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Biological Characteristics and Tissue Structure of a Crustose Coralline *Lithophyllum* Alga

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The disappearance of seaweed flora in some rocky areas, which is known as algal whitening, barren ground, coralline flats, or deforested areas, is associated with some species of coralline algae. To determine the biological characteristics of a representative species of crustose coralline alga, the 18S rDNA gene was sequenced to identify the genus *Lithophyllum*. According to its morphological and distributional characteristics, it was deduced to be *L. yessoense*. Viability was measured using triphenyl tetrazolium chloride and showed high viability from December to February. Culture conditions of 16°C, a 16 hr light, 8 hr dark cycle, and 30 $\mu E/m^2/s$ light intensity were optimal for maintaining the viability of the alga for up to five days. Included in the fatty acids was 9.7% ω-3 eicosapentaenoic acid. An electron microscopy scan of the surface structure revealed round craters about 3.6 μm in diameter, which were covered with rough, irregular, and angular polygon-shaped structures about 1.0 to 3.7 μm in size. Based on the composition and structure found in our study, biomimetic coralline alga might become an environmentally friendly antifouling material against the attachment of soft foulants.

Key words : Crustose alga, fatty acid, *Lithophyllum*, tissue structure, viability

Introduction

Seaweeds play a major role in marine ecosystems. They provide nutrients for animals - either directly when fronds are eaten, or indirectly when decomposing parts break down into fine particles and are taken up by filter-feeding animals. Beds of seaweed provide shelter and habitat for scores of coastal animals for all or part of their lives. Coralline red algae, both of crustose (nongeniculate) and articulated corallines, abound in intertidal rocky shore areas and strongly influence the benthic community. Crustose coralline algae are a major calcifying component of the marine benthos from tropical to polar oceans at all depths within the photic zone [20]. When the coralline algae are growing, the rock surfaces appear pink, while the fleshy seaweed flora disappears from the rocky areas. In marine environments, this phenomenon is generally called algal whitening, barren ground [22], coralline flats, or deforested areas. It is now recognized as a natural hazard adversely affecting marine ecosystems and damaging commercial fishing areas. Although biological [2, 11, 23] and physical [8, 15] factors may be sufficient to prevent the recruitment of fleshy seaweeds, allelopathic bromoform [16] and fatty acid [10, 14] substances may also inhibit the settlement or germination of seaweed spores. Consequently, coralline algae may prevent fouling by fleshy seaweeds. Alternatively, biomimetic coralline alga material might become an environmentally friendly antifouling material. In nature, most coralline algae are pink and have almost indistinguishable shapes, those causes confusion in their identification. Therefore, in an attempt to make a biomimetic coralline alga material, we have to identify the species, select the best conditioned tissues as standard material, analyze the fatty acid composition, and observe the fine surface structure.

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Materials and Methods

Plant material

Crustose coralline algae were collected monthly from the rocky intertidal area at Cheongsapo (35°09′28" N, 129°11′47" E), on the east coast of Busan, Korea. The samples were transported in a container with seawater to the laboratory. After rinsing well with autoclaved seawater to remove epiphytes and debris, the encrusted tissues were sonicated three times with 30-s pulses of an ultrasonic water bath (low-intensity frequency of 90 kHz) to remove other micro-epiphytes.

Molecular identification

Approximately 0.2 g of the coralline alga was chopped into very tiny pieces and placed in a microtube. DNA was extracted using LiCl, following Hong et al. [7]. The 18S rDNA gene, encoding the ribosomal RNA in the small subunit of eukaryotic cytoplasmic ribosome, was amplified by polymerase chain reaction (PCR) with the universal primers 18sF (5′CAACCTGGTTGATCCTGCCAGT3′) and 18sR (5′GATCCTTCTGCAGGTTCACCTACGGAA3′) [4]. The PCR cycling parameters consisted of 94°C for 5 min, 30 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 1.5 min, and a final 72°C for 10 min. The amplification products were sequenced using the same primers (SolGent, Daejeon, Korea). The sequences were edited and manipulated using MEGA3 [12]. Phylogenetic trees were inferred using the neighbor-joining algorithm [19] in MEGA3 with bootstrap analysis of 1,000 bootstrap replications.

Viability assay

To measure the viability of the coralline tissue, the assay of Park et al. [17] was used. Briefly, 1 ml of 0.8% 2,3,5-triphenyltetrazolium chloride (TTC) in seawater containing 50 mM Tris-HCl buffer (pH 8.0) was added to 50 mg of tissue in a 1.5-ml microtube and incubated in darkness for 1.5 hr at 20°C under mineral oil. The triphenyl formazan that formed in the tissue was extracted with 0.6 ml of 0.2 N KOH in 75% ethanol by heating for 15 min at 60°C. The triphenyl formazan was quantified by measuring the absorbance at 475 nm. To determine the optimal conditions for maintaining viability, 0.25 g of the tissue was cultured in 50 ml of seawater under 30 $\mu E/m^2/s$ light intensity on a 16 hr light:8 hr dark cycle at 16°C for 5 days as the standard conditions.

Scanning electron microscopy

Healthy tissue collected on February 9, 2012 was washed with Milli-Q water (Millipore, Billerica, MA) and dried under vacuum before scanning electron microscopy (SEM) analysis. For SEM images, tissues were mounted on conductive carbon tabs of a SEM post (Ted Pella, Inc., Redding, CA), sputter-coated using a Desk-II coater equipped with a gold target (Alfa Aesar, Ward Hill, MA), and imaged in a scanning-electron microscope (JSM-6700F; JEOL, Tokyo, Japan). To determine the elemental composition of parts of the tissues, the tissues were analyzed using energy-dispersive X-ray spectroscopy. The standards for carbon, oxygen, sodium, chlorine, and calcium were calcium carbonate, silicon dioxide, albite, potassium chloride, and wollastonite, respectively.

Fatty acid analysis

Fatty acids were determined by gas chromatographic quantification of their methyl esters (FAMEs), which were prepared using slightly modified method from the AOAC [3]. Total lipid was extracted from the dried samples using a Soxhlet extractor. Then, FAMEs were prepared with 5 ml of methylation solution (1 H_2SO_4:20 CH_3OH:10 toluene) and heated at 100°C for 1 hr. Gas chromatography-mass spectroscopy (GC-MS) analysis was conducted using a 6890 network GC system with a 5973N Mass Selective Detector (MSD) (Agilent Technologies, Palo Alto, CA). The oven was started at 50°C and held for 1 min, and then ramped up to 320°C at 5°C/min. The MSD was operated based on electron ionization.

Results and Discussion

A dominant crustose coralline alga from barren grounds was pinkish to reddish and generally characterized by their encrusted calcareous composition. They typically colonized rocky substrates and formed smooth and flat crust in the intertidal area exposed to waves. It is known that many areas of the rocky shorelines of Korea and Japan are dominated by crustose coralline algae such as *Lithophyllum yessoense* Foslie [9, 21]. The group's internal taxonomy is still in a state of flux; molecular studies are proving more reliable than morphological methods in approximating relationships within the group [5]. To ascertain the species identification, we determined partial 18S rDNA gene sequence (NCBI submission # 1609660). They were then aligned and analyzed

using the neighbor-joining method to construct a dendrogram. The 1,391-bp 18S rDNA sequence from base 274 to 1,664 was compared to the sequences of 13 species of coralline algae obtained from the NCBI database to infer the phylogenetic relationship (Fig. 1). The 18S rDNA sequence showed the closest to the sequence of *Lithophyllum incrustans*. The sequence matched 99% homology with 15 base difference from the *L. incrustans* sequence (GenBank accession # AF093410.1). Meanwhile, the *L. incrustans* is described as thick, dull chalky, yellowish, pink or lavender calcareous crusts forming irregular concretions, to 40 mm thick, margins ridged where crusts meet, in the AlgaeBase [6]. The alga used in this study has typically no margins ridged where crusts meet, and is not much thick, dull chalky and yellowish. Thus, the alga was identified to be belonging to the crustose coralline *Lithophyllum* genus using the 18S rDNA sequence, but not able to confirm the species level. By the morphological shape, the crustaceous thalli spread irregularly like a pinky patch, non-verrucose on surface. It is distributed in warm current sea along the coasts of Jeju and Busan, Korea. From the morphological and distributional characteristics, it may be deduced to *L. yessoense* [22].

Using the TTC method, we measured the viability of crustose coralline tissues collected at the same site throughout the year (Fig. 2) and quantified viability as the absorbance at 545 nm. The tissues collected in February had the greatest viability, which then decreased gradually in the spring and summer. Some tissues remained pinky crust, and some disappeared from the rock surface. In autumn, they started to grow, and most tissues recovered their viability. In December and February, the tissues again had the healthiest pink structure and greatest viability. Therefore, this period would be the best season to use as a model structure of a biomimetic antifouling material. To keep healthy tissues, we optimized the maintenance conditions using the TTC viability assay (Fig. 3). The optimal temperature for incubation was 16°C, the optimal light intensity was 30 μE/m^2/s with white fluorescent light, and the optimal light period was a 16 hr light:8 hr dark cycle. Under these conditions, the tissues maintained the best viability for up to 5 days in a standing flask containing natural seawater. Therefore, the tissues were kept at the optimized conditions and used within 5 days.

Among the factors preventing the settlement or germination of fleshy seaweed spores, we found that polyunsaturated fatty acids (PUFAs) have potent lytic activity against algal spores [14]. When preparing biomimetic coralline alga material, the antifouling activity might be enhanced by adding bioactive PUFAs. Therefore, we determined the major fatty acid composition of healthy coralline tissue (Table 1). Of the fatty acids, 23.4% were PUFAs, with ω-3 eicosapentaenoic acid (EPA; C20:5) comprising 9.7% and ω-6 arachidonic acid (AA; C20:4) comprising 5.1%; the ω-6:ω-3 ratio was 1.42. In the previous study [14], EPA and AA showed strong lytic activity against algal spore with LC_{50} = 2.1 and 1.8 μg/ml, respectively. Therefore, the preparation of biomimetic calcium carbonate nanoparticles with EPA and/or AA will have potent antifouling activity as an envi-

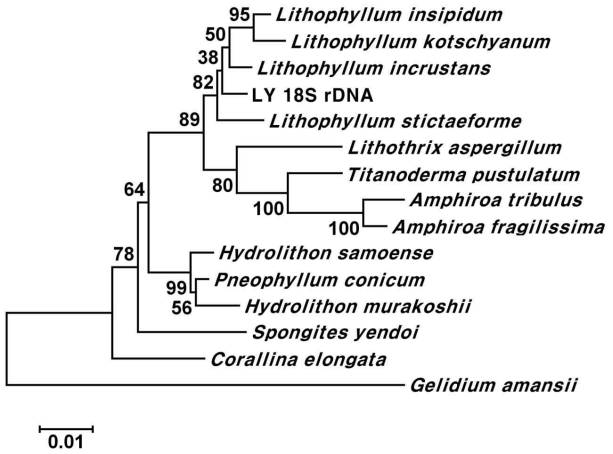

Fig. 1. Phylogenetic dendrograms of the crustose coralline alga based on 18S rDNA sequence, and constructed using the neighbor-joining method. Numbers at nodes indicate the level of bootstrap support (1,000 replicates).

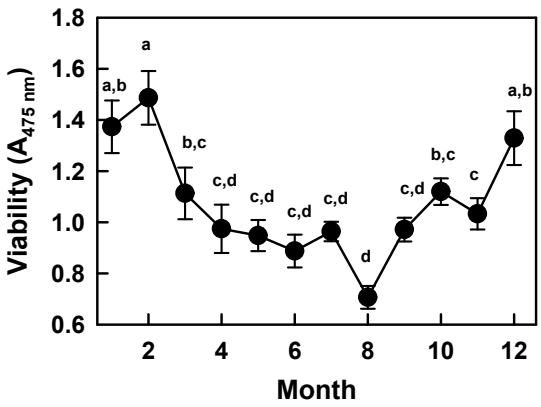

Fig. 2. Seasonal variation in the viability of the crustose coralline tissue. The viability was quantified using the absorbance at 474 nm, and the values are the mean±SD of at least five independent assays. Mean values with different letters (a-d) are significantly different by Duncan's multiple range test (p<0.05).

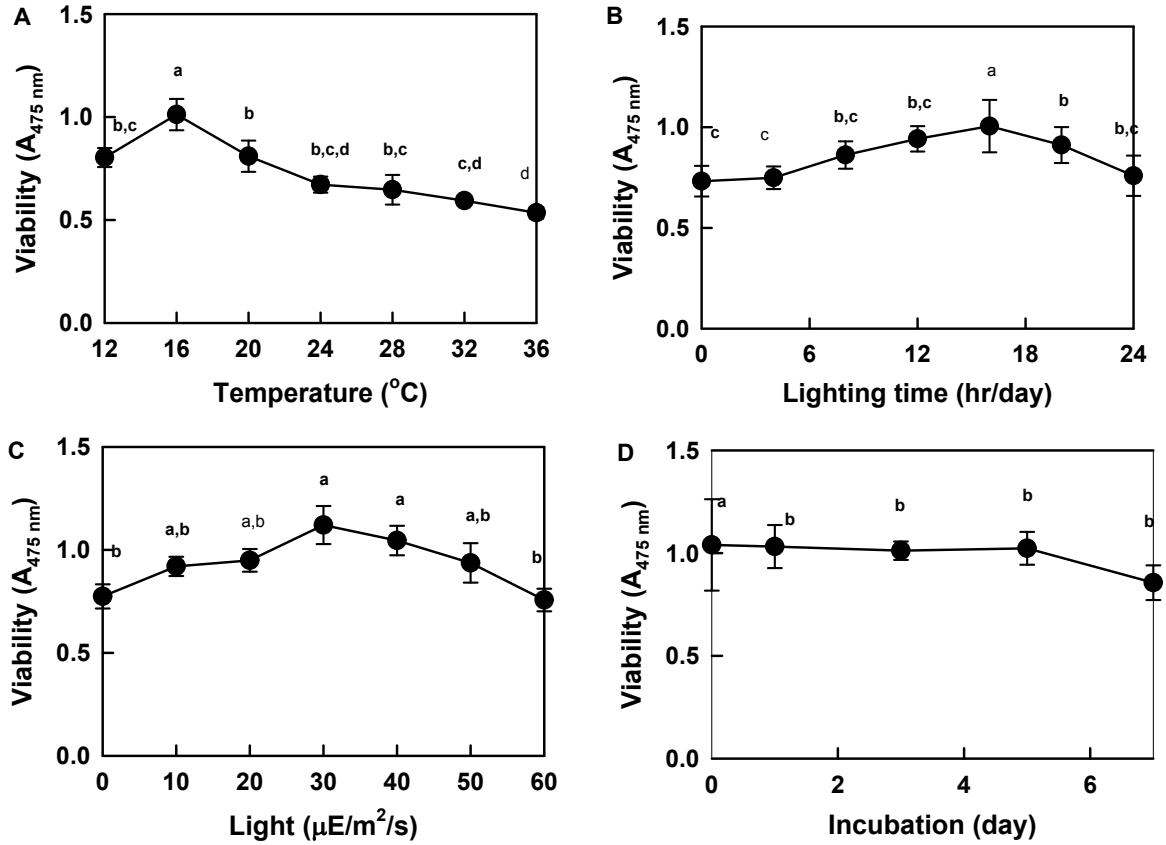

Fig. 3. Effect of various parameters on the optimal maintenance of crustose coralline tissue: (A) incubation temperature, (B) amount of light per day, (C) light intensity, and (D) incubation period under the optimized conditions. The viability was measured using the absorbance at 474 nm, and the values are expressed as the mean±SD of at least five independent assays. Mean values with different letters (a-d) are significantly different by Duncan's multiple range test (p<0.05).

Table 1. Profile of the major fatty acids (% of total fatty acids) in the crustose coralline tissue collected on February 9, 2012

Fatty acids	Relative amount (%)
C16:0	36.9
C18:0	11.6
C18:1 ω-9	1.2
C18:2 ω-6	3.2
C18:3 ω-6	1.5
C20:0	1.7
C20:4 ω-6	5.1
C20:5 ω-3	9.7
C22:0	11.4
C22:2 ω-6	3.2
C24:0	3.3
Saturated fatty acids	74.0
MUFAs	2.6
PUFAs	23.4

ronmentally friendly bio-control.

The surface structure of the crustose coralline tissue was examined using SEM. The tissue surface was covered with round craters about 2.5 to 5.0 μm in diameter at the surface (Fig. 4A). Most of these crater-shaped structures were irregular circles of average 3.6 μm in diameter. The upper rough tissue was covered with irregular and angular polygon-shaped structures about 1.0 to 3.7 μm in size (Fig. 4B). The average is about 2.1 μm in size. Some corallines slough off a surface layer of epithallial cells, which in a few cases may be an antifouling mechanism which serves the same function as enhancing herbivore recruitment [8, 15]. This also affects the community, as many algae recruit on the surface of a sloughing coralline, and are then lost with the surface layer of cells. This can also generate patchiness within the community. Sloughing in this case is probably a means of eliminating old reproductive structures and grazer-damaged surface cells, and reducing the likelihood of surface penetration by burrowing organisms. SEM-based energy-dispersive X-ray spectroscopy showed that the relative elemental composition was 50% carbon, 39%

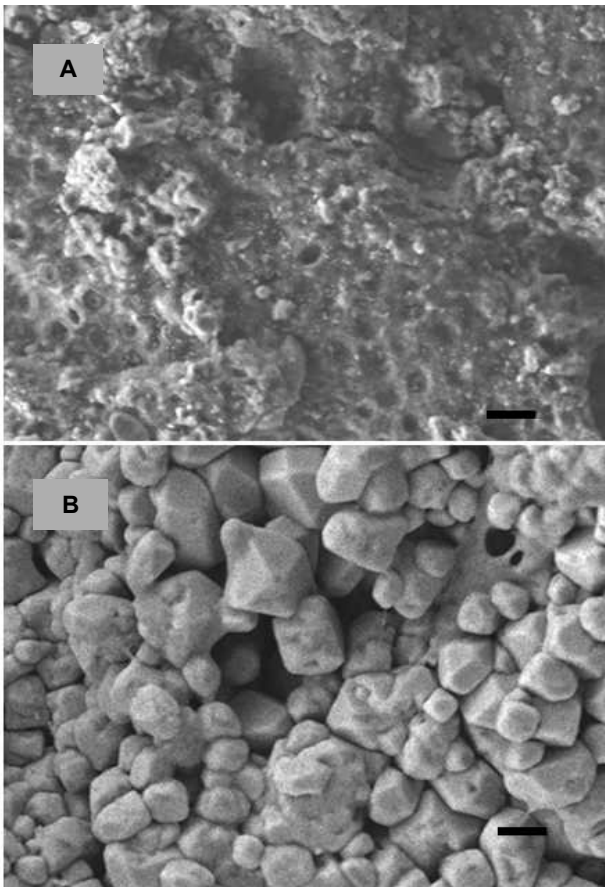

Fig. 4. Tissue of the articulated coralline alga. Scanning electron micrograph of the coralline tissue by 1,000 (A) and 5,000 (B) magnitude. The bars in A and B indicate 10 μm and 2 μm, respectively.

oxygen, 3% sodium, 2% chlorine, and 6% calcium by atomic percentage, or 37% carbon, 38% oxygen, 5% sodium, 5% chlorine, and 15% calcium by weight. The major mineral content was calcium carbonate. Calcification by crustose coralline algae is crucial to the formation and maintenance of coral reefs [13]. Coralline algae bind adjacent substrata and provide a calcified tissue barrier against erosion. They also serve as food for grazers - notably parrot fish, urchins, and starfish [1]. Coralline algae provide hard substrata for settlement and metamorphosis in a large diversity of marine invertebrate larvae, including abalone [18]. We found that allelopathic fatty acid substances prevented the settlement or germination of fleshy seaweed spores [10, 14]. Bromomethane released by the articulated coralline alga can eliminate epiphytic organisms, especially microalgae on the surface, and might induce the continuation of coralline flats in marine environments [16]. Crustose coralline algae are thus capable of limiting the local abundance of fleshy seaweed by reducing recruitment success. Furthermore, biomimetic materials derived from the crustose coralline algae, containing antifouling substances, might be developed to protect against the attachment of soft foulants, especially micro- and macroalgae.

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초록 : 해조류 무절산호조 혹돌잎의 생물학적 특성 및 조직구조

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연안 암반지역에서 해조류 군락의 소실 즉 백화 혹은 갯녹음현상은 산호조류와 관련성이 있다. 대표적인 무절산호조의 생물학적 특성을 파악하기 위하여 18S rDNA 유전자를 분석한 결과 혹돌잎(*Lithophyllum*) 속에 속하는 것을 확인하였고 그 형태적 특성으로 보아 *L. yessoense* 종인 것으로 유추된다. Triphenyl tetrazolium chloride로서 활력을 측정한 결과 12월에서 2월 사이가 가장 높았으며, 조직 활력을 유지하기 위하여는 16°C, 16:8 시간 명암광주기, 30 μE/m^2/s 광도에서 5일간 최적상태를 보였다. 지방산 조성에서는 EPA가 가장 많은 고도불포화지방산으로서 9.7%를 차지하고 있다. 주사형전자현미경에 의한 표면구조를 보면 평균 3.6 μm 직경의 둥근 함몰 분화구 모양을 이루며 그 위에 1.0 내지 3.7 μm의 비정형 다각형 구조물들이 덮여져 있다. 이 같은 조성과 구조를 바탕으로 한 생체모방 산호조는 해조류 등에 대한 환경친화적 방오소재로서 활용되어질 수도 있을 것이다.