Isolation of symbiotic bacteria and bioactive proteins from the marine sponge, *Callyspongia diffusa*

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Received 3 April 2008; revised 3 November 2008; accepted 28 January 2009

The marine sponge, *Callyspongia diffusa*, collected near Mumbai coast, was studied for symbiotic bacterial association and biologically active proteins. Four bacterial species (*Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio parahaemolyticus* and *V. cholera*) were found associated with the sponge. Crude protein from the sponge was extracted at concentration of 1.43 mg/mL in methanol and 1.62 mg/mL in aqueous extract. Antibacterial activity of the extracts against the four symbiotic bacteria inhibited the growth of only *V. cholera*. Both extracts exhibited hemolytic activity on chicken erythrocytes at 6.99 HT/mg for methanolic and 8.64 HT/mg for aqueous extracts. On SDS-PAGE, the crude protein yielded eight bands in methanolic extract and five bands in aqueous extract, with molecular weight ranging from 14.4 to 116 kDa with three well defined bands of 19.5, 39.0, 66.2 kDa in both the extracts.

**Keywords:** Marine sponge, symbiotic bacteria, bioactive proteins, hemolytic assay, *Callyspongia diffusa*

**Introduction**

It is generally accepted that sessile, soft-bodied marine invertebrates (sponges, corals, tunicates, etc.), which lack physical defenses, produce toxic chemicals to protect themselves in hostile environments. These most toxic chemicals have been developed in the oceans for thousands of years\(^1\). Sponges, exclusively aquatic and mostly marine, are found from the deepest oceans to the edge of the sea. There are approximately 15,000 species of sponges in the world, of which, 150 occur in freshwater, but only about 17 are of commercial value\(^2\). About 486 species of sponges have been identified in India so far\(^3\). During the last few decades there has been an increase in research activities being performed on the marine sponges for biomedical compounds. A variety of natural products from the marine sponges have been found to exhibit remarkable antitumour and anti-inflammatory activities\(^4\). The sponges do harbor symbiotic microorganisms, which may account for up to 60% of the sponge biomass\(^5\), and they also can produce novel bioactive compounds as defense mechanism. An attempt has been made here to find out the symbiotic bacteria and bioactive proteins from the marine sponge, *Callyspongia diffusa*

**Materials and Methods**

The study area was located at Khardhanda Beach near Juhu, Mumbai coast. It is a sandy beach towards the north and precedes southwards as rocky beach. Specimens of *Callyspongia diffusa*, were collected at low tide by hand picking and brought to the laboratory with seawater for the isolation of associated bacteria. Subsequently they were preserved at low temperature. The sponge tissue was boiled with concentrated HNO\(_3\) to extrude the spicules, and its identity was confirmed as *C. diffusa*, based on the characters as proposed by Thomas\(^3\). The tissues of fresh sponges, cut by sterile blade, were homogenized using a pestle and mortar. After serial dilution, the homogenized samples were used for the isolation of microorganisms by pour plate method using different media such as MacConkey’s agar (MAC), Vibrio agar (VA), Nutrient agar medium (NAM) and Eosin methyl blue (EMB). The bacterial associates were identified using Bergey’s manual\(^6\).

**Extraction of Crude Toxin**

**Methanolic Extraction**

Crude toxin was extracted following the method of Bakus and Green\(^7\). The sponge was dried in air for 2 d, and then 10 g sponge tissue was soaked in 200 mL of methanol for 5 h. The solvent was removed after squeezing the sponge and filtered through Whatman filter paper No. 1. The solvent was
evaporated at low pressure using a Buchi Rotavapor at 60°C and the extract was stored in refrigerator for further use.

**Aqueous Extraction**

The aqueous extract of sponge was prepared by squeezing the sand-free specimens in triple distilled water. The resultant solution was filtered and dialyzed, using Sigma dialysis membrane-500 (flat width-24.26 mm, diameter-14.3 mm and capacity approx.-1.61 mL/cm) against D-glucose to remove the excess water. The supernatant obtained was lyophilized using the Labcono Freeze dry system and stored at 4°C in a refrigerator for further use as crude aqueous extract.

**Antimicrobial Activity**

Petri dishes with nutrient agar were inoculated with four isolated bacteria. Sponge extracts were sterilized by passing each through a 0.22 µm Millipore GV filter (Millipore, USA). Round paper discs (0.8 cm radius) were dipped into 0.001 mL of each sponge extract and placed in the centre of inoculated Petri dishes. Bacterial colonies were allowed to grow overnight at 37°C, then the inhibition zone around the disc was measured.

**Partial Purification of Crude Extract**

Partial purification of the crude extract was carried out using DEAE cellulose anion exchange chromatography according to the method of Stempion.

**Protein Estimation**

Protein estimation was done as described by Lowry and Lopez, using bovine serum albumin as the standard at concentrations ranging from 0.1 to 1 mg/mL. 5 mL of alkaline copper reagent was added, mixed well and allowed to stand for 15 min at room temperature. Then 0.5 mL of diluted Folin’s phenol reagent was added and mixed well. The mixture was incubated for 30 min at room temperature. The absorbance at 650 nm was read spectrophotometrically. A standard graph was obtained by plotting absorbance against concentration. The protein concentration of *C. diffusa* was estimated using the standard graph.

**Hemolytic Activity**

The hemolytic activity of crude toxin on chicken blood was tested by micro hemolytic method as proposed by Venkateshwaran. The micro hemolytic test was performed in 96 well ‘V’ bottom microtitre plates. Different rows were selected for chicken blood. Serial 2-fold dilutions of the crude toxin were made in 100 mL of normal saline and this process was repeated up to the last well and 100 µL RBC was added to all the wells. Appropriate controls were included in the test. The 1% RBC suspension and 100 µL normal saline served as negative control. The plate was gently shaken and allowed to stand for two hours at room temperature and the results recorded. Uniform red coloured suspension in the wells was considered as positive hemolysis and a button formation at the bottom of the wells was considered as lack of hemolysis. Reciprocal of the highest dilution of the crude toxin showing pattern was taken as 1 Hemolytic Unit (HU).

**SDS-PAGE**

One-dimension sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out following the modified method of Laemmli. SDS-PAGE was run on vertical slab gel system. Proteins were electrophoresed on 12% separating gel (0.75 mm thickness) overlaid with 5% stacking gel.

**Statistical Analysis**

Statistical analysis was performed using one way analysis of variance (ANOVA) by SPSS software package, version-13.0 followed by Duncan’s multiple range test (DMRT).

**Results and Discussion**

Morphological and physiological characteristics of bacteria isolated from the sponge species are given in Table 1. Four types of Gram negative bacteria, namely *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio parahaemolyticus* and *Vibrio cholera* were found predominantly associated with the sponge tissue.

<table>
<thead>
<tr>
<th>No.</th>
<th>Bacteria</th>
<th>Colony character</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>P. aeruginosa</em></td>
<td>Growth on NAM light - grey colonies of 1.5 mm diam</td>
</tr>
<tr>
<td>2</td>
<td><em>E. coli</em></td>
<td>Metallic sheen colonies on EMB agar</td>
</tr>
<tr>
<td>3</td>
<td><em>V. parahaemolyticus</em></td>
<td>Grey colonies on NAM, MAC, 1-2 mm diam., swarm across plate</td>
</tr>
<tr>
<td>4</td>
<td><em>V. cholera</em></td>
<td>Colonies are round smooth 2-3 mm, greenish-grey on VA</td>
</tr>
</tbody>
</table>
Methanolic extract (500 g) of marine sponge, *Callyspongia diffusa*, yielded 5.4 g of crude extract, whereas aqueous extract yielded 5.09 g. The protein content of the crude extract from *Callyspongia diffusa* was found to be 1.62 mg/mL in methanolic extract and 1.43 mg/mL in aqueous extract (Table 2).

The aqueous and methanolic extracts at different concentrations (5, 10 and 15 mg/mL) were tested against *P. aeruginosa*, *E. coli*, *V. parahaemolyticus*, and *V. cholera*. None of the extracts inhibited the test bacteria except the aqueous extract did inhibit *V. cholera*.

The crude methanolic extract induced hemolysis on chicken erythrocytes. The hemolytic titre in methanolic extract was 14 and its specific activity was estimated at 8.64 HU/mg of protein. The hemolytic titre of aqueous extract was found 10 and its hemolytic activity was 6.99 HU/mg of protein.

The SDS-PAGE on 12% gel, crude protein toxins yielded 6 bands in the aqueous extract and 8 bands in the methanolic extract of *Callyspongia diffusa*, ranging from 14.4 to 116 kDa molecular weight with 3 well-defined bands of 19.5, 39.0, 66.2 kDa in both the extracts (Fig. 1).

Extraction of 500 g sponge yielded 5.09 g crude extract in methanol and 5.4 g in water. Richet obtained 5.8 g of crude extract from 490 g fresh weight of the marine sponge, *Suberea preatense*\(^1\). The crude protein content was found 1.62 mg/mL in methanolic extract and 1.43 mg/mL in the aqueous extract. Corresponding data on protein content of sponge toxin are not available in literature for comparison. Both the methanolic and aqueous extracts of *C. diffusa*, induced pronounced hemolysis on chicken erythrocytes, with an activity of 14 HU in aqueous extract and 10 HU in methanolic crude. The results are in accordance with earlier studies. Stempein reported hemolytic activity of halitoxin obtained from the genus *Haliclona*\(^8\). Fuseta supervised that the sterol derivatives *viz.*, halistanol sulphate and sokotrasterol sulphate, obtained from Halichondridae sponges possessed hemolytic activity\(^13\). Mebs reported the haemagglutination and hemolytic activity of the aqueous extract obtained from 48 tropical sponge species\(^14\). The present study indicates a very strong hemolytic activity in both the methanolic and aqueous extracts of *Callyspongia diffusa*. Hemolytic activity being indicative of cytotoxicity makes the extract of the sponge species worthwhile for further studies on their anti-tumour/anti-neoplastic activities, which might ultimately lead to the detection of anticancer compounds.

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Nature of sample</th>
<th>Total activity (100 mL)</th>
<th>Total protein (100 mL)</th>
<th>Specific activity HT/mg</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>Crude</td>
<td>9.20</td>
<td>1.2</td>
<td>7.02</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Partial purification</td>
<td>13.99</td>
<td>1.62</td>
<td>8.64</td>
<td>135</td>
<td>1.23</td>
</tr>
<tr>
<td>Methanolic</td>
<td>Crude</td>
<td>4.77</td>
<td>0.91</td>
<td>5.24</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Partial purification</td>
<td>9.99</td>
<td>1.43</td>
<td>6.99</td>
<td>157</td>
<td>1.33</td>
</tr>
</tbody>
</table>

Values are average from 4 replicates.
Values not showing a common superscript differ significant at P<0.05 (DMRT)
The aqueous extract of the crude toxin of *C. diffusa*, inhibited the growth of *V. cholera*, which was isolated from marine sponge. Burholder isolated 2 bromo-compounds from *Verongia fistularies* and *V. vauliformis* that inhibited the growth of Gram-positive and Gram-negative bacteria. Bergquist and Bedford suggested that the antibacterial agents produced by sponges may have a role in enhancing the efficiency with which sponge retains bacterial food and also reported that the activity is higher in temperate species than in tropical species (87% as opposed to 58%). Generally, marine sponges have antimicrobial activity against the marine bacteria, but in the present study, no activity against the bacteria has been observed thus confirming that the isolated bacteria are symbiotic to marine sponge species studied.

The SDS-PAGE analysis revealed the presence of three protein bands viz. 19.5, 39.0 and 66.2 kDa. The presence of one band at 66.2 kDa in the present study might be a stress induced protein and this type of stress protein has been reported to occur in marine cat-fish.

Acknowledgement
The authors are thankful to the Director, C A S in Marine Biology for his encouragements and the authorities of Annamalai University for providing the facilities.

References