**In-vitro** modulation of Na\(^+\), K\(^+\), Mg\(^{++}\) ATPases and AchE by sea anemone toxic proteins

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The crude as well as partially purified protein fractions of the sea anemone species viz. *Heteractis magnifica*, *Stichodactyla hadoni* and *Paracodylaactis sinensis*, were collected from the Gulf of Mannar, southeast coast of India. These were found to modulate the functioning of the sodium pump (Na\(^+\) K\(^+\) Mg\(^{++}\) ATPase) at the presynaptic complex of neurotransmission, thereby affecting ATP hydrolysis. They promote cholinesterase activity at the post synaptic complex by modulating the activity of the enzyme Acetyl Choline Esterase (AchE). Modulation was dose dependent. The proteins were toxic to mice. Histopathological observations reveal that the toxins affected the anatomy of the brain. Toxin injected mice showed various behavioral changes including manner shivering of fore limbs, loss of balance, opaque eyes, convulsions, paralysis, micturition, flexing of muscles, prodding (insensitive to stimulii), dragging of hind limbs, rolling of tail, foaming from mouth and exophthalmia. This could be possibly correlated with CNS dysfunction.

[Keywords: Sea anemones, Proteins, Sodium pump, Acetylcholine, Neurotoxins, Enzyme].

**Introduction**

Sea anemones (Phylum Cnidaria, Class Anthozoa) are known to possess neurotoxins of polypeptide nature. Many such proteins have become important tools in neurobiological research. They act by modifying the frequency and duration of action potentials, interact with membrane-ion-channels and receptors; and also disrupt synaptic vesicles. Optimal channel function requires ATP binding and hydrolysis. The Na\(^+\) K\(^+\) ATPase (sodium pump) is a pre-synaptic membrane protein of higher organisms which hydrolyses cytoplasmic ATP, interacts with neighboring membrane proteins and organized cytosolic cascades of signaling proteins to send messages to intercellular organelles. Physiologically, the ionic transport of the sodium pump is essential for electrolyte movement across epithelial cells. When Na\(^+\) K\(^+\) is inhibited by ouabain, Mg\(^{++}\) replaces the action of the above ions coupled with ATPase in the sodium pump. Dysfunction of the pump leads to neuronal dysfunction, renal abnormalities, heart disease and hypertension. Another enzyme involved in neuro-transmission is Acetyl Cholinesterase (AchE), which hydrolyzes acetylcholine (Ach) in the neuromuscular junctions and other cholinergic synapses to terminate neuronal signal. Cholinesterase inhibitors block the function of AchE and cause excessive Ach to accumulate in the synaptic cleft leading to neuromuscular paralysis and death by asphyxiation.

Such neuromodulating proteins have been reported from sea anemones viz. *Anemonia sulcata*, *Anthopleura xanthogrammica*, *Entacmaea actinostoloids*, *Radianthus macrodactylus*, *Radianthus paumotensis*, *Bolocera tuediae*, *Calliactis parasitica*, *Stichodactyla helianthus and Condylactis gigantean*, *Bundostoma granulifera*, *Actinia erythraea*, *Anthopleura fuscoviridis* and *Bundostoma caisserianum*, *Doliena armata* and *Entacmaea ramsayi* and *Anthopleura sp.*

Despite these explorations, toxic proteins of sea anemones from the Indian waters have been poorly studied for their neuromodulatory potential, which could further lead to the formulation of neuro-stimulant and/or suppressant drugs. Present study is an attempt to determine the modulation in ATP hydrolysis by Na\(^+\) K\(^+\) ATPase and Mg\(^{++}\) ATPase (sodium pump) and Ach hydrolysis by acetylcholinesterase in mitochondrial nerve endings; isolated from mouse brain, over the influence of the toxins extracted from sea anemones *viz.* *Heteractis magnifica* (Quoy and Gaimard, 1833), *Stichodactyla*...
Materials and Methods

The crude protein from the sea anemones *H. magnifica*, *S. haddoni* and *P. sinensis* was extracted with methanol and fractionated in a DEAE-cellulose anion exchange chromatographic column. Ten fractions from the crude (5 mg/mL) were collected in a step-wise gradient with 0.1 to 1M NaCl in Phosphate Buffer Saline (PBS).

Protein content was estimated with bovine serum albumin (BSA) as the standard and was read spectrophotometrically at 280 nm.

Mice bioassay for lethality—Lethality studies were conducted on Kausauli strain male albino mice of 20±2 g weight procured from M/s. Haffkine Biopharma, Mumbai, maintained in a healthy condition in the animal house, following the codal formalities of Central Institute of Fisheries Education, Mumbai, in accordance with the norms of Animal Welfare Ethics.

Stability of toxin—Toxicity bioassays (with lethal dose of toxic protein) were conducted to ascertain the stability of the crude anemone proteins under varying conditions of (i) heat (50, 60, 80 and 100°C) (ii) different pH (3.0-8.0), and (iii) storage at −20°C for more than one year.

Histopathology—Histopathological observations were made on the Brain of mice that died upon envenomation while ascertaining the toxicity of the anemone extracts.

In vitro evaluation of the effect of the toxins on the mouse brain AchE enzyme.

*P2 Fraction Preparation*- *P2* fraction (mitochondrial nerve endings) from the brain of male albino mouse (20 ± 2 g) was prepared. Brain was homogenized in ice cold sucrose solution (0.32 M) and centrifuged (Sorvall Super T-20 Refrigerated centrifuge) at 2,500 rpm for 15 min at 4°C to remove the cell debris, nuclei and plasma membrane fragments. Again, the supernatant was centrifuged at 15,000 rpm for 20 min at 4°C. Then the supernatant was discarded, the pellet was dissolved in sucrose solution and again respun at 15,000 rpm for 15 min. It was washed once again in the same fashion and the resultant pellet was dissolved in sucrose solution depending upon the pellet size and kept in deep freezer as enzyme source and the protein content was estimated.

ATPase Assay-ATPase assay was carried out following standard method. For total ATPase reaction mixture, 0.8 mL of Imidazole buffer (0.135 mM) with 100 mM NaCl, 20 mM KCl and 5 mM MgCl2, were taken in each test tube and 0.1 mL enzyme was added and stirred. To this mixture, 0.1 mL of crude toxin with different concentrations (250 μg, 500 μg, 750 μg, and 1000 μg) or 0.1 mL fractionated protein were added immediately using micropipettes.

For Mg++ATPase reaction mixture, 0.07 mL Ouabain (1 mM) was added as inhibitor for Na+ K+ ATPase in addition to the above mixture; 0.1 mL of triple distilled water was added to the total ATPase reaction mixture and 0.03 mL of triple distilled water was added to the Mg++ATPase mixture to bring the reaction mixture to a total volume of 1.05 mL.

The reaction was started by adding 50 μl of ATPase substrate (4.5 mM) in each tube. All the tubes were gently shaken and incubated at 37°C for 30 min in a water bath. By adding 0.5 mL of 10% TCA, the reaction was stopped and the contents of all tubes were centrifuged and the supernatants were taken. To this supernatant, 0.3 mL of 0.1 N sodium acetate solution followed by 0.4 mL of ammonium molybdate (1%) and H2SO4 (0.05N) were added to each tube. The colour developed was read at 800 nm in a spectrophotometer after 15 min. Control experiments were also run simultaneously with 100 μl of triple distilled water instead of toxins.

In vitro evaluation of the effect of the toxins on the mouse brain AchE enzyme

Preparation of Enzyme Source- In vitro effect of the toxins on the mouse brain AchE enzyme was evaluated. Brain isolated from the mouse was homogenized with 0.25 M ice cold sucrose solution and 2% (w/v) tissue homogenate was prepared in the same solution and stored in the freezer as enzyme source.

Phosphate buffer (3 mL) (pH 8.0) was taken in each tube to which 0.1 mL of enzyme source was added and stirred. Then 100 μl of 0.01 M DTNB (5-5-dithiobis-2 nitrobenzoic acid) was added and the initial colour was measured spectrophotometrically at 412 nm. The test solution of crude toxin (100 μl) in different concentrations such as 200, 400, 600, 800, and 1000 μg were added. For the fractions, 100 μl of sample was used. Control experiment was run simultaneously with 100 μl of PBS, instead of toxins.

To start the reaction, 20 μl of Acetyl Thio Choline Iodide (ACTI) (0.075 M) was added to each tube as substrate and then the reaction was allowed to continue for 15 minutes at room temperature. The colour
developed was measured spectrophotometrically as final reading at 412 nm.

Results & Discussion

Protein content of the crude and partially purified sea anemone extracts, its toxicity to mice and associated histopathological implications, including brain, among other vital organs and stability of toxic proteins under varying conditions of pH, temperature and storage have been published elsewhere. To avoid duplication in the presentation, such results have not been presented in this paper, though relevant and corroborative.

Crude extracts as well as the partially purified fractions of all the three anemone species (H. magnifica, S. haddoni and P. sinensis) affected the neuronal system functioning of mice. The results are presented in Tables 1 & 2. The fact that these anemone toxins affect the brain of mice has been established by histopathological observations. The findings from the brain of mice that died upon the above sea anemone toxin envenomation were: the capillaries were enlarged and glial nodules - the foci of microglia in degenerating neurons were found, particularly in the cerebrum; mild congestion of capillaries and pycnotic nuclii, a condition formed by the condensation of chromatin in the nucleus of a cell undergoing necrosis, were found in the cerebellum.

In-vitro effect of toxins on the sodium pump (Na\(^+\) K\(^+\) ATPase and Mg\(^{++}\) ATPase) - Crude protein of H. magnifica showed a dose dependent effect on the activity of the sodium pump (Figs 1 & 2). Enzyme responsible for the hydrolysis of ATP had higher activity at lower concentrations of toxins and the activity diminished when the toxin concentration increased. Crude toxic proteins of S. haddoni showed a reverse response compared to the toxin of H. magnifica, with respect to Na\(^+\) K\(^+\) ATPase activity (Fig 1), but Mg\(^{++}\) ATPase activity showed higher activity at lower concentration and lower activity when the concentration increased (Fig 2). Strong dose dependent neuromodulatory effect on Mg\(^{++}\) ATPase by TTX has been reported. Crude toxin of Protonibea diacanthus, Otolithoides biauritus and Muraenesox talabonoides is reported to elevate the Mg\(^{++}\) ATPase activity. It is also reported that the

<table>
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<tr>
<th>Fractions</th>
<th>Level of modulation of Na(^+)K(^+) ATPase activity (%)</th>
<th>Level of modulation of Mg(^{++}) ATPase activity (%)</th>
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<tr>
<td>H. magnifica</td>
<td>S. haddoni</td>
<td>P. sinensis</td>
</tr>
<tr>
<td>1</td>
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</tr>
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<thead>
<tr>
<th>Fractions</th>
<th>Moles of Ach hydrolysed/mg protein/hr</th>
<th>Level of modulation (%)</th>
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<tr>
<td>H. magnifica</td>
<td>S. haddoni</td>
<td>P. sinensis</td>
</tr>
<tr>
<td>1</td>
<td>0.024±0.002</td>
<td>0.010±0.001</td>
</tr>
<tr>
<td>2</td>
<td>0.021±0.001</td>
<td>0.011±0.001</td>
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<tr>
<td>3</td>
<td>0.016±0.001</td>
<td>0.050±0.002</td>
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<tr>
<td>4</td>
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<td>0.015±0.003</td>
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<tr>
<td>5</td>
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<td>10</td>
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Horse shoe crab extracts enhanced the mouse brain Mg\textsuperscript{++} ATPase\textsuperscript{35}. In the case of \textit{P. sinensis}, sodium pump action got lowered with the increase in concentration of toxins. Effect of fractionated proteins on the mouse brain sodium pump activity is given in Table 1. Most fractions inhibited the action of the sodium pump except the fractionated proteins (9 fractions) of \textit{P. sinensis} which increased the ouabain resistant Mg\textsuperscript{++} ATPase activity. It is interesting to note that the crude protein of all the three anemone species has stimulated the action of the sodium pump in a dose dependent manner, except the crude protein of \textit{S. haddoni}, which was inhibitory at lower concentration and turned to be stimulatory at higher concentrations. But on the contrary to the crude proteins, many of the fractionated proteins inhibited the action of the sodium pump. These results reveal that the sea anemone toxins act in the pre-synaptic region of neuromuscular transmission\textsuperscript{2,14,22,23,36-38} and the response is dose dependent. Like that in the present study, it is reported that BTTX II, a toxic protein from \textit{Bolocera tuediae}, a sea anemone, influences the activation as well as inactivation of the sodium channel\textsuperscript{39}. Also, there are reports that sea anemone toxins act on the nervous system and affect the functioning of the ion pumps\textsuperscript{19,40,24}. Hence, the present study reveals that the toxins of the test sea anemones altered the hydrolysis of ATP by Na\textsuperscript{+} K\textsuperscript{+} ATPase in the pre-synaptic region thereby possibly affecting signaling to intercellular organelles\textsuperscript{5} and electrolyte movement across epithelial cells\textsuperscript{6}. This could be the reason for few or some of the intoxicated animal behavior such as lying on belly with widespread forelimbs, running around the cage in an exited manner, escape reaction, prolonged palpitation, closed eyes, grooming, shivering of fore limbs, loss of balance, opaque eyes, squeaking, tonic convulsions, gasping for breath, arching of body backwards, paralysis, micturition, flexing of muscles, prodding (insensitive to stimuli), diarrhoea, lethargy, dragging of hind limbs, rolling of tail, foaming from mouth and exophthalmia observed during the present study.

\textit{In vitro effect of sea anemone toxins on AchE activity}-The crude as well as all fractionated proteins of the three anemones except very few, showed increase in the mouse brain AchE activity (Table 2 & Fig. 3). Activity was dose dependent in the case of crude toxin and increased with increase in concentration. Characteristic nature of toxins to act on the post synaptic region in neuromuscular transmission, thereby affecting AchE activity has been reported earlier\textsuperscript{33-35}. Few of the behavioral changes enlisted above such as flexing of muscles, tonic convulsions, arching of body backwards, paralysis might be due to the accumulation of AchE and total termination of neuronal signaling\textsuperscript{9}. Also, increase in AchE is corroborated with neuromuscular paralysis and death by asphyxiation\textsuperscript{10}. Present study reveals that the toxins of the sea anemones \textit{viz. Heteractis magnifica, Stichodactyla haddoni and Paracodylactis sinensis} act variably on the presynaptic sodium pump but promote cholinesterase activity at the post synaptic complex of neuromuscular transmission. Hence, such proteins could be used against cholinesterase inhibitors and neuromodulatory drugs can be developed from sea anemones, taking into consideration the dose at which it is beneficial and also growing further knowledge in the specific subject.

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References


