Review

Macroalgae as the Source for Environmental Assessment

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Abstract Macroalgal tissues can be used as indicating materials for environmental assessment using several algal biotechnology techniques. As bioassay test organisms, macroalgal tissues are required as an axenic state for suitable biological indicators. Callus formation and blade regeneration under suitable culture conditions are also useful for the tests. Quantitative method using tetrazolium chloride or alamarBlue™ is devised on a rapid assessment of the seaweed viability. The use of RT-PCR especially differential display technique should provide the means for the detection and isolation of the responding genes induced by the environmental stress. Seaweed thriving in more environmental changes might contain more diverse biologically active substances.

Key words : Macroalgal, environmental assessment, RT-PCR

Introduction

Changes of environmental factors may increase the risk of abrupt and non-linear changes in many ecosystems, which would affect their composition, function, biodiversity, and productivity [3]. Multiple stressors including pollutants, eutrophication, altered habitat, and hydrological regimes as well as floods and droughts can impact resources through single, cumulative or synergistic processes, lowering the overall system stability [4]. Responses of biota to these environmental stressors are the integrated result of both direct and indirect processes which can be manifested as changes in abundances, diversity and fitness of individuals, populations and communities [1]. Distinguishing and integrating the effects of natural and anthropogenic stressors is an essential challenge for understanding and managing coastal biotic resources [15]. Assessment of natural competition or man-made pollution levels has been determined by analysis of macroalgal or seaweed tissues collected from the sea, sometimes following transplantation. Much greater use has been made of macroalgae tissues as test organisms in laboratory culture [5]. For the tissue culture or cellular responses, the material tissues at first needed as an axenic bacteria-free state from callus culture or axenic treatment from field collection. Then the tissues can be used to evaluate their environmental damage quantitatively. The damage-induced mRNA also can be picked up using the differential display technique. Some biologically active substances such as PCR inhibitors, anti-viral substances and anti-tumor substances were more frequently occurred from seaweed collected in environmentally diverse area, namely crossing area of cold current and warm current rather than seaweed in cold current only.

Axenic tissue culture as a material supply

As bioassay test organisms, macroalgal tissues are required as an axenic state for suitable biological indicators. The choice of test algae could be largely determined by its ecological dominance in habitats, rapid growth rate, easy culture and handling, and economical significance. For those criteria, the seaweed *Enteromorpha prolifera* could be recommended as one of the best species in this local area. The long thallus was brushed and sonicated (28 kHz) twice for 1 min to eliminate epiphytes. The tissue was immersed in an autoclaved seawater containing 1% Betadine and 2% Triton X-100 for 3 min. After rinsing with seawater,

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Table 1. Ratio of seaweed that have anti-PCR, anti-viral and anti-tumor activities*

	Numbers of active species/total species collected in Korea	Numbers of active species/total species collected in Canada
Anti-viral seaweed	15/27 (56%)	17/60 (28%)
Anti-tumor seaweed	5/27 (19%)	4/30 (13%)
Anti-PCR seaweed	8/27 (30%)	3/30 (10%)

*Anti-PCR seaweed was screened for the presence of *Taq*DNA polymerase inhibitors. Anti-viral seaweed was tested against Herpes simplex DNA virus and Sindbis RNA virus. Anti-tumor seaweed was screened for the presence of inhibitors against tumor-related protein kinases. Those substances were more frequently occurred from Korean seaweed collected in the crossing area of cold current and warm current rather than Canadian seaweed in cold current only.

it was then sterilized in antibiotics mixture at 18°C for 1 day [17]. In the growth tests, we placed 5 intercalary segments of 5 mm length in one ml of PES medium [18] containing 5 μL of test sample of methanol extract (40 mg/mL), and incubated the cultures at 18°C under 40 μmol/m^2 /s for 1 week. The percentage increase in length of the segments compared with the control was then used to assess the toxicity of thetest sample [6].

It has been widely known that a certain excised part of algal tissue has excellent powers of callus formation and blade regeneration under suitable culture conditions. Maintaining stock cultures on agar medium is usually free of contaminants, and the material can be available throughout the year with minimal genetic variation. Although a wide range of algae can be used for the tests, the brown alga *Hizikia fusiformis* was one of the most convenient and responsive species. Callus or blade regeneration of the *H. fusiformis* was formed differently depending on gelling agents under axenic culture conditions [10]. Excised cylindrical pieces (5 mm) of the holdfast were cultured on several gelling agents including PESI under 40 μmol/m^2/s at 18°C for 30 day. Usually the assessment of growth is based on the percentage number of segments showing signs of callus or blade regeneration in comparison to the control. Then forty-seven % of holdfast pieces produced callus on the PESI solid medium supported by 2% high gel strength agar among 7 gelling agents (Fig. 1). None of callus was formed in the liquid medium. Blades were formed in PESI liquid medium at the rate of 45%, meanwhile a solid medium supported by 0.5% high gel strength agar showed 30% of axenic blade formation asthe best gelling agent for solid support. Thus it is advisable to carry out the axenic bioassay tests for callus cell growth on 2% high gel strength agar or for blade regeneration on 0.5% high gel strength agar. Meanwhile, in the case of *Laminaria japonica*cultured on same condition, fifty-two % of stipe tissues produced callus on the PESI solid medium supported by 0.5% Phytagel among 7 gelling agents (unpublished data).

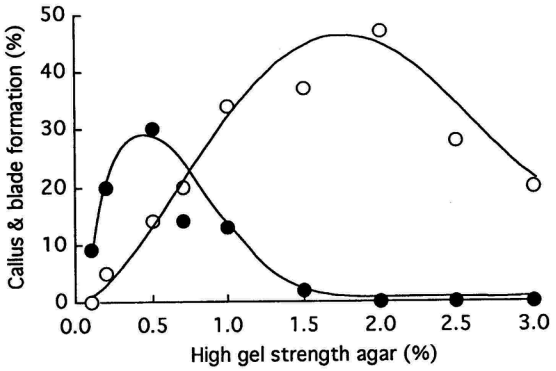

Fig. 1. Formation rate of callus (open circle) and blade (closed circle) from excised explants of the *H. fusiformis* rhizoid. Explants were cultured at 18 °C under 40 μmol/m^2/s for 1 month on PESI solid medium supported by high gel strength agar.

Viability assay for seaweed tissues using tetrazolium chloride

Instead of waitingfor a growth period, a number of test procedures such as bleaching effect, plasmolysis technique and viable dye technique have been devised which are based on a rapid assessment of the seaweed viability [5]. More reliable and quantitative methods based onthe metabolic activity of the tissue as a whole need to be developed. Thus we have modified an enzymatic reduction procedure of colorless 2, 3, 5-triphenyltetrazolium chloride (TTC) to a red-colored triphenylformazan (TPF), that was used to test viability in land plants quantitatively [19]. To allow accurate quantification of the TPF in the seaweed thallus, tissues are extracted with 0.2N KOH in 25% ethanol after incubation with TTC reagent in the dark at 20°C for 1 hr under oil layer. Then TPF is partitioned away by prompt addition of hexane and vortexing. By this proce-

dure, we observed nearly complete separation of TPF from seaweed pigments, moreover observed good spectrophotometric discrimination between TPF and other hexane-soluble pigments at 545 nm [14; 16]. The assay has proved applicable to a wide range of seaweed taxa including *Porphyra yezoensis*. In summing up; (1)Put 0.2 g of tissue (wet wt) into tube. (2) Add 4 mLof 0.8% TTC in seawater containing 50 mM Tris-HCl, pH 7.6. (3) Incubate in the dark at 20°C for 1 hr under a mineral oil layer. (4)Rinse tissues with seawater 3 times. (5) Extract with 2 mLof 0.2N KOH in 25% EtOH by heating for 15 min at 60°C. (6) Partition TPF into 2 mL of hexane. (7) Measure absorbance at 545 nm.

Another method for measuring cell viability and proliferation has become available using alamarBlue™. As with tetrazolium salts, alamarBlue™ monitors the reducing environment of the cells. AlamarBlue™ is soluble, stable in culture medium, and is non-toxic. The continuous monitoring of cells in culture is therefore permitted [2]. Because alamarBlueTM is non-toxic, the cells under study can be returned to culture or used for other purposes including histological studies. Viability or proliferation measurements may be made spectrophotometrically by monitoring the absorption of alamarBlueTM supplemented cell culture media at two wavelengths of 570 nm (reduced red) and 600 nm (oxidized blue). To calculate the percentage of reduced alamarBlue in culture: Viability (%) = $[A_{570} - (A_{600} \times R_0)]$ x 100, where $R_0 = A_{570}/A_{600}$ at 0 time.

Differential display of mRNA

Measurement of the body burden by environmental contaminants has been useful in establishing their uptake and impact in the organisms themselves. To understand the biological-chemical interactions within the cells, the use of RT-PCR especially differential display technique should provide the means for the detection and isolation of the responding genes induced by the environmental stress [8; 13]. This approach has been used in the study of *P. yezoensis*responding to acid treatment. An acid-labile mRNA in the tissue was identified by comparing the differential display to the control with arbitrary primers. Total RNA was extracted by the LiCl-guanidinium method from tissues treated in 0.05% hydrogen chloride (pH 3.0) for 5 min. After culture in PES medium for 30 min or 1 hr, the first-strand cDNA was synthesized by reverse transcription of total extracted RNA with random hexamers and amplified by PCR with an arbitrary primer OPA-1 (CAGGCCCTTC). The acid-shocked tissue revealed the disappearance of the acid-susceptible gene expression of dethiobiotin synthase (E.C. 6.3.3.3) (Fig. 2). Genetic responses of the seaweed *Ulva pertusa* to pine needle ash have been compared using differential display technique [11]. By DNA sequence analysis, an ash-inducible gene (342 bp) and an ash-suppressed gene (1690 bp) were identified as hypothetical proteins (data not shown).

Occurrence of biologically active substances

Fifty-seven species of marine macroalgae from the coasts of British Columbia, Canada, and Korea have been screened for the presence of PCR inhibitors, namely inhibitors of *Taq* DNA polymerase. 30% of Korean seaweed and 10% of Canadian seaweed species displayed some inhibitory activities at the concentration of 5 μg of methanol or water extract in 25 μL reaction mixture of PCR containing 1.5 unit of *Taq* DNA polymerase [9]. For antiviral activity, various concentrations of methanol extracts were tested against 100 plaque forming units of Herpes simplex DNA virus and Sindbis RNA virus in Vero cell monolayers. Among 89 seaweed species tested, 11 species inhibited both viruses, while 22species were active against only one of the viruses. Thus in total of 37 % of the species were active, and only two of these extracts showed cytotoxicity at the concentrations tested. The antiviral activities were proportionately more frequent in the Korean sea-

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5′-CAGGCCCTTCTACCACGACATGATCGACTTTTTCGGTCAGGTTTGCC
AGGCCGTTTGAAATGAGGGTGTAATTGATTGGGCAACTGTGCGCCACGCT
ACTTTCTTCTTCGCTTAACGCGATAGGATTAACTGCTTCATAAGGCAGTT
CGATGGTTGAAACACTCTGCAACACCAGGGCATCTTTATTACGCAGCCCT
TCGGGAGTCTCTTTGCTCCCCTTCGCTACGGGTTTATATCCCGCAACCGT
TTTTCCCTGGGAGGCTAACGCTTGTAGCAATGCGCGGGAAACCACCGTTT
TCCCTACAGAAGTGTCTGTACCGGTAATAAAGAAACGCTTCAGCATCACT
AACTCCACCTAAATGCTTCACAAATATAAACCAGGAAAATAATTAACCTT
GAAAGTCTAAGTTATGCTTTCCTGGCCTAAATTGAGATAGCGCAAATTTT
GGTAGAACAGTTAGAAAATGTTAACCCTGCAACAGACGAATCAACAAAGA
ACCGTTATACATCGCGTCTTTTACCAGTGCAGCGCCTGCCATCGTGCCCT
GGTTAGAAAACTGAGTACTCTCAACGCTGATGTGCTGACTATACGCAGGA
AAGGGCTG-3′
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Fig. 2. Nucleotide sequence of the acid-labile gene fragment (605 bp). The fragment was identified as a part of gene for dethiobiotin synthase (E.C. 6.3.3.3) by database similarity search. This gene expression was transiently repressed in the seaweed *P. yezoensis* by acid shock treatment at pH 3.0 for 5 min.

weeds (56%) compared to 27% of the Canadian seaweeds [12]. For antitumor activity, methanol extracts of seaweed have been screened for the presence of inhibitors against tumor induction-related protein kinases such as CDC-2, CK-II, ERK-1, and GSK-3b.The activity of these protein kinases increases in the disease state, e.g., cancer and diabetes. Among 70 seaweed species tested, 19% of Korean seaweed and 13% of Canadian seaweed species showed some activities against a certain protein kinase [7]. Most of the Korean seaweed have been collected at near Pusan and Pohang in Korea, those areas are in the crossing place of cold current and warm current. Canadian seaweed has been collected in the area of cold current only. From those results, we dare imagine that the seaweed thriving in more environmental changes contain more diverse biologically active substances. But still we need more experiments to confirm this kind of environmental impact on the occurrence of biologically active substances.

Acknowledgments

This research was supported by a grant from the Marine Bioprocess Research Center of the Marine Bio 21 Project funded by MOMAF, Korea. We thank the Brain Busan 21 program for graduate support (HQL).

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