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Regeneration of protoplasts isolated from the sporophyte of *Cladosiphon okamuranus* Tokida (Chordariaceae, Phaeophyta)

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The process of the regeneration of protoplasts was studied for the edible marine brown alga *Cladosiphon okamuranus*. The protoplasts were prepared from sporophytes by enzymatic degradation of the cell wall in the presence of EGTA, a calcium-specific chelating agent. Regeneration of the protoplasts followed three different patterns. Most of the protoplasts grew into filamentous or clumpy germlings, which matured to release plurispores. The resulting discoid germlings developed into normal, erect, sporophyte thalli. Some protoplasts divided to form cell aggregations, which did not grow further. A few protoplasts remained as single cells which gradually enlarged and became poorly pigmented. Results provide a method for the production of sporophyte thalli from protoplasts of this commercially important species.

Key Index Words: Cladosiphon—*EGTA—enzyme degradation—plurispore—protoplast—regeneration.*

The preparation and culturing of protoplasts are useful basic techniques for the breeding of marine algae. These techniques make it possible to produce many clones from seaweed strains which have valuable characteristics for mariculture, and also to attempt somatic cell fusion for breeding purposes. There have been several reports on the isolation of protoplasts from marine algae by enzymatic degradation of the cell wall (Saga and Sakai 1984, Fujita and Migita 1985, Fisher and Gibor 1987, Yamaguchi *et al.* 1988, Butler *et al.* 1989, Kloareg *et al.* 1989, Chen 1989). However, the culturing of isolated protoplasts has not always been successful. For the Phaeophyta, few studies have succeeded in regenerating a normal thallus from an isolated protoplast (Ducreux and Kloareg 1988).

The phaeophyte *Cladosiphon okamuranus* Tokida is an economically important species and is cultivated along the coasts of Japan's southwestern islands. There have been some reports on the life cycle and ecology of this species (Shinmura 1974a, 1974b, 1975), but biotechnological and morphogenetic studies have not been conducted.

In the present study, protoplasts of *C. okamuranus* were isolated, and the process of regeneration into normal thalli was investigated for future investigations in breeding and morphogenesis.

Materials and Methods

Plant material

Unialgal cultures of *Cladosiphon okamuranus* were obtained by culturing plurispores released from a parent thallus collected in May 1991 from a commercial farm for this species located in the town of Tatsugo in Kagoshima prefecture. Cultures were incubated in a photoperiod of 15L: 9D under cool-white fluorescent lamps (ca. 36 $\mu E/m^2/s$ at the surface of the culture vessels) at 20°C. ESI (Tatewaki 1966) was used as a culture medium and was replenished at intervals of 30–40 days. Cultures were grown in cylindrical glass vessels (ϕ5.5 cm × 8 cm) with a 120 ml of medium or in Erlenmeyer flasks with a 0.5 *l* of medium. Thalli grown to 1–2 cm in height were used as materials for protoplast

preparation.

Protoplast preparation

Protoplasts were prepared by enzyme degradation. To make cell walls accessible to the enzymes, EGTA (ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid), a calcium-chelating agent, was added to the enzyme solution. According to Butler *et al.* (1989), EGTA improved the protoplast yield of *Laminaria saccharina* and *L. digitata* which may have resulted from the dissolution of the alginate gel by removal of calcium from the polygluronate linkages. The procedure for protoplast preparation is shown in Fig. 1. The cultured thalli (80-140 mg fresh weight) were maintained for 10 min in 10 ml of solution I (Table 1) which was prepared using ASP12NTA (Provasoli *et al.* 1957) as a basal solution. Then, the thalli were cut into small pieces (ca. 1 mm square) followed by the enzyme treatment. For the enzymatic degradation of cell walls and intercellular substances, the pieces of thalli were incubated with 5 ml of solution II (Table 1) for 1 hour at 20-22°C with reciprocal shaking (30 strokes min^{-1}). After incubation, the digested tissue was filtered through 20 μm nylon mesh to remove tissue fragments. The protoplast suspension thus obtained was settled for 30 min. Then the supernatant was replaced with solution I, followed by gentle shaking. In the same manner, washing was repeated 4 times with solution III (Table 1) reducing the sorbitol concentration to 0.7, 0.5, 0.2, and 0 M in the process. The number of protoplasts was counted with a haematocytometer.

Protoplast culture

Protoplasts were cultured in plastic dishes (ϕ35 mm × 10 mm) with 4 ml of medium or in multi-well plates (ϕ16 mm × 17 mm, 24 wells) with 2 ml of medium in each well. To observe the fate of individual protoplasts, they were isolated into separate wells. The culture medium was modified ASP12 NTA, in

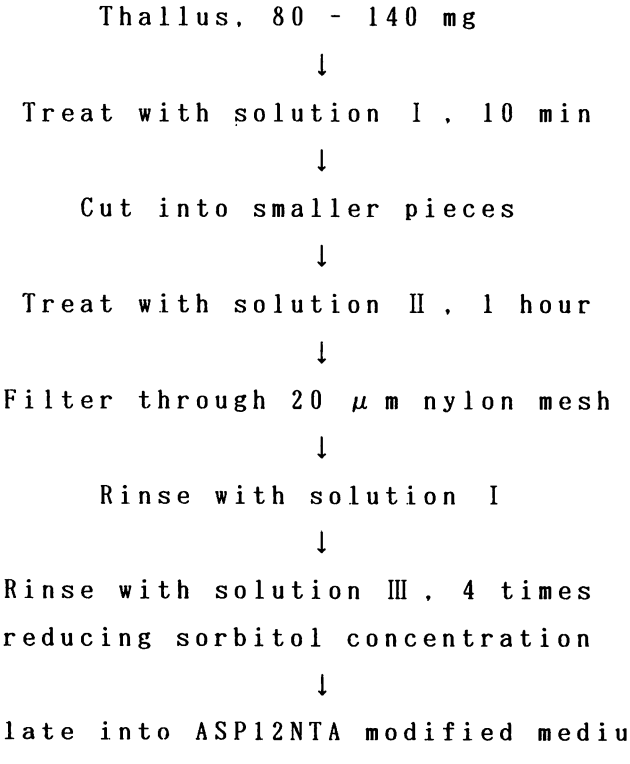


Fig. 1. Method for preparation of protoplasts of *Cladosiphon okamuranus*.

Table 1. Composition of the enzyme solution and washing solutions for protoplast preparation

Component	Solution I	Solution II	Solution III
NaCl	280 mg	280 mg	280 mg
$MgSO_4 \cdot 7H_2O$	70 mg	70 mg	70 mg
$MgCl_2 \cdot 6H_2O$	40 mg	40 mg	40 mg
KCl	7 mg	7 mg	7 mg
$CaCl_2$	—	—	11 mg
$NaNO_3$	1 mg	1 mg	1 mg
KH_2PO_4	64 μg	64 μg	64 μg
Sodium glycerophosphate	100 μg	100 μg	100 μg
P II metals*[1]	0.1 ml	0.1 ml	0.1 ml
Vitamin B_{12}	2 ng	2 ng	2 ng
Thiamine	1 μg	1 μg	1 μg
Biotin	10 ng	10 ng	10 ng
Tris*[2]	—	—	10 mg
MES*[3]	43 mg	43 mg	—
EGTA*[4]	38 mg	38 mg	—
Sorbitol	1.27 g	1.27 g	0–1.27 g
AAP*[5]	—	100 mg	—
Cellulase*[6]	—	50 mg	—
Macerozyme*[7]	—	50 mg	—
Dextran sulfate	—	100 mg	—
pH	6.5	6.5	7.5
Total	10 ml	10 ml	10 ml

*[1] Provasoli *et al.* (1957), *[2] Tris hydroxymethyl aminomethane, *[3] 2-(N-Morpholino) ethanesulfonic acid, *[4] Ethylene glycolbis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid, *[5] Abalone acetone powder (Sigma), *[6] Cellulase Onozuka RS (Yakult), *[7] Macerozyme R-200 (Yakult)

which K_3PO_4 was replaced with KH_2PO_4 keeping phosphorus at the same concentration and from which Na_2SiO_3 was omitted. Other culture conditions were the same as described under 'Plant material'.

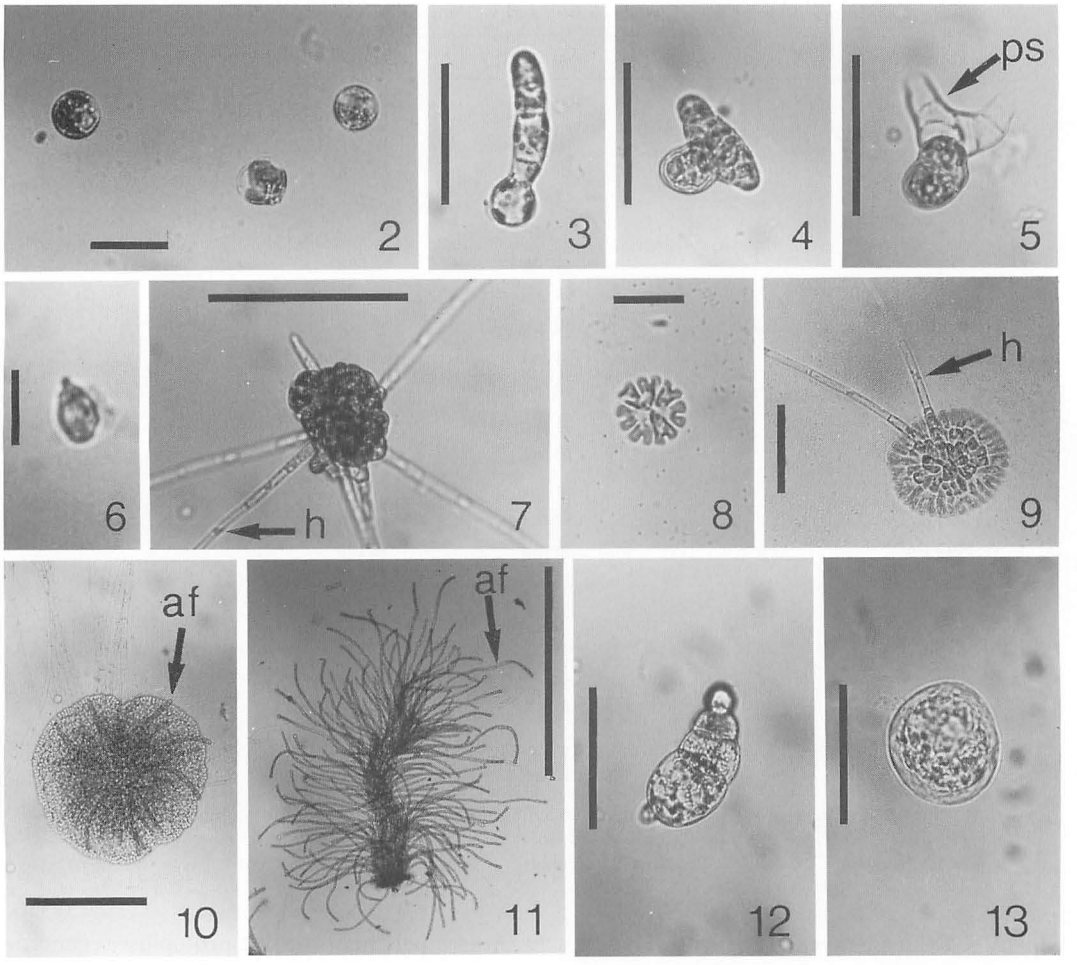
Results

Protoplast preparation

The yield of protoplasts in solution II was 9.4×10^4/100 mg fresh weight. The protoplasts were brownish and spherical; their size was 9–18 μm in diameter (Fig. 2). Judging from their color and the size, most of them had been released from the assimilatory filaments. The cells of other portions such as the subcortex, the medullary layer, and the cortex hairs were weakly pigmented and large, whereas the cells of the assimilatory filaments were densely pigmented and small. Thus, these experiments on the protoplast regeneration focused on protoplasts released from the assimilatory filaments.

Protoplast regeneration

Three regeneration patterns were observed for the protoplasts isolated from the assimilatory filaments of this species. About 30% of all protoplasts divided into two cells 4–16 days after isolation and developed into filamentous or clumpy germlings (Figs. 3, 4). These microthalli formed plurilocular sporangia after 7–20 days in culture and released plurispores (Figs. 5, 6) which were biflagellate and pear shaped. Some of the microthalli grew further to form cell aggregations with colorless hairs (Fig. 7), releasing plurispores. These cell aggregations never grew into normal thalli. After settling on the bottom of the vessels, most of the plurispores developed int-



Figs. 2–13. Protoplast regeneration of *Cladosiphon okamuranus*. Fig. 2. Protoplasts released from the assimilatory filaments. Scale bar=20 μm. Fig. 3. A filamentous germling 5 days after the isolation of a protoplast. Scale bar=40 μm. Fig. 4. A clumpy germling 11 days after the isolation of a protoplast. Scale bar=40 μm. Fig. 5. A clumpy microthallus after releasing plurispores. Scale bar=40 μm. Fig. 6. A plurispore. Scale bar=10 μm. Fig. 7. A cell aggregation developed from a germling, producing colorless hairs. Scale bar=100 μm. Fig. 8. A discoid germling from a plurispore. Scale bar=20 μm. Fig. 9. A disc producing colorless hairs. Scale bar=50 μm. Fig. 10. A disc producing assimilatory filaments 13 days after germination of a plurispore. Scale bar=200 μm. Fig. 11. An erect, sporophyte thallus 35 days after germination of a plurispore. Scale bar=1 mm. Fig. 12. A cell aggregation with large and poorly pigmented cells. Scale bar=40 μm. Fig. 13. A single large cell with poorly pigmented cell 40 days after isolation of a protoplast. Scale bar=100 μm. ps, plurilocular sporangium; h, hair; af, assimilatory filament.

discoid germlings (Fig. 8), although some of them formed cell clumps which matured to release plurispores again. In some cases, the cell contents were extruded from the cells of the microthallus and developed in the same way as the plurispores. The discs became larger through several cell divisions, producing colorless hairs (Fig. 9). Within 20 days after the settlement of the spores, the discoids began to develop erect filaments from their central areas (Fig. 10). They continued to elongate and differentiated to form the normal erect thalli of the sporophytes (Fig. 11).

A small number of protoplasts (less than 3% of the total) formed cell aggregations by successive cell division (Fig. 12). When the germlings became 6-10 celled masses, cell division ceased and the cells began to enlarge and

become poorly pigmented. The aggregations neither developed further nor formed any reproductive cells.

Unlike these two types of regeneration, a few protoplasts (less than 3% of the total) remained as single cells even 40 days after the isolation, becoming larger and poorly pigmented (Fig. 13). They resembled medullary cells in color and size.

Discussion

Most of the viable protoplasts of *Cladosiphon okamuranus* obtained in these experiments gave rise to filamentous or clumpy germlings which matured to release biflagellate plurispores. In some members of the Chlorophyta, however, the individual protoplast enlarges to form a sporangium directly without forming a multicellular germling. For example, the protoplasts of *Ulva pertusa* transform into zoosporangia, and the protoplasts of *Monostroma nitidum* develop into gametangia (Fujita and Migita 1985). Besides these regeneration patterns, it is known for various species of marine algae that protoplasts can grow directly into intact thalli (Fujita and Migita 1985, Kitoh 1985, Ducreux and Kloareg 1988, Saga and Kudo 1989). In the present case, the protoplasts of *C. okamuranus* never developed into intact thalli directly, but only through the formation of reproductive cells.

A low percentage of *C. okamuranus* protoplasts grew to form filamentous cell aggregations characterized by large and poorly pigmented cells. They formed neither intact thalli nor reproductive cells. A similar result has been reported for *Monostroma angicava* by Saga and Kudo (1989). In that case, a small number of protoplasts prepared from a female gametophyte grew into callus-like cell aggregations which never developed into leafy thalli nor produced any reproductive cells. The third pattern of the protoplast regeneration in *C. okamuranus* was that the protoplasts remained as single cells, increasing in size. Thus, three different types of protoplast regeneration were observed in this species. It has been reported for several algal species that protoplasts isolated from the same individual followed several regenerative processes. This is reasonable considering that even a simple leafy thallus such as *Porphyra* has different types of cells (Polne-Fuller and Gibor 1984). The protoplasts of *C. okamuranus* prepared in the present study were released mostly from the assimilatory filaments. Therefore, it is probable that the cells of the filaments differentiate to a certain extent, and that protoplasts from the subcortex and the medullary layer follow different regeneration processes from those demonstrated here. Chen (1989) reported that protoplasts of *Porphyra linearis* showed several regeneration patterns and that a cell-suspension culture could be established from the protoplast-derived cells which did not regenerate into thalli. Furthermore, it was shown that these cells in a cell-suspension culture regenerated into organized thalli by altering culture conditions including temperature, photoperiod and irradiance. It is worth to examine the effect of culture conditions on the protoplast regeneration of *C. okamuranus*. Another possible explanation for the variety of regeneration patterns of algal protoplasts relates to differences in coexisting bacteria and their effects on the developmental pattern of the protoplasts (Uchida *et al.* 1992). It has been reported that growth and morphogenesis of some algal species, such as *Porphyra tenera* (Tsukidate 1977), *Ulva lactuca* (Provasoli and Pintner 1980) and *Monostroma oxyspermum* (Tatewaki *et al.* 1983), are affected by bacteria. The study of protoplast regeneration using axenic cultures is important for assessing the bacterial effects on the pattern of regeneration.

The present investigation provides a method for the production of sporophyte thalli from protoplasts of *Cladosiphon okamuranus*. This may serve as the basis for future studies on breeding and morphogenesis of this species.

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内田卓志・有馬郷司：オキナワモズク胞子体から作出したプロトプラストの再生

鹿児島県竜郷町地先で得たオキナワモズク胞子体からプロトプラストを作出し，その再生を観察した。プロトプラストの再生には次のような3通りのタイプが観察された。最も高率で生じたタイプではプロトプラストが数回細胞分裂を繰り返して糸状あるいは不定型の細胞塊となった後，成熟して遊走子を放出した。遊走子は細胞分裂を繰り返して盤状体を形成し，胞子体に成長した。また別のタイプでは，プロトプラストは細胞塊を形成したが，細胞分裂は数回で停止し，細胞の肥大化及び色調の薄くなる傾向がみられた。他には，プロトプラストは細胞分裂を行わず，細胞の肥大化のみられるタイプが観察された。(739-04 広島県佐伯郡大野町丸石2-17-5 水産庁南西海区水産研究所)