Research Paper

In vitro hemolysis and lipid peroxidation-inducing activity of the tentacle extract of the sea anemone (*Paracondylactis indicus* Dave) in rat erythrocytes

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ABSTRACT

Objective: *In vitro* hemolytic activity of the tentacle extract of *Paracondylactis indicus* (Dave), a sea anemone found in the eastern coastal region of West Bengal (India), was determined in rat erythrocytes.

Materials and Methods: Acute toxicity study was carried out with the tentacle extract followed by detailed biochemical studies to evaluate the direct and indirect hemolytic activities of the extract. The effects of pH, temperature, divalent cations and chelating agents on hemolysis were also evaluated. Efforts were also made to elucidate the mechanism of the hemolytic activity of the extract.

Results: The tentacle extract of *P. indicus* (Dave) produced significant hemolysis (both direct and indirect) in washed rat erythrocytes. The direct hemolytic activity of the tentacle extract was found to be both temperature- and pH-dependent. Divalent cations (Ca^{2+}, Mg^{2+}) produced significant enhancement of direct hemolytic activity. Hemolysis (both direct and indirect) was significantly diminished in the presence of Zn^{2+}, EDTA and *p*-bromophenacyl bromide. The tentacle extract was found to induce lipid peroxidation in washed erythrocytes, which was inhibited by chlorpromazine. The tentacle extract demonstrated significant proteolytic activity, which was found to decrease in the presence of protease inhibitors (EDTA, EGTA and PMSF).

Conclusion: *P. indica* tentacle extract (and in particular, the 60% cut fraction) probably contains enzymatic components like phospholipase(s) along with proteases, which may be the contributing factors for its observed hemolytic activity.

KEY WORDS: Hemolysis, *Paracondylactis indicus*, sea anemone, tentacle.

Introduction

The venoms produced by sea anemones to overwhelm their prey and / or repel predators are located on the tentacles containing specialized cellular organelles called nematocysts. The hemolytic activities of various cytolysins and their specific binding to various membrane phospholipids have been well documented.[1] However, the exact mechanism(s) of action of these cytolysins remain unclear. Some reports indicate that these venoms disrupt the biological membranes by forming pores.[2] Lipid peroxidation is regarded as one of the main manifestations of oxidative damage and has been attributed as one of the major pathways for explaining the toxicity of many xenobiotics. During the peroxidative damage of lipid membranes, there is loss of polyunsaturated fatty acids, decreased lipid fluidity, alteration in membrane permeability, release of membrane-bound enzymes, alteration in ion transport and generation of lipid hydroperoxides.[3] Many studies conducted with *in vitro* and *in vivo* models have shown that during oxidative stress, several parameters are actively involved such as changes in permeability of erythrocyte membranes to ions,[4] increased lipid peroxidation[5] and activation of proteolysis.[6]

The present biochemical study (using *in vitro* assay models) on a sea anemone species from the West Bengal coastline is preliminary in nature and also the first of its kind conducted on *Paracondylactis indicus* (*P. indicus*) tentacle extract (venom).

Materials and Methods

Preparation of the tentacle extract

Sea anemones (*P. indicus*) were collected from the coastline of West Bengal, (South 24 Parganas, India), during the month of January. The samples were authenticated by the Zoological Survey of India, Kolkata. The tentacles were separated and homogenized in phosphate-buffered saline (pH 7.2) and centrifuged at 10,000 r.p.m, at 4˚C for 30 min. The brownish

supernatant was collected (hereafter referred to as "tentacle" or "crude" extract) and preserved at -70˚C till use. Concentration of the tentacle extract in various experimental models has been expressed in terms of protein equivalent.

Protein estimation

Protein content of the crude extract and different fractions (obtained following ammonium sulphate saturation) were estimated following the method of Bradford[7] using bovine serum albumin as standard.

Animals used

All pharmacological experiments were conducted using rats of Charles foster strain (each weighing 120-180 g) and mice of Swiss Albino strain (each weighing 18-22 g). The animals (obtained from the Indian Institute of Chemical Biology (IICB), Kolkata 32) were acclimated to the laboratory environment for seven days. According to the ethical guidelines, the animals were housed in standard polypropylene cages (at 24 ± 2˚C) and provided *ad libitum* access to food and water.

Acute toxicity study (determination of LD_{50} dose)

The LD_{50} dose was determined according to the method of Litchfield and Wilcoxon.[8] For acute toxicity studies, groups of male mice (ten in each group) were injected (*i.p.*) with different doses of the tentacle extract and mortality was recorded up to 24 h.

Ammonium sulphate precipitation of the tentacle extract

The crude tentacle extract was saturated with different concentrations of ammonium sulphate (30%, 60% and 80% saturation). After centrifugation at 10,000 r.p.m. for 30 min, the precipitates were subjected to dialysis and kept aside for future experimentation.

Direct hemolytic activity

Hemolytic effect of the tentacle extract was examined using washed rat erythrocytes (red blood cells, RBCs). Blood samples were collected in citrated tubes, repeatedly washed with 20 mM Tris-HCl containing 144 mM NaCl (pH 7.4). Thereafter, 1% RBC suspension was prepared and the subsequent erythrocyte suspension was incubated at 37˚C for 30 min (Incubator Shaker, Model KMC-8480 SL, Vision Scientific) with different concentrations of the tentacle extract or respective controls (referred to as "test samples"). Following incubation, the samples were centrifuged at 2000 r.p.m. for 5 min and the absorbance of the supernatant was read at 540 nm in a Hitachi U-2000 spectrophotometer. Percentage hemolysis was determined following comparison with 100% lysed erythrocytes. In order to test the possible involvement of free radical-mediated oxidative damage in the observed hemolytic activity, the antioxidant glutathione (GSH) (50 µM) was added to additional tubes containing the RBCs and crude extract.

Time course hemolysis of the tentacle extract

One percent RBC suspension along with the tentacle extract was incubated for different time intervals (37˚C) and the time period necessary for 100% hemolysis (measured at 540 nm) was determined.

Effect of temperature on the hemolytic activity

The test samples were preincubated at 40˚C, 60˚C and 80˚C respectively for 20 min. These heat-treated samples were then incubated with 1% RBC suspension and the hemolytic activity was determined at 540 nm.[9]

Effect of pH on the hemolytic activity

The test samples were incubated with washed erythrocytes at different pH (3.5, 5.5, 7.5 and 9.5) for 45 min[10] and the absorbance was measured at 540nm.

Effect of different divalent cations and protease inhibitors on the hemolytic activity

Different divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}) and ethylenediamine tetraacetic acid (EDTA), a protease inhibitor (20 mM) were incubated with the test samples for evaluating their effects on erythrocyte hemolysis.[10] The degree of hemolysis in the reaction mixture was measured at 540 nm.

Effect of P. indicus tentacle extract (venom) on lipid peroxidation

The peroxidative effect of the tentacle extract on rat erythrocytes was assessed by the estimation of the thiobabituric acid-reactive substances (TBARS). Aliquots (1 ml) of 2% RBC suspension preincubated with the extract were mixed with 2 ml of TBA (thiobarbituric acid) reagent (containing 0.375 g TBA, 15 g tricholoroacetic acid (TCA) and 2.5 ml HCl). The solutions were heated in a boiling water bath for 30 min and then centrifuged at 3000 rpm for 15 min. Optical density was then measured at 532 nm. Results are expressed as nmol TBARS/ mg protein/ ml.[11]

Effect on indirect hemolysis

Indirect hemolysis involves the conversion of lecithin to lysolecithin and fatty acids, which are known to induce hemolysis of erythrocytes.[12] Erythrocyte suspensions were incubated with the tentacle extract (0.05-0.2 mg/ml) in the presence of 30 µg/ml lecithin and 1.25 mM $CaCl_2$. Reaction was stopped by the addition of ice-cold saline. Samples were centrifuged for 10 min at 4000 r.p.m. and the absorbance of the supernatant was measured at 540 nm.

The same procedure (incubation steps described above) was repeated in the presence of Zn^{2+}, EDTA or *p*-bromophenacyl bromide (*p*-BPB, a phospholipase A2 [PLA_2] inhibitor), the tentacle extract and the fractions (obtained from ammonium sulphate saturation).

Proteolytic activity

The process of casein digestion by the tentacle extract was used as a measure of its proteolytic activity.[13] A solution of 1% (w/v) casein in 20 mM phosphate buffer (containing 150 mM NaCl, pH 8) was incubated with different concentrations (protein mg/ml) of the tentacle extract for 1 h at 37°C. Thereafter, 0.5 ml of 10% TCA was added and the protein concentration in the supernatant was determined at 625nm.[14] The same assay procedure was used to determine the effects of different protease inhibitors-EDTA (50 mM), ethyleneglycol-bis-N,N'-tetraacetic acid (EGTA; 10 mM) and phenylmethylsulphonyl fluoride (PMSF; 50 mM). Proteolytic activity was calculated using trypsin as a standard protease.

Statistical analysis

All values are presented as Mean ± standard error (SE) (n = 6). Statistical analyses were performed with analysis of variance (ANOVA) followed by post-hoc Dunnett's test and 't' test.

Results

Acute toxicity

LD_{50} of the extract was found to be158.5 mg/kg *i.p.* in mice.

Direct hemolytic activity

The tentacle extract produced a concentration-dependent (50-400 µg/ml of protein) direct hemolytic response in rat erythrocytes. Complete lysis (100%) of the erythrocytes was observed after 3 h of incubation with the *P. indicus* tentacle protein. However, the addition of GSH to the reaction mixture produced significant reduction in erythrocyte lysis [Figure 1].

Effect of temperature on hemolytic activity

The *P. indicus* tentacle protein showed variable hemolytic activity when exposed to a temperature range of 30-80˚C (42.16 ± 1.26% at 30˚C, 52.71 ± 2.01% at 40˚C, 31.32 ± 0.88% at 60˚C and 21.16 ± 1.63% at 80˚C respectively). The maximum hemolytic activity was recorded at 40˚C.

Effect of pH on hemolytic activity

The hemolytic response of the extract was found to be influenced by the pH of the reaction medium. The optimum pH for the hemolytic activity of this venom was found to be 7.5 [Figure 2].

Effects of different ions (divalent) on hemolytic activity

Different divalent cations produced remarkable changes in the hemolytic activity of the tentacle extract. The presence of Ca^{2+} and Mg^{2+} significantly increased the hemolytic activity of the extract while EDTA (a divalent ion chelator) produced attenuation of the hemolytic response. Interestingly, the presence of Zn^{2+} in the reaction mixture effectively inhibited the direct hemolytic activity of the extract [Table 1].

Lipid peroxidation activity

The tentacle extract of *P. indicus* showed a concentration-dependent (50-200 µg/ml of protein) increase in lipid peroxidation measured as TBARS (expressed in nmol TBARS/ mg protein/ ml). Preincubation of the tentacle extract with chlorpromazine (100 µM) produced a significant reduction in lipid peroxidation [Figure 3].

Effect of different inhibitors on indirect hemolysis using washed rat erythrocytes

The crude tentacle extract and also the 60% cut (protein) fraction produced significant indirect hemolysis of the washed erythrocytes. The addition of Zn^{2+}, EDTA or *p*-BPB

Figure 1: Histogram showing inhibitory effect of GSH on *P. indicus* tentacle extracts -induced direct hemolytic activity (values are expressed as mean ± SE, n=6). (ANOVA: F =374.8,d.f. =11, *P*<0.0001),**P* < 0.001 by post-hoc Dunnet's test (vs., saline control) and (ANOVA: F=176.9,d. f. =11, *P*<0.0001),***P*<0.001(vs. GSH control). Control (20 mM Tris-HCl with 144 mM NaCl; pH-7.4). GSH control (50 µM GSH in 20 mM Tris-HCl Buffer; pH-7.4)

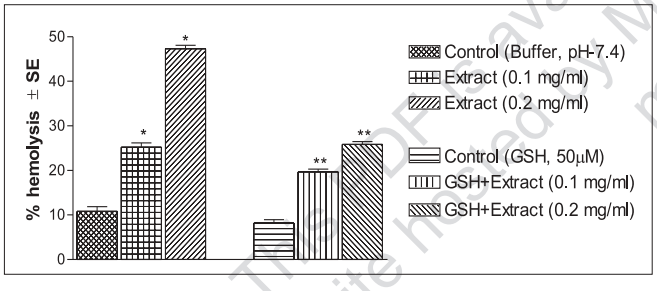


Figure 2: Histogram showing the effect of pH on *P. indicus* tentacle extract - induced hemolytic response (values are expressed as mean % hemolysis ± SE, n=6). '*P*' value (vs. respective control) by 't' test: **P*<0.001. Control 1 and 2 (100mM Citric acid buffer), Control 3 (100mM Phosphate buffer), Control 4 (100mM Carbonate buffer)

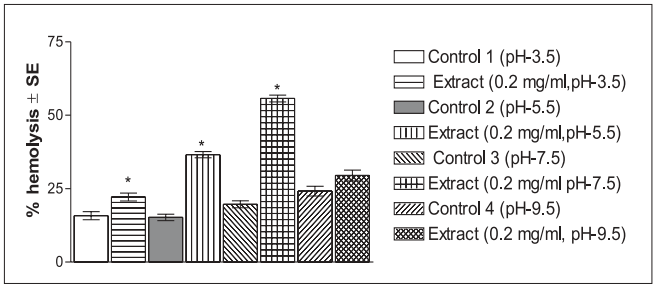


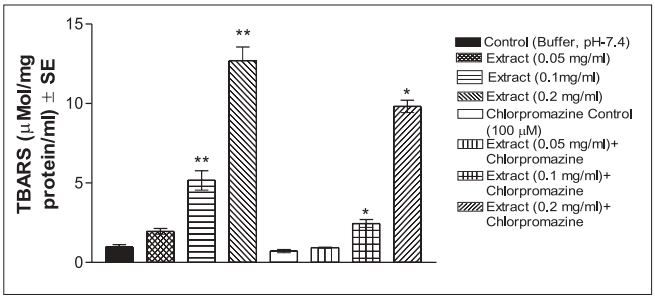
Table 1

Effects of different divalent cations and inhibitors on *P. indicus* (tentacle protein)-induced direct and indirect hemolysis (*n* = 6; mean ± SE)

Drug	*Direct hemolysis (%)*	*Indirect hemolysis (%)*
PBS	5.25 ± 0.13	6.12 ± 1.13
Crude (Tentacle protein)	50.79 ± 3.04	48.67 ± 1.24
Crude + Ca^{2}+	75.61 ± 2.30*	- NT -
Crude + Mg^{2}+	71.78 ± 2.17*	- NT -
Crude + Zn^{2}+	10.78 ± 2.56*	21.39 ± 0.83*
Crude + EDTA	32.5 ± 2.03*	34.26 ± 1.10*
Crude + BPB	- NT -	11.02 ± 1.04*
60% ammoniumsulphate cut fraction	- NT -	41.56 ± 1.25
60% cut fraction + Zn^{2}+	- NT -	14.15 ± 0.63**
60% cut fraction + EDTA	- NT -	30.60 ± 1.36**
60% cut fraction + BPB	- NT -	8.60 ± 0.89**

'*P*' values by 't' test: **P* < 0.001 (*vs*. crude protein control), ***P* < 0.001 (*vs*. 60% ammonium sulphate cut fraction control)

Figure 3: Histogram showing the effect of chlorpromazine on in vitro lipid peroxidation activity of *P. indicus* tentacle extract on washed rat erythrocytes. (values are expressed as mean % hemolysis ± SE, n=6). (ANOVA: F =99.73,d.f. =19, P<0.0001), ***P* < 0.001 by post-hoc Dunnet's test (vs., saline control) and (ANOVA:F=334.6,d.f.=19, *P*<0.0001), **P*<0.001(vs. Chlorpromazine control). Control (20 mM Tris-HCl with 144 mM NaCl; pH-7.4). Chlorpromazine control (100 µM chlorpromazine in 20 mM Tris-HCl Buffer; pH-7.4)



(a phospholipase A_2 [PLA_2] inhibitor) to the reaction mixture (containing either the tentacle extract or the 60% cut fraction) produced significant attenuation of the mean per cent hemolysis when compared to the respective controls [Table 1].

Proteolytic activity

The tentacle extract and the different cut fractions were assayed for their proteolytic activity. The 60% ammonium cut fraction showed the highest proteolytic activity (56.71%) when casein was used as a substrate.

In another study, the test samples (crude tentacle extract; 60% and 80% ammonium sulphate cut fractions; trypsin) were preincubated with inhibitors like EDTA, EGTA and PMSF. EDTA displayed maximum inhibitory activity against the crude tentacle extract (55.48%), 60% cut fraction (71.92%) and 80% cut fraction (85.54%). However, PMSF produced maximum inhibition (96.01%) of trypsin.

Discussion

Sea anemones contain a range of active biological compounds including some potent toxins. It has been reported that the venoms present in different species of sea anemones are known to possess potent hemolytic properties.[15] It has been observed in most of the reported cases that such toxic properties are attributed to the presence of secretory phospholipases.

A detailed literature survey reveals inadequate scientific information about the biological activities of *Paracondylactis indicus* (Dave), an endemic species found in abundance in the eastern coastal regions of West Bengal, India. It is noteworthy to mention that most of the earlier reports claim that *in vitro* hemolysis can serve as a sensitive test to express the degree of cytotoxicity, necessary for characterization of the toxic cytolysins.[16] Hence, we tried to evaluate the *in vitro* hemolytic activity of this tentacle extract in addition to conducting an acute toxicity study. In our present study with the *in vitro* hemolytic model, the observed hemolytic concentration (HC_{50}) was found to be 210 µg (protein/ml) as compared to the LD_{50} dose of 158.5 mg/kg determined from an acute toxicity study in mice.

Hemolytic activities observed with animal venoms can be categorized in to two groups: (i) those which lyse erythrocytes directly and (ii) those which possess an indirect action (*i.e.*, requiring the presence of lecithin or free fatty acids) in order to induce hemolysis.[17] The tentacle extract was found to possess both direct and indirect hemolytic activities in washed rat erythrocytes. Apart from the crude venom extract, we have also evaluated the direct and indirect hemolytic activities of three different cut fractions (*i.e.*, 30%, 60% and 80% ammonium sulphate saturated protein precipitates). It was observed that unlike the crude tentacle extract, none of these fractions produced direct hemolysis (data not shown). However, the 60% cut protein fraction was found to produce indirect hemolytic (data related to other fractions have not been depicted) activity as compared to the other ammonium sulphate cut fractions.

The observed hemolytic activity of *P. indicus* tentacle extract (derived from the tentacles of sea anemone) was found to be influenced by both pH and temperature. The hemolytic activity was found to increase up to a temperature of 40˚C but thereafter, it decreased with increase of temperature and was completely lost when the temperature was raised to 80˚C. Optimum activity was best expressed in a slightly alkaline pH of 7.5. This was in agreement with an earlier report about sticholysin II (St-II), a cytolysin from *Stichodactyla helianthus*, (a basic 20 kDa polypeptide) possessing potent hemolytic effect against cell membranes.[18] It has been reported that alteration of pH induces some conformational changes in the protein structure, thereby modifying the binding of these toxins to the target membranes, finally leading to hemolysis.[19] It was found that exposure to divalent cations affected the hemolytic (both direct and indirect) activity of the *P. indicus* venom extract, where it was found to be enhanced by Ca^{2+} and completely inhibited by Zn^{2+} ions. This finding is consistent with earlier reports on hemolytic activity of other species of sea anemones.[20-22]

The sea anemone, *Aiptasia pallida* contains a multi-component venom, possessing both hemolytic and cytolytic activities. The hemolytic action of *A. pallida* crude venom was attributed to at least three synergistic protein components including a major PLA_2 component, which was required for the cytolytic activity.[23] This particular PLA_2 component was found to be activated in the presence of Ca^{2+} and a slight alkaline pH (7.7). It was also observed that this enzyme component was inactivated in the presence of Zn^{2+}, thereby suggesting a probable binding of Zn^{2+} to the Ca^{2+} binding site in the PLA_2 catalytic domain.[24] The *A. pallida* nematocyst venom was found to be unstable at temperatures above 40°C and at least 50% of the hemolytic activity was lost after exposure at 56°C. Our results show a distinct similarity to the observations made with *A. pallida* nematocyst venom, thereby indicating the probable presence and involvement of a phospholipase A-like enzymatic component in the *P. indicus* tentacle venom extract. Furthermore, from our studies related to proteolytic (caesinolytic action) activity, it was also evident that both the *P. indicus* tentacle extract and the 60% cut fraction possess strong proteolytic activity and these proteases present in the extract probably contribute towards erythrocyte membrane degradation.[25]

Currently, lipid peroxidation is regarded as one of the most important organic expressions of oxidative stress induced by the formation of free radicals during various types and levels of xenobiotic exposure. Erythrocytes are found to be the common targets of such oxidative damage because they are rich in high concentrations of unsaturated lipids as well as iron (in the hemoglobin). These cells cannot replace the damaged cellular components and hence, oxidative damage may induce a permanent alteration in the red cell membranes.[26] In addition, erythrocyte membrane proteins are susceptible to covalent damage including cross-linking and aggregation by oxygen radical-induced lipid peroxidation.[27] It has also been suggested that the fatty acids / phospholipids present in the RBC membranes may be the preferred substrates for phospholipases, leading to the distinct phases of initiation (formation of catalytic levels of lipid hydroperoxides) and propagation (extension of lipid hydroperoxide formation upon degradation) during membrane damage.[3]

In our study, the hemolytic activity of the extract was found to be dose-dependent and similarly, studies related to the lipid peroxidation assay also showed a concentration-dependent

response in washed erythrocytes. The presence of GSH produced significant reduction of the extract (venom)-induced direct hemolysis indicating the possible involvement of free radical formation during hemolysis. Free radical formation during hemolysis can in turn induce a reduction in membrane fluidity and increase erythrocyte membrane fragility.[3] Therefore, our observation in the lipid peroxidation models suggests that the peroxidation process might at least partially contribute to the hemolytic effect, a finding which is similar to an earlier observation made with *Bartholomea annulata*, another species of sea anemone.[26]

Beckman *et al.*[28] demonstrated the probable involvement of phospholipase A in lipid peroxidation and the accumulation of TBARS in rat liver microsomes. In our studies on lipid peroxidation, chlorpromazine, an inhibitor of phospholipase A,[28] strongly inhibited the tentacle extract-induced lipid peroxidation. Our observation with chlorpromazine was similar to earlier observations where chlorpromazine significantly inhibited phospholipase-induced lipid peroxidation.[28]

Furthermore, indirect hemolytic assay with the sea anemone tentacle preparation revealed that when the tentacle preparation (crude and 60% cut fraction) was preincubated with 3.3 mM *p*-BPB (a phospholipase A_2 blocker), there was a significant reduction in indirect hemolysis. The present study was similar to earlier observations recorded with a phospholipase enzyme from *Naja kaouthia* venom,[13] where significant inhibition of phospholipase activity was observed following preincubation with 3 mM of *p*-BPB.

Therefore, it may be concluded that the *P. indicus* tentacle extract (and in particular the 60% cut fraction) probably contains enzymatic components like phospholipase(s) along with proteases, which may be the contributing factors for the observed cytotoxicity.

Further studies related to bioactivity-guided purification and characterization of the hemolytic peptide(s) are currently under progress in our laboratory.

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