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Toshiyoshi Araki and Tatsuo Morishita: Isolation and regeneration of protoplasts from *Bangia atropurpurea*[1)]

Key Index Words: Bangia atropurpurea—*β-1,4,-mannanase—porphyranase—protoplast—β-1,3-xylanase.*

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The establishment of the method for culture of experimental marine algae in laboratory and protoplast isolation will do a major service to the expand of marine algae biotechnology. A red alga, *Bangia atropurpurea* (Geesink 1973), belonging to the same family as *Porphyra* releases monospores and the fronds required in experiment are obtained easily by culturing the monospores in all seasons. This alga is, therefore, expected as a useful experimental material for the basic study on marine algae biotechnology. There have been some papers on protoplast isolation from *Porphyra* which is a popular edible alga in Japan and Korea (Saga and Sakai 1984, Poline-Fuller and Gibor 1984, Fujita and Migita 1985, Chen 1987) but no report on that from *Bangia*. Both cell walls of *Bangia* and *Porphyra* are composed of three kinds of unique polysaccharides, β-1,4-mannan, β-1,3-xylan, and porphyran (McDowell 1967). For isolation of protoplasts from their fronds are, therefore, required three kinds of enzymes capable of degrading these constituent polysaccharides. We have also published on isolation and regeneration of protoplasts from *Porphyra yezoensis* and *P. tenera* (Hatate *et al.* 1986, Araki *et al.* 1987a) by using the cell wall-digestive enzyme solution which contains β-1,4-mannanase, β-1,3-xylanase, and porphyranase prepared from *Aeromonas* sp. F-25 (Araki and Kitamikado 1981), *Vibrio* sp. AX-4 (Araki *et al.* 1987b), and *Vibrio* sp. AP-2 (Aoki *et al.* 1990) isolated from natural habitats, respectively.

In this paper, we have attempted to isolate protoplasts from *B. atropurpurea* by using the cell wall-digestive enzyme discribed above. The fronds of *B. atropurpurea* used in this experiment were collected at the mouth of a river flowing into coastal sea in Fukuoka Prefecture. The fronds were cultured in a ASP_{12} (NTA) medium of Provasoli (Provasoli *et al.* 1957) at 17°C under a 9 h light/15 h dark cycle. The artificial light intensity was adjusted to 48 $\mu Em^{-2} s^{-1}$ on the outer surface of the Petri dish. The fronds have so far been maintained in our laboratory by culturing monospores and fronds repeatedly since January 1990. The process for protoplast isolation from *Bangia atropurpurea* are as follows. The fronds (3–5 cm in length) were placed into 30 ml of a papain solution (20 mM MES buffer, pH 7.5, containing 2% papain and 0.7 M mannitol) in 100 ml-beaker, and incubated at 22°C for 15 min with shaking. After washed with ASP_{12} (NTA) medium containing 0.7 M mannitol several times, they were cut into a few mm in length with a razor. The fragments were placed into 8 ml of the cell wall-digestive enzyme solution (1 unit each β-mannanase, β-xylanase, and porphyranase, and 0.7 M mannitol in 20 mM MES buffer, pH 6.0) in 30 ml-Erlenmeyer flask and incubated at 22°C for 60 min with agitation. One unit of β-mannanase, β-xylanase, and porphyranase was defined as the amount of enzyme that produces reducing sugar correspond to 1.0 μmol of D-mannose, D-xylose, and D-galactose per min from β-1,4-mannan, β-1,3-xylan, and porphyran, respectively. As shown in Fig. 1-A, a large number of protoplasts were observed. The protoplast suspension which passed through 40 μm of a nylon mesh was centrifuged at

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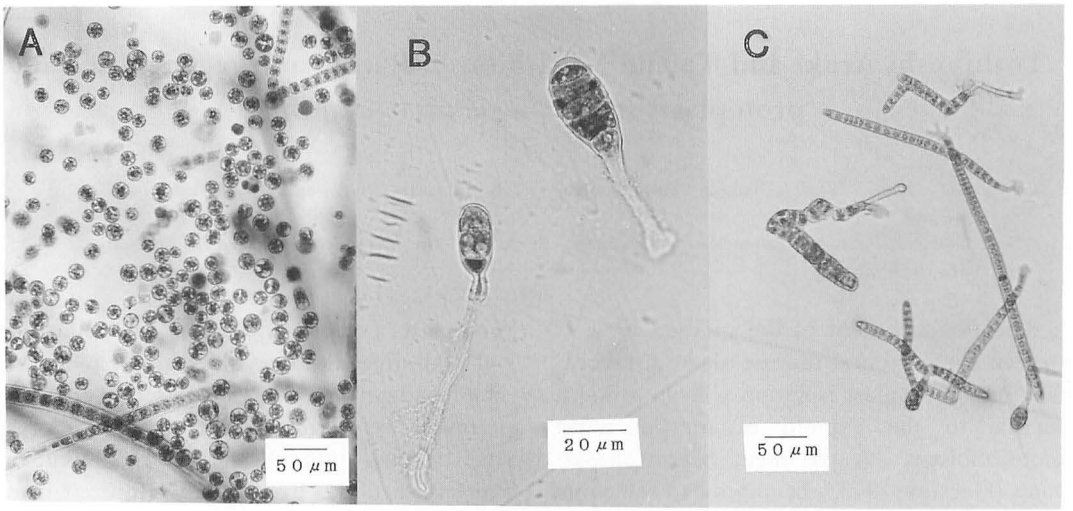

Fig. 1. Regeneration of protoplasts from *Bangia atropurpurea*. A, protoplasts from the fronds; B, dividing cells after 10 day-culture; C, regeneration fronds after 18 day-culture.

2,000 rpm for 5 min. The collected protoplasts counting was done with a Thoma Hemacytometer so that $(2.8 \pm 0.05) \times 10^6$ protoplasts were found to be isolated from the razor cut fragments (about 100 mg in wet weight). The protoplasts were, continuously, washed in order with ASP_{12} (NTA) medium containing mannitol of 0.7 M, 0.5 M, and 0.2 M by centrifugation. Protoplasts were pipetted onto some glass slides (26 × 26 mm) in a 9-cm Petri dish containing 50 ml of ASP_{12} (NTA) medium with 0.2 M mannitol and incubated in the dark at 17°C for 2 days. The protoplast-attaching glass slides were transferred into a Petri dish containing ASP_{12} (NTA) medium and exposed to the artificial light of about 16 $\mu Em^{-2} s^{-1}$ on a 9 h light/15 h dark cycle. The cell wall and rhizoid were formed 1 day and 2-5 days after lighting, respectively. Cell division was also observed after 2-5 days in lighting culture. Then they were cultured with gentle agitation under about 48 $\mu Em^{-2} s^{-1}$ (Fig. 1-B). They developed into small plantlets of 100-600 μm long 2-3 weeks after culture with agitation (Fig. 1-C) and grew up over 10 cm long after 8-9 weeks. The majority of them grew up fronds of regular shape but some became irregular shape. The rate of protoplasts which regenerated into plantlets was about 40% in the ASP_{12} (NTA) medium. The regenerate fronds of irregular or regular shape released monospores after approximate 5 weeks culture and most of them grew into regular fronds.

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荒木利芳・森下達雄：ウシケノリ (*Bangia atropurpurea*) からのプロトプラストの分離と再生

室内で年間を通して容易に培養できる海藻の開発とプロトプラスト単離法の確立は海藻バイオテクノロジーの基礎研究を行なう上で有益であろう。紅藻ウシケノリ (*Bangia atropurpurea*) は単胞子とそれから生長した葉状体を繰り返し培養することにより，人工気象器内で3年以上葉状体を維持することができた。また，自然界から単離した3種類の細菌の酵素（β-1,4-マンナナーゼ，β-1,3-キシラナーゼ，ポルフィラナーゼの各1 unitの混合液）を用いて，22°C，60分間処理することにより本藻から $(2.8\pm0.05)\times10^6$ のプロトプラストを得ることができた。プロトプラストを ASP_{12} (NTA) 培地中，温度17°C，48 $\mu Em^{-2}s^{-1}$，1日9時間照明で培養すると，8－9週間後には10 cm以上の葉状体に生長した。（514 津市上浜町1515　三重大学生物資源学部）

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