微細藻類が産生する生物活性物質の検索

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Screening of Biologically Active Compounds in Microalgae*1

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As part of the investigation on useful constituents in microalgae, we searched for biologically active compounds in the water-soluble and the fat-soluble fractions of twenty five species and strains of microalgae by means of the antimicrobial activity test and the inhibition test of development of fertilized echinoderm eggs. We found eleven and eight active species by the former and latter test, respectively, and five species to have both activities. The activity existed exclusively in the fat-soluble fractions. For example, a marine cyanophyte *Oscillatoria* sp.-2, a freshwater diatom *Cyclotella* sp., and a marine dinoflagellate *Goniodoma pseudogoniaulax* showed strong inhibition against the fungus *Mortierella ramannianus*. In particular, the fat soluble fraction of *G. pseudogoniaulax* inhibited the growth at a concentration of 0.1 mg/m*l*. The fraction also had a strong inhibitory effect on the fertilized eggs of the sea urchin *Hemicentrotus pulcherrius*, stopping the first cleavage at a concentration of as low as 1 μg/m*l*.

Microalgae are an assemblage of a great number of species of microorganisms that constitute major oceanic and freshwater primary producers. Despite the abundance and species diversity of microalgae, there are still limited studies of their biologically active compounds. It has been known that microalgae produce such secondary metabolites as antibiotics, algicides, toxins, and plant growth regulators, but most research has centered on toxin production by blue-green algae and dinoflagellates.[1-10] Metting and Pyne[11] recently reviewed biologically active compounds from microalgae and suggested that microalgae might become economic sources of new drugs and other specialty chemicals because they are amenable to fermentation and mass culture. We therefore started to search for new biologically active compounds in microalgae. As the first step we have carried out the screening on more than twenty species by the assay methods of antimicrobial activity and inhibition of development of echinoderm embryos, and detected several promising species. This paper reports the results.

Materials and Methods

Algal Samples

The following species were collected from Japanese waters, locations of which were indicated in the parentheses, isolated to a unialgal state by the procedure according to Nishizawa and Chihara,[12] and cultured: freshwater cyanophytes *Microcystis* sp. (Teganuma), *Microcystis* sp. (Inbanuma), *Phormidium* sp. (Teganuma), *Phormidium* sp. (Hinuma), *Anabaena* sp. (Suginamiku), and *Oscillatoria* sp. (Teganuma); marine cyanophytes *Oscillatoria* sp.-1 (Gokashowan) and *Oscillatoria* sp.-2 (Gokashowan); freshwater chlorophytes *Micractinium* sp. (Teganuma), *Micractinium* sp. (Inbanuma), *Scenedesmus* sp. (Kitaura), *Scenedesmus* sp. (Teganuma), *Chodatella* sp. (Teganuma), *Crucigenia* sp. (Kitaura), *Golenkinia* sp. (Kitaura), and *Pediastrum* sp. (Kitaura); marine chlorophyte *Chlorella minutissima* (Setonaikai); freshwater diatom *Cyclotella* sp. (Teganuma); marine haptophyte *Isochrysis galbana* (Mieken); and marine prasinophyte *Tetraselmis tetrathele* (Mieken).

Freshwater organisms were grown in a modified C medium[13] containing $Ca(NO_3)_2 \cdot 4H_2O$ (15 mg), KNO_3 (10 mg), $MgSO_4 \cdot 7H_2O$ (4 mg), β-Na_2 glycerophosphate (5 mg), vitamin B_{12} (0.01 μg), biotin (0.01 μg), thiamin HCl (1 μg), PIV metals (0.3 m*l*), Bicine (50 mg) and distilled water (99.7 m*l*) at pH 7.5. The cultures in 5 *l* flasks were placed under illumination of 80 μE/m^2/s on a 12: 12 h LD cycle at 20°C. Marine cyanophytes were grown in ES medium[14] containing $NaNO_3$

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(10 mg), Na_2HPO_4 (2 mg), soil extract (5 m*l*) and sea water (100 m*l*), and the haptophyte and the prasinophyte were cultured in Sweeney medium[15] containing KNO_3 (20.2 mg), K_2HPO_4 (3.5 mg), $FeCl_3$ (97 μg), EDTA (1 mg), $MnCl_2 \cdot 4H_2O$ (9.5 μg), vitamin B_{12} (1 μg), sea water (75 m*l*) and distilled water (25 m*l*) under the same conditions as above. The marine chlorophyte was grown under the same conditions in a medium containing the following components (mg/*l* filtered sea water) $(NH_4)_2SO_4$ (100), K_2HPO_4 (15), and FeEDTA (5).

The algal cells harvested at stationary phase by continuous flow centrifugation at 7000 rpm or by filtration with filter paper (TOYO ROSHI No. 2) were lyophilized and stored at −20°C until analyzed. Freeze-dried preparations of freshwater chlorophytes *Botryococcus braunii* Austin and *B. braunii* Berkeley and halotolelant chlorophyte *Dunaliella bardawil*, and a spray-dried preparation of freshwater cyanophyte *Spirulina maxima* were donations from Professor H. Iwamoto of Meiji University, Professor A. Ben-Amotz of National Institute of Oceanography, Israel, and Japan Spirulina Co., Ltd., respectively. A marine dinoflagellate *Goniodoma pseudogoniaulax* was collected from unialgal blooms in rockpools at Jogashima Island, Kanagawa Prefecture,[16] lyophilized, and stored at −20°C unitl analysis.

Preparation of Test Fractions

The algal samples were homogenized and refluxed two times with 10–100 volumes of 70% ethanol, and filtered immediately. The 70% ethanolic extracts, after removal of most of ethanol under reduced pressure, were shaken three times with equal volumes of diethyl ether.[17] The aqueous layer was dried under vacuum, weighed, and used as the water-soluble fraction for bioassay. The residue of the 70% ethanolic extraction was further extracted with hot ethanol. The ethanolic extract was combined with the ethereal layer, dried under vacuum, weighed, and used as the fat-soluble fraction for bioassay.

Antimicrobial Assay

Antimicrobial tests were performed against three fungi *Candida albicans*, *Penicillium chrysogenum*, and *Mortierella ramannianus*; three Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus aureus*, and *Mycobacterium smegmatis*; two Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* that were obtained from Institute of Applied Microbiology of our university.

Test solutions were prepared by dissolving the water- and the fat-soluble fractions in water and diethyl ether, respectively, to a concentration of 10 mg/m*l*. Eight mm-diameter filter paper disks saturated with the test solutions were placed on agar plates seeded with the microorganisms. Assays for the fungi were carried out in the yeast agar medium; 0.2% yeast extract, 1% saccharose, and 1.1% agar. Assays for the bacteria except for *M. smegmatis* were performed in the beef extract medium; 1% Bacto-pepton, 1% beef extract, 0.5% NaCl, and 1.2% agar. Assays for *M. smegmatis* were done in the glycerol-containing Czapek medium; 1% glycerol, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.005% $MgSO_4$, 0.0001% $FeSO_4$, and 1% agar. The plates were incubated at 27°C for the fungi and at 37°C for the bacteria. Diameters of zone of inhibition were measured by a scale.

Fertilized Echinoderm Egg Assay

The assay was performed by using fertilized eggs of the starfish *Asterina pectinifera* or the sea urchin *Hemicentrotus pulcherrinus* according to the literature.[18–21] The fertilized eggs were obtained by adding sperms to oocytes matured with 10^{-6}–10^{-8} M 1-methyl adenine for starfish and to eggs released with 0.5 M KCl for sea urchin. The fertilized eggs were then immersed in test solutions which were prepared by dissolving the water-soluble fraction or by suspending the fat-soluble fraction in filtered seawater to a concentration of 100 μg/m*l*. The development of the embryos was examined under a microscope to judge the activity of test solutions by comparison with that of control embryos. Ten-fold dilution of the test solutions was repeated until the activity was not observed.

Results

Antimicrobial Activity

The results are shown in Table 1. The activity was found in the fat-soluble fractions of several species but not in the water-soluble fractions of any species. *Oscillatoria* sp.-2 (Gokashowan), *Cyclotella* sp. (Teganuma), and *G. pseudogoniaulax* showed strong inhibition against the fungus *M. ramannianus*. In particular, the fat-soluble fraction of *G. pseudogoniaulax* inhibited the growth at a concentration of as low as 0.1 mg/m*l*. The activity against the Gram-positive bacterium *M.*

Table 1. Antimicrobial activity of the fat-soluble fractions extracted from microalgae

Microalgae	Test microorganisms*							
	Can	Pen	Mor	Bac	Sta	Myc	Esc	Pse
Microcystis sp. (Teganuma)	−	−	−	−	−	+	−	−
Phormidium sp. (Teganuma)	−	−	−	−	−	+	−	−
Phormidium sp. (Hinuma)	−	−	−	−	−	++	−	−
Oscillatoria sp. (Teganuma)	−	−	−	−	−	+	−	−
Oscillatoria sp.-2 (Gokashowan)	−	−	++	−	−	−	−	−
Micractinium sp. (Teganuma)	−	−	−	−	−	+	−	−
Scenedesmus sp. (Kitaura)	−	−	−	+	+	+	+	+
Crucigenia sp. (Kitaura)	−	−	−	−	−	+	−	−
Golenkinia sp. (Kitaura)	−	−	−	−	−	+	−	−
Cyclotella sp. (Teganuma)	−	−	+++	−	+	−	−	−
Goniodoma pseudogoniaulax	+	−	+++	−	−	−	−	−

* Can: *Candida albicans*, Pen: *Penicillium chrysogenum*, Mor: *Mortierella ramannianus*, Bac: *Bacillus subtilis*, Sta: *Staphylococcus aureus*, Myc: *Mycobacterium smegmatis*, Esc: *Escherichia coli*, Pse: *Pseudomonas aeruginosa*

Diameter of inhibition zone: +++ more than 30 mm, ++ 20–30 mm, + less than 20 mm, − no inhibition zone was formed.

Table 2. Inhibition of development of fertilized echinoderm eggs by fat-soluble fractions extracted from microalgae

Microalgae	Activity (μg/ml)
Phormidium sp. (Hinuma)	100
Anabaena sp. (Suginamiku)	100
Oscillatoria sp.-1 (Gokashowan)	<1
Oscillatoria sp.-2 (Gokashowan)	100
Micractinium sp. (Teganuma)	100
Scenedesmus sp. (Kitaura)	10
Pediastrum sp. (Kitaura)	10
Goniodoma pseudogoniaulax	<1

smegmatis was detected in many of the microalgae listed in Table 1. In addition, *Scenedesmus* sp. (Kitaura) exhibited no activity against the fungi but inhibited the growth of all the bacteria tested.

Inhibition of Development of Fertilized Echinoderm Eggs

The results are shown in Table 2. The activity was detected in the fat-soluble fraction of the eight species of microalgae; *Phormidium* sp. (Hinuma), *Anabaena* sp. (Suginamiku), *Oscillatoria* sp.-1 (Gokashowan), *Oscillatoria* sp.-2 (Gokashowan), *Micractinium* sp. (Teganuma), *Scenedesmus* sp. (Kitaura), *Pediastrum* sp. (Kitaura), and *G. pseudogoniaulax*, but not in the water-soluble fraction of all the species examined. Although *Anabaena* sp., *Oscillatoria* sp.-1, and *Pediastrum* sp. had no antimicrobial activity, they inhibited the development of echinoderm eggs. Among them, the activity of *Oscillatoria* sp.-1 was strongest, being effective even at a concentration of 1 μg/ml. The effect, however, differed in a dose-dependent manner; in high concentrations of about 100 μg/ml the fat-soluble fraction induced the delay of development and arrested the cell division at 4–6 cell stage, but in low concentrations around 1 μg/ml, the cells developed normally until blastula-stage but then followed by cytolysis. *G. pseudogoniaulax* which showed a potent antimicrobial activity against the fungus *M. ramannianus*, also had a strong inhibitory effect on the fertilized eggs, stopping the first cleavage at a concentration of 1 μg/ml.

Discussion

In the screening of biologically active substances in microalgae by means of the antimicrobial activity test and the inhibition test of development of fertilized echinoderm eggs, we detected eleven and eight active species by the former and latter test, respectively, among twenty five species and strains assayed. Furthermore, five species possessed both activities. The activity was found exclusively in the fat-soluble fractions in accordance with our preliminary work.[22] These results suggest that microalgae produce fat-soluble active substances in a high incidence.

Among fourteen active species found in the present work, seven belong taxonomically to cyanophytes, five to chlorophytes, and one each to diatom (chrysophyta) and dinoflagellate (pyrrophyta). It has already been known that cyanophytes produce a variety of toxic substances but some species elaborate biologically active compounds other than toxins.[2,23,24] Cardellina *et al.*[25] reported the isolation and structure determination of an antibiotic in the methanolic extract of a shallow water variety of the marine blue-

green alga *Lyngbya majuscula*, which is active against *M. smegmatis* and *Streptococcus pyogenes*. A deeper water variety of *L. majuscula* was shown to produce a novel cyclic depsipeptide that inhibits the growth of a number of fungal plant pathogens.[26] A freshwater blue-green alga *Scytonema hofmanni* was found to produce a secondary metabolite which inhibits the growth of other algae,[27] and the structure of the active principle was determined.[28] Antialgal compounds were obtained also from *Hapalosiphon fontinalis*.[29] Furthermore, the production of herbivore deterrents by *Anabaena flos-aquae*,[30] antitumor compounds by *Hormothamnion pseudohofmanni*,[31] an antitumor agent by *Tolypothrix byssoidea*,[32] and an antialgal compound by *Lyngbya aestuai*[33] has been reported.

In contrast to the occurrence of diverse bioactive molecules in blue-green algae, only a few papers[34,35] have been published as to biologically active substances in chlorophytes. It is generally accepted that diatoms are not toxin producers, but some species do produce antimicrobial constituents.[36-41] Although toxin production by dinoflagellates has long been recognized and extensive investigations have been done,[11] information on their antimicrobial substances is still limited. Sharma *et al.*[42] reported the isolation and properties of an antifungal substance obtained from a bloom of *Goniodoma* sp. collected in Puerto Rico. The presence of strong antifungal activity was also confirmed for *G. pseudogoniaulax* in our investigation.

Microalgae are a very diverse group of largely photoautotrophic microorganisms and their taxonomic diversity is reflected in their metabolic diversity which suggests the occurrence of many secondary metabolites of pharmacological interest. The finding in the present work supports this concept. We are attempting the mass culture as well as the axenic culture of the promising species found and have already succeeded in the isolation of some active principles from *Oscillatoria* sp.-1, *Scenedesmus* sp., *Pediastrum* sp., and *G. pseudogoniaulax*. In addition, we have determined the structure of goniodomin A, the main antifungal substance in *G. pseudogoniaulax*.[43] Further results will be published elsewhere.

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