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Isolation and culture of protoplasts from the marine green alga *Monostroma angicava*

Naotsune Saga & Toshihiko Kudo
[1] *Hokkaido Regional Fisheries Research Laboratory, Kushiro, Hokkaido 085, Japan*

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Abstract

Protoplasts were isolated enzymatically from gametophytes of the marine green alga *Monostroma angicava*. The protoplasts regenerated in PES medium after gradual reduction of the osmoticum. Three types of developmental process were recognized in the protoplast regeneration: an original type, in which the protoplasts regenerated into leafy gametophytes; an apogamic type, in which they regenerated into sporophytes; a callus type, in which they regenerated into callus-like tissues. The resulting gametophytes and apogamic sporophytes became fertile in successive cultures.

Introduction

Seaweeds have been used for many purposes and are important natural resources. Nowadays they are regarded as promising marine biomass for energy production (North 1980). The development of basic techniques for farming of economically valuable seaweeds has been expected for a long time. Protoplast isolation from seaweeds has been achieved in nine genera and 16 species to date (Saga & Sanbonsuga, 1988). Several studies on enzymatic protoplast isolation from marine green algae are known (Millner *et al.*, 1979; Zhang, 1983; Saga, 1984; Fujita & Migita, 1985; Saga *et al.*, 1986), but there are few reports concerning protoplast regeneration in seaweeds (Zhang, 1983; Fujita & Migita, 1985).

The present paper deals with the isolation and regeneration of protoplasts from the edible seaweed *Monostroma angicava*.

Materials and methods

Plant materials

The unialgal cell cultures used in this study were initiated from a female gametophyte of the marine green alga *M. angicava* collected from the beach of Charatsunai, Muroran, Hokkaido, Japan during the spring of 1980. The cell cultures were subcultured every 3 months for several years in PES medium (Provasoli, 1968) in 200-ml Erlenmeyer flasks in an incubator at 14 °C and illuminated with cool white fluorescent lamps at 38 μE m^{-2} s^{-1} with a 14 : 10 LD photoperiod. They were transferred to 10 °C and a 14 : 10 LD photoperiod before use.

Protoplast isolation

Protoplasts were isolated according to a method described previously (Saga, 1984). Infertile parts

of leafy thalli were cut into pieces 1–2 mm square with a knife, and 1 g of material (fresh weight) was maintained for 10 min in 1.0 M mannitol solution, pH 6.0, in order to obtain plasmolysis. They were incubated in 10 ml enzyme solution containing various concentrations of Cellulase Onozuka R-10 (Kinki Yakult Mfg. Co. Ltd., Nishinomiya, Japan) and 1.0 M mannitol, and the pH was adjusted to 6.0.

Incubations were carried out at various temperatures and continued for 1–10 h with reciprocal shaking (60 strokes min^{-1}). Isolated protoplasts were separated from debris by filtration through 30-μm nylon mesh. They were collected by centrifugation at 500 × *g* for 15 min and washed several times with 100 mM Tris buffer containing 1.0 M mannitol, pH 8.0. The number of protoplasts was counteed with a hemocytometer. Viability of protoplasts obtained was confirmed by the ability to exclude Evans Blue, which indicates membrane semipermeability (Saga, 1984). Evans Blue (Sigma Chemical Co., St. Louis, MI, U.S.A.) was used as 1% solution in the hypertonic seawater.

Protoplast culture

The resulting cleaned pellet was resuspended in 10 ml hypertonic seawater (supplemented with 0.7 M mannitol, pH 8.0) and maintained in a refrigerator for 1 h. Then the osmoticum was reduced gradually (reduction speed, 0.1 M h^{-1}) and finally the protoplasts were adapted to seawater excluding mannitol. They were collected by centrifugation for 15 min and resuspended in autoclaved seawater at a density of 10^6 protoplasts ml^{-1}. Then 0.01 ml suspension was inoculated into a 60 × 15 mm petri dish containing 10 ml liquid medium. The culture medium employed was PES (Provasoli 1968), and it was renewed weekly. The culture was usually maintained in an incubator at 14 °C and illuminated with cool white fluorescent lamps at 38 $\mu E\, m^{-2}\, s^{-1}$ with a 14:10 LD photoperiod except as noted. Cell wall regeneration was detected by means of staining with Calcofluor White M2R (American Cyanamid Co., Wayne, N.J., U.S.A.) and checked under a fluorescence microscope: microscope, Olympus HLS-1; filters, DV-1 & BG-12; wave length of UV, 320–400 nm (Nakazawa *et al.*, 1969).

Results

Effect of various factors on protoplast isolation

The effects of 1%, 2%, 3%, 5% and 10% enzyme concentrations on the isolation of protoplasts were examined. Treatment with 2–5% Cellulase Onozuka R-10, for 5 h at 20 °C was very effective, and released protoplasts showed a good yield (over 4 × 10^6 from 1 g fresh weight of cell cultures). The yield of protoplasts decreased with enzyme concentrations over 5%, indicating that this enzyme was somewhat toxic (Fig. 1).

The effect of temperature on the isolation of protoplasts was also examined. Treatment at 15–25 °C, with 2% Cellulase Onozuka for 5 h was very effective; 20 °C was most effective, and

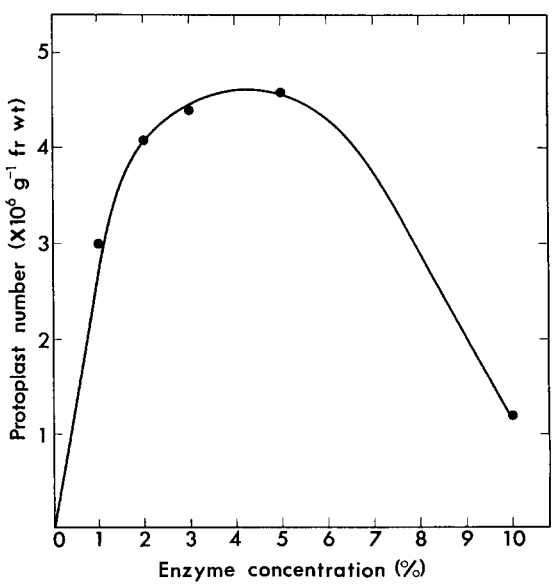

Fig. 1. Effect of enzyme concentration on the yield of protoplasts isolated from *Monostroma angicava*. Each enzyme solution contained 1–10% Cellulase Onozuka R-10, and 1.0 M mannitol at pH 6.0. The cell cultures were incubated on a reciprocal shaker (60 strokes min^{-1}) at 20 °C.

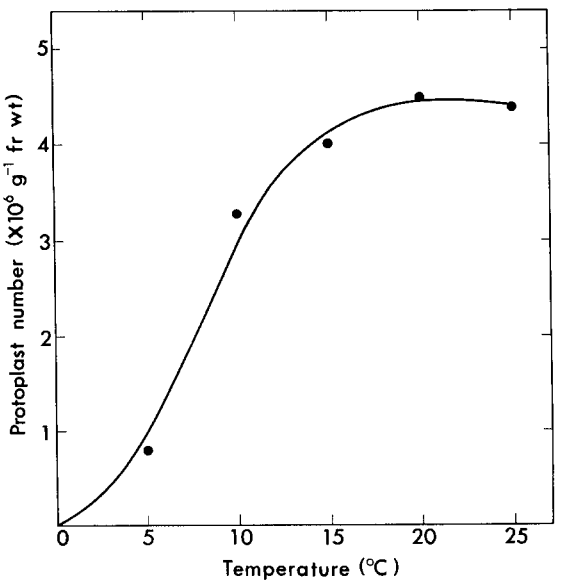

Fig. 2. Effect of temperature on the yield of protoplasts isolated from *Monostroma angicava*. The enzyme solution contained 2% Cellulase Onozuka R-10, and 1.0 M mannitol at pH 6.0. The cell cultures were incubated on a reciprocal shaker (60 strokes min^{-1}) for 5 h at 5–25 °C.

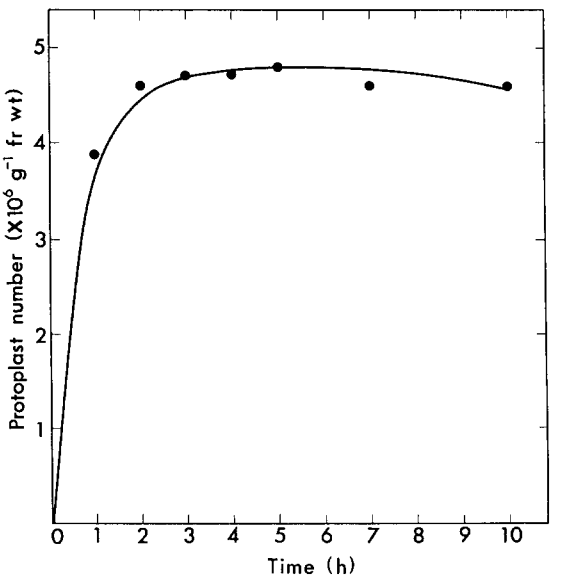

Fig. 3. Time course of protoplast release from *Monostroma angicava*. The enzyme solution contained 2% Cellulase Onozuka R-10, and 1.0 M mannitol at pH 6.0. The cell cultures were incubated on a reciprocal shaker (60 strokes min^{-1}) at 20 °C for up to 10 h.

released protoplasts with a high yield (4.5×10^6 from 1 g fresh weight of cell cultures). The yield of protoplasts decreased at 25 °C, indicating that temperatures above 20 °C were harmful.

Figure 3 shows a time course of the enzymatic release of protoplasts. The yield of protoplasts increased during the first few hours, and reached a maximum (4.8×10^6 from 1 g fresh weight of cell culture) at about 5 h.

The course of protoplast regeneration

From these results, an enzyme solution containing 2% Cellulase Onozuka R-10 and 1.0 M mannitol in distilled water at pH 6.0 was adopted for further studies. Incubation for 1 h at 20 °C in this solutions was sufficient for the isolation of protoplasts in significant amounts.

Freshly isolated protoplasts were green, spherical, measured 10–28 μm in diameter and contained one discoid chromatophore per protoplast (Figs 4a, b). The yield of protoplasts was $3.5–4.0 \times 10^6$ and more than 80% of them were viable.

Within 1 day of inoculation into the culture medium, the protoplasts began to regenerate cell walls and within 2 days most of them had produced cell walls (Fig. 5a). The succeeding development of the protoplasts followed 3 different types; an original type, in which protoplasts regenerated into leafy gametophytes; an apogamic type, in which they regenerated into cyst-like apogamic sporophytes; a callus type, in which they regenerated into callus-like tissues.

The development of the original type was as follows. Within 4 days, most of the walled cells which originated from the protoplasts divided into two cells (Fig. 5b). By successive cell divisions (Figs 5c, d) and branching, the creeping filaments developed into small discs within 1 month (Fig. 5e). When they were transferred to 10 °C, 14 : 10 LD photoperiod, the discs began to produce primordia of erect thalli from the central part, and within 3 months they developed into saccate thalli (Fig. 5f). By progressive growth of the thalli, they developed into leafy thalli and matured, producing biflagellate female gametes.

The development of the apogamic type was as follows. Within 1 month, the cells developed into

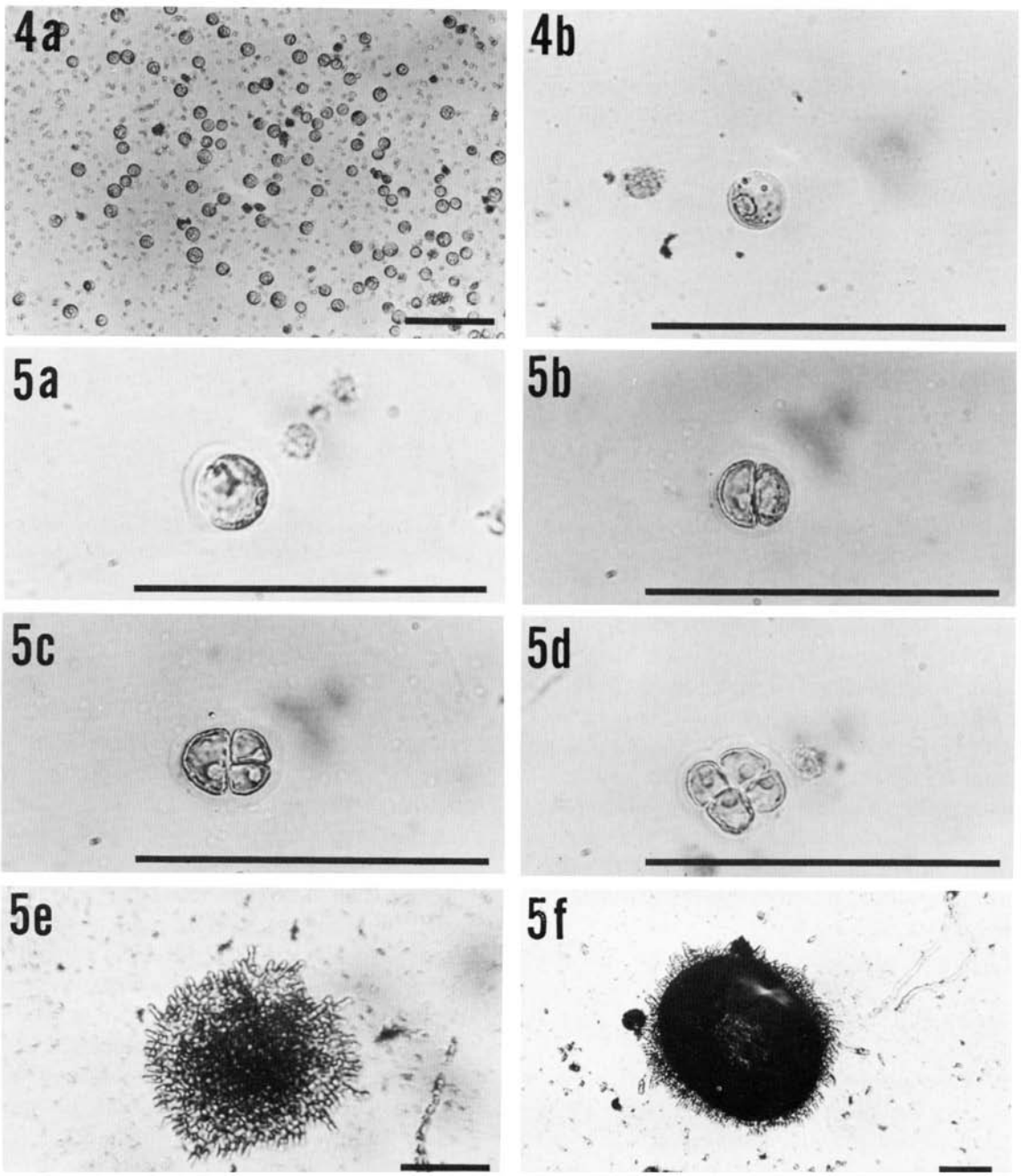

Fig. 4. Freshly isolated protoplasts from cell cultures of *Monostroma angicava*. (a) Photograph with low magnification. (b) Photograph with high magnification. Scale bars = 100 μm.

Fig. 5. Protoplast regeneration in *Monostroma angicava*, the original type. (a) Two-day-old cell originating from a protoplast; it has already regenerated a cell wall. (b) Two-cell stage (4 days old). (c) Three-cell stage (5 days old). (d) Four cell stage (6 days old). (e) One-month-old prostate thallus (vertical view). (f) Three-month-old erect thallus (vertical view). Scale bars = 100 μm.

dark green, thick-walled cysts, measuring 40–60 μm in diameter (Fig. 6a) at 14 °C, 14 : 10 LD photoperiod. When transferred to 10 °C, 10 : 14 LD photoperiod, they began to produce slender tubes. Within 2 months of the transfer, they became fertile and produced quadriflagellate spores (Fig. 6c).

The development of the callus-like type was as

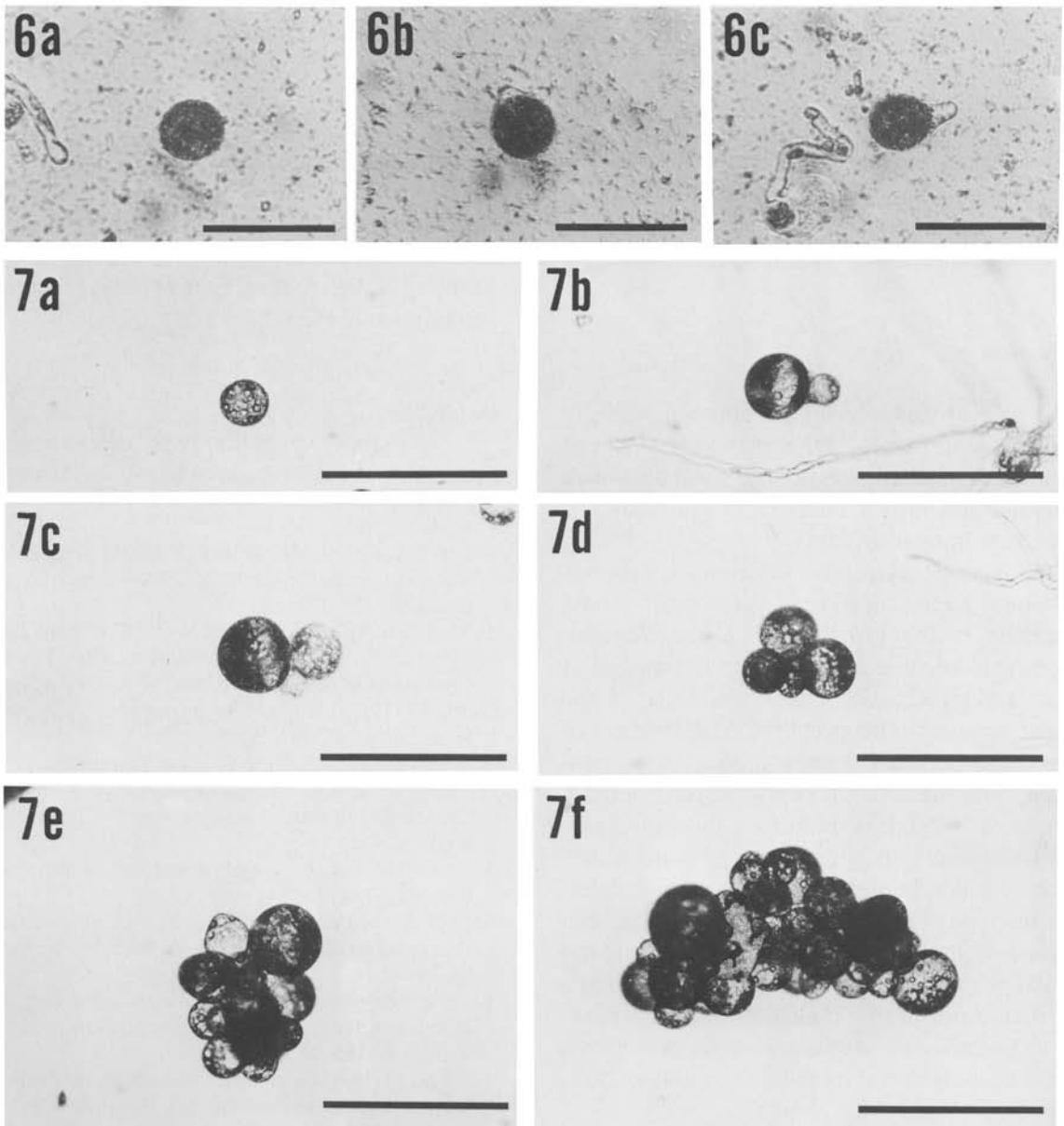

Fig. 6. Protoplast regeneration in *Monostroma angicava*, the apogamic type. (a) One-month-old cyst. (b) Two-month-old cyst beginning to produce a slender tube. (c) Three-month-old fertile cyst. Scale bars = 100 μm.

Fig. 7. Protoplast regeneration in *Monostroma angicava*, the callus-like type. (a) Ten-day-old round cell. (b) Two-cell stage (15 days old). (c) Two-cell stage (20 days old). (d) Four-cell stage (1 month old). (e) Callus-like cellular aggregate (2 months old). (f) Callus-like cellular aggregate (3 months old). Scale bars = 100 μm.

follows. By progressive growth of the walled cells, they increased in size up to 20–30 μm diameter (Fig. 7a). Within 15 days, they began to divide into two round cells (Fig. 7b). By successive abnormal cell divisions (Figs. 7c, d, e), they developed into callus-like cellular aggregates (Fig. 7f); they never formed leafy thalli or thick-walled cysts.

The apogamic type was dominant (usually more than 80%) as a regeneration pattern in this study while the original type was usually below 20%. In some cases, the callus type was observed, usually representing only a few percent of regenerating cells.

Discussion

In the present study, high yield and high viability of protoplasts from *M. angicava* have been obtained. Enzymatic protoplast isolation with high yield and high viability from seaweeds has never been known to date.

After osmotic adaptation to normal conditions for several hours, most viable protoplasts could regenerate in the usual PES culture medium. Three types of development were recognized in protoplast regeneration. This life cycle of the present species is heteromorphic alternation of generations between macroscopic leafy gametophytes and microscopic cyst-like sporophytes (Tatewaki, 1969). It is interesting that some protoplasts regenerated into apogamic sporophytes, which should be haploid. The factors which determine the type of the generation have not yet been clarified. In the present study, the developmental process of the protoplasts could not be regulated and such a polymorphic phenomenon is a problem to be solved in the future. Algal protoplasts should become useful tools for the study of morphogenesis or life cycle regulation.

Genetic manipulation of protoplasts will offer new means of breeding seaweeds. Somatic hybridization using protoplasts provides a method of combining the genomes of different plants that are sexually incompatible. Also, protoplasts are essential materials for transformation involving direct gene transfer of foreign DNA using plasmids as vectors. This is the first report of high yield isolation and high rate regeneration of protoplasts in multicellular seaweeds. The present successful results will contribute to many fields of applied phycology.

Acknowledgements

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