Isolation and Purification of Protoplasts from *Porphyra tenera* Thalli*

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김엽체에서의 원형질체분리와 정제

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ABSTRACT

Protoplasts were isolated enzymatically from thalli of *Porphyra tenera* in relation to its utilization in breeding. As the concentration of the enzymes and incubation period increased, so did the yield of protoplasts. When a 250 mg fresh weight of the thalli was incubated in the 12% abalone digestive enzyme mixture at 22 ℃ for 3 hours, about 2.5×10^6 protoplasts were released. The size of protoplasts ranged between 9 μm and 25 μm, with an average of 14.5 μm.

요 약

김 (*Porphyra tenera*)의 생물공학적인 육종법개발을 위해 그의 엽체를 효소처리하여 원형질체를 분리하고 정제하였다. 원형질체의 수율은 효소의 농도와 효소액에 의한 조직의 처리시간에 비례하였다. 12%의 전복 내장 건조 분말로 조제된 효소액으로 22℃에서 3시간동안 처리했을때 약 2.5×10^6 개의 원형질체가 분리되었다. 원형질체들의 크기는 9~25 μm로 다양했으며 평균 14.5 μm였다.

INTRODUCTION

In recent years considerable attention has been paid to the development of protoplast technology. The absence of cell walls around protoplasts provides an opportunity for genetic modification by inducing the uptake of foreign particles, like DNA and RNA molecules, intact virus particles, microbes and organelles on daughter cells and subsequently regenerated plants. Moreover, the ability of plasma membrane to fuse under certain conditions allows the fusion of similar or contrasting cell types through the process of somatic hybridization ultimately leading to the production of somatic hybrids and cybrids.

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With the world population expanding and energy needs increasing, it is inevitable that the ocean surface as well as the land will have to be used for cultivation of energy rich biomass (North, 1980). Since the marine multicellular algae (seaweeds) is now being seriously considered as promising solar-energy converters, we aspire to convert them from seaweeds to sea crop plants. It is, therefore, greatly expected that protoplast technology will be a necessary adjunct tool in breeding program of seaweeds. Although considerable success of the protoplast technique in higher plants has been already achieved, little has been reported on protoplast of seaweeds to date (Saga et al., 1986).

The red seaweed *Porphyra* is one of the important edible seaweeds and its cultivation is a major aquacultural enterprise in Korea. It is, therefore, hoped that protoplast technology can be applied to improve the genetic qualities of the cultivated *Porphora* strains. Only recently fundamental attempts for isolation of *Porphyra* protoplast have been made (Polne-Fuller et al., 1984 ; Saga et al., 1986) and fusion experiments were assessed (Fujita and Migita, 1987 ; Araki et al., 1987). Here we wish to report our experimental results on the isolation and purification of protoplasts from cultivated *Porphyra tenera* thalli.

MATERIALS AND METHODS

Vegetative thallus of *Porphyra tenera,* which is the most popular species in Korea, was used as a source of all experiments. The plants were harvested from a cultivated population at Yeosu, in November, 1988.

The thalli for protoplast isolation were washed three times with sterile sea water and pretreated in 50 mM Tris-HCl buffer, pH 7.4, containing 2.5% papain (Sigma) and 0.7 M mannitol at 22℃ for 15 min. After this period the tissue were rinsed 3 times with 25 mM MES buffer containing 0.7 M mannitol, 5 mM $CaCl_2$, 2% NaCl, 0.5% potassium dextran sulfate at pH 6.0. The thalli were drained semi-dry condition on sterile filter paper, sliced into small pieces of segments about 1~2 mm using a No. 11 scalpel and about 250 mg fresh weight was transferred to 60×15 mm plastic petri dishes containing 5 mℓ solution of an abalone digestive enzyme mixture. The six different enzyme mixtures were prepared as follows. 3 g, 6 g and 12 g of abalone acetone powder (Sigma) were dissolved in 100 mℓ of two different extraction solutions of which those were 25 mM MES buffer and sea water containing 0.7 M mannitol, 5 mM $CaCl_2$, 2% NaCl, 0.5% potassium dextran sulfate at pH 6.0, and followed by maintained for 20 h at 4℃. After centrifugation at 17,000 rpm for 20 min, the supernatant was sterilized with 0.22 μm membrane filter and stored at 4℃ until use. The incubations were carried out at 22℃ on a reciprocating shaker a speed of at 60 rpm. After 1~3 hr digestion plates were swirled to release protoplasts and the solution was removed with a pasteur pipet to a 52 μm stainless steel sieves over 15 mℓ conical centrifuge tubes. Protoplasts were pelleted at 700 rpm for 5 min and the pellet was washed 3 times by repeated suspension and centrifugation in ASP_{12} medium (Provasoli, 1968) containing 0.5 M mannitol. Protoplast number was estimated with a hemocytometer and it was maintained in Nunclon plastic petri dishes at a

density of 5×10^5 cells/㎖ in ASP_{12} medium containing 0.2 M mannitol. Dishes were maintained at a temperature of 18 ℃ in 12 h photoperiods under fluorescent light of 1000 lux.

RESULTS AND DISCUSSION

Protoplasts are easily isolated in many higher plant species by enzymatic removal of cell walls as pioneered by Cocking (1960). It has become possible by the availability of a number of commercial cell wall degrading enzymes. But the cell walls of *Porphyra* thallus have been proved to be resistant to the same enzymes used for dissociation of higher plants. These results may be caused by their specific cell wall composition, i.e., β-1, 4 mannan, β-1, 3 xylan, and porphyran (Kito, 1985). Although the purified enzymes for degrading the *Porphyra* cell wall components are not commercialized until now, *Porphyra* thallus protoplasts have been obtained using the abalone acetone powder which have the activity of porphyranase, β-1, 4 mannanase, and β-1, 3 xylanase capable of degrading the cell wall of *Porphyra* (Araki et al., 1987). The cell structure of thalli was different from each other by the age of tissue (Fig. 1a-c). The more the thalli was old, the more cell size was large. An additional obstacle to be removed for protoplast isolation from the thalli is the protein layers on the surface of a thallus which prevents the penetration of the enzymes. In the present experiments we used only the thalli of about 10 cm in length for isolation of protoplasts. The pretreatment of 2.5% papain solution was very effective for softening *Porphyra* thalli as described by previously (Araki et al., 1987). The cuticle layers were partially dissolved (Fig. 1d) and will be helpful to penetration of cell wall digesting enzyme solution. In this experiment, six different enzyme mixtures were assessed for protoplast isolation from *Porphyra* thallus. Protoplasts were readily isolated from the tissue treated with the enzyme mixture described. Protoplasts began to be released about 30 minutes after shaking the tissue in the enzyme solution (Fig. 1e). Most treatments were effective on protoplast isolation. However, protoplast release was poor if the abalone-digestive enzyme mixtures were extracted for short time (below 5 hours). After 3 hours of incubation, protoplast-enzyme mixtures shown a large population of protoplasts (Fig. 1f). If the proposed techniques of seaweed improvement using protoplasts are to be applied to the *Porphyra*, an important prerequisite is the efficient production of protoplasts from thalli. Fig. 1g shows a typical protoplast preparation following the purification procedures. This method would appear to have general applicability for isolation and purification of protoplasts from *Porphyra* thallus. Yields of the protoplasts were significantly influenced by a variety of factors. In general, as the concentration of the enzymes and incubation period increased, so did the yield of protoplasts (Fig. 2). Using a 12% abalone solution extracted with MES buffer, 2.5×10^6 protoplasts were obtained from 250 mg fresh weight of the thallus. In contrast, when using the enzyme mixture prepared with sea water, the yield was significantly decreased as 5.5×10^5 cells. Under a suitable condition it was demonstrated that 1 g abalone acetone powder exhibited the enzyme activity of 1.4 unit porphyranase, 12 unit mannanase and 0.77 unit xylanase (Araki et al., 1987). They suggested, therefore, that 12% abalone enzyme will be sufficient

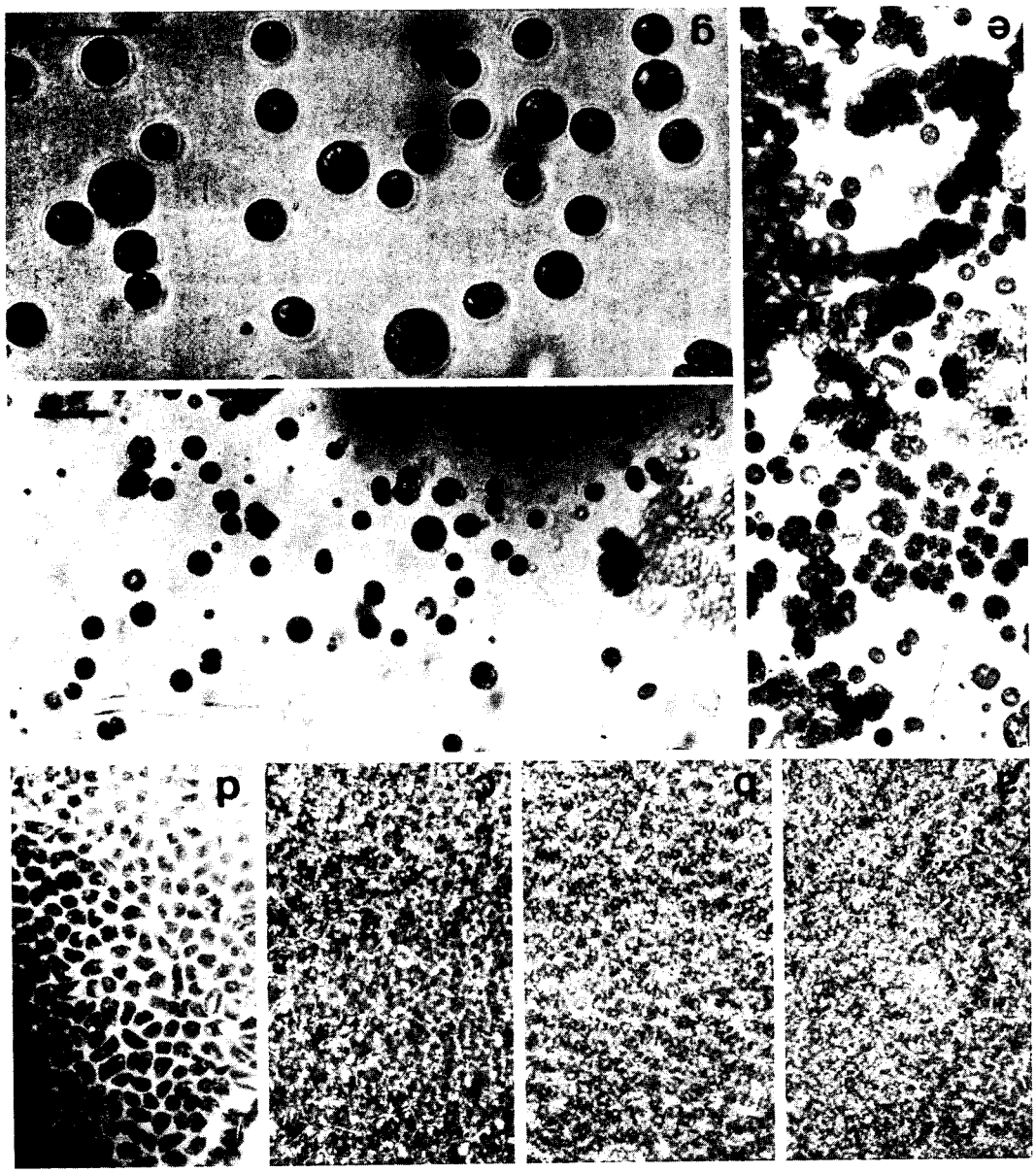

Fig. 1. Photographs of thalli and isolated protoplasts. Protoplasts were isolated using 6% abalone enzyme mixture from 10 cm long thallus. a-c, Microscopical thalli of 5 cm, 10 cm and 15 cm long respectively. d, Thalli pretreated with 2.5% papain solution for 15 min. e, Protoplasts isolating from intact tissue. f, Freshly isolated protoplasts. g, Purified protoplasts in liquid ASP_{12} medium. Same magnification in a-f. The horizontal bars represent 50 μm.

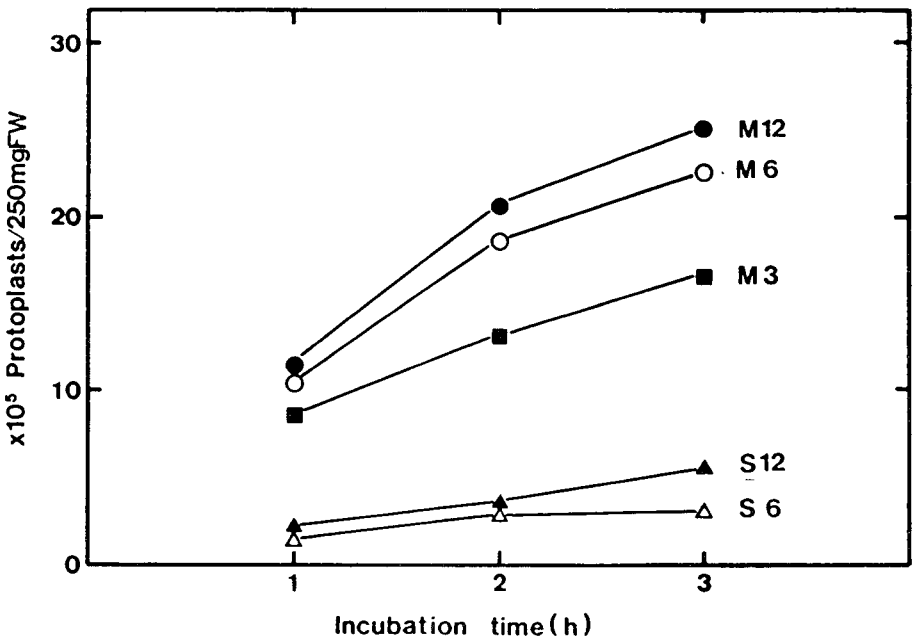

Fig. 2. Effects of enzyme mixture and incubation time required on efficiency of protoplast isolation from *Porphyra* thalli. M (MES) and S (sea water) represent extraction solution and numbers mean the concentration of abalone powder. Each point represents the mean of 3 trials.

for isolation of protoplasts from *Porphyra* thalli. In this experiment we could identify 6% abalone enzyme also can be applicable, but the protoplast yield was significantly decreased using 3% abalone enzyme. The protoplasts examined microscopically were found to vary considerably in size but characteristically spherical (Fig. 3). The size

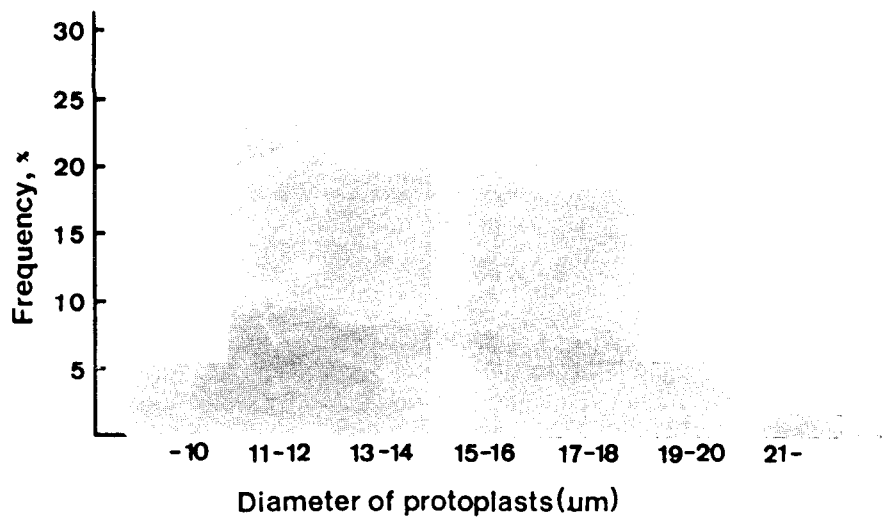

Fig. 3. Size distribution of protoplasts isolated from 10 cm long thallus. Frequency represents the percentage of protoplasts in a random population of 200.

of protoplasts ranged between 9 μm and 25 μm, with an average of 14.5 μm and the majority were 11~18 μm in diameter. It is anticipated that protoplasts will serve as a material for genetic improvements of marine algae. As the first step in an attempt to apply cell culture technique in *Porphyra* breeding, isolation and purification of protoplasts were successful in this experiments. The results will facilitate the use of protoplasts in studies on genetic manipulation of *Porphyra*. As a result, these protoplasts are now being used in experiments on in vitro regeneration which will be detailed in future reports.

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