protoplasts were resuspended in SMS or PS, and incubated at 28°C as described previously7). The cell wall regenerated on protoplasts was stained by Calcofluor White and the reversion types of the protoplasts, budding type or germ-tube type1,7), were checked under an inverted microscope. The percent of mycelial colonies reversed from protoplasts were estimated on a basis of the number of the total protoplasts added.

Fractionation of protoplasts. The protoplasts incubated in SMS with the enzymes were fractionated by the two phase system with 0.6 M mannitol and 0.6 M sucrose as shown in Fig. 3; 2 volume of the synthetic medium containing 0.6 M mannitol was laid on 1 volume of the protoplasts incubated in SMS with the enzymes in a test-tube and centrifuged at 50×g for 15 min. The upper (mannitol) phase and the bottom (sucrose) phase were separated with a pipet and centrifuged at 550×g for 10 min respectively. The precipitated protoplasts in the bottom phase were resuspended in SMK and centrifuged again to remove the enzymes. Finally, the precipitated protoplasts in both the phase were resuspended in SMS.

Determination of metabolic activities in protoplasts. Protoplasts (ca. 10⁶ to 10⁷ protoplasts) resuspended in SMS were incubated at 28°C with 0.1 μCi 14C-amino acids mixture, 0.1 μCi 14C-uridine, 0.1 μCi 14C-thymidine, 0.2 μCi 14C-acetate, and a mixture of 0.5 μCi 3H-N-acetylglucosamine and 0.2 μCi 14C-glucose, respectively. After 1 hr of incubation, the reaction was stopped with cold TCA (5%, final). Acid insoluble materials were trapped on a glass filter (Whatman GF/C) and washed successively with 5% TCA solution and EtOH. Incorporations of 14C or 3H-precursors into acid insoluble fractions were determined with a liquid scintillation counter (Packard Model 3320) using the xylene-based scintillator7). Incorporation of 14C-acetate into lipid fraction was determined after extraction of lipids with the
modified procedure of Bligh and Dyer\(^4\).

RESULTS

**Time-Course of occurrence of the giant protoplasts**

Many of the protoplasts were derived from one cell between septa. Sometimes several protoplasts were isolated from one cell. The diameter of the protoplast just after isolation ranged from 2 to 9 $\mu$m. When the isolated protoplasts were incubated in SMS or PS with the enzymes, the protoplasts kept spherical shape and sensitivity to osmotic change, and became larger in size. We define two words tentatively, the giant protoplast and the normal protoplast; the former is defined as a protoplast with more than or equal to 12 $\mu$m in diameter and the latter as that with less than 12 $\mu$m. The giant protoplasts occurred about 6 hr after incubation and gradually increased with the passage of incubation time (Fig. 1). Twenty-four hr after incubation, more than half of the protoplasts became double or more in size, properly being the giant protoplasts (Fig. 1 and Plate I). At that time both the giant and the normal protoplasts were not stained at all by Calcofluor White, but stained by FDA and/or neutral red at range of 50 to 80% without significant difference between them (Plate I-2 and I-3).

In this system, total number of the protoplasts scarcely decreased and apparently the protoplasts individually became larger under a continuous observation. Vacuole was specifically enlarged in most of the giant protoplast. Protoplasts derived from germ-tube became larger in the same manner in this system. And protoplast incubated in SMS with the enzymes hardly differed from those in PS with the

![Fig. 1. Histogram of the diameter of the P-2 protoplast after incubation in SMS with the enzymes for a given period. 0: just after isolation; the other figures indicate the hours after incubation.](image-url)
enzymes in occurrence of the giant protoplasts, although the occurrence in the former medium was slightly slower.

Effect of IBP, kasugamycin and polyoxin D on formation of the giant protoplasts and their viability

Addition with IBP and kasugamycin in this system inhibited formation of the giant protoplast and also decreased their viability in proportion to their concentration and sensitivity of the parent isolate to the fungicides (Fig. 2). However, polyoxin D scarcely inhibited the formation of the giant protoplast, but decreased their viability in protoplast from the sensitive P-2.

A field resistant isolate H13-6 and G4-5 to IBP, and a resistant mutant RM P-2 to polyoxin D were usually slower in mycelial growth. The protoplasts derived from these isolates became giant more slowly and the size was relatively smaller in this incubation system even without addition with any fungicides.

Fractionation of the protoplasts

The two phase system with 0.6 M mannitol and 0.6 M sucrose made possible to fractionate the protoplasts incubated in SMS or PS with the enzymes.

In the 1st centrifugation, the giant protoplasts were concentrated at the interface between sucrose and mannitol phases and the normal protoplasts were shared into both of the phases. Finally, the bottom sucrose phase included only the normal protoplasts and the upper mannitol phase included the giant protoplasts (25-40%) mixed with the normal protoplasts.
Table 1. Metabolic activity of the isolate P-2 protoplasts in the giant-rich fraction (G+N) and the normal protoplast fraction (N) by the two phase system with 0.6 M sucrose and 0.6 M mannitol after incubation in SMS with the enzymes for 24 hr

<table>
<thead>
<tr>
<th>Precursor</th>
<th>dpm incorporated/10⁶ of protoplasts</th>
<th>Ratio of G+N/N (G/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein: [U-¹⁴C] amino acid mixture</td>
<td>40634 (111780)</td>
<td>2324</td>
</tr>
<tr>
<td>RNA: [2-¹⁴C] uridine</td>
<td>18709 (51989)</td>
<td>789</td>
</tr>
<tr>
<td>DNA: [2-¹⁴C] thymidine</td>
<td>2330 (6024)</td>
<td>342</td>
</tr>
<tr>
<td>Lipids: [U-¹⁴C] sodium acetate</td>
<td>10111 (26124)</td>
<td>1489</td>
</tr>
<tr>
<td>Chitin: [1-³⁵H] N-acetylglucosamine</td>
<td>44971 (122763)</td>
<td>3083</td>
</tr>
<tr>
<td>Glucan: [U-¹⁴C] glucose</td>
<td>3060 (6482)</td>
<td>1217</td>
</tr>
</tbody>
</table>

a) Figures in parenthesis indicate the estimated values of the giant protoplast on the basis of the data in “N” fraction.

Viability and metabolic activity of the giant protoplasts

Metabolic activity in the above-mentioned fractions was determined. The protoplasts in the giant-rich fraction had higher activity in all biosynthesis, compared with those in the normal protoplast fraction (Table 1). The activity was equal to or slightly higher than that in protoplasts just after isolation as described in the previous report. Provided the normal protoplasts in the giant-rich fraction have the same activity as those in the normal protoplast fraction, the giant protoplast will be estimated to have more high activity (Table 1).

Protoplasts just after isolation regenerated like as germination of spore at 60 to some 70% and reversed to colonies at 50 to some 60%. Protoplasts after incubation in SMS with the enzyme for 24 hr kept the ability to regenerate and reverse, although the ability slightly decreased in comparison with protoplasts just after isolation (Table 2 and Plate I-4).
Table 2. Ability of protoplasts to regenerate cell wall and reverse to mycelial colonies

<table>
<thead>
<tr>
<th></th>
<th>Regenerating germination</th>
<th>Reversion to colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>G+N fraction(^a)</td>
<td>59 (%)</td>
<td>53 (%)</td>
</tr>
<tr>
<td>G(^b)</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>N(^c)</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>N fraction(^d)</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>Protoplasts just after isolation</td>
<td>72</td>
<td>60</td>
</tr>
</tbody>
</table>

\(^a\) The giant-rich (G+N) and the normal protoplasts (N) fraction were fractionated by the two phase system with 0.6 M sucrose and 0.6 M mannitol after incubation in SMS with the enzymes for 24 hr.
\(^b\) In regenerating germination test, the giant and the normal protoplasts in “G+N” fraction were individually checked under an inverted microscope.

DISCUSSION

As long as we continuously observed the protoplasts in this system, they sometimes agglutinated but did not happen to fuse each other. Then, it was not likely that the giant protoplast resulted from natural fusion. When the diameter of a protoplast becomes double, the surface area will increase by 4 times and the cubic volume by 8 times. Accordingly the formation of the giant protoplast may require a newly synthesized cell membrane at least. Since the formation of the giant was inhibited by IBP as an inhibitor of phospholipid or chitin biosynthesis and by kasugamycin as an inhibitor of protein biosynthesis, it was more proper that at least (phospho) lipid and protein biosynthesis functioned normally in the protoplasts and were involved in the formation of the giant. And then it was not likely that the giant protoplast resulted only from its swelling, although vacuole was specifically enlarged in the giant protoplast. Furthermore, considering the data of the metabolic activity (Table 1) and the ability to regenerate and reverse (Table 2), this protoplasts incubation system with the enzymes may be not so poisonous that the protoplast could keep viability and grow without cell wall, and consequently become giant. In other words, all biosynthesis may function normally in the protoplasts but the cell wall synthesized should be digested by the enzymes added in this system.

Wakabayashi et al.\(^9\) have already reported a similar incubation system in mushroom protoplasts, *Pleurotus cornucopiae*, and they applied the giant protoplasts for electrofusion\(^6\), although they did not demonstrated the giant protoplasts kept viability in their system. Our incubation system for protoplasts of *P. oryzae* also makes possible to apply for a micromanipulation in this fungus, such as injection of foreign DNA into protoplasts or protoplast fusion for genetic analysis in *P. oryzae*. Likewise, Kim et al.\(^5\) reported that etiolated oat protoplasts were swollen by treatment with cAMP and irradiation with red light, and studied a possible function of cAMP. Similarly, our incubation system may be available to study an unknown mechanism of action or resistant mechanism of a fungicide for rice blast disease, because the protoplasts can be most easily transferred to a cell-free system by osmotic change at a given point after treatment.

In this study, we were not sufficiently able to separate only the giant protoplasts from the normal by the two phase system with 0.6 M sucrose and 0.6 M mannitol. Hereafter, it must be necessary to develop the fractionation system to separate only the giant protoplast in order to apply the giant more widely. The other fractionation system, such as the iso-osmotic gradient centrifugation as described by Harms and Potrykus\(^2\) or Percoll system, may be available to do so.

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Literature cited


Explanation of plate

Plate I

1. Protoplasts incubated in SMS with the enzymes for 24 hr. G: the giant protoplast with 12±μm in diameter. Vacuole (v) was specifically enlarged in the giant. N: the normal protoplast with 12>μm in diameter. Bar represents 10μm.

2. Protoplasts stained with Neutral red and FDA after incubation in SMS with enzymes for 24 hr. V: viable protoplast (stained); D: dead protoplasts (not-stained). Bar represents 10 μm.

3. The same visual field as Plate I-2 under a fluorescent microscopy. Bar represents 10 μm.

4. Regenerating protoplasts like as germination of spore, stained with Calcofluor White. A: budding type; B: germ-tube type. Bar represents 20 μm.