Two Distinct Steps for Spontaneous Generation of Subprotoplasts from a Disintegrated Bryopsis Cell

Jun Yong Pak1, Carmen Solorzano2, Masayoshi Arai, and Takeshi Nitta*
Laboratory of Biology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan

ABSTRACT

The unusual nature of protoplasm to generate subprotoplasts spontaneously from disintegrated Bryopsis cells was examined. Protoplast extruded from algal cells aggregated rapidly in cell sap which was derived mainly from huge central vacuoles of the cells. Electron microscopic observations revealed extensive agglutination of algal cellular membranes in the protoplasmic masses, suggesting that this is of primary importance for the wound-healing ability of the alga. Seawater caused spheration of the resultant protoplasmic aggregates. Gelatinous sheaths were formed temporarily surrounding the spherical protoplasmic masses before reformation of cell membrane. Staining with phosphotungastic and chromic acids suggested that new cell membranes were formed by fusion of the disintegrated original cell membrane with cytoplasmic vesicles on the surfaces of the protoplasmic masses. Both pH and salts were found to be essentially important at the two steps of subprotoplast generation. The newly formed cell membranes were responsible for subsequent notable plasmolysis of the wounded cells in seawater. Thus, it is suggested that unicellular marine algae Bryopsis spp. naturally contain effective materials for agglutinating and fusing particular cellular membranes through the sequential aid of acidic cell sap and alkaline seawater after disintegration of the giant cells.

Algae have long evolved and some of them have developed unusual peculiarities, and thus many coenocytic marine algal cells have a recognized ability to heal wounds (2, 4, 7, 9, 16, 25). This wound healing has been suggested to be the result of cascade events in these algal cells. When the cells are wounded in seawater, the protoplast does not flow away from them after its aggregation and spheration at the injury site. Subsequently, the cells cause intense retraction of the entire protoplasm primarily from the site of injury (7, 9, 16, 17). In time, the retracted protoplasm recovers its original volume and shape and then new cell wall is formed repairing the wounded wall. These unusual protoplasmic reactions efficiently prevent loss of the algal cell contents caused by wounding. Although contractile protein systems were suggested to play an important role in the protoplasmic motility after wounding (17–19), a full explanation has not been offered of the peculiar activity of the coenocytic algal cells in healing wounds, especially in repairing disrupted plasma membrane.

Surprisingly, when some coenocytic algae are cut and squeezed in seawater, numerous protoplast-like structures are spontaneously generated (8, 13–15, 17, 30). The mechanism of this unusual phenomenon is also poorly understood. The phenomenon reflects the unusual nature of the algal protoplasm to aggregate and to reform plasma membrane at an injury site, and thus is useful for clarifying the important early events in wounded algal cells. We examined the sequential steps of subprotoplast generation in Bryopsis (Derbesiales and Chlorophyta). Because most of the protoplast-like structures from a Bryopsis cell contain nuclei and generate new algal plants (13–15, 30), they are tentatively referred to as subprotoplasts, discriminating them from cytoplasts which are lacking in nuclei and from protoplasts which contain all original cell contents except for cell walls. Applying the mechanisms of spontaneous subprotoplast formation from Bryopsis cells to higher plant cells appears interesting and promising.

MATERIALS AND METHODS

Plant Materials and Protoplast Preparation

Bryopsis maxima Okamura and B. sp. were collected from Tokyo Bay at Chiba from October to June, 1987 to 1989. The algal thalli were cut into pieces of about 1 cm length with scissors and were squeezed through nylon mesh (90 μm in pore size) to form protoplasmic masses. Sizes of the resultant protoplasmic masses depended on experimental conditions; the structures were unstable to mechanical shocks and their sizes were significantly controlled by pipetting or gently stirring the extruded cell contents.

Protoplastic Aggregation and Spheration

Experimental conditions for aggregation of algal protoplast were examined by promptly diluting a 0.25 mL aliquot of the extruded cell contents with 20 volumes of a test solution containing 0.5 M sorbitol in a plastic Petri dish of 3 cm diameter. Aggregation activity of a test solution was judged by separately counting aggregated and isolated chloroplasts (more than 200) in micrographs taken 30 min after the dilution. Protoplastic masses of 50 to 200 μm diameter were usually formed under the current experimental conditions.

Protoplastic spheration was examined by gently adding 20 volumes of a test solution containing 0.5 M sorbitol to a 0.25 mL aliquot of the protoplasmic masses of 50 to 200 μm diameter which were aggregated for 30 min in vacuolar sap.

1 Permanent address: Faculty of Education, Korea University, Kodaia, Tokyo 187.
2 On leave from Tissue Culture Laboratory, Plant Science Department, Autonomous University of Chapingo, Mexico.
Spherical protoplasts and nonspherical protoplasmic masses (more than 100) were recognized under a microscope 30 min after the addition. The generated subprotoplasts were cultured in enriched seawater (29). To maintain the pH of a test solution, 50 mM acetate (pH 4-6) or Tris-HCl buffer (pH 7-9) was used. Only chloride salts were tested throughout the experiments.

**Electron Microscopic Observations**

Algal subprotoplasts were fixed with 3% (w/v) glutaraldehyde in 50 mM phosphate buffer of pH 7 containing 0.5 M sorbitol at 4°C for 2 h and then with 2% (w/v) osmium tetroxide in the buffer at 4°C for 4 h. The fixed materials were embedded in 1.5% (w/v) agar (27) and were dehydrated in an acetone series. The sectioned materials were stained with 1% (w/v) uranylacetate in 50% (v/v) ethanol and then with 1% (w/v) lead nitrate. Cell membrane was stained with phosphotungstic acid-chromic acid according to Rolánd et al. (26).

For preparation of freeze-fracture replications, spherical protoplasmic structures were fixed with 2.5% (w/v) glutaraldehyde in the phosphate buffer at 4°C for 2 h. After washing, the materials were soaked in 30% (v/v) glycerol of 50 mM phosphate buffer (pH 7) at 4°C for 2 h and were frozen in liquid nitrogen. The samples were cracked and the obtained surface was shadowed with platinum and covered with carbon using a JEOL JFD-7000. The samples were observed with a JEOL 100C electron microscope.

**RESULTS AND DISCUSSION**

**Sequential Effects of Vacuolar Sap and Seawater on Extruded Protoplast**

When giant *Bryopsis* cells were cut and extruded, extraordinarily large chloroplasts of about 10 μm diameter were observed to aggregate rapidly, together with nuclei and mitochondria, to form irregularly shaped masses in a large volume of cell sap derived mainly from huge central vacuoles, but spherical subprotoplasts were never formed in the sap (Fig. 1, A and B). When the aggregated protoplasmic masses were transferred to seawater, they became spherical within several minutes (Fig. 1C). However, when the extruded protoplasm was promptly transferred to seawater before protoplasmic aggregation occurred in the extruded vacuolar sap, neither protoplasmic masses nor subprotoplasts were observable (Fig. 1D). New subprotoplasts were never generated after once-generated subprotoplasts were mechanically disintegrated in seawater.

Thus, two steps were apparently involved in the spontaneous generation of algal subprotoplasts: protoplasmic aggregation was necessary before subprotoplast generation. Algal vacuolar sap first aggregates protoplasm while seawater makes the resultant protoplasmic masses spherical. Most of the subprotoplasts through the two steps generated new algal plants in enriched seawater as reported by Tatewaki and Nagata (30).

These sequential reactions appeared to be introduced naturally in protoplasm at injury sites of algal cells; rapid aggregation of the protoplasm was first induced probably in vacuolar sap and then spheration of the aggregated protoplasm was caused in seawater. When the algal cells were in air, protoplasmic aggregation was not followed by spheration at injury sites of the algal cells.

**Experimental Conditions for Subprotoplast Generation**

First, we examined experimental conditions of the two steps. These two distinct reactions were able to be induced in...
simple definite solutions. Protoplasmic aggregation in a test solution was examined by taking a count of chloroplasts aggregated and nonaggregated to protoplasmic masses. Prompt dilution of extruded algal cell contents with a test solution of 20 volumes prevented protoplasmic aggregation by the inevitably contaminating vacuolar sap. Neither more nor less dilution of the cell contents was found to be useful; dilution with a larger volume of the test solution did not cause effective aggregation of the extruded protoplasm. On the other hand, the extruded protoplasm was aggregated fully in the vacuolar sap before it was transferred to a spheration-test solution. Thus, we found that, in addition to osmotica of more than 0.25 M, both pH and salt concentration were important for the two steps of aggregation and spheration of the algal protoplasm (Fig. 2). The optimum pH for aggregating cell organelles was 5 to 6, the same as the pH of algal vacuolar sap (Fig. 2A), while the optimum pH for spheration of the protoplasmic masses was 8 to 9, just like the pH of seawater (Fig. 2C). The optimum pH to aggregate the organelles differed slightly between Na+ and Ca2+ containing media.

Neither protoplasmic aggregation nor spheration was induced in a medium lacking salts even at the optimum pH. The effective ranges of salt concentrations were different between the two subprotoplast generating steps (Fig. 2, B and D). In concentration ranges up to 0.5 M, K+ and Na+ had the same efficiency for both steps and Mg2+ had almost the same efficiency as Ca2+. A high concentration of Ca2+ or Mg2+ inhibited the protoplasmic aggregation induced by monovalent ions. In contrast, Cs+, Li+, Zn2+, and Mn2+ at least up to 0.5 M were ineffective for protoplasmic aggregation and were not inhibitory for the aggregation induced by other cations. A chelating agent (10 mM EDTA) did not inhibit the protoplasmic aggregation caused by monovalent cations, but completely inhibited the spheration caused by them. The presence of a small amount of endogenous divalent cations, therefore, appears essential for the protoplasmic spheration induced by free exogenous cations, although the endogenous ones alone cannot cause the spheration.

Thus, the experimental conditions were distinct between the two steps in generating subprotoplasts; both vacuolar sap and seawater afford the experimental conditions for osmotic potential, pH, and salts essential to the two steps. Experimental conditions under which extruded algal protoplasm does not cause aggregation have been utilized to isolate intact cell organelles by differential centrifugation after squeezing algal cells (1, 23).

Known cellular contractile proteins do not explain the distinct effects of salts and pH on generation of subprotoplasts. It is noted that these conditions are well comparable to those found for artificial fusion of cell membrane introduced by a large amount of an exogenous effector such as dextran (10, 11) or polyethylene glycol (12).

![Figure 2](image.png)

**Figure 2.** Effects of pH (A and C) and salts (B and D) on aggregation (A and B) and spheration (C and D) of extruded *Bryopsis maxima* protoplasm. Percentages of aggregated chloroplasts (A and B) or spherical protoplasmic masses (C and D) in total ones are shown. Dotted lines with open circles show effects of Ca2+ and unbroken lines with circles effects of Na+. The test solutions to examine pH ranges of protoplasmic aggregation contained 0.3 mM NaCl or 0.05 mM CaCl2 (A) and those for protoplasmic spheration contained 0.2 mM NaCl or CaCl2 (C). To examine effects of salts, test solutions of pH 5.5 were used for protoplasmic aggregation (B) and those of pH 8.5 for spheration of protoplasm (D). For experimental details, see text.

**Electron Microscopic Observations on Aggregation of Extruded Protoplasm**

The unusual aggregation of *Bryopsis* protoplasm was then examined by electron microscopy. Agglutination of cellular membranes was remarkable in the protoplasmic masses (Fig. 3, A and B). Cell organelles associated with each other directly or indirectly by extended and/or circled membranes in the masses. Such extensive agglutination of cellular membranes was never observed in intact algal cells (2-4). The extensive agglutination of cellular membranes will result in prevention of loss of soluble cell contents, although the resultant protoplasmic masses had no enclosing membranes.

On the other hand, prompt transfer of the extruded cell contents to seawater did not cause the extensive agglutination of the cellular membranes, although tiny spherical protoplasmic masses with enclosing membranes were often generated (Fig. 3C).

The characteristic agglutination of cellular membranes causes effective aggregation of the extruded algal protoplasm. Very recently, the significant role of microtubules in organelle movement in extruded *Bryopsis* protoplasm has been proved
(22), although the array of the contractile proteins are expected to be disorganized after squeezing algal cells. The stated hypothesis on the proteins for particular motility of the extruded algal protoplasm and aggregation of cell organelles to generate subprotoplasts does not appear to reconcile with the observations on remarkable agglutination of algal cellular membranes. However, at the least, the newly induced arrays of the contractile proteins in the extruded algal protoplasm might accelerate the protoplasmic aggregation cooperatively with agglutinated cellular membranes.

Alterations in Enclosing Structures of Protoplastic Masses during Spheration

When the aggregated protoplasmic masses in vacuolar sap were transferred to seawater, their spheration was induced. The just-generated spherical masses fused effectively to the others to form bigger masses. Unexpectedly, electron microscopic observations showed that sheaths of about 50 nm width first enclosed the spherical protoplasmic masses under the current experimental conditions (Fig. 4. A–C). The sheaths appeared to be similar to elastic plug walls formed rapidly at injury sites of coenocytic algal cells; a large amount of a gelatinous material released from the huge central vacuoles aggregates temporarily to generate thick plug walls separating protoplasm from seawater (2, 4–6, 20, 21). The role of the plug material in wound-healing events of algal cells remains to be explained. The sheaths enclosing the spherical protoplasmic masses disappeared within several hours and the enclosing structures were replaced by continuous smooth membrane (Fig. 4D). After reformation of the enclosing membrane, the subprotoplasts no longer fused to each other.

Changes in the surface structures of protoplasmic masses were also shown by freeze-fracture method (Fig. 4, E and F). First, lipid bilayer surfaces were not conspicuous on the fractured surface of the spherical protoplasmic masses after their transfer to seawater (Fig. 4E); they then appeared on this surface, suggesting generation of the enclosing membrane (Fig. 4F). However, light microscopic observations did not easily discriminate the spherical protoplasmic masses enclosed by gelatinous sheaths from those enclosed by the reformed cell membrane. New cell wall was recognized 2 d after the culture of subprotoplasts in enriched seawater (Fig. 4, G and H).

Regeneration of Cell Membrane

It is quite evident that the enclosing membrane of subprotoplasts results from the fusion of small membranes in the protoplasmic masses, because comparable large membranes are never found in the extruded protoplasm prior to spheration (Figs. 3 and 4). Fusion of the small membranes appeared to occur actively on the surfaces of the protoplasmic masses. Both chloroplasts and nuclei often associated close together or close to cytoplasmic vesicles in the extruded vacuolar sap, but, after their transfer to seawater, they did not fuse but separated from each other within the generated subprotoplasts (Fig. 4). The fusion specificity of the algal cellular membranes remains to be elucidated.

The presence of numerous small vesicles in cytoplasm is a characteristic of Bryopsis cells (2–4), and hence most of the agglutinating vesicles in the protoplasmic masses are not derived from cell membrane disintegrated by squeezing the algal cells. In order to identify the original cell membrane, the protoplasmic masses were stained with phosphotungstic and chromic acids at a low pH (26). As reported in some other algal cells (28), the acids stained Bryopsis cell membrane but neither numerous small cytoplasmic vesicles nor other cellular membranes of this alga (data not presented). Stainable and unstainable vesicles were found within the aggregated protoplasmic masses in vacuolar sap after extrusion, suggestive of their different origins (Fig. 5A). The acids showed the existence of partially stained cell membranes in just-generated subprotoplasts in seawater (Fig. 5B). The membranes enclosing subprotoplasts were thus the result of random fusion of the two kinds of cellular membrane: the unstainable membrane moieties are derived from cytoplasmic vesicles and the stainable ones from disrupted original cell membrane.
Figure 4. Electron micrographs of spherating protoplasmic masses of Bryopsis sp. 10 min (A), 1 h (B and E), 2 h (C), 6 h (D and F), 24 h (G), and 48 h (H) after incubation of the aggregated masses in seawater, suggesting that the enclosing structures (shown by arrows) are temporarily made by gelatinous walls (A–C) and then by membranes mainly from cytoplasmic vesicles (D). The surface structures were observed by freeze-fracture method (E and F). Cp, chloroplasts; N, nucleus; Mt, mitochondria; V, vesicle; CW, cell wall. Bars = 0.5 μm.

Figure 5. Electron micrographs of sections of phosphotungstic acid-chromic acid stained Bryopsis sp. protoplasmic masses in vacuolar sap (A), subprotoplasts incubated in seawater for 3 h (B) or 12 h (C), and those in seawater containing 100 μg/mL cycloheximide for 12 h (D) or in seawater without the reagent for 12 h (E). Arrows show reformed cell membranes of subprotoplasts. Bars = 0.5 μm.
The cell membranes were stained homogeneously by the acids 1 d after the subprotoplast generation (Fig. 5C). Cycloheximide (10–100 μg/mL) effectively inhibited the recovery of full stainability of the enclosing membrane (Fig. 5D). The inhibition was reversible when the subprotoplasts were transferred to seawater without the reagent (Fig. 5E). Therefore, the change in staining properties of the cell membrane came from incorporation of newly synthesized proteins. The stainability of plant cell membrane by the acids probably depends upon the presence of some membrane-specific proteins.

Osmolarity-Resistant Function of Repaired Cell Membrane

Intense contraction of the entire protoplasm follows the early protoplasmic reactions at injury sites of coenocytic algal cells (7, 9, 16, 17), while the phenomenon was not so striking in newly generated Bryopsis subprotoplasts of smaller protoplasmic volumes. Therefore, subprotoplasts were not useful for examining the late step of wound healing and we, instead, examined the phenomenon in wounded algal cells.

Repair of the disrupted cell membranes was found to be essential to the subsequent contraction of wounded protoplasm; when the algal cells were wounded in an acidic solution or in air, rapid protoplasmic aggregation was properly introduced at the injury site, but neither protoplasmic spheration nor subsequent intense retraction was induced in those cells lacking in repaired cell membranes (data not presented). Whenever wounded algal cells were kept in a solution with osmotica of less than 0.25 m, the whole protoplasm failed in intense retraction, more convincingly indicating that the late step depended on osmotic pressure of a surrounding medium (data not presented). It is reasonable that wound-healing algal cells with new cell membranes derived mostly from cytoplasmic vesicles which lack proteins specific to genuine cell membranes are compelled to react differently from intact cells toward an osmotic environment. Finally, we concluded that seawater where intact algal cells never cause plasmolysis induced plasmolysis of the wound-healing algal cells with newly repaired plasma membrane.

Recovery of the plasmolyzed Bryopsis cells was observed to be inhibited by the presence of 10 to 100 μg/mL cycloheximide (data not presented). As previously described, this reagent inhibited acid stainability of reformed cell membranes in subprotoplasts. Incorporation of particular protein moieties will change the reformed chimeric cell membranes so that they function in the same way as genuine cell membranes resistant to the osmolarity of seawater.

The presence of cycloheximide did not inhibit the algal plasmolysis following wounding, but, unexpectedly, we found that prolonged treatment of 10 to 100 μg/mL cycloheximide effectively caused plasmolysis of unwounded algal cells even in seawater (Fig. 6, A and B). Recovery from the plasmolysis was observed when the treated algal cells were transferred to fresh seawater without the reagent (Fig. 6, C and D). Hence, the membranous proteins responsible for resistance to the osmotic pressure are suggested to have considerably rapid turnover rates in Bryopsis cells. Because terrestrial plants have lost their tolerance to seawater in the course of evolution from marine algae, the osmolarity regulatory membranous proteins might prove themselves beneficial in improving resistance in salinity-sensitive crops.

Concluding Remarks

No protoplasm from other plants and animals generated such protoplast-like structures spontaneously under the current experimental conditions; the algal cells must contain a particular and effective material for agglutinating and fusing algal cellular membranes with the sequential aid of vacuolar sap and seawater. In fact, when separated from vacuolar sap by repeated centrifugation (12,500g, 30 min), the algal protoplasm no longer aggregated nor spherated even under the optimum conditions of pH and salts. Isolation and characterization of the effective material occurring naturally in Bryopsis cells are under way.

Thus, our final hypothesis is that a large amount of a certain effective material is contained in the huge central vacuoles, and this introduces a series of wound-healing reactions through agglutination of the particular cellular mem-

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Figure 6. Plasmolysis of Bryopsis sp. cells kept for 12 h (A) or 24 h (B) in seawater containing 100 μg/mL cycloheximide and their recovery when cultured in fresh seawater without the reagent for 24 h (C) or 48 h (D) after being kept in the reagent-containing seawater for 24 h. Because Bryopsis thallus cells are long and gigantic, their plasmolysis often gives rise to fragmented spherical masses of algal protoplasm and their recovery from plasmolysis causes fusion of the fragmented protoplasm as shown. Bar = 100 μm.
branes through the sequential aid of acidic vacuolar sap and
then alkaline seawater. Although membrane recycling is a
common phenomenon in animal and plant cells, Bryopsis
cells apparently have unique membrane systems to protect
their gigantic unicellular organization from injury, as seen in
clathrin-coated membranes of Boergesenia cells to produce
aplanospores by wounding (7, 24).

Kobayashi and Kanaizuka (13, 14) separated extruded
Bryopsis cell contents into chloroplast and nonchloroplast
fractions, together to make subprotoplasts, and thus at-
tempted application of the wound-healing algal ability to
artificial cell construction. Subprotoplast regeneration from
disintegrated higher plant cells was partially successful using
vacuolar sap and cytoplasmic vesicles of this alga under the
experimental conditions of the two essential step reactions.
The detailed experimental results will be reported elsewhere.

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