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Application of an Alginate Lyase from *Alteromonas* sp. for Isolation of Protoplasts from a Brown Algae *Laminaria japonica*

Tomoo Sawabe,* Yoshio Ezura,* and Takahisa Kimura*

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An alginate lyase from *Alteromonas* sp. H-4 was used for the preparation of protoplast from young sporophytes of *Laminaria japonica*. Optimum conditions for the isolation of protoplasts were as follows: a) fine pieces of the sporophytes chopped by a razor were incubated in an enzyme mixture for 3 to 5 h at 15°C with shaking, b) the composition of the enzyme mixture was purified alginate lyase (30 U/m*l*) with cellulase Onozuka R-10 (1.5%) in a hypertonic solution (0.7 M mannitol, 25 mM $MgCl_2$, and 5 mM HEPES in 50% seawater, pH 7.8). Yields of protoplast were 10^6–10^7 cells/g (sporophytes) and protoplast viability was more than 75%. Protoplasts were brown-greenish and spherical with a diameter of 10–25 μm. The purity of alginate lyase from the strain H-4 scarcely influenced the yield of protoplast, but purified enzyme produced the highest viability of cells.

Some Laminariales plants, such as *Laminaria*, *Undaria*, and *Macrocystis*, are economically valuable seaweeds for food[1] and are good sources of fine chemicals,[2] medicine,[3] and for energy production.[4] Recently, biotechnological approaches, *i.e.* tissue culture, hybridization by fusion of protoplasts, and genetic recombination have been applied in the field of cultivation and breeding of these algae.[5] Protoplast is a useful tool in breeding and genetic engineering. Protoplast isolation from brown algae have been reported in genera of *Laminaria*,[6,7] *Undraira*,[8-10] *Macrocystis*,[11,12] *Fucus*,[13] *Sargassum*,[4,11,14] *Dictyota*,[15] and *Sphacelaria*.[16] In most cases, alginate lyase from marine molluscs or marine echnoderms have been used in order to degrade cell walls for protoplast isolation from brown algae, but high yield and viability of isolated protoplasts have not always been achieved. Mixed alginate lyases from marine molluscs and marine bacteria have been used in a few studies,[7,8] but alginate lyase from marine bacteria has not been used singly.

We have previously reported a marine bacterium, *Alteromonas* sp. H-4 which produced an active alginate lyase, from decaying thalli of *Laminaria japonica*.[17] The purified enzyme was able to effectively degrade alginate and required salt for its activation of the same level as that in seawater.[18] Accordingly, we expected to obtain a high yield and viability of protoplasts from *L. japonica* by using this enzyme.

Materials and Methods

Preparation of Alginate Lyase

Alginate lyase from *Alteromonas* sp. H-4 was purified from the culture supernatant by the following method as previously reported[18]: ultrafiltration, gel filtration with Sephadex G-100, and anion-exchange chromatography with DEAE-Sephadex A-50. For isolation of protoplasts, each active fraction of alginate lyase in the purification process was dialyzed against a hypertonic solution containing 0.7 M mannitol, 25 mM $MgCl_2$, and 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) in 50% seawater (pH 7.8) overnight at 0–4°C, prior to use.

Preparation of Enzyme Mixture

The enzymes used were cellulase (Cellulase Onozuka R-10, Yakult) and alginate lyase as described above. The enzyme mixture of cellulase and alginate lyase was prepared in 5 m*l* of the above-mentioned hypertonic solution, giving a final concentration for each enzyme at 0, 1.5, 3.0% and 0, 15, 30 U/m*l*, respectively. One unit of alginate lyase was defined as previously described.[18]

* Laboratory of Microbiology, Faculty of Fisheries, Hokkaido University, Minato, Hakodate, Hokkaido 041, Japan (澤辺智雄，絵面良男，木村喬久：北海道大学水産学部微生物学講座).

Sporophyte of Laminaria japonica

The young sporophytes (0.5–1.0 cm in length) used in this study were obtained from Osatsube kombu nursery facilities in Hokkaido on October 1989 and cultured in PESI medium[5] at 15°C under a photopeirod of 14: 10 h LD, 1000 lx for 1–2 days, prior to use.

Isolation of Protoplast

The young sporophytes of *L. japonica* were chopped into fine pieces by a razor, and the pieces were rinsed several times with the hypertonic solution mentioned above. Approximately 0.5 g of the sample was added to 5 m*l* of the enzyme mixture in a 50-m*l* Erlenmeyer flask or a plastic dish (50×10 mm; Terumo Co., Ltd.). The flask was gently shaken on a rotary shaker (40–50 strokes/min) for 3–15 h at 15°C. After enzyme treatment, the contents of the flask were gently filtrated through an 80 μm mesh nylon filter.

The number of protoplasts was determined using a haemacytometer. To confirm digestion of cell walls, protoplasts were stained with 0.1% Calcoflour White M2R (Sigma Chem.) in a hypertonic solution and were observed using an epifluorescence microscope (Olympus, Vanox-AHB) equipped with an appropriate UV filter system.[19]

Cell viability of the protoplasts was confirmed by staining cells with 0.02% neutral red (Kanto Chem.) or 0.02% Evans blue (Sigma Chem.) for 30 min at 15°C. Viability was assessed by the ability of the cells to incorporate neutral red and exclude Evans blue.[20]

Protoplast yield and viability were calculated as the average of duplicate samples in all conditions tested.

Results

Effect of Concentration of the Purified Alginate Lyase and Cellulase Onozuka in the Isolation of Protoplasts

The results of protoplast isolation from the sporophytes by the treatment of various enzyme mixtures for 3 h at 15°C are presented in Table 1. The single use of individual enzymes did not release any protoplast. Protoplasts were obtained only when both enzymes were combined. The highest yield of protoplasts was 1.2×10^6 cells/g in an enzyme mixture containing 1.5% Cellulase Onozuka and 30 U/m*l* purified alginate lyase, and in this case, the viability of isolated protoplasts was more than 80%. Viability was slightly higher with a low concentration of both enzymes and the yield was in reverse. The number of viable protoplasts released was highest with the use of 30 U/m*l* alginate lyase with 1.5% Cellulase Onozuka R-10 (Table 1).

The protoplasts isolated from the sporophytes by the treatment of enzymes were brown-greenish, spherical, and 10–25 μm in diameter (Fig. 1). The absence of Calcoflour fluorescence, which is present in sporophytes with cell walls, was observed in isolated cells. Therefore, we confirmed the removal of the cell wall in the protoplasts.

Effect of Purity of Alginate Lyase for Isolation of Protoplasts

Based on the above results, the optimum production of protoplasts was attained by combining 30 U/m*l* of alginate lyase and 1.5% Cellulase Onozuka in the hypertonic solution for 3-h treatment at 15°C. Then, the effect of purity of alginate lyase for protoplast production was confirmed. The yield and viability of protoplasts are summarized in Table 2. The yields of proto-

Table 1. Effect of alginate lyase and cellulase concentrations on protoplast isolation from *L. japonica* sporophytes

Purified alginate lyase (U/m*l*)	Cellulase Onozuka R-10 (%)	Yields*1 (cell/g)	Viability*2 (%)	Number of viable protoplasts*3 (log)
—	3.0	0	—	—
30.0	—	0	—	—
30.0	1.5	1.2×10^6	81.8	5.99
30.0	3.0	6.5×10^5	87.5	5.75
15.0	1.5	5.5×10^5	90.0	5.69

*1 Number of protoplasts derived from 1 g sporophytes (fresh weight).
*2 Ratio of viable protoplasts (%).
*3 Calculated by the following formula: log (yield × viability/100).
Enzyme treatment was operated at 15°C for 3 h.

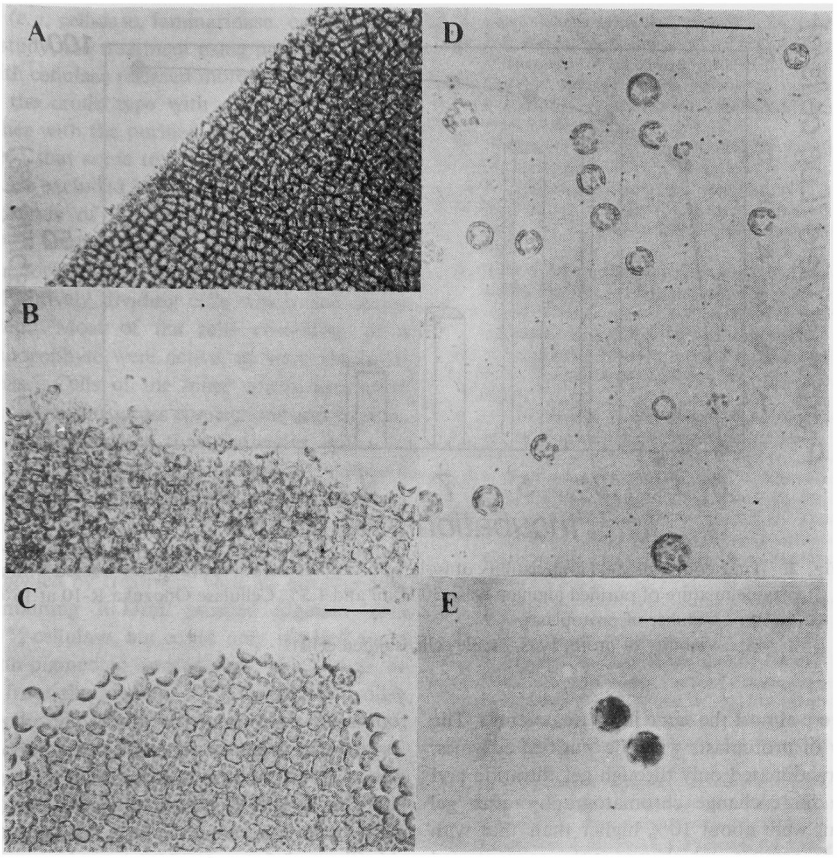

Fig. 1. Isolation of protoplasts from sporophytes of *L. japonica* treated with purified alginate (30 U/m*l*) and 1.5% Cellulase Onozuka R-10 at 15°C.
(A): Untreated sporophyte tissue.
(B): Sporophyte treated with enzymes for 1 h.
(C): Sporophyte treated with enzymes for 3 h.
(D): Protoplasts released from sporophyte after 5-h treatment.
(E): Viable protoplasts taking up neutral red.
Scale of bar is 50 μm.

Table 2. Effect of purification level of the alginate lyase on protoplast isolation from *L. japonica* sporophytes

	Purification step*1	Yields*2 (cells/g)	Viability*3 (%)	Number of viable protoplasts*4 (log)
(1)	Ultrafiltration	2.5×10^8	72.0	6.26
(2)	Gel filtration: Sephadex G-100	2.5×10^8	82.9	6.32
(3)	Ion-exchange: DEAE-Sephadex A-50	3.9×10^8	81.4	6.50

*1 30 U/m*l* of each step lyase was combined with 1.5% Cellase Onozuka R-10.
*2 Number of protoplasts from 1 g of sporophytes (fresh weight).
*3 Ratio of viable protoplasts (%).
*4 Calculated by the following formula: log (yield × viability/100).
Enzyme treatment was operated at 15°C for 3 h.

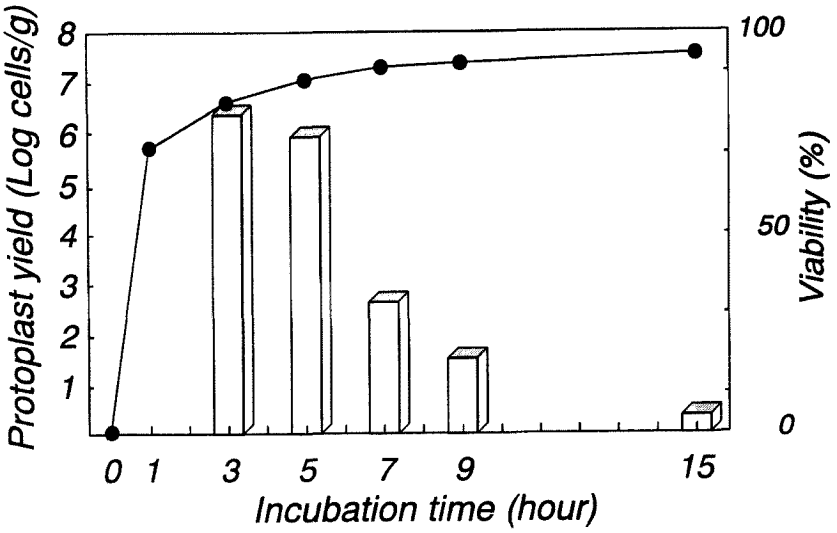

Fig. 2. Time course of yield and viability of isolated protoplasts from sporophytes treated with an enzyme mixture of purified alginate lyase 30 U/m*l* and 1.5% Cellulase Onozuka R-10 at 15°C.
●: Number of protoplasts.
□: Viability of protoplasts (viable cells/isolated cells).

plasts were almost the same in all treatments. The viability of protoplasts with the purified enzymes, which fractionated only through gel filtration and through ion-exchange chromatography after gel filtration, were about 10% higher than that with the crude enzyme, which was only concentrated in the culture solution with ultrafiltration, and the yield was slightly higher in the highest purified enzyme, through gel filtration and ion-exchange chromatography.

Time Course of Yield and Viability of Protoplasts

The time course of yield and viability of protoplasts in the optimum enzyme mixture as described above is shown in Fig. 2. For 5-h treatment at 15°C, the yield of protoplasts was 1.3×10^7 cells per 1 g of the young sporophyte. Numbers increased to give a final yield of 2.4×10^7 cells/g until 15 h, but for longer incubation periods the viable protoplasts began to show signs of deterioration, and viability was less than 20% after 9 h and less than 10% after 15 h incubation.

Discussion

Protoplasts were isolated from the young sporophytes of *L. japonica* by enzyme treatment with the bacterial alginate lyase. In this study, optimum conditions for the isolation of viable protoplasts were as follows: the composition of the enzyme mixture was purified alginate lyase (30 U/m*l*) with Cellulase Onozuka R-10 (1.5%) in a hypertonic solution (0.7 M mannitol, 25 mM $MgCl_2$, and 5 mM HEPES in 50% seawater, pH 7.8), and the incubation time was 3–5 h at 15°C. The yields of protoplasts and their viability were higher than in a previous report.[6]

Kloareg and Quatrano[21] have suggested that the cell wall of Phaeophyta have complex structures and that cellulose chains are organized in crystalline microfibrils which are embedded in a three-dimensional continuous alginate network. Alginate and cellulose may be linked by glycoprotein. No protoplast was obtained by single treatment with alginate lyase or cellulase, but the cooperated interaction of purified alginate lyase and cellulase permitted the isolation of protoplasts from sporophytes of *L. japonica* (Table 1 and Fig. 1). It was assumed that both enzymes were required to break a three-dimentional network of cell wall and to isolate protoplasts.

Butler *et al.*[7] reported that purified mannuronate specific lyase released more protoplasts of *L. saccharina* than the crude type. But cell wall materials were degraded more rapidly with crude alginate lyase which might have many kinds of

enzyme (*e.g.* cellulase, laminarinase, or protease). In this study, the treatment using purified alginate lyase with cellulase released more protoplasts than that of the crude type with cellulase. Viability was higher with the purified type (Table 2). It is speculated that some toxic materials in the crude enzyme are excluded by purification.

The fronds of *L. japonica* have three tissue layers:[22] a central medulla, an inner cortex, and an outer cortex. The outer cortex layer consists of small actively dividing cells which are deeply pigmented. Most of the cells consisting of a young sporophyte were active, as were the outer layer cells. Cells of the inner cortex are larger and bear numerous cross connections and hyphae, and are less pigmented than the outer cells. In the medulla, elongated cells are spread out in a mucilage. Cells of the medulla do not have any pigments.

We tried to isolate protoplasts from adult thalli of *L. japonica* by treatment with an enzyme mixture containing 30 U/m*l* purified alginate lyase with 1.5% cellulase, but could only isolated some large non-pigmented protoplasts, which may be derived from the medulla. We could not isolate pigmented cells from the outer layer (data not shown). To regenerate the protoplasts, actively dividing cells should be isolated from the outer layer of the adult thallus or yound sporophyte, which have a high capability of regeneration. The young sporophytes tissue seems to be more amenable to the enzyme system than that of the adult plant, and most cells were actively dividing and pigmented. Therefore, to establish the culture conditions of the *Laminaria* protoplasts, young sporophytes are an adequate material.

Further study will also be required to isolate the protoplasts from adult thalli of *Laminaria* sp. by this enzyme system. Then we will try to regenerate protoplasts which are released from young sporophytes and adult thalli.

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