Andrimid, An Antimicrobial Substance Produced by *Vibrio* Sp. Bacterium Associated with in the Marine Sponge *Hyatella* Sp.

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Abstract

The bacterial isolate, M22-1, belonging to the genus Vibrio *was obtained from a homogenate of the sponge,* Hyatella Sp. *The bacterium was cultured in marine agar and was found to produce an antimicrobial compound. The substance was chemically identified as andrimid, a peptide-like antibiotic. The same substance was found in sponge extract, suggesting that the anti-Bacillus activity observed in the homogenate of the sponge body is derived from a product of the associated microorganism.*

Key Words: andrimid, *Vibrio, Hyatella, Bacillus,* marine sponge peptide antibio

Introduction

Marine sponges live as filter feeders and strainers of microorganisms, cyanobacteria and microplankton. These organisms form part of the sponge diet (Reiswig, 1975). Such adaptations, may during evolution, produce resistance to the action of organisms which are found in the canal systems and porous bodies. This adaptive mechanism may bring about the production of substances which facilitate rejection of invading organisms. Bergquist (1978) suggested the possibility that the antimicrobial properties of marine sponges are due to the symbiotic microorganisms which they harbor. It was observed that fluids of freshly collected sponges showed a high degree of antibacterial activity (Nigrelli, 1952). Until recently, there has been little experimental evidence as to whether these thriving microorganisms are the sources of active substances or whether the sponge itself synthesizes the compound (Fenical. 1993), because methods for cultivation of marine sponges under laboratory conditions had not been developed. Therefore, it was difficult to obtain sufficient amounts of the active compounds assumed to be produced by the associated microorganisms. The present investigation has demonstrated that a culture of a bacterium isolated from the sponge contained the same substance as the one purified from the extract of the host sponge.

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Materials and Methods

Collection of marine sponges

Several marine sponges were collected along the coast of Oshima Island, Miyazaki, Japan during a field expedition in April 1991 on board the research vessel Toyoshio-maru, Hiroshima University. The samples were harvested using a self-contained underwater breathing apparatus at depths ranging from 15 to 10 meters. Aliquots of the samples were kept alive in aquaria which were supplied with running sea water. The remaining sponge specimens were frozen on board.

Isolation and characterization of bacteria associated with sponges

Employing strict aseptic procedures, live sponge specimens were washed thoroughly with sterile artificial sea water (ASW) to remove contaminants. They were then cut into cubes of 0.5 cm length and homogenized in sterile ASW. The homogenate was serially diluted with sterile ASW and inoculated immediately into marine agar (Difco 22216) plates. The plates were incubated for 6 days at 20°C and after that distinct colonies were picked up separately for single colony isolation. This process was repeated two or three times to ensure the homogeneity of each colony. Characterization of the strain was carried out using differential and selective media according to the procedures as described by (Krieg, andd Holt (1984). Pure cultures were stocked in marine broth with the addition of an equal volume of 25% glycerol and kept at -85° C. All bacterial cultures were tested for antibiotic activities in the same manner as that of the sponge extracts.

Assay for antimicrobial activity

In order to determine which of the sponge samples contained antimicrobial compounds, 1 g of a frozen sample of each species was taken and extracted with 3 ml of methanol. To determine which cultures of the bacteria associated with the marine sponges contained antimicrobial compound, 2 to 4-day old broth cultures of the respective bacteria were obtained and centrifuged to separate the supernatants from the mycelia. The resulting supernatant fluids together with the sponge extracts were then examined for antimicrobial properties using the following test microorganisms: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Saccharomyces cerevisciae* and *Aspergillus niger.* Bouillon's agar (BA) plates were used for bacterial culture and yeast peptone glucose (YPG) agar plated for the culture of fungi (Collins, 1967). To ensure uniformity of inoculum in each assay plate the top agar technique was employed. A filter paper disc-diffusion technique was used for the evaluation of antimicrobial activities (Collins, 1967). For every filter paper disc (8 mm in diameter), 30 ul of extract was impregnated.

Preparation of extracts from selected bacteria

Among the isolates, M22-1 strain was selected because its broth fluid exhibited the same inhibition pattern as that of the extract of its host *Hyatella sp.* The strain was then cultured in marine agar plates for 2-4 days at 20° C. The agar was harvested and macerated

by passing through a steel wire mesh with an aperture of about 3 mm. The pH of the macerated agar was adjusted to 8.9 by adding 0.1 N NaOH, after which, 1 liter of ethyl acetate was added and the mixture was stirred gently for 2 hours. Then, the agar was separated and the solvent was evaporated in vacuo to afford a crude residue.

Preparation of extracts from Hyatella species

In order to obtain the active substance from the host sponge, Hyatella sp., a frozen sample of about 60 g was macerated and 180 ml of methanol was added. The mixture was kept at room temperature with occasional stirring for 2 hours, after which it was filtered through a Whatman filter paper (#1). The extraction procedure was repeated twice, the extracts were combined and a crude residue was obtained after evaporating the solvent to dryness.

Purification of active substance from bacteria and host sponge

The purification of active substances from both the bacterial culture and the host sponge was guided by an anti-*Bacillus* assay and the activity of the substance was compared with chloramphenicol, a known broad-spectrum antibiotic. Chromatography was carried out using a silica gel (Unisil, Nakarai, Kyoto, Japan) column (1.9 x 25 cm). The first step, the crude substance obtained by ethyl acetate extraction of the marine agar cultures of the bacterial isolate was loaded into the column, eluted in a stepwise manner with 30, 40, and 50% ethyl acetate in benzene.

For further purification, the active fractions which were recovered from 40% ethyl acetate in benzene were then loaded into an octadecyl silica (ODS) column (1.9 x 19.5 cm) which was eluted isocratically with 90% aqueous methanol. The resulting active fraction was finally isolated to homogeneity by HPLC using a Shimadzu chromatography model LC-6AD equipped with a UV-Vis spectrophotometric detector. A silica type Wakosil 5Sil (Wako, Osaka, Japan) column (0.75 x 25 cm) was used and elution was carried out using 40% n-hexane in methanol or 10% methanol in chloroform, monitoring at 254 nm at a flow rate of 1 ml/min.

Mass spectrometry

The high resolution fast atom bombardment mass (HRFABM) spectrum was recorded on a Jeol JMS-Sx 102A mass spectrometer unit with polyethylene glycol matrix. The secondary ion mass spectra (SIMS) were obtained by detecting positive ions with an Hitachi M-80B double focusing spectrometer equipped with an M-8086 Xenon beam generating system (matrix: glycerol; accelerating voltage, 3 KeV). Assignment of mass number was achieved by comparing the spectra with mass spectrum of CsI.

Nuclear magnetic resonance spectroscopy

1H- and 13C-nuclear magnetic resonance (NMR) spectra were obtained at 500 and 125 MHz, respectively, on a Jeol GSX-500 spectrometer operating in the Fourier transform mode.
Circular dichroism spectrometry

The CD (circular dichroism) spectra were measured on a Jasco J-600 circular dichrometer instrument at 25 C in a quartz cell of 1.0 cm path length.

Amino acid analysis

The purified active substance (8 mg) was hydrolyzed in a sealed, desiccated tube containing 6 N HCl at 100 C for 24 h. After evaporating the solvent, the residue was dissolved in distilled water and chromatographed by HPLC using and ODS (inertsil, GL Science, Tokyo, Japan) column. Continuous elution was made with linear gradient 0-100 aqueous CH3CN. Each UV absorbing fraction (210 nm) was analyzed by NMR spectroscopy to obtain fragments of the active compound.

Other analytical methods

The UV spectra were obtained on a Shimadzu UV - I 60A spectrometer using a 1.0 cm cell. The IR spectra were determined as films in CaF2 with Jasco FT/Ir 5300 spectrometer. Optical rotations were measured in methanol solution with a Jasco DIP-370 digital polarimeter.

Results

Antimicrobial activity of extracts of sponges and bacterial isolates

Sponges belonging to the following genera were collected: *Plakorites, Cribrochalina, Spirastrella, Agelass, Hyatella, Jaspis, and Theonella.* Their methanolic extracts were examined for antimicrobial activity using the filter-paper disc-diffusion technique. To each paper disc each sponge extract was impregnated. All of the extracts prepared from these samples showed varying degrees of inhibition in at least one of the test microorganisms. From the living sponge specimens, bacteria found in their porous bodies were aseptically separated. It was found that broth filtrates obtained from bacterial isolates showed several antimicrobial activity. The sponge, *Hyatella sp.* yielded four bacterial isolates and the broth of two isolates exhibited antimicrobial activity against *P. aeruginosa* and *B subtilis.* The bacterial isolate, M22-1, showed a similar inhibition pattern to that of the host sponge extract. It suppressed the growth of *B. subtilis* without affecting that of *E. coli, P. aeruginosa, S. aureus, S. cerevisceae* and *A. niger* . The other isolates manifested inhibitions which were different from those of their respective host sponge species.

Characterization of isolate M22-1

Employing standard biochemical characterization and using selective or differential media, the isolate M22-1 was shown to possess the following characteristics: Gram-negative, slightly curved rod-shaped, and motile with polar flagella. The pure culture of the bacterium produced white colonies when cultured on marine agar plates. The Hugh-Leifson (HL) test showed that the organism was a facultative anaerobe. It required 1-6% NaCl for growth and grew best between 20 and 30° C producing amylase, catalase, oxidase, and indole. Utilization of glucose yielded acids but did not produce gases. All these character-

Discussions

The results of the present study revealed that *Vibrio sp.* that is associated with the body of the sponge *Hyatella sp* produces andrimid which was obtained from the culture of broth of an *Enterobacter sp* (Fredenhagen et al., 1987 and Kenny et al., 1989). Andrimid exhibits potent antimicrobial activity against *Xanthomonas campestris pv. oryzae*, the pathogen causing bacterial blight in rice plants. The antimicrobial spectrum of andrimid is very specific; among phytopathogens tested, only the bacterial blight pathogen and few others such as *X. campestris pv. incanae, Erwinia milletiae,* and *X. campestris pv. oryzacola* are susceptible to andrimid (Fredenhagen et al., 1987 and Kenny., et al 1989). In the present study it was found that andrimid exerts inhibitory effect on the growth of *Bacillus subtilis*. Although it is unlikely that *B. subtilis* is associated with the body of *Hyatella sp.*, it is possible that andrimid affects the growth of specific bacteria, thereby, affecting the population of the bacteria harboring the sponge body.

In general, bacteria often symbionts with marine sponges (Sara and Vacelet, 1973). Indeed, Vacelet (1975) postulated that there were three broad categories of bacterial symbiosis based on electron microscopic observations of sponge bodies, namely (1) small populations of non-specific bacteria similar to those of ambient water, (2) large mesohyl populations of symbiotic bacteria which appeared to constitute a specific flora, and (3) small populations of specific symbiontic intracellular bacteria. Wilkinson (1978a, 1978b) noted that symbiotic bacteria were associated with marine sponges in three ways: the majority were free-living in mesohyl, large aggregates occured in cyanocytes, and a few were present in digestive vacuoles. Fenical (1993) cited a successful isolation of a bacterium, *Vibrio sp.* from an Indian ocean sponge, *Dysthea sp.* In culture, the bacterium appeared to produce small amounts of bis (dibromophenyl)-ether. The bis (bromophenyl)-ethers are typical products isolated from *Dysthea* sponges, and it has been speculated that they may be of bacterial origin. This is the first report which provides the experimental lines of evidence to support the hypothesis that some of the materials found in sponge extracts are produced by associated bacteria. In several other cases, bacteria have been isolated from sponges and their metabolites identified. Stierle et al., (1988) showed that *Micrococcus sp.* produced diketopiperazines composed of various amino acids. Since the diketopiperazines were also isolated from the sponge, this was presented as evidence of the accumulation of symbiont's products in the host sponge. However, such evidence is not conclusive because more than 90% of all the Gram-negative bacteria produce these diketopiperazines when grown in nutrient-rich media (Fenical, 1993). In the present study, the antimicrobial compound andrimid was produced by *Vibrio sp.* Previous work carried out by Fredenhagen (1987) showed that andrimid was produced by Enterobacter sp., an intracellular bacterial symbiont found in the eggs of a brown planthopper, *Nilaparvata lugens*. Therefore, it is possible that andrimid could be produced by a bacterial symbiont, *Vibrio sp.* present inside the cell of the sponge, *Hyatella sp.* Whether the bacterium is a real symbiont or just an occasionally associated microorganism still remains to be elucidated and further investigation is obviously required to settle the issue.

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