Formation and Purification of Protoplasts from *Rhizoctonia solani*

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ABSTRACT

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A high yield of protoplasts from *Rhizoctonia solani* was obtained with a combined enzyme system containing cellulase "onozuka" R-10 from *Trichoderma viride,* macerozyme R-10 from *Rhizopus* sp., and β-glucuronidase extracted from gut juice of *Helix pomatia.* When 1 g (fresh weight) of 15-hr-old mycelium was incubated with this enzyme mixture in 0.6 M mannitol at pH 5.2, 6.0×10^7 protoplasts were obtained within 3 hr. The age of the mycelium strongly affected the yield of protoplasts. Intact protoplasts were separated from mycelial fragments and cell debris in an aqueous two-phase system, which consisted of 0.6 M sucrose-0.6 M mannitol. Purified protoplasts produced colonies after 48–72 hr at 25 C.

Recently, an increasing number of research workers have become interested in using protoplasts obtained from mycelial cells for physiological, biochemical, and genetic studies of fungi (2,11).

Digestive enzyme preparations from the gut of *Helix pomatia,* which contain some 30 or more different hydrolases, have been shown to be effective for cell maceration in studies of several filamentous fungi (1,3,4,6,7,18). Morris (12) found that the addition of glusulase to the Trichoderma lytic enzyme increased the rate of release of protoplasts from *Aspergillus nidulans,* and the maximal yield of protoplasts from *Penicillium chrysogenum* was obtained with a mixture of enzymes containing cellulase and sulfatase from *H. pomatia* gut juice (1). De Vries and Wessels (20) reported that an enzyme preparation from *Trichoderma viride* released spheroplasts from all species of basidiomycetes tested

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TABLE 1. Influence of different enzymes on the release of protoplasts from *Rhizoctonia solani* mycelium

Enzyme	Source of enzyme	Yield (protoplasts/g)[a]
Cellulase type 1	*Aspergillus niger*	0
Cellulase "onozuka" R-10	*Trichoderma viride*	1.7×10^6
Cellulase	mold	0
Chitinase	*Streptomyces griseus*	0
Driselase	mold	1.1×10^4
β-Glucuronidase	*Helix pomatia*	1.9×10^6
Hemicellulase	*Aspergillus niger*	0
Macerozyme R-10	*Rhizopus* sp.	0
Pectolyase Y-23	*Aspergillus japonicus*	0
Pectinase	*Aspergillus niger*	0

[a] Fresh weight of mycelia.

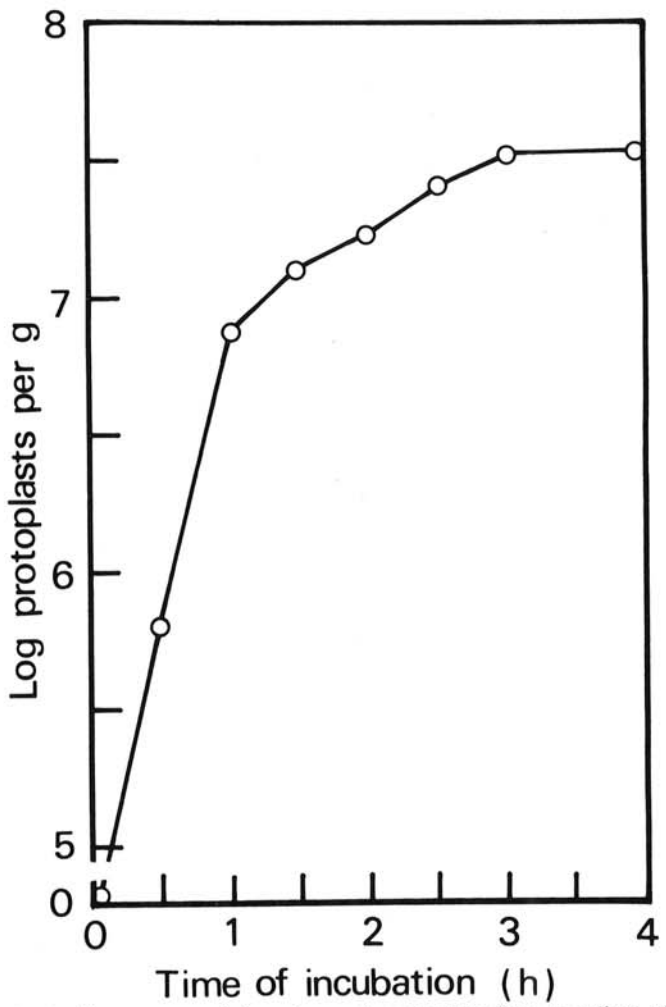

Fig. 1. Time course of the release of protoplasts from mycelium of *Rhizoctonia solani*.

except *Rhizoctonia solani*. To our knowledge, attempts to prepare protoplasts from *R. solani* have not been reported in detail.

The present investigations show that high yields of protoplasts from *R. solani* can be obtained by using a combined enzyme system containing cellulase "onozuka" R-10, macerozyme R-10, and β-glucuronidase.

MATERIALS AND METHODS

Organisms and culture conditions. The isolate of *Rhizoctonia solani* Kühn (isolate 0-0) used in this study was obtained from infected rice plants in early September, 1970, by the method described previously (8–10). The fungus is synonymous with the *R. solani* anastomosis group 1 (AG-1) of Ogoshi (13,14) and Parmeter et al (15). Cultures were maintained on potato-sucrose agar medium (200 g potato, 20 g sucrose, 20 g agar, and 1,000 ml water). In experiments for the isolation of protoplasts, plates containing 20 ml of potato-sucrose liquid medium were inoculated with mycelial fragments from the margin of a colony of the fungus precultured on potato-sucrose liquid medium for 2 days. All plates were incubated at 25 C.

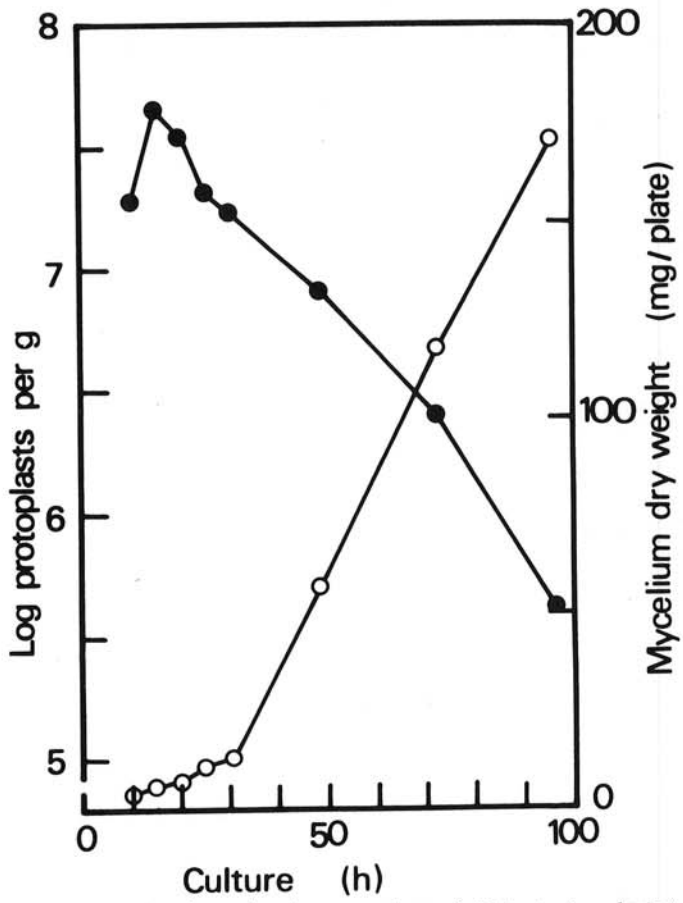

Fig. 2. Protoplast formation from mycelium of *Rhizoctonia solani* at different stages of growth. o = Dry weight of mycelium; • = protoplast yield.

TABLE 2. Comparison of effectiveness of several enzymes to release protoplasts from *Rhizoctonia solani* mycelium

Enzyme	Combination									
	1	2	3	4	5	6	7	8	9	10
Cellulase "onozuka" R-10	+	−	−	+	+	+	+	+	−	+
Macerozyme R-10	−	+	−	+	−	+	+	−	+	+
Driselase	−	−	+	−	+	+	−	+	+	+
β-Glucuronidase	+	+	+	−	−	−	+	+	+	+
Yield (protoplasts/g)[a]	3.0×10^7	2.8×10^7	2.6×10^7	2.2×10^6	2.3×10^6	2.4×10^6	6.0×10^7	3.2×10^7	3.0×10^7	6.6×10^7

[a] Fresh weight of mycelia.

Enzyme solutions to release protoplasts. The enzyme solutions to release protoplasts contained cellulase "onozuka" R-10 (20 mg/ml), macerozyme R-10 (5 mg/ml), driselase (10 mg/ml), pectoyase Y-23 (1 mg/ml), cellulase (20 mg/ml), chitinase (20 mg/ml), hemicellulase (20 mg/ml), pectinase (20 mg/ml), and β-glucuronidase (0.06 mg/ml), in 0.6 M mannitol (pH 5.2). The enzymes were tested singly or in combinations. The pH of the solution was adjusted to pH 5.2 by adding 0.1 N KOH after the addition of enzyme.

Sources of enzymes. Macerozyme R-10 from *Rhizopus* sp. and cellulase "onozuka" R-10 from *T. viride* were obtained from Yakult Biochemicals Co., Ltd. (Nishinomiya, Japan) and driselase from basidiomycetes and cellulase from mold were obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). β-Glucuronidase type H-2 from *H. pomatia,* chitinase from *Streptomyces griseus,* pectinase, hemicellulase, and cellulase type 1 from *Aspergillus niger* were obtained from Sigma Chemical Co. (St. Louis, MO 63178). Pectolyase Y-23 from *Aspergillus japonicus* was obtained from Kikkoman Shoyu Co., Ltd (Noda, Chiba, Japan).

Protoplast formation. One gram (fresh weight) of the mycelium grown for 15 hr in 20 ml of potato-sucrose liquid medium was harvested on Toyo No. 2 filter paper, washed with distilled water, and suspended in 10 ml of the enzyme solution in a 100-ml Erlenmeyer flask. The flask was placed on a reciprocal shaker at 75 strokes/min at 36 C for 3 hr. The culture was filtered through a 150-μm (35-mesh) steel sieve to remove mycelial fragments, and the filtrate was centrifuged at least twice at 700 *g* for 5 min to remove the enzymes. The pellet obtained consisted of intact protoplasts, broken protoplasts, undigested tissues, and cellular organelles. The pellet was resuspended in 2 ml of 0.6 M mannitol.

Purification of protoplasts. A liquid-liquid two-phase system of 0.6 M sucrose and 0.6 M mannitol was developed for the separation of intact protoplasts from broken protoplasts, undigested tissues, and cellular organelles. Two milliliters of the protoplast suspension were layered onto 4 ml of 0.6 M sucrose, 1 mM $CaCl_2$, 5mM HEPES-KOH (pH 7.0) and centrifuged in a swinging bucket rotor at 200 *g* for 5 min at room temerature. The intact protoplasts, which were located at the interphase of the two-phase system, were removed with a Pasteur pipette and resuspended in mannitol. Broken cells were found in the lower phase of the two-phase solution. The protoplasts were diluted with 0.6-M mannitol solution and centrifuged at 700 *g* for 5 min. The pellet was resuspended in the mannitol solution and taken as the protoplast preparation.

Protoplast counting. Protoplast preparations were diluted in 0.6 M mannitol and counted in a Burkel-Turk hemacytometer.

RESULTS

Protoplast formation. To find a suitable enzyme system, 10 commercially available enzyme preparations were screened for lytic activity against *R. solani* mycelium. *A. niger* cellulase, chitinase, pectinase, hemicellulase, pectolyase Y-23, and macerozyme R-10 were inactive. Some lytic activity was observed after a 3-hr incubation with driselase. Cellulase "onozuka" R-10 from *T. viride* and β-glucuronidase from *H. pomatia* were more active and yielded 1.7×10^6 and 1.9×10^6 protoplasts per 1 g (fresh weight) of mycelia after 3 hr, respectively (Table 1).

Effectiveness of various combinations of enzymes was compared as shown in Table 2. The enzyme mixtures containing cellulase "onozuka" R-10, macerozyme R-10, driselase, and β-glucuronidase, or cellulase "onozuka" R-10, macerozyme R-10, and β-glucuronidase were significantly more active and yielded 6.6×10^7 and 6.0×10^7 protoplasts per 1 g (fresh weight) of mycelia after 3 hr, respectively (Table 2).

The maximum release of protoplasts was attained after incubation for 3 hr (Fig. 1). Prolonged treatment beyond 3 hr did not increase the yield of protoplasts.

Increasing the volume of the enzyme-mannitol solution from 10 to 20 ml for incubation of 1 g (fresh weight) of mycelia did not influence the yield of protoplasts.

Mycelia from cultures of different ages were tested for protoplast production. The age of the mycelium strongly affected the yield of protoplasts. Yields were greatly reduced when the mycelium had been grown for more than 20 hr (Fig. 2). Yields of protoplasts prepared from 3- to 4-day-old mycelium were very low.

Purification of protoplasts. The crude protoplast preparation obtained from the mycelial fragments of *R. solani* contained undigested tissues, broken protoplasts, and various cellular organelles in addition to protoplasts (Fig. 3A).

In a preliminary experiment, crude protoplast preparations from the mycelium were suspended in a solution of 0.6 M sucrose, and attempts were made to collect the protoplasts by flotation. No protoplasts floated to the top of the sucrose solution during centrifugation at 200 *g* for 5 min.

An apparently homogeneous suspension of protoplasts was obtained by purifying the crude protoplast preparation with a sucrose-mannitol system (Fig. 3B). When necessary, further purification was obtained by repeating the two-phase separation. The protoplasts remained viable in 0.6 M mannitol solution for at least 4 days at 4 C.

Regeneration of protoplasts. When appropriately diluted suspensions of purified protoplasts (10^5/ml) were incubated on water agar made 0.6 M with respect to mannitol, between 10 and

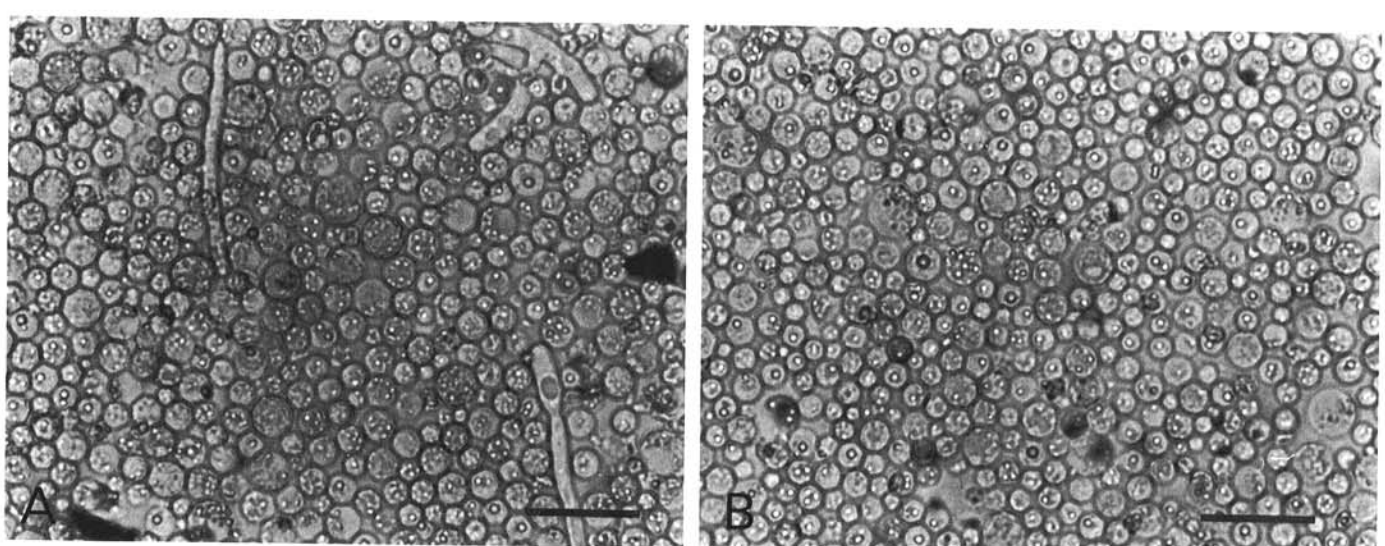

Fig. 3. A, Light micrograph of crude protoplast preparation obtained from mycelium of *Rhizoctonia solani* (×780). **B,** Light micrograph of intact protoplasts of *R. solani* after purification with the two-phase system (×780). Bar represents 20 μm.

20% of the protoplasts produced visible colonies after 48–72 hr at 25 C.

In liquid medium, the first stage of regeneration of protoplasts of *R. solani* was observed after 6 hr, when a small protuberance was noticed on some of them (Fig. 4A). After 12–24 hr, several stages of regeneration were observed. Some protoplasts produced short chains of cells (Fig. 4D). One of these cells developed into a germ tube (Fig. 4E and F). Most frequently, however, one hyphal tube emerged from the protoplasts (Fig. 4B and C). These hyphae branched and formed a microcolony after 48–72 hr of regeneration.

DISCUSSION

Protoplasts have been obtained from fungi belonging to all the major taxonomic groups (16,19,20). However, there are no reports on isolation of protoplasts from *R. solani* that has proved satisfactory in quality for physiological and genetic study.

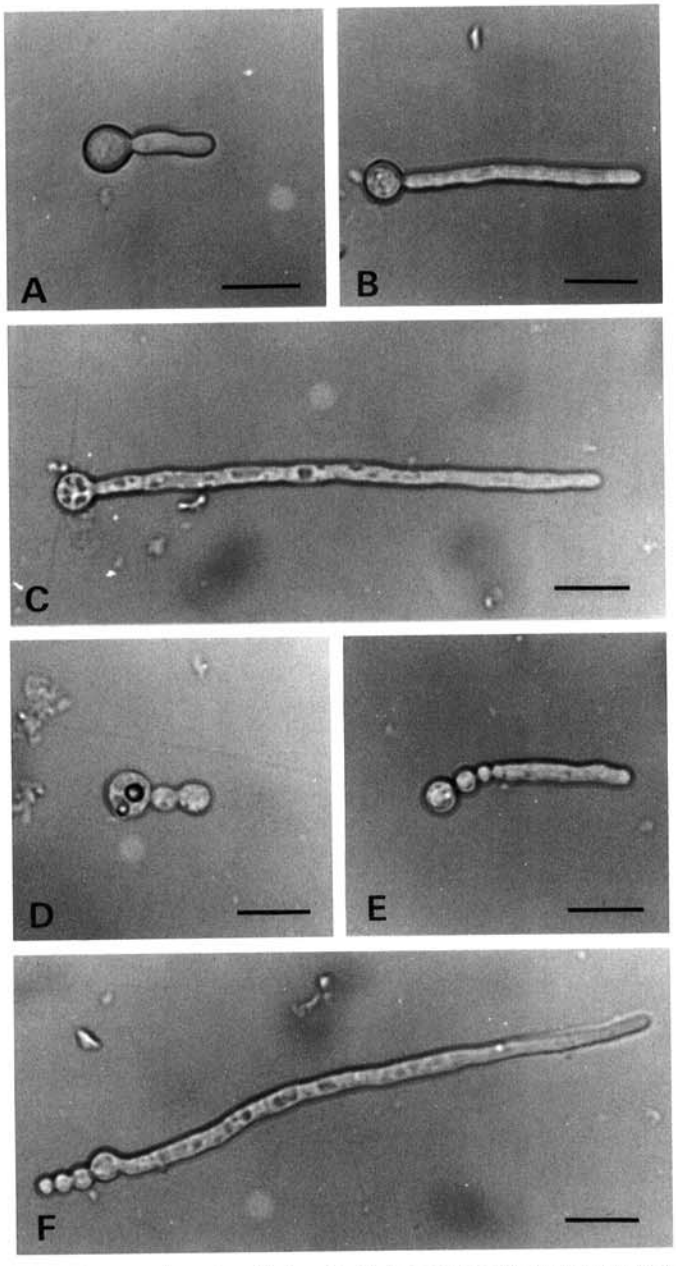

Fig. 4. Regeneration of purified protoplasts, incubated on water agar made 0.6 M with respect to mannitol. **A,** a protoplast developing a small protuberance after 6 hr of regeneration; **B and C,** a regenerating protoplast after 12 and 24 hr, respectively; **D,** some protoplasts produced short chains of cells; and **E and F,** short chains of cells developed into a germ tube. Bar represents 10 μm.

We found that high yields of protoplasts from *R. solani* could be obtained by using a combined enzyme system. Compared with other methods, this technique offers the advantage of reproducibly yielding a large quantity of viable protoplasts. The evidence that cellulase "onozuka" R-10 and macerozyme R-10 were effective is strengthened by the fact that this preparation was also active in combination with β-glucuronidase from *H. pomatia.* Snail gut digestive enzyme preparations are undefined mixtures containing several enzymes. Similarly, cellulase "onozuka" R-10 and macerozyme R-10 are complex mixtures of enzymes. The effect of the combinations of these enzyme mixtures in enhancing protoplast release was presumed to result from synergistic action of the major components.

The components of the lytic enzyme mixtures involved in fungal protoplast release are known in only very few cases. Anne et al (1) also reported that maximum yield (3×10^7/g) of protoplasts from *P. chrysogenum* were obtained with a combined enzyme system containing cellulase plus sulfatase of *H. pomatia.* Peberdy (16) suggested that enrichment of lytic complexes with more of the specific polysaccharase constituents can enhance the yield of protoplasts obtained or the rate of protoplast release.

Some researchers have reported purification of intact protoplasts from some plants by flotation on solutions of sucrose (5,17). Flotation on sucrose solutions may be applicable with protoplasts that have large vacuoles and a low concentration of organelles. We have found that intact protoplasts of *R. solani* have a strong tendency to partition at the interphase of the mannitol-sucrose two-phase system, leaving broken protoplasts in the lower phase; in systems with sucrose <0.5 M, both intact and broken protoplasts sedimented into the lower phase.

The introduction of a reproducible and effective method for obtaining protoplasts from *R. solani* may be of great advantage for further investigations with this organism.

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