

Life without a cell membrane: regeneration of protoplasts from disintegrated cells of the marine green alga *Bryopsis plumosa*

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SUMMARY

When the multi-nucleate giant cells of the green alga *Bryopsis plumosa* (Huds.) Ag. are injured, the protoplasm is extruded from the cells and can generate spontaneously numerous new cells. The cell organelles aggregate rapidly in seawater and become covered with a gelatinous envelope within 15 minutes. A lipid cell membrane is formed inside the envelope within 9 to 12 hours and about 15% of the original cell membrane is recycled to make the membrane of new protoplasts. Cytochemical studies using Nile Red and various enzymes revealed that the primary envelope is initially composed of polysaccharides, and then transformed into a polysaccharide-lipid complex.

Fluorescein diacetate staining showed that the primary envelope has some characteristics of a cell membrane including semi-permeability and selective transport of materials. The aggregation of cell organelles appears to be mediated by two kinds of materials, one present in vacuolar sap and the other on the surface of the cell organelles. About a thousand new cells were generated from a single disintegrated branch and 40% of them eventually developed into mature plants.

Key words: Cell membrane, *Bryopsis*, Protoplast regeneration, Algae.

INTRODUCTION

The ability of many coenocytic green algae to repair partial rupture to their cell membrane has been well studied (e.g. La Claire, 1991). When cells are damaged in seawater, they quickly seal the wound with one or more insoluble plugs composed of proteins, polysaccharides or both to prevent loss of the cell contents (Mariani-Colombo et al., 1980; Pak et al., 1991). Subsequently, the cell recovers its original volume and shape and a new cell wall is formed to repair the wounded wall. Considering the large size of these cells, the importance of wound healing in coenocytic green algae is obvious, however, a full explanation of how these cells repair disrupted cell membrane remains to be determined.

An interesting wound response has been reported in a coenocytic green alga, *Bryopsis plumosa* of the order Derbesiales. When the cytoplasm of the algal cell is squeezed out, after cutting the cell, new protoplasts are formed in seawater and the protoplasts make new cell wall within several hours (Tatewaki and Nagata, 1970; Kobayashi and Kanaizuka, 1985). Although there are some descriptive studies on the reunification of cell fractions (e.g. Kobayashi and Kanaizuka, 1985) and the ultrastructure of protoplasts in this alga (Pak et al., 1991), knowledge of the extent and functions of protoplast regeneration is extremely fragmentary (Mine et al., 1996; Bück and Schnetter, 1997; Togashi et al., 1998).

It is hard to imagine that such a sophisticated structure as a cell membrane could be regenerated in such a simple process. Moreover, our results show that the gelatinous envelope initially formed on the protoplasmic masses is not temporary

but lasts for several hours and acts in a similar way to a cell membrane until a new cell membrane develops. In this study, we provide a full description of how the extruded cytoplasm regenerate new cells in vitro for the first time, and discuss the implications of these observations in understanding the extent and functions of cell membrane recycling in this alga.

MATERIALS AND METHODS

Plant material

Vegetative plants of *Bryopsis plumosa* (Huds.) Ag. were collected from Kachon, on the southern coast of Korea and maintained as unialgal cultures in IMR medium (Kim and Kim, 1999). Plants were grown at 25°C in 16:8 light and dark cycles with 30 µmol photons m⁻² s⁻¹ provided by cool-white fluorescent bulbs.

Survival and pH experiment

Survival rates and the effects of pH on regeneration were studied using artificial seawater. The composition of artificial seawater is as follows: 100 ml distilled water, 2.63 g NaCl, 0.609 g MgCl₂, 0.193 g MgSO₄, 0.074 g KCl, 0.11 g CaCl₂, 0.1 g Tris. pH was adjusted with HCl or NaOH. The algal thalli were cut into pieces in a cell counter containing artificial seawater and were squeezed to form protoplasmic masses. The size of sub-protoplasts was measured 30 minutes after wounding. The number of sub-protoplasts with a primary envelope was counted over a time course.

Estimation of membrane surface area

Total membrane surface area and cell volume were estimated from photographs of entire intact cells and of the sub-protoplasts regenerated from the extruded protoplasm. The initial plants chosen

were nearly cylindrical, so the planar area determined by the image analysis system was multiplied by π to give the total cell-membrane surface area in the intact cells. The sub-protoplasts were spherical, so their two-dimensional areas (πr^2) were summed for each cell by the computer and multiplied by 4 to give the total surface area. Accuracy of the values and assumptions were checked through direct geometrical analysis (by hand) of the micrograph.

Enzyme experiment

The enzymes were first dissolved in phosphate-buffered saline (PBS) and then diluted in enriched seawater to final concentrations: 20 mg/ml cellulase (Sigma Chemical Co., St Louis, MO, USA), 5 mg/ml lipase (Fluka Chemie), 0.05 mg/ml α -mannosidase (Sigma), 0.8 mg/ml pectinase (Sigma), 0.3 mg/ml β -glucosidase (Sigma), 3 mg/ml β -glucuronidase (Sigma), 10 mg/ml proteinase K (Sigma). Regenerated sub-protoplasts attached to the coverslip were transferred to seawater that contained an enzyme, left for 10 minutes and then examined under a light microscope.

Application of fluorescent probes

For intracellular lipids and membrane staining, 10 mg/ml stock solution of Nile Red (Nile Blue A Oxazone, Sigma) in acetone was diluted in artificial seawater at pH 8 to a final concentration of 3 mM (Oparka and Read, 1994). The fluorochrome was applied to the sub-protoplasts at various times after wounding and cells were examined while still immersed in the stain. For cellulosic cell wall staining, Calcofluor White M2R (Sigma) was diluted in artificial seawater at pH 8 to a concentration of 0.1 mg/ml, and was used for 5–10 minutes. After a water wash, sub-protoplasts were mounted in enriched seawater and examined under a UV filter. For 'live staining' with fluorescein diacetate, one drop of the stock solution of fluorescein diacetate (Sigma; 1–5 mg/ml in acetone) was diluted in artificial seawater at pH 8 and applied to the regenerated sub-protoplasts over time. For 'dead' staining, 1–5 mg/ml stock solution of propidium iodide (Sigma) dissolved in distilled water was diluted to a concentration of 100 μ g/ml with enriched seawater. The fluorochrome was applied to the sub-protoplasts for 5 minutes, and then they were examined under a UV filter, while still immersed in the stain. For nuclei staining, 1 mg/ml stock solution of DAPI (4',6-diamidino-2-phenylindole, Sigma) dissolved in distilled water was applied to the sub-protoplasts at a concentration of 5 μ g/ml for 5–10 minutes, and then they were examined under a UV filter.

Microscopy

For fluorescence microscopy, an Olympus BX-50 microscope was used. Additional studies of fluorescein diacetate staining of sub-protoplasts were performed with a Fluoview II (Olympus) confocal microscope. Photographs were taken with 400 ASA Kodak T-max film.

RESULTS

Sequential process of protoplast regeneration

Bryopsis plumosa is a green alga that consists of a coenocytic cell with distichous branches and grows up to 10 cm high (Fig. 1A). When the multinucleate giant cells were cut in seawater, the extruded cell organelles aggregated rapidly to form numerous irregularly shaped masses (Fig. 1B,C). The aggregated protoplasmic masses became spherical within several minutes and were covered with a gelatinous envelope in 10–20 minutes (Fig. 1D). When the aggregation process of the extruded cell components was studied with time-lapse photography, an active swirling movement of cell organelles was observed during the first few minutes (Fig. 2). The binding between the organelles became tighter over time and some

gelatinous material was squeezed out. The gelatinous material spread along the surface to form an envelope within several minutes. A narrow gap developed between the protoplasmic mass and the gelatinous envelope (Fig. 2). A lipid-based membrane (secondary membrane) developed inside the gelatinous envelope 12 hours after wounding and the cell began to expand (Fig. 1E). We have termed the protoplasmic mass enclosed by a gelatinous envelope, the sub-protoplast, to distinguish it from the protoplast with a lipid-based membrane.

Size of sub-protoplast required for survival

The plants were chopped with a blade and protoplasm was squeezed out in a cell counter containing enriched or artificial seawater. The size of newly formed sub-protoplasts was mostly 30–40 μ m in diameter regardless of the wounding conditions (Fig. 3). This size is almost the same as that of spores of this species. Survival of sub-protoplasts was partially dependent on their size. No sub-protoplast less than 10 μ m in size survived to form a secondary membrane. The survival rate of sub-protoplasts did not increase with size after 30 μ m. Sub-protoplasts larger than 100 μ m often divided into several small sub-protoplasts or degenerated. The sub-protoplasts of 30 μ m in size usually contained 3–5 nuclei (Fig. 4A). A dramatic decrease in number of intact sub-protoplasts was observed regardless of the size, between 9 hours and 12 hours after

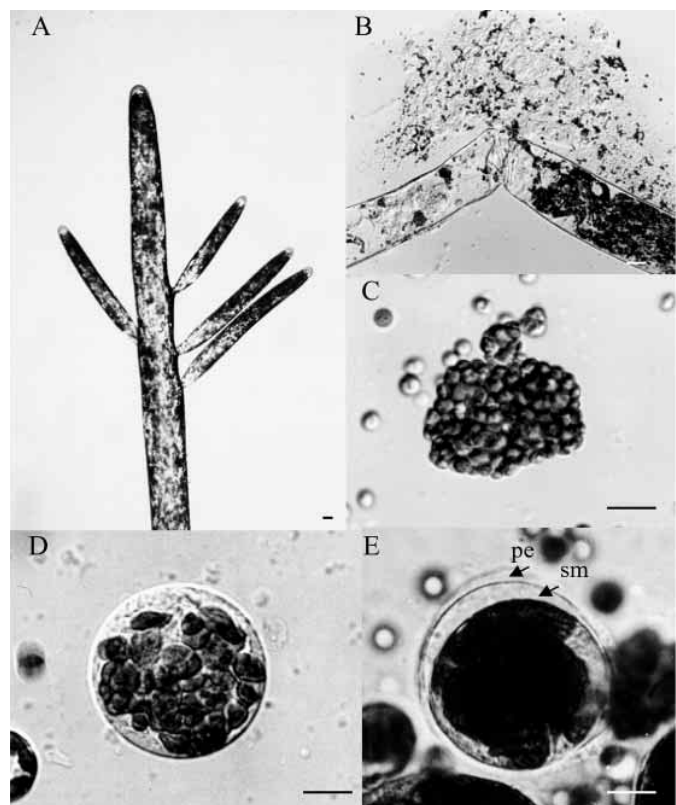


Fig. 1. The sequential process of sub-protoplast regeneration from disintegrated cells of *Bryopsis plumosa*. (A) Vegetative plant with distichous branches. (B) Protoplasm comes out from the wounded cell and spreads in seawater. (C) Aggregation of the extruded cell organelles in seawater. (D) Regenerated sub-protoplast with a primary envelope 20 minutes after wounding. (E) The secondary lipid-based membrane inside the primary envelope 12 hours after wounding (pe, primary envelope; sm, secondary membrane). Bars, 10 μ m.

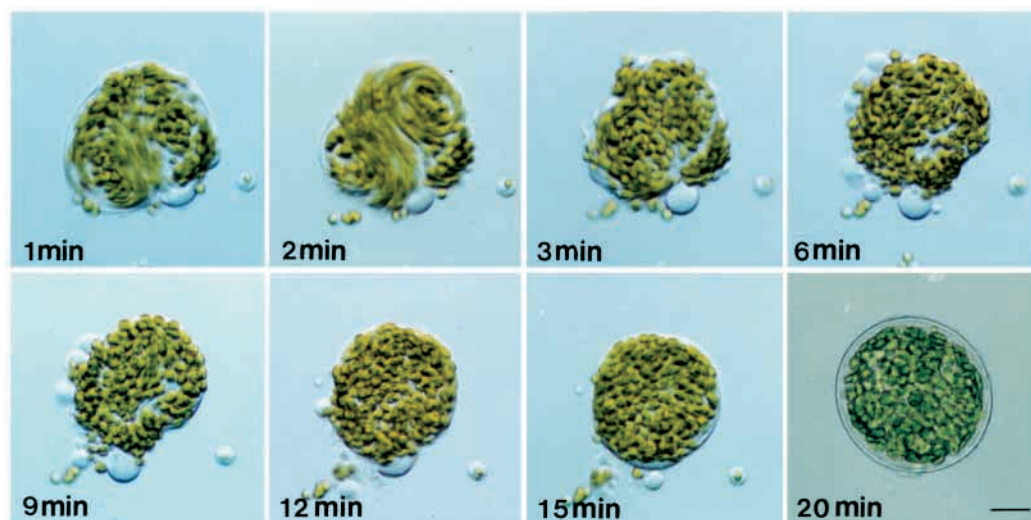


Fig. 2. Time-lapse photography of early sub-protoplast regeneration. Bar, 10 μm .

wounding, just before cell membrane formation. About 50% of sub-protoplasts degenerated during that time. Once the sub-protoplasts developed cell membranes, most (>80%) survived and began to grow in enriched seawater. Therefore, the development of the cell membrane appears to be a critical factor for survival of sub-protoplasts.

To examine the effect of water-soluble substances in the protoplasm on survival of sub-protoplasts, extruded protoplasm was centrifuged directly at 12,000 g for 10 minutes and the supernatant (>95% of total volume) was removed. When the pellet was washed with mild centrifugation (1200 g for 2 minutes) and resuspended in seawater, sub-protoplasts formed and about 40% survived to become mature plants. Therefore, the amount of water soluble substances in the original protoplasm does not appear to be critical for development or survival of sub-protoplasts.

Total membrane surface area of individual cells was estimated by image analysis of intact cells and the sub-protoplasts regenerated from the extruded protoplasm (Table 1). On average, the total membrane surface area of the sub-protoplasts formed from a single wounded cell was 85% less than that of the original cell before wounding.

Effects of pH on the aggregation of cell organelles

Protoplasmic aggregation was pH dependent. When we isolated protoplasm in air, we observed the aggregation of cell organelles in vacuolar sap of pH 5.8, but prompt dilution of the protoplasm with seawater at the same pH prevented aggregation. By contrast, when the plants were directly chopped and extruded in artificial seawater the cell organelles aggregated best at pH 8-9, the normal pH of seawater. The swirling movement and tight binding of cell organelles was not observed at a pH lower than 6 or higher than 11. Therefore, we hypothesized that there are at least two kinds of materials involved in the

Table 1. Total membrane surface area in five sizes of *Bryopsis* cells before and after extrusion of protoplasm and formation of sub-protoplasts

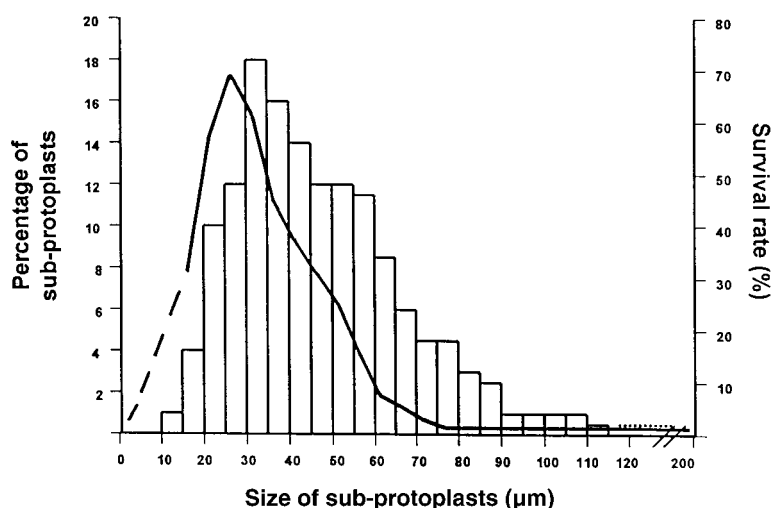
Cell	Total membrane surface area (mm^2)		No. of sub-protoplasts	Decreased surface area (%)
	Intact cell	Sub-protoplast		
1	22.1	3.4	1016	84.7
2	30.1	4.5	1798	85.1
3	26.4	3.7	1310	86.1
4	13.8	1.6	580	88.4
5	34.3	6.0	2115	82.5
Mean				85.4

The number of sub-protoplasts was counted 1 hour after wounding.

aggregation of cell organelles, one in vacuolar sap with an activity that was optimal at pH 5-6 and the other on the surfaces of cell organelles with an optimum of pH 8-9.

Vacuolar-sap-free cell organelles were obtained by repeated centrifugation and washing of protoplasm under high salt conditions (12,000 g , 10 minutes, three times; 900 mM NaCl). All the cell organelles were detached after this treatment and no envelope formation was observed. However, when the isolated

Fig. 3. The size distribution and survival rate of the regenerated sub-protoplasts. Size distribution (line) was studied 1 hour after wounding. Survival rate according to the size of sub-protoplasts (bars) was calculated using the equation $\% = (\text{number of cells after 24 hours} / \text{number of cells after 1 hour}) \times 100$.



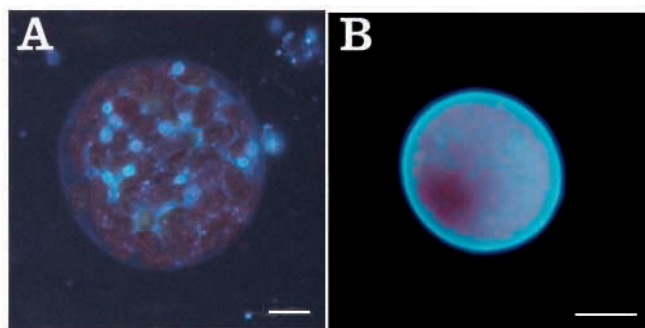


Fig. 4. DAPI staining and Calcofluor White staining of the regenerated sub-protoplasts. (A) Nuclei stained with DAPI, fluorescence blue. (B) Calcofluor White staining shows accumulation of cellulose on the surface of the primary envelope at 6 hours after wounding. Bars, 10 μ m.

cell organelles were resuspended in artificial seawater at pH 8-9 they aggregated again. The isolated cell organelles did not aggregate in artificial seawater at a pH lower than 6 or higher than 11. Therefore, the material involved in agglutination appears to be located on the membrane surfaces of cell organelles.

Depletion of calcium from the artificial seawater showed no effect on the aggregation of cell organelles and the formation of the primary envelope.

Formation of the primary envelope and its composition

After several minutes, when the swirling movement of protoplasmic masses stops, they become spherical in shape and covered with a gelatinous envelope. No primary envelope was formed in artificial seawater at a pH lower than 6, but when the pH of the solution was increased to 8, the protoplasmic masses became enclosed within an envelope. The primary envelope was not formed in artificial, low salt (<80 mM NaCl) seawater, but it appeared when more salt was added to the solution. Once

the envelope was formed it was stable at low pH (5-6) or salinity (<80 mM NaCl).

Initially, Nile Red staining showed that there was active movement of lipid membranes towards the interior of the protoplasmic masses (Fig. 5A,B). When swirling finished, a large mass of lipid material was observed in the center of the protoplasmic masses (Fig. 5B). When the protoplasmic masses became spherical and covered with a gelatinous envelope, the membranes of cell organelles became stained with Nile Red but no staining of the envelope was observed (Fig. 5C). Some lipid material, as indicated by Nile Red staining, began to be incorporated into the envelope 1 hour after sub-protoplast formation (Fig. 5D), and by 6 hours after wounding the outer membrane was strongly stained with Nile Red (Fig. 5E,F).

To determine the composition of the gelatinous envelope, various digestive enzymes were applied to the sub-protoplasts over time (Table 2). Most of the polysaccharide-digesting enzymes were effective in removing the envelope up to 6 hours after wounding. α -mannosidase was the most effective in digesting the envelope in the first 1 hour. Proteinase K had no visible effect throughout the regeneration process. Lipase had little effect on sub-protoplast regeneration in the first hour, but became effective at dissociating sub-protoplasts 3-6 hours after wounding. All the digesting enzymes had no visible effect after 12 hours, the time when formation of the secondary membrane was complete and the cell wall had developed (Table 2; Fig. 4B).

Characteristics of the primary envelope

The primary envelope was semi-permeable. The sub-protoplasts swelled in low salt conditions (200 mM NaCl) and shrank in high salt conditions (600 mM NaCl). Repeated exposure to low and high salt conditions for short periods of time (<5 minutes) did not affect the integrity of the envelope or the viability of the sub-protoplasts. The sub-protoplasts, surrounded by primary envelopes, fused easily with each other in seawater to form larger sub-protoplasts.

Some viability-testing probes were applied to sub-protoplasts

Fig. 5. Nile Red staining (orange colour) of lipid and membranes at various times during the regeneration of sub-protoplast. (A) Active movement of lipid material towards the interior of the protoplasmic mass occurred during the first few minutes. (B) The swirling movement of the cell organelles stopped and lipid material was localized to the centre of the protoplasmic mass. (C) The sub-protoplast became spherical and covered with primary envelope, but the envelope is not stained at this stage. (D) The lipid material begins to be incorporated into the envelope at 1 hour after wounding. (E-F) Strong labelling of the primary envelope and secondary membrane. Bars, 10 μ m.

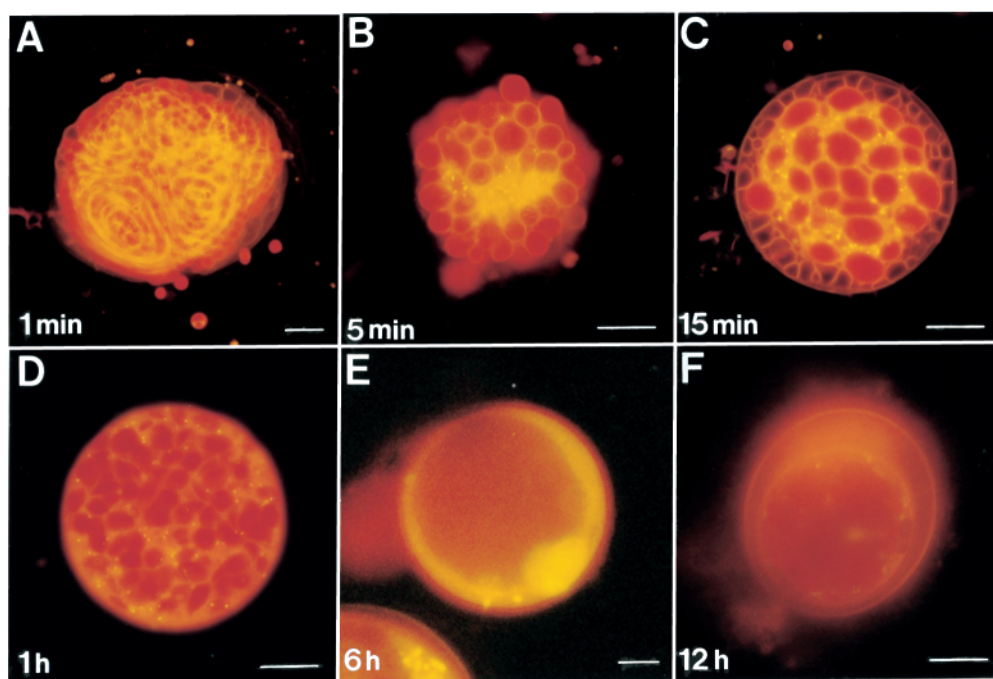


Table 2. Percentage of sub-protoplasts with membrane after treatment with various enzymes

Enzyme	Time of treatment (hours)					
	1	3	6	9	12	24
Control	56.4±2.0	59.5±5.9	56.8±2.0	59.0±6.1	32.0±2.4	26.0±2.3
α-mannosidase	9.8±0.7*	26.5±4.0*	36.2±0.5*	43.1±2.9*	32.6±3.9	29.3±4.8
Pectinase	25.8±4.1*	22.6±4.5*	25.8±4.1*	54.4±6.2	28.3±2.6	29.2±4.2
β-glucosidase	32.4±8.4*	22.3±2.5*	32.4±8.3*	28.6±10.7*	32.0±2.4	27.9±4.5
Cellulase	22.5±2.1*	24.4±3.7*	22.5±2.1*	53.3±3.5	29.9±3.5	28.5±4.6
β-glucuronidase	41.8±2.4*	55.2±5.3	50.1±2.4	57.0±4.8	33.6±2.9	28.4±5.4
Proteinase K	57.6±6.0	59.6±7.2	57.6±6.0	50.0±4.1	28.6±3.5	27.1±1.5
Lipase	58.8±4.8	38.3±6.1*	15.8±3.2*	48.1±4.8	30.7±2.8	25.4±2.3

The enzymes were applied to the regenerated sub-protoplasts over time.

Data are the means of multiple comparisons by the Tukey method, $n=10$, * $P<0.001$.

to determine the characteristics of the primary envelope. Fluorescein diacetate has been used as a means of vital staining of cells to monitor the integrity of cell membranes. Fluorescein diacetate is essentially a non-fluorescent, non-polar molecule that is passively taken up into the cell. After cleavage by cellular esterases in cytoplasm, fluorescein diacetate fluoresces green when excited. When the sub-protoplasts were embedded in seawater containing fluorescein diacetate, the labelling appeared within the sub-protoplast by 5 minutes (Fig. 6). Fluorescein accumulated inside the sub-protoplast over time but was observed only in the peripheral region of the sub-protoplasts (Fig. 6). This may be because of a large mass of lipid material in the central region of the sub-protoplast, as shown by Nile Red staining (Fig. 5B). The sub-protoplasts were not stained with a dead-cell stain, propidium iodide, which indicated that the primary envelope was not defective (data not shown). These results show that the sub-protoplast can uptake a substrate across the primary envelope.

Regeneration of the cell wall began around 4 hours after sub-protoplast formation. Calcofluor White staining showed the accumulation of cellulose on the surface of the primary envelope (Fig. 4B). The accumulation of cellulose component began prior to the development of secondary membrane. Therefore, the sub-protoplast has the ability to transport cellulose components across the primary envelope.

DISCUSSION

There are four stages in the spontaneous generation of *Bryopsis* sub-protoplasts: aggregation of cell organelles, changes in protoplasmic masses to a spherical shape, primary envelope formation and secondary membrane development. Our data indicate that at least two types of molecules are involved in the aggregation of cell organelles, one acts at an optimum of pH 5-6 and the other on the surfaces of cell organelles acts at an optimum of pH 8-9. This result was surprising because it is contrary to previous results (Pak et al., 1991). Pak et al. suggested that the most effective material for agglutination of cell organelles might be contained in the vacuolar sap and, therefore, the sequential action of vacuolar sap (pH 5-6) and seawater would be very important for the aggregation of cell organelles. They also reported that prompt dilution of extruded algal cell contents with 20 volumes of seawater prevented aggregation. The discrepancy

between our results and those of Pak et al. may be due to experimental conditions. Pak et al. initially extruded protoplasm in air and diluted the protoplasm with 20 volumes of a test solution containing 0.5 M sorbitol and then counted protoplasmic masses between 50 µm and 200 µm in diameter. In our study, the plants were chopped directly in seawater and all the viable sub-protoplasts larger than 15 µm were counted.

Depletion of calcium from the artificial seawater showed no effect on the aggregation of cell organelles and formation of primary envelope. Therefore, the protoplast regeneration process of *Bryopsis plumosa* appears different from the wound healing process observed in sea urchin egg, which is highly calcium sensitive (McNeil et al., 2000).

The change of protoplasmic masses to a spherical shape appears to result from an accumulation of lipid material in the center of the protoplasmic masses. This stage occurs only when the protoplasmic masses are exposed to seawater. Higher levels of salinity in seawater compared with levels in the cytoplasm may explain the swirling movement in protoplasmic masses. A high saline solution is more hydrophobic than a low saline solution and may provide a stronger force to compress the hydrophobic lipid material to the center of protoplasmic masses. Hydrophilic materials, including polysaccharides,

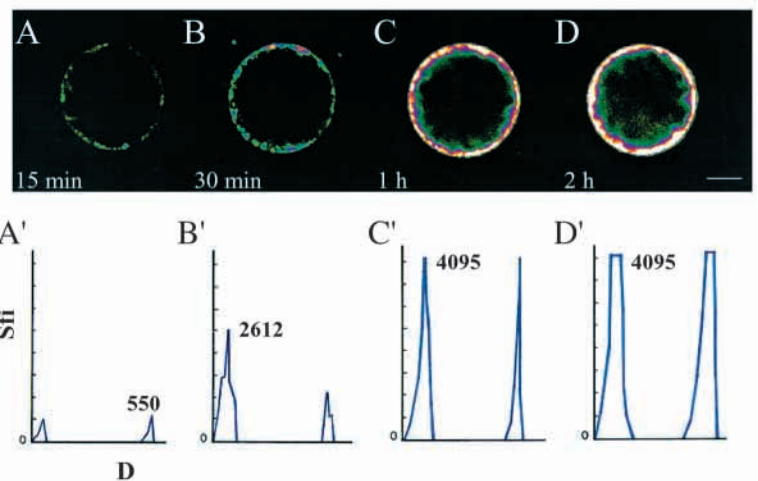


Fig. 6. Fluorescein diacetate staining in the regenerated sub-protoplast. (A-D) Confocal laser scanning micrographs of the sub-protoplast regeneration taken at various times post-wounding. Artificial colours indicate an increase of fluorescence intensity inside the sub-protoplast over time. (A'-D') Quantitative analysis of the fluorescence intensities, measured along a line through the central portion of the sub-protoplast (D, distance; Sfi, subtracted fluorescence intensity). Bar, 10 µm.

inside the protoplasmic masses are pushed out during the process and spread along the surface to make the primary envelope. When we put the extruded protoplasm in a solution close to isotonic with cytoplasm (<80 mM NaCl) neither swirling movements nor the development of the primary envelope was observed. This supports our hypothesis.

Our data from experiments using Nile Red and various enzymes indicate that the primary envelope is not a lipid membrane. Initially it is composed of polysaccharides and then changes to a polysaccharide-lipid complex over time, finally it is substituted by a lipid-based membrane. Polysaccharides seem to be important in maintaining the integrity of the primary envelope, at least for the first 6 hours. This is interesting because the primary envelope is temporary and initially not a lipid membrane but, nevertheless, has many characteristics of a cell membrane. The sub-protoplasts swelled or shrank according to the salinity of the solution, indicating that the primary envelope is semi-permeable. Moreover, fluorescein diacetate staining showed that the primary envelope allowed entry of this substrate into the sub-protoplast. The esterase activity, as shown by fluorescein diacetate staining, increased over time. Because the sub-protoplast could survive when we removed almost all the water soluble substances from the cytoplasm, and as esterases are water-soluble proteins, some protein might be synthesized in the sub-protoplasts. Fluorescein accumulation in the peripheral region of the sub-protoplasts suggests that their cytoplasm becomes polarized as soon as the primary envelope is formed.

Our final hypothesis is that the remnant of the original cell membrane is compressed in the centre at first and incorporated into the primary envelope over time. On average, the total membrane surface area of the sub-protoplasts formed from a single wounded cell was 85% less than that of the original cell before wounding. Therefore, there is a lipid source for regeneration of the secondary membrane. Electron microscopic studies reported significant agglutination of cellular membranes in sub-protoplasts, and suggested that the new cell membrane might be formed by fusion of the original, disintegrated cell membrane with cytoplasmic vesicles on the surfaces of the protoplasmic masses (Pak et al., 1991). Such extensive agglutination of cellular membranes was never observed in intact *Bryopsis* cells (Burr and West, 1970; Burr and West, 1971; Burr and Evert, 1972). Therefore, the lipid material in the centre of the protoplasmic masses as shown by Nile Red staining may be the remnant of the original cell membrane, which appears to be incorporated into the polysaccharide primary envelope over time.

Wound response has been studied for many years in many different groups of algae, but it has been considered only to be a healing process to maintain the integrity of the plants (Kim and Fritz, 1993; Kim et al., 1995; Menzel, 1988; Nawata et al., 1993; O'Neil and La Claire, 1984; Shihira-Ishikawa, 1987; Waaland, 1990). The process of sub-protoplast formation in *Bryopsis* species, however, can be interpreted as a method of propagation because one branch of a plant could generate hundreds of viable new cells spontaneously in seawater. It has been shown that even dissociated cell fractions make new living cells when the fractions are mixed together under certain experimental conditions (Kobayashi and Kanaizuka, 1985). Considering the large size of the cell in this species, the

importance and benefit of such a propagation method is obvious, however, more studies at an ecological level are necessary for a better understanding. Regeneration of protoplasts from the sub-cellular fractions may be an important model system for the study of such processes as the interaction of various cell organelles, formation of various hybrid cell types and especially, evolution of cell membranes.

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