Biological activity of sea anemone proteins: II. Cytolysis and cell line toxicity

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Received 1 May 2008; revised 12 July 2010

Potent cytolytic activity was exhibited by proteins extracted from three sea anemones viz. Heteractis magnifica, Stichodactyla haddoni and Paracodylactis sinensis by affecting the red blood corpuscles (RBC) and the mouse fibroblast cell line (L929) and leukemia cell line (P388). Crude toxin of all the three anemone species induced spontaneous hemolysis of chicken, goat and human erythrocytes. The crude toxin of H. magnifica (0.98 mg/ml) elicited hemolysis at levels of 4096, 512 and 4096 HU (hemolytic unit) in chicken, goat and human erythrocytes respectively. Subsequently, the crude toxin of S. haddoni (0.82 mg/ml) exhibited a hemolytic activity of 256, 128 and 512 HU and that of P. sinensis (0.60 mg/ml) had a hemolytic activity of 128, 4096 and 512 HU. Most of the partially purified proteins of these anemones also exhibited the activity against the three different erythrocytes. The viability of L929 and P388 was adversely affected on adding the crude toxins. The symptoms of toxicity shown by the cells were rounding, lysis and detachment from the substratum. These effects were the least in S. haddoni, as compared to those the crude toxins of the other two species. Inhibition of growth of L929 exhibited by the toxin of the three species ranged between 61.08 and 93.38%. Similarly, inhibition of the growth of P388 ranged between 51.32 and 86.16%. The present investigation reveal the cytotoxic nature of anemone toxins.

Keywords: Anti-cancer, Cytolytic, Erythrocytes, L929, P388, Sea anemone protein

Cytolysins are proteins and peptides that can lyse the cells mainly by the formation of pores in the targeted membrane or by increasing the membrane permeability1.

More than 32 sea anemones have been reported to produce cytolytic peptides exhibiting various biological activities including haemolysis and cytotoxicity2-4. The molecular weight of these cytolysins ranges between 5-80 kDa. Some thoroughly studied sea anemone cytolysins2-4 are: Actinoporins, extracted from family Actiniidae and Stichodactylidae; Equinotoxins, Sticholysins and Magnificalysins from Stichodactyla helianthus and Metridiolysin from Metridium senile.

The sea anemone cytolysins are water soluble polypeptides, exhibiting the unique property of inserting and accommodating spontaneously into membranes. Due to their lytic capacity and the possibility to address them to specific tissues, cytolysins have been evaluated as promising anti-tumor agents5,6. They attract more attention for potential use in treating tumors and killing parasites7. These toxins exhibit strong cytotoxicity against U251, NSCLC, BEL 7402 and NIH Swiss mouse fibroblast cell lines7.

Hence the present study has laid focus at extracting cytolysins from the three tropical sea anemones viz. Heteractis magnifica (Quoy and Gaimard, 1833), Stichodactyla haddoni (Saville-Kent, 1893) and Parachondylactis sinensis Carlgren, 1833 collected from Gulf of Mannar, Southeast coast of India8.

The proteins extracted from the target anemone species have been tested for their cytotoxic effect on chicken, goat and human blood cells as well as in the mouse fibroblast cell line (L929) and leukemia cell line (P388).

Materials and Methods

The crude protein from the sea anemones tissue was extracted9 with methanol and fractionated10 in a DEAE-cellulose anion exchange chromatographic column. Ten fractions from the crude (5 mg/ml) were collected in a stepwise gradient with 0.1-1M NaCl in phosphate buffer saline (PBS).

The protein content was estimated11 with bovine serum albumin (BSA) as the standard and was read spectrophotometrically at 280 nm.
The haemolytic activity of crude and fractionated protein extracted from the three anemone species on chicken, goat and human erythrocyte was tested by micro haemolytic method.

*Haemolytic assay*—The micro-haemolytic test was performed in 96 well microtitre plates. Different rows were selected for chicken, goat and human erythrocyte suspensions. Serial two-fold dilutions of the crude toxin were made in 100 µl of normal saline. Then 100 µl of 1% erythrocyte was added to all the wells. Appropriate controls were included in the test. To the 1% RBC suspension, 100 µl of distilled water was added, which served as a positive control and 100 µl of normal saline served as negative control. The plate was gently shaken and allowed to stand for 2 h at room temperature and the results were recorded. Uniform red color suspension in the wells was considered as positive haemolysis and a button formation in the bottom of the wells was considered as lack of haemolysis. Reciprocal of the highest dilution of the crude toxin showing the haemolytic pattern divided by the protein content was taken as 1 haemolytic unit (HU).

*Cell line toxicity assay*—The glassware used in the experiment were of neutral glass of Borosil make and non-toxic to the cell cultures. They were soaked overnight in a mild HCl solution. Sterile disposable plasticwares such as tissue culture flasks, petridishes, multiwell plates of tissue culture grade (Tarsons, India) were used for the experiment.

Mouse fibroblast cell line (L929) and Leukemia cell line (P388) were procured from the National Center for Cell Sciences (NCCS), Pune, India. The cells were sub-cultured into a fresh flask containing Eagles Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS). The cells were rinsed in serum free medium and trypsin (0.1%) was added to remove the cells from the substratum. When the monolayer had detached from the flask, the activity of trypsin was stopped by adding serum-containing medium (serum contains the trypsin inhibitor α-1 antitrypsine). The cells were counted and the flask concentration was adjusted to 10^4-10^5 cells/ml and seeded on to a 96 well microtitre plate at the rate of 200 µl/well and incubated at 37°C.

When the wells reached 80% confluence, they were used for cytotoxicity assay with toxins of the target species. The crude samples were diluted in EMEM at the rate of 1 mg/ml and serial ten-fold dilutions were made in sterile tubes with 2% fetal calf serum. The dilutions without the toxins served as the control. The plate was incubated at 37°C for 48 h. The cells were observed under an inverted microscope for changes in their morphology.

The toxin treated plates after 48 h of incubation were taken for MTT (3, [4, 5- dimethylthiazole-2, 4] - 2-5-diphenyl tetrazolium bromide) cell viability test. MTT stock solution was prepared (1 mg/ml) and filtrates were sterilized in 0.22 µm membrane filter. MTT stock solution (50 µl) was added to each well. The plates were incubated in a humidified incubator at 37°C for 4 h. The medium was replaced with 200 µl DMSO and incubated for 10 min on a micro plate shaker to dissolve the formazan crystals of MTT. Sorensen's glycine buffer (25 µl; 0.1M glycine and 0.1M NaCl) was added to each well. The plates were then read at 570 nm against a reference of 690 nm in an ELISA reader. Plates were read within 30 min after the addition of DMSO. Samples were tested thrice for confirmation.

**Results and Discussion**

The crude as well as the partially purified protein fractions of all the three anemone species viz. *H. magnifica*, *S. haddoni* and *P. sinensis* exhibited pronounced haemolytic activity on chicken, goat and human erythrocytes. Their lytic potencies varied with variation in the protein content and the type of erythrocyte used for the experiment. The results are presented in Table 1. More fractions exhibiting haemolysis in chicken, goat and human erythrocytes were observed in *H. magnifica*. Following this, fractions of *P. sinensis* exhibited potent haemolysis in chicken and human erythrocytes but it showed weak haemolysis in goat erythrocytes. In *S. haddoni*, lesser number of fractions (2) showed potent haemolysis in goat erythrocytes and the rest of them had failed to elicit haemolysis. Weak haemolysis was observed in chicken and human erythrocytes. Thus, variations in haemolysis have been observed in different erythrocytes. The results reveal that all the sea anemone species have potent haemolytic activity, a fact that has been amply supported.

The cytolytic toxins RM-I and RM-II have been found to possess a haemolytic activity, comparable with the values exhibited by the partially purified proteins of the anemone species in the present study. The crude extract of *Radiantthus macrodactylus* is reported to possess a haemolytic activity of 1400 HU and this is comparable with the haemolytic activity exhibited by some of the fractions of
**H. magnifica**, such as fraction 4 in human erythrocytes and fractions 3 and 5 in goat erythrocytes in the present study.

Haemolytic property of the anemone extracts varied greatly on purification on a DEAE-cellulose chromatographic column. Some partially purified proteins retained the activity while others showed reduction/increase in the activity when compared with the crude (Table 1). Such variations in the haemolytic activity have also been reported from the experiments\(^{14}\) on extracts of *Radianthus macrodactylus* at various levels of purification through various columns. Thus the results of the present study are in conformity with earlier studies\(^{14}\), emphasizing that there will be variations in the haemolytic activity on purification of proteins.

The viability of mouse fibroblast cell line (L929) and leukemia cell line (P388) was adversely affected on the addition of the crude proteins extracted from all the three anemone species (Tables 2 and 3). The cells showed rounding, lysis and detachment from the substratum. However, in both L929 and P388 cell lines, the inhibition was dose dependent (i.e) higher inhibition at lower dilutions and lower inhibition at higher dilutions (Tables 2 and 3) of the crude proteins of the three sea anemone species. The level of inhibition was maximum in *H. magnifica*, followed by *P. sinensis* and the lowest level of inhibition was recorded in *S. haddoni*, in both the crude and fractionated proteins. These findings lend support to earlier findings\(^{15}\) which reports the cytotoxic effect of equinatoxin on Ehrlich ascites, leukemia cell line (L1210) and lung macrophages\(^{16}\), equinatoxin II exhibited cytotoxicity to the fibroblast cell line V-79-379\(^{17}\) cytolsin from *S. helianthus* caused cytotoxicity to human T-acute Lymphoblastoma (CEM), cell line of haematopoetic origin (K562) and human breast carcinoma (MDA-MB-134)\(^{18}\), besides being cytotoxic to MDA-MB-231\(^{15}\).

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### Table 1—Haemolytic activity of crude and partially purified fractions of the sea anemones on chicken, goat and human blood [Values are mean of triplicate sets]

<table>
<thead>
<tr>
<th>Species</th>
<th>Crude/Fraction</th>
<th>Protein content (µg/ml) (mean±SE)</th>
<th>Haemolytic Unit (HU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chicken</td>
</tr>
<tr>
<td><em>H. magnifica</em></td>
<td>Crude</td>
<td>981.1±0.32</td>
<td>4096</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>70.5±0.11</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>160.7±0.25</td>
<td>4096</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>70.3±0.18</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>80.6±0.17</td>
<td>4096</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20.3±0.13</td>
<td>4096</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>30.7±0.21</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10.9±0.17</td>
<td>2</td>
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<tr>
<td></td>
<td>8</td>
<td>10.7±0.08</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>20.4±0.15</td>
<td>4096</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.2±0.10</td>
<td>4096</td>
</tr>
<tr>
<td><em>S. haddoni</em></td>
<td>Crude</td>
<td>820.4±0.37</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30.3±0.24</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30.6±0.19</td>
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<td>3</td>
<td>140.8±0.27</td>
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<td>4</td>
<td>10.2±0.13</td>
<td>4</td>
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<tr>
<td></td>
<td>5</td>
<td>20.5±0.11</td>
<td>4</td>
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<td></td>
<td>6</td>
<td>60.4±0.23</td>
<td>2</td>
</tr>
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<td></td>
<td>7</td>
<td>10.1±0.09</td>
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<td>8</td>
<td>10.2±0.10</td>
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<td></td>
<td>9</td>
<td>10.3±0.06</td>
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<td></td>
<td>10</td>
<td>10.4±0.11</td>
<td>-</td>
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<tr>
<td><em>P. sinensis</em></td>
<td>Crude</td>
<td>605.3±0.31</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>110.6±0.33</td>
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<td></td>
<td>2</td>
<td>10.3±0.14</td>
<td>256</td>
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<td></td>
<td>3</td>
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<td>10.4±0.11</td>
<td>128</td>
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<td></td>
<td>10</td>
<td>10.2±0.11</td>
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</table>

### Table 2—Effect of crude protein of the sea anemones on MTT cell viability test in mouse fibroblast cell line (L929) [Values are mean of triplicate sets]

<table>
<thead>
<tr>
<th>Level of dilution</th>
<th><em>H. magnifica</em></th>
<th><em>S. haddoni</em></th>
<th><em>P. sinensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>10(^0)</td>
<td>93.38</td>
<td>73.78</td>
<td>89.53</td>
</tr>
<tr>
<td>10(^-1)</td>
<td>91.27</td>
<td>70.09</td>
<td>87.20</td>
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<tr>
<td>10(^-2)</td>
<td>90.61</td>
<td>69.52</td>
<td>86.33</td>
</tr>
<tr>
<td>10(^-3)</td>
<td>89.50</td>
<td>67.29</td>
<td>84.79</td>
</tr>
<tr>
<td>10(^-4)</td>
<td>87.96</td>
<td>66.34</td>
<td>84.40</td>
</tr>
<tr>
<td>10(^-5)</td>
<td>85.15</td>
<td>65.92</td>
<td>80.68</td>
</tr>
<tr>
<td>10(^-6)</td>
<td>84.97</td>
<td>65.19</td>
<td>79.83</td>
</tr>
<tr>
<td>10(^-7)</td>
<td>84.03</td>
<td>61.08</td>
<td>77.12</td>
</tr>
</tbody>
</table>

### Table 3—Effect of crude proteins of the sea anemones on MTT cell viability test in mouse leukemia cell line (P388) [Values are mean of triplicate sets]

<table>
<thead>
<tr>
<th>Level of dilution</th>
<th><em>H. magnifica</em></th>
<th><em>S. haddoni</em></th>
<th><em>P. sinensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>10(^0)</td>
<td>86.16</td>
<td>64.75</td>
<td>76.31</td>
</tr>
<tr>
<td>10(^-1)</td>
<td>85.28</td>
<td>63.30</td>
<td>75.79</td>
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<tr>
<td>10(^-2)</td>
<td>83.59</td>
<td>61.10</td>
<td>75.37</td>
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<tr>
<td>10(^-3)</td>
<td>80.93</td>
<td>59.90</td>
<td>74.80</td>
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<tr>
<td>10(^-4)</td>
<td>80.24</td>
<td>56.35</td>
<td>72.07</td>
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<td>10(^-5)</td>
<td>78.13</td>
<td>55.62</td>
<td>71.49</td>
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<td>10(^-6)</td>
<td>77.25</td>
<td>53.37</td>
<td>69.54</td>
</tr>
<tr>
<td>10(^-7)</td>
<td>75.19</td>
<td>52.32</td>
<td>67.90</td>
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Thus, results of the present study on the cytotoxicity of sea anemone extracts on different erythrocytes, mouse fibroblast cell line (L929) and leukemia cell line (P388) indicates the potential for extraction of cytotoxic compounds from sea anemones. Detailed studies in future could evolve out a compound that could be used for cancer therapeutics.

Acknowledgement
This paper is a tribute to the co-author (Late) Dr. K. Venkateshvaran, Principal Scientist, CIFE, Mumbai. The authors thank the authorities of Centre of Advanced Study in Marine Biology, Annamalai University, Paragipettai and Central Institute of Fisheries Education, Mumbai, for facilities.

References