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Efficient Isolation and Culture of Viable Protoplasts from *Laminaria longissima* Miyabe (Phaeophyceae)

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Abstract

A method for isolating viable protoplasts from young sporophytes of *Laminaria longissima* using commercial cell-wall degrading enzymes and suitable conditions for cell culture are described. Cell cultures were carried out at 5, 10, 15 and 18°C; low temperatures (especially 5°C) were more suitable for survival and cell division. This method is an easy and stable way to produce and culture protoplasts from Laminariales plants.

Key words: Cell culture, Cell division, Cell-wall regeneration, Enzyme, Isolation, *Laminaria longissima*, Protoplast

Introduction

Protoplasts of brown algae were first isolated using commercial cell-wall degrading enzymes and crude extracts of the digestive organs of marine herbivorous invertebrates. These enzymes have been used to isolate protoplasts of the following species: *Laminaria japonica* (Saga and Sakai, 1984), *Undaria pinnatifida* (Fujita and Migita, 1985; Tokuda and Kawashima, 1988), *Macrocystis pyrifera* (Saga et al., 1986), *Sargassum muticum* (Saga et al., 1986; Fisher and Gibor, 1987; Polne-Fuller and Gibor, 1987), and *Dictyota dichotoma*, *Dictyopteris prolifera* and *D. undulata* (Kajiwara et al., 1988). However, the number of isolated protoplasts was low, ranging from 5×10^3 to 2×10^4 g^{-1} (fr.wt.), and viability was also low in most species. A large number of protoplasts (10^6-10^8 g^{-1} fr.wt.) from *L. japonica*, *L. saccharina*, *L. digitata* and *M. pyrifera* have been obtained using crude or purified enzymes (e.g. alginate lyases; mannuronate lyase or guluronate lyase) produced by bacteria that decompose the cell walls of brown algae (Butler et al., 1989; Kloareg et al., 1989; Sawabe et al., 1993). However, in Laminariales plants, cell culture of isolated protoplasts generally has not been successful. Easy and efficient isolation and culture methods of protoplasts are needed to facilitate future studies of cell biology. In this paper, I described a method using commercial enzymes to isolate protoplasts from young sporophytes of *L. longissima* and to culture these protoplasts.

Material and Methods

Young sporophytes of *L. longissima* (5～10 mm long) were used for protoplast

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preparation. The sporophytes were rinsed several times with sterilized seawater and then incubated in 50-ml bottles with filter-sterilized seawater containing 12.5 mg penicillin-G potassium, 12.5 mg streptomycin sulfate and 1 mg nystatin for 0.5 ~1 hour at 5°C in the dark (Fig. 1). The sporophytes were then washed several times with sterilized seawater and cut into small pieces (1~4 mm^2) with a sterilized razor blade. Before enzymatic degradation of the cell walls, the pieces (0.5 g in fresh weight) were incubated in 10 ml of Solution I (Table 1) for 30 minutes at 17°C in the dark.

Then, the pieces were incubated in Solution II (5 ml) under gentle rotary shaking (20 strokes $min.^{-1}$) for 2 hours at room temperature (17°C). After incubation, undigested tissues and protoplasts were separated through an 80-μm nylon net. The protoplast suspension was centrifuged at 1,500 × g for 10 minutes in 10-ml conic tubes, and then debris and the enzyme solution were removed with a Pasteur pipette. Protoplasts were resuspended in 10 ml of Solution III (Butler et al., 1989) and centrifuged at 1,500 × g (for 10 minutes) three times to remove completely all the enzymes.

Protoplasts were cultured at 5 and 10°C under a 12 : 12 (L : D) photoperiod, and at 15 and 18°C under a 14 : 10 (L : D) photoperiod under cool-white 20 W fluorescent lamps (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in 10 days culture and 20~40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ after 11 days culture) in 21 and 55 cm^3 plastic tissue culture flasks (Corning Sterile 25010 and 25020). Protoplasts from ca. 1.77×10^6 cells were resuspended in Solution III (10 ml), and then 50% ESS medium (Saga and Gibor, 1986) containing 5 mM $CaCl_2$ (2 ml : Culture medium I) was added to the mixture. These suspensions were immediately incubated at four different temperatures. To reduce the osmotic pressure gradually, 2 ml of Culture medium I was renewed several times every day for 10 days. Protoplasts were then transferred into 50% ESS medium and cultured for 20 days (medium changed every other day), after which they were transferred to ESS medium (medium changed every 3 days).

The number of protoplasts was estimated by counting cells with a

Table 1. Composition of the hypertonic and enzyme solutions.

Component	Solution I	Solution II	Solution III
NaCl	40.908 g	0.430 g	42.953 g
$MgCl_26H_2O$	6.099 g	0.061 g	6.099 g
$MgSO_47H_2O$	7.394 g	0.111 g	11.092 g
KCl	1.491 g	0.011 g	1.119 g
$CaCl_2$		0.001 g	0.111 g
MES*1		0.039 g	3.904 g
EGTA*2	7.61 g		
Cellulase Onozuka RS (Yakult)		0.2 g	
Abalone acetone powder (Sigma)		0.2 g	
pH	5.5	6.5	6.5
DW	1,000 ml	10 ml	1,000 ml

*1 2-(N-Morpholino) ethanesulfonic acid

*2 Ethylene glycolbis (β-aminoehyle ether) N, N, N′, N′-tetraacetic acid

hemocytometer. The viability of protoplasts was examined by staining cells in 0.02% solution of Evans Blue (Sigma) or in 0.02% solution of Neutral Red (Kanto Chem.). Cell-wall regeneration was checked with a fluorescence-microscope (Olympus, Vanox) after staining with 0.01% w/v Calcofluor White M2R (Sigma).

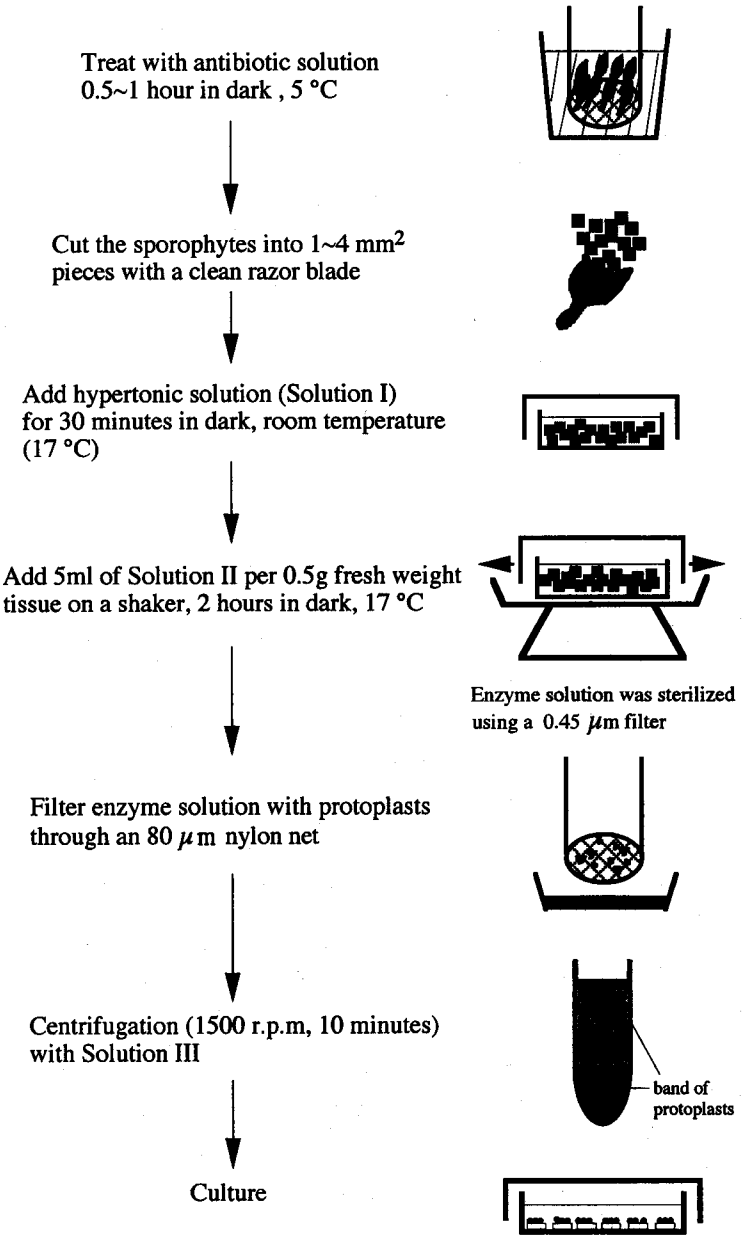

Fig. 1. Method for isolation and culture of protoplasts of *Laminaria longissima*.

Results and Discussion

Immediately after incubation in the hypertonic solution (Solution I), chloroplasts in cells moved toward the cell membrane (Fig. 2-(1)). When the pieces were incubated in Solution II, the cells became almost round in shape, and after 15～30 minutes, they began to release protoplasts gradually (Fig. 2-(2)). After 2 hours, about 8.83×10^6 protoplasts/0.5 g (fr.wt.) were extracted from various portions of the sporophyte tissue using this mixture enzyme (Solution II) (Fig. 2-(3)), and the survival rate of protoplasts was more than 95%.

Isolated protoplasts were varied widely in size and pigmentation. They were spherical and 8～60 μm in diameter (Fig. 2-(3)). Judging from size and pigmentation, 8～20 μm protoplasts were derived from the epidermis (yellowish-brownish), 20～30 μm protoplasts were derived from the outer cortex (pale yellowish-brownish), and 35～60 μm protoplasts were derived from the inner cortex (a few chloroplasts and large vacuoles).

Most protoplasts settled on the substrate in 24～48 hours after culture. After settlement, some protoplasts regenerated cell walls within 3 days culture (Fig. 2-(4), (5)), and most viable protoplasts of all sizes regenerated within at least 15 days culture at 5°C, 10°C and 15°C. However, at 18°C, most protoplasts died without regenerating cell walls within 15 days culture. Cell-wall regeneration was fastest at 10°C.

Survival rates in 30 days culture were ca. 2.7% (ca. 4.7×10^4 cells) at 5°C, 0.8% (ca. 1.46×10^4 cells) at 10°C and 0.35% (ca. 6.2×10^3 cells) at 15°C.

Cell division was observed in 15～25 μm cells (Fig. 2-(6), (7)), suggesting that most of these cells were derived from the epidermis and outer cortex of the young sporophytes. Most cells from the inner cortex (35～60 μm in size) died without dividing. First cell division was observed after 8 days culture at 10°C, 12 days at 5°C and 13 days at 15°C. The cells cultured at 5～15°C divided at high rates of more than 85% in 60 days culture.

In complex multicellular brown algae, such as Laminariales, plant regeneration generally has not been successful (Saga and Sakai, 1984; Tokuda and Kawashima, 1988; Wu, 1988; Butler et al., 1989; Kloareg et al., 1989). Also, the effect of water temperature on protoplast culture has not been considered in previous works. However, water temperature appears to be an important factor for plant regeneration. The present results show that low temperatures were more favorable than high temperatures for survival (especially at 5°C), cell-wall regeneration and cell division, and that cells of this alga are intolerant of high temperature (18°C). These results indicate that protoplasts should be cultured at low temperature (especially 5°C), as 3～8°C is suitable for growth of wild sporophytes of *L. longissima*.

In the present study, many viable protoplasts from young sporophytes of *L. longissima* were obtained. These protoplasts continued to survive and divide, suggesting that this simple procedure is suitable for isolating viable protoplasts. This method will facilitate the isolation of protoplasts and cell-culture experiments, and may be applicable to other Laminariales plants. Morphogenesis can now be examined using this method to obtain fundamental knowledge for new and useful cultivation of Laminariales plants.

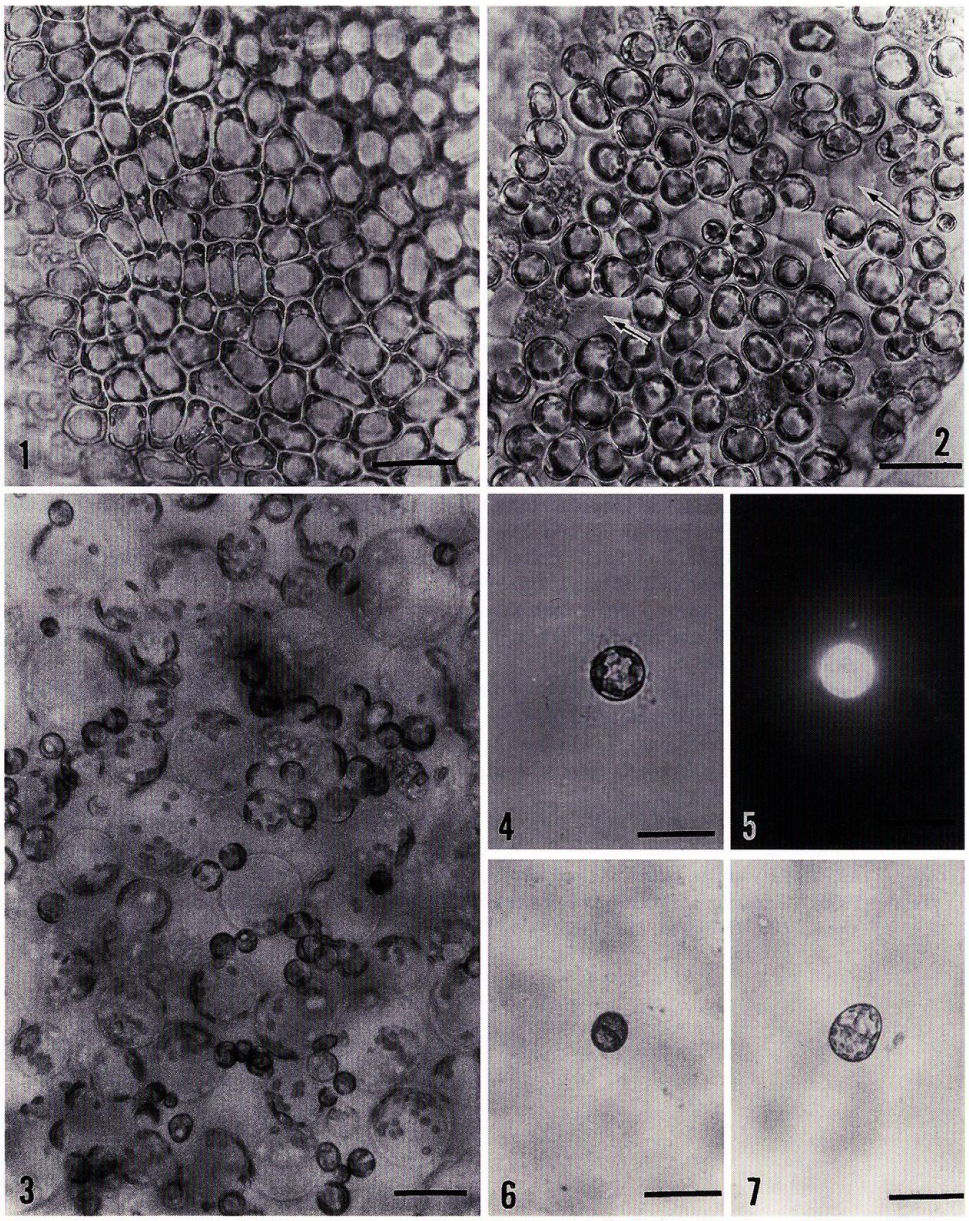

Fig. 2. Isolation and regeneration of protoplasts. Scale bar: 30 μm. (1) Pieces of sporophyte-tissue of *L. longissima* in Solution I. (2) Round cells in Solution II. Arrows show where protoplasts were released. (3) Protoplasts isolated from young sporophytes of *L. longissima*, showing epidermal cell (smaller) and cortical cell (larger). (4) and (5) Protoplasts regenerating cell wall stained with Calcofluor White in 3 days culture, under bright field (4) and with U.V. excitation (5). (6) Initial cell division of a protoplast-derive cell in 10 days culture. (7) Sporophyte of three-cell stage in 10 days culture.

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