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Antibiotic Substance Produced by a Newly Isolated Marine Microalga, *Chlorococcum* HS-101

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Marine algae can grow autotrophically using light, CO_2 and minerals;indeed algae are the primary producers of organic matter in the sea. In order to compete with other organisms, algae are expected to secrete toxic and/or growth-inhibitory substances under certain circumstances. This was first observed by Harder(1917). Later, Prat et al.(1942) found antibacterial activity in a freshwater green alga, Chlorella sp. Since then, many bioactive compounds have been isolated from various algae (Ishitsuka et al. 1988; Berland 1972; Mason et al. 1982), including fatty acids, terpenes, carbonyl compounds, bromophenols and halogenated compounds(Rosell and Stivastava 1987; Higgs 1981; Fujimoto et al.1985). However, most of these compounds have been purified from marine algae, mainly red or brown, and few have been isolated from green algae. Matsueda et al.(1988) purified a heat-stable glycoprotein having antitumor activity from Chlorella vulgaris. Lustingmann (1988) compared four strains of Dunaliella sp. and observed substantial production of antibacterial substances by Dunaliella sp. isolated from the seawater of San Francisco Bay.

We report here the first findings of the properties of an antibiotic substance extracted from a marine green alga, Chlorococcum HS-101, which exhibited the highest activity among 68 strains of isolated marine microalgae.

MATERIALS AND METHODS.

Marine algae were isolated from an enriched culture of marine samples (seawater, seaweeds and sediment) collected from the coastal areas of western Japan, and used throughout this study. These algae were purified

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by the agar-plate method. For reproducible laboratory studies, the medium for Chlorococcum HS-101 used a modification of BG-11 medium (pH 8.0), by the addition of NaCl 20 g, 100 μ g of vitamin B_1 and 1 μ g of vitamin B_{12} in 1.0 L of the BG-11 medium(ATCC Medium #616). The alga was grown at 25 °C in 1.5-L Roux flasks containing 1.0 L of the medium and harvested at late-log phase. The algal cultures were illuminated continously by a bank of fluorescent lamps at a light intensity of 25 W/m^2. The cultures were sparged continously with air containing 1 % CO_2 at a flow rate of about 300 mL/min.

Crude extracts were prepared for activity testing from 68 algal cultures. Each algal culture was centrifuged (4000 x g, 10 min) at 0° C. The algal concentration was adjusted to 0.1 g fresh wt/mL by the addition of distilled water to the pellets. The algal suspension was then sonicated on ice by using an ultrasonic generator and centrifuged. The supernatant was filtered through a 0.22-μ m Millipore membrane filter. The filtrate was designated as the crude extract.

The crude extracts were examined for antimicrobial activity against eight bacteria and four fungi by observing inhibition of their growth at 25° C on nutrient-broth (Difco) agar plates for 24-48 hr; 40 μ L of algal crude extract (0.1 g fresh wt/mL) was transferred to an 8-mm paper disk, which was then applied to a plate seeded with a test strain. After incubation at 25° C for 24 hr, the resulting zone of inhibition was measured. Distilled water was used as a control, as well as samples treated under various experimental conditions. No inhibition zone was observed with any of the control disks.

To investigate the pH stability of the antibiotic activity in the crude extract of Chlorococcum HS-101, sonicated algal cells were exposed to pH 2, 4, 6, 7, 9 and 12 at 25° C for 1 hr. Each sample was brought to pH 7, adjusted to the initial concentration (0.1 g fresh wt/mL). After centrifugation and filtration, the antibiotic activity of the filtrate was measured by means of the disk test described above. The temperature stability was also investigated. Each sample of sonicated algal cells was incubated at 15, 25, 40, 60, 70, 80 and 100° C for 1 hr, followed by centrifugation, filtration and testing as described above. To determine the effects of various inhibitors, samples of sonicated algal cells were mixed with EDTA, diethyl pyrocarbonate, p-chloromercuribenzonic acid or $HgCl_2$, and incubated at 25° C for 1 hr. The procedure after that was the same as in the case of the pH stability experiment.

To choose suitable solvents of antibiotic substance in Chlorococcum HS-101, algal cells(0.1 g fresh wt/mL) were sonicated and freeze-dried. We then attempted to dissolve the antibiotic substance in the freeze-dried samples in different solvents. After centrifugation, the supernatants were freeze-dried and dissolved in distilled water, followed by filtration and testing as described above.

RESULTS AND DISCUSSION.

As part of a search for new antibiotic substances, marine algae were collected in the western coastal areas of Japan, and unicellular microalgae were grown and isolated on an enriched seawater medium. Antibiotic activities in the crude extracts of 68 algal isolates were tested against a gram-negative bacterium, Escherichia coli and a gram-positive bacterium, Staphylococcus aureus. Although all crude extracts of 68 algal isolates exhibited no activity toward E. coli, the growth inhibition of S. aureus was observed in the crude extracts of five algal isolates, HS-101, HS-109, HS-110, HS-364 and HS-366(Table 1). We found that one algal strain, HS-101, exhibited greater antibiotic activity toward S. aureus than did the others. This strain was a green alga belonging to the genus Chlorococcum. Finally, we selected Chlorococcum HS-101, and the crude extract from Chlorococcum HS-101 was also tested against another six bacteria, Salmonella typhimurium, Enterobactor aerogenes, Vibrio parahaemolyticus, Bacillus subtilis, Bacillus cereus and Micrococcus luteus, or four fungi, Claosporium cladosporides, Penicillium funiculosum, Paecilomyces variotii and Aspergillus niger. Marked inhibition of the growth of B. subtilis, B. cereus and E. aerogenes was observed, but growth of the fungi was not inhibited.

As strong growth inhibition of S. aureus was observed, the activity was compared with that of an authentic antibiotic substance, ampicillin. By using this relationship, the activity of crude extracts was represented in terms of an equivalent amount of ampicillin. The optimum conditions for activity of the antibiotic substance in this strain were investigated. Figure 1 shows the effect of pH on antibiotic activity in the crude extract of Chlorococcum HS-101. The activity was stable in the range of pH 6 to pH 12 with an optimum at pH 7, whereas the activity in the acidic range of pH 2 to pH 4 was depressed. The effect of temperature on the antibiotic activity in the crude extracts of this strain was also investigated (Fig. 2). The activity of this substance was very stable up to 60° C, and 70 % of the maximum activity was observed even after treatment at 100° C for 1 hr.

Table 1. Antibiotic activities of 68 marine algal isolates.

Strain #	Genus	Antibiotic activity against S.aureus	Strain #	Genus	Antibiotic activity aginst S.aureus	Strain #	Genus	Antibiotic activity against S.aureus
	(Cyanophyceae)		HS-434	Synechococcus sp.	-	HS-440	Chlorella sp.	-
HS-9	Synechococcus sp.	-	HS-435	Synechococcus sp.	-	HS-443	?	-
HS-23	Phormidium sp.	-	HS-437	?	-	HS-445	Chlorella sp.	-
HS-26	Phormidium sp.	-	HS-444	?	-	HS-557	?	-
HS-40	Synechococcus sp.	-	HS-527	Phormidium sp	-	HS-601	Carteria sp.	-
HS-57	?	-	HS-564	Phormidium sp	-	HS-608	?	-
HS-69	?	-	HS-610	?	-	HS-615	Carteria sp.	-
HS-86	Anabena sp.	-	HS-691	Ocillatoria sp.	-		(Bachillariophyceae)	
HS-107	?	-		(Chlorophyceae)		HS-36	Nitzchia sp.	-
HS-108	Calothrix sp.	-	HS-56	Chlorella sp.	-	HS-123	?	-
HS-112	?	-	HS-58	?	-	HS-224	?	-
HS-263	?	-	HS-90	Chlamydomonas sp.	-	HS-327	Skeletonema sp.	-
HS-301	Phormidium sp.	-	HS-100	?	-	HS-328	Skeletonema sp.	-
HS-304	Phormidium sp.	-	HS-101	Chlorococcum sp.	+++	HS-368	Chaetoceros sp.	-
HS-310	?	-	HS-106	?	-	HS-441	Rhizoteria sp.	-
HS-320	Ocillatoria sp.	-	HS-109	Chlorella sp.	+	HS-442	Rhizoteria sp.	-
HS-332	Phormidium sp.	-	HS-110	Chlorella sp.	++	HS-569	?	
HS-333	?	-	HS-113	Dunaliella sp.	-	HS-673	Nitzchia sp.	-
HS-334	Ocillatoria sp.	-	HS-214	?	-		(Rhodophyceae)	
HS-363	Synechococcus sp.	-	HS-228	?	-	HS-366	Phorphyridium sp.	+
HS-364	Synechococcus sp.	+	HS-329	Chlamydomonas sp.	-		(Dinophyceae)	
HS-370	?	-	HS-341	?	-	HS-273	Prorocentrum sp.	-
HS-371	?	-	HS-342	Chlamydomonas sp.	-		(Rhaphidophyceae)	
HS-372	?	-	HS-355	?	-	HS-111	Chattanella sp.	-
HS-374	Ocillatoria sp.	-	HS-356	?	-			

‡ Antibiotic activity was indicated as this: - no inhibition; + 9-12 mm diameter of circle of inhibition; ++ 13-16 mm diameter of circle of inhibition; +++ 16-20 mm diameter of circle of inhibition.
‡‡ 68 marine algal isolates were grown at 20 °C or 25 °C in an enriched seawater medium with sparging air containing 1 % CO_2.

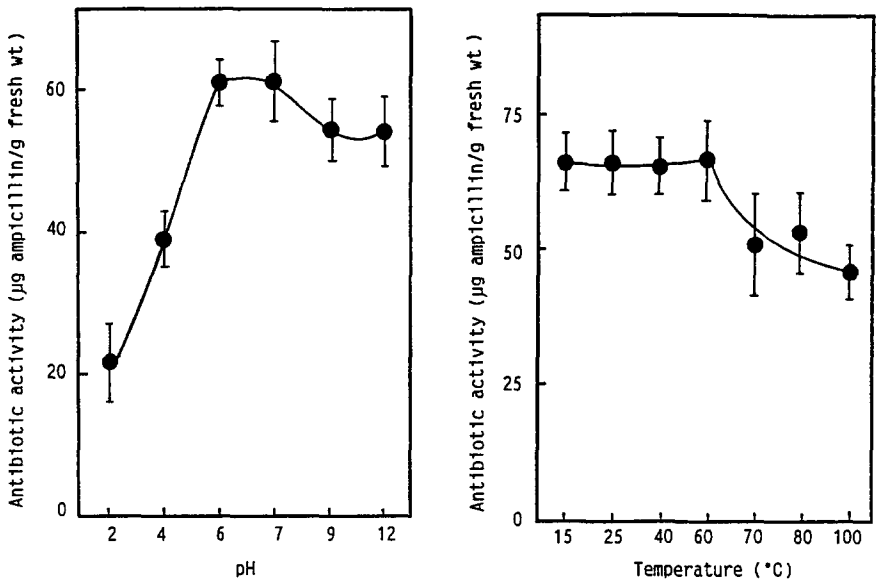

Figure 1. Effect of pH on antibiotic activity of crude extract from Chlorococcum HS-101. Points are the mean ± s.d. of three experiments.

Figure 2. Effect of temperature on antibiotic activity of crude extract from Chlorococcum HS-101. Points are the mean ± s.d. of three experiments.

Table 2. Effect of inhibitors on antibiotic activity of crude extract from Chlorococcum HS-101.

Inhibitor	Concn. (mM)	Residual activity (%)
None		100 ± 15.1 **
EDTA *	1.0	92 ± 11.8
	2.0	55 ± 10.8
DEP *	1.0	108 ± 16.2
	2.0	100 ± 13.0
PCMB *	1.0	108 ± 16.0
	2.0	100 ± 15.8
$HgCl_2$	0.04	92 ± 19.2
	0.1	100 ± 17.0

* EDTA, Ethylenediaminetetraacetate; DEP, Diethyl pyrocarbonate; PCMB, p-chloromercuribenzoic acid
**100 % activity is equivalent to 136.2 μg ampicillin/g fresh wt.
Values are the mean ± s.d. of three experiments.

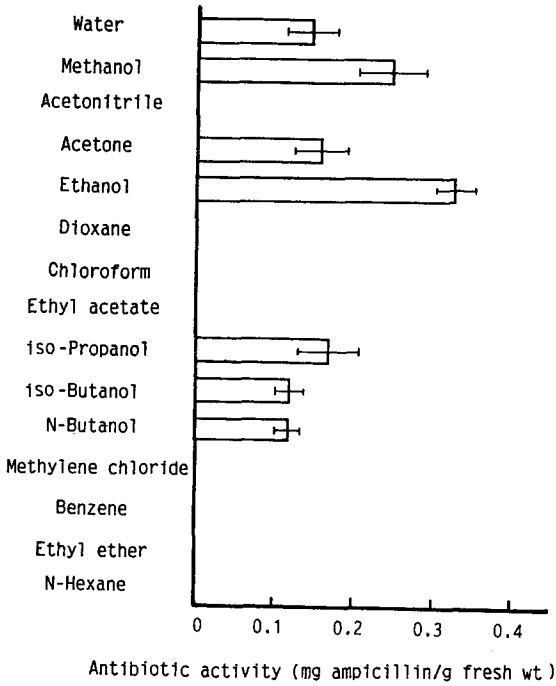

Figure 3. Solubility and stability of antibiotic substance extracted with different solvents from Chlorococcum HS-101. Values are the mean ± s.d. of three or more experiments.

Table 2 shows the effect of inhibitors on the antibiotic activity of crude extract of Chlorococcum HS-101. A chelator of metal ions (EDTA) and SH-blocking agents (DEP, PCMB and $HgCl_2$) were used as inhibitors. The activity was inhibited to 50 % by 2 mM EDTA, but was unaffected by the other agents. This indicates that the expression of antibiotic activity involves a metal ion, and is probably not due to an enzyme reaction.

It is essential to choose a suitable solvent for extracting and further purifying the antibiotic substance in this strain. As shown in Fig. 3, the solubility and stability of this substance were tested in various solvents. Independent of the solvent polarity, high activities were observed in alcoholic solvents and no activity was observed in other solvents except water and acetone. From the above results, we conclude that this substance is likely to be a heat- and base-stable non-protein material.

In general, the role of an antibiotic substance in marine organisms is considered to provide a

competitive advantage in their growth environment (Lustingman 1988; Berland 1972). We observed that the extract of this strain depressed the growth of a marine diatom, Nitzschia sp. (data not shown). Further, it was reported that β -lactum antibiotic production of the fungus Cephalosporium sp. was enhanced in the presence of Chlorella sp. (Khang et al. 1988). Therefore, we may be able to induce higher levels of antibiotic production by modifying the algal environment. Further work is planned to purify and identify this antibiotic substance from Chlorococcum HS-101.

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