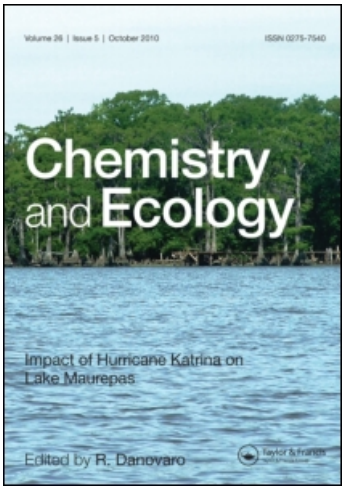

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HEMOLYTIC EFFECTS OF CRUDE VENOM FROM *AIPTASIA MUTABILIS* NEMATOCYSTS

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The aim of this paper was to focus on the toxinological aspects of microbasic-mastigophore nematocysts isolated from acontia of *Aiptasia mutabilis*, an Anthozoan collected in the Strait of Messina, by performing hemolytic assay on human, chicken, and rabbit red blood cells in suspension. The hemolytic effects of single isolated nematocysts were achieved by checking the lytic pattern after discharge. Crude extract from a population of isolated nematocysts was obtained by sonication on ice. Hemolysis induced by crude extract was detected spectrophotometrically. The results of the extraction method used show that crude venom that can be obtained is effective in inducing hemolysis of erythrocytes from the different sources. Rabbit erythrocytes in particular seem to be more sensitive to this crude venom. The hemolytic activity is dose-dependent and is inhibited if heated to 70 °C. Further investigations will better focus these results leading to venom characterization.

Keywords: Nematocyst; Crude extract; Hemolysis; *Aiptasia mutabilis*; Cnidaria

1 INTRODUCTION

Cnidarians (Coelenterates), in evolutionary terms the most primitive of Metazoa, developed a special mechanism for self-defence and attacking prey, based on nematocyst discharge. Nematocysts are complex organelles, contained in specialized cells, the nematocytes, and are considered one of the most complex cellular secretory products.

A nematocyst, which has a capsule wall comprised of three layers, contains an inverted tubule and a matrix with the capsular fluid, in which toxins are stored. When an adequate chemico-physical stimulus is applied, the thread is rapidly everted (discharge), adhering to or penetrating the integuments of prey. Toxins are then injected. Nematocyst discharge is one of the most rapid biological phenomena known to date, since it occurs with an initial acceleration of 40,000*g* and takes place within 3 msec (Holstein and Tardent, 1984). Different theories have been put forward to explain discharge such as the pressure exerted by apical flaps or operculum (Yanagita and Wada, 1954), an increase in pressure due to a water influx (Lubbock and Amos, 1981), the activation of contractile structures (Cormier and Hessinger, 1980), a reduction of mechanical resistance of the capsule contents allowing the shrinkage of the wall and consequently the thread eversion (Salleo, 1984). Mechanical

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stimulus, produced by a swimming prey, can be combined with a chemical one, such as organic compounds delivered by either prey or aggressor. Since the nematocyte has a kinocilium, it could be considered a mechanoreceptor, while chemoreception could be controlled by sensory cells or supporting cells (Watson and Hessinger, 1989; 1992).

Moreover, nematocysts discharge seems to be a Ca^{2+} activated phenomenon (Salleo *et al*., 1994a; 1994b) since Ca^{2+} influx occurring in supporting cells or sensory cells provokes NO release and leads to the discharge (Salleo *et al.*, 1996). More recently, the ability of thiol compounds to induce the discharge has been clearly shown (La Spada *et al.*, 2002).

As regards the toxinological aspects of capsular fluid, a large variety of Cnidarians specimens have been investigated so far and toxins have been extracted by several methods. In particular those of the Portugese Man-of-war (*Physalia physalis*) (Edwards and Hessinger, 2000), the sea wasp (*Chyronex fleckery*) (Tibbals *et al.*, 1998), the sea nettle (*Chrysaora quinquecyrrha*) (Bloom *et al.*, 2001), *Carukiia barnesi* (Wiltshire *et al.*, 2000), and *Chrysaora achlyos* (Radwan *et al.*, 2000) have been studied.

Because Anthozoans are easily maintained in aquaria, unlike Schyphozoans, many sea anemones toxins are well known. In general, toxinological aspects of Cnidarians have been studied because of their considerable impact on public health. In fact, accidental contact of bathers with Cnidarians, especially with Schyphozoans or Hydrozoans, induce severe local and systemic pathologies (Williamson *et al.*, 1996). Cnidarians envenomation produces immediate and sharp localized skin pain, but more severe symptoms can follow, such as vasospasm, cardiac irregularities, peripheral neuropathy and even death.

Observation of the toxic effects on organisms treated with Cnidarians toxins led to further investigation into the biochemical characteristics of the single toxins in the capsular fluid. Sea anemones are a rich source of biologically active peptides and proteins as hemolysins (Macek, 1992), so that specimens of *Actinia tenebrosa* (Simpson *et al.*, 1990), *Stichodactyla helianthus*, and *Heteractis magnifica* (Mebs, 1994) have been thoroughly investigated. It is known that toxins can affect ion transport of sodium and calcium in particular (Norton, 1991; Zorec *et al.*, 1990), induce channels or pores in neural and muscular cellular membranes (Nishio *et al.*, 1991; Anderluh and Macek, 2002), alter the structure of membranes, and organelles and release mediators of inflammation. The activity of Cnidarian toxins is assessed mainly from the lytic effect on various cells, especially on erythrocytes (Long-Rowe and Burnett, 1994; Malpezzi and Freitas, 1991).

Hemolysis assays are extremely simple quantitative procedures for detecting cytolysins which act by making the cell membrane permeable to ions. Simply by looking it is possible to determine if the proper range of venom dilutions has been selected in order to obtain a complete dose–response curve.

The purpose of our study was to assess the possible hemolytic activity of crude venom extracted by either a chemical or a physical method from isolated nematocysts of the Anthozoan *Aiptasia mutabilis* living in the Strait of Messina. Our investigations attempted to determine the toxicity of crude extracts of isolated nematocysts thus excluding other biologically active compounds of tissues. The hemolytic test was carried out on fresh human red blood cells (RBCs). Chicken and rabbit RBCs were also tested.

2 MATERIALS AND METHODS

Specimens of *A. mutabilis* were collected from the Strait of Messina. They were maintained in closed-circuit aquaria at a temperature of 18–22 °C and fed weekly on shrimp.

Nematocysts were isolated as follows: acontia were cut out from the specimens, washed several times in low Ca^{2+}-concentrated artificial sea water (ASW, whose composition in $mmol/l^{-1}$ is the following: NaCl 520; KCl 9.7; $CaCl_2$ 10; $MgCl_2$ 24; $MgSO_4$ 28; imidazole 5; pH = 7.65; $\pi = 1100\,mOsm/kg_{H_2O}$), collected in a tube and treated with a 1 M sodium citrate solution. After 10 min of incubation, the acontia were gently shaken with a pipette to allow nematocysts to be detached and then the isolated capsules were repeteadly washed in ASW.

Isolated nematocysts were either immediately used or stored at −20 °C. Thawed nematocysts were exhaustively rinsed with ASW before use. They were anatomically intact and maintained their discharge capability. The responsiveness of the capsules was expressed as a percentage of discharged capsules in a population of >50 capsules during a period of 15 min. It was tested by counting under a light microscope the number of nematocysts discharged after the addition of the discharging agent (553 mM NaSCN plus Ca^{2+} 10 mM, pH = 7.65).

Stored capsules were used whenever specimens of *Aiptasia* were unavailable because of bad weather.

Since acontia mostly contain microbasic-mastigophore capsules, the crude venom was obtained exclusively from this type of nematocyst.

For the hemolytic assays, fresh human erythrocytes were centrifuged from citrate blood, washed three times with isotonic buffer (NaCl 0.9% plus Tris–HCl 0.01 M, pH = 7.4) and resuspended in the same buffer to a final concentration of either 5% or 0.5%.

To test the hemolytic activity of single isolated nematocysts, a slide was prepared with 5% fresh human RBCs suspension in an isotonic buffer. An equal volume of isolated resting nematocysts was added together with two volumes of the discharging agent. A cover slip was put in place and hemolysis around discharged nematocysts was then seen under a light microscope (Leica DMLS, ×200). Control conditions were maintained by treating 5% RBCs suspension with the discharging agent, without nematocysts.

In order to extract crude venom from isolated nematocysts, capsules suspended in ASW, were first counted in a Burker chamber, resuspended in an appropriate volume of isotonic buffer (pH = 7.4) and immediately sonicated on ice (Sonoplus) 25 times for 20 sec, at 70 mHz. Care was taken not to keep intact nematocysts too long in the isotonic buffer since 145 mM NaCl could induce discharge, as previously described by Salleo *et al.* (1983; 1984).

Separation of crude venom from crushed capsules was achieved by centrifugation and the extracted venom was kept on ice until use, and never for longer than 1 h from sonication. Each preparation of crude venom was tested for the hemolytic activity on 0.5 volumes of a 5% fresh human RBCs suspension.

Aliquots of crude venom were employed to measure the protein concentration by BCA protein assay (Pierce).

Crude venom-induced hemolysis was also spectrophotometrically detected, samples of 0.5% fresh human or chicken or rabbit RBCs suspension being treated with different aliquots of the crude venom. The total volume of each sample was 1 ml and the venom doses employed (final concentration) were respectively 1%, 2%, 5% and 10% (v/v). Samples were placed in microfuge tubes and incubated at room temperature for 60 min, with gentle shaking. They were then centrifuged (3000*g*) and the supernatant was spectrophotometrically read (540 nm). Total hemolysis (100%) was obtained by centrifuging the same amount of RBCs and suspending them in the same volume of distilled water.

Heat treatment of crude venom was carried out by exposing it at different temperatures, in a thermostat-regulated double boiler for different periods of time with the following protocol: 4, 20, 40, 60 and 70 °C for alternatively 5 min, 30 min and 1 h.

3 RESULTS

Intact nematocysts of *A. mutabilis* were obtained by sodium citrate isolation (Fig. 1) so that 553 mM NaSCN plus Ca^{2+} 10 mM was effective in inducing capsules discharge: such nematocysts could be successfully used in hemolytic assays.

Protein concentration of crude venom obtained from a sample of 200 nematocysts/μl was 1.7 μg/μl.

The discharge of isolated nematocysts on human RBCs suspension induced crude venom release so that within 1 h of nematocyst discharge a hemolytic area was observed around the everted tubule of each fired capsule (Fig. 2).

Isolated crude venom (0.34 μg/μl) induced total hemolysis of 5% fresh human RBCs suspension placed on a slide within 10 min of venom treatment. The microscope observations revealed that after venom addition the RBCs membrane remained intact and the cytoplasm became transparent as a result of hemoglobin release. Ghosts were then observed.

The lytic action of crude venom extracted from nematocyst of *A. mutabilis* on erythrocytes from various animal sources is shown in Figure 3, where the hemolysis percentage and the different crude venom dilutions are plotted. Table I shows the doses of crude venom (μg/μl 0.5% RBCs suspension) needed for 50% hemolysis to occur.

As shown, notable hemolysis, comparable with total hemolysis, was induced by crude venom at a final concentration of 0.102 μg protein/ml of 0.5% RBCs suspension, on erythrocytes from the three different sources, given that values ranging between 83% and 96% of hemolysis were reached. It is worth noting that 68% hemolysis was already induced on rabbit RBCs by the lowest dose, that is 0.0051 μg protein/ml of RBCs suspension, whereas the same dose induced 20% and 10.55% hemolysis on human and chicken RBCs, respectively. The difference between hemolysis provoked by the lowest doses and the highest doses was significant ($p < 0.05$) for both chicken and human RBCs but not for rabbit RBCs. In the latter case no difference was observed between each dose given (Fig. 3).

As previously mentioned, fresh crude venom at 0.34 μg/μl completely lysed a 5% human RBCs suspension within 10 min of treatment.

The same result was noted with venom (0.34 μg/μl) stored at 4 °C respectively for 30 min and one 1 h after extraction. An aliquot of the same crude venom maintained at 20 °C for

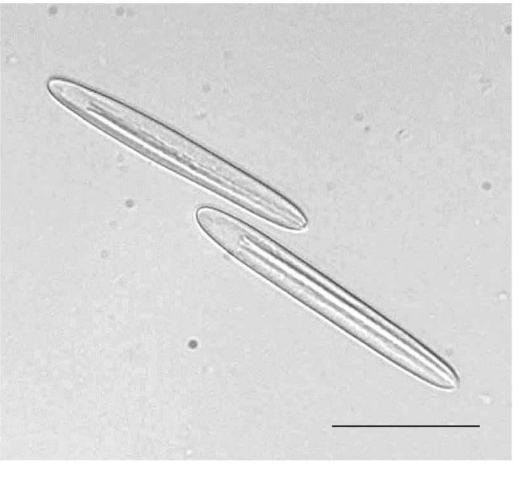

FIGURE 1 Isolated resting microbasic-mastigophore nematocysts from acontia of *A. mutabilis*. Light microscope observation (bar: 30 μm).

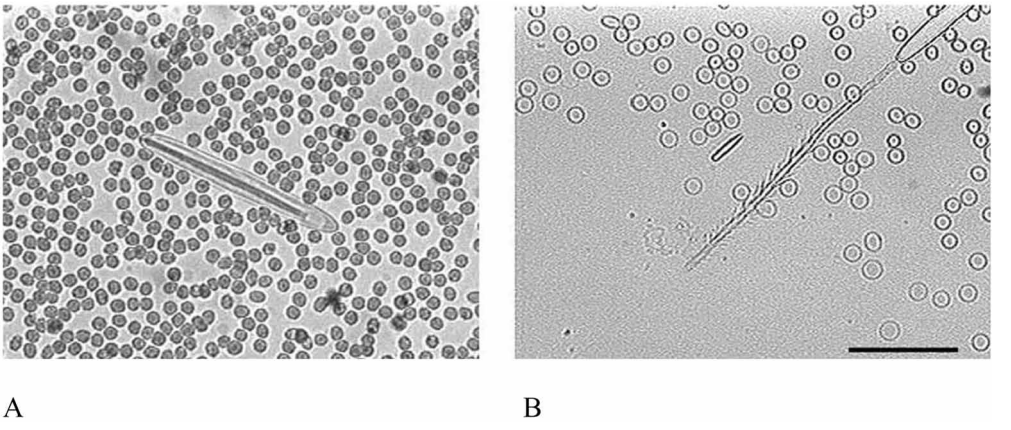

FIGURE 2 Hemolytic activity of a single microbasic-mastigophore nematocyst discharging in SCN^- solution on human 5% RBCs suspension. (A) Resting nematocyst; (B) fired nematocyst and hemolytic area around the everted tubule after 30 min of discharge (bar: 30 μm).

5 and 30 min was still able to induce hemolysis, while storage at 20 °C for 1 h led to the loss of the hemolytic power and only erythrocytes swelling was noted in this case.

Hemolytic power was not affected by treatment of crude venom for 5 min at 40 °C, whereas storage at the same temperature for 30 min prevented hemolysis.

Finally, hemolysis was significantly reduced ($p < 0.05$) when crude venom was treated for 5 min at 60 °C, completely prevented by the same treatment for 30 min and by treatment at 70 °C for 5 min.

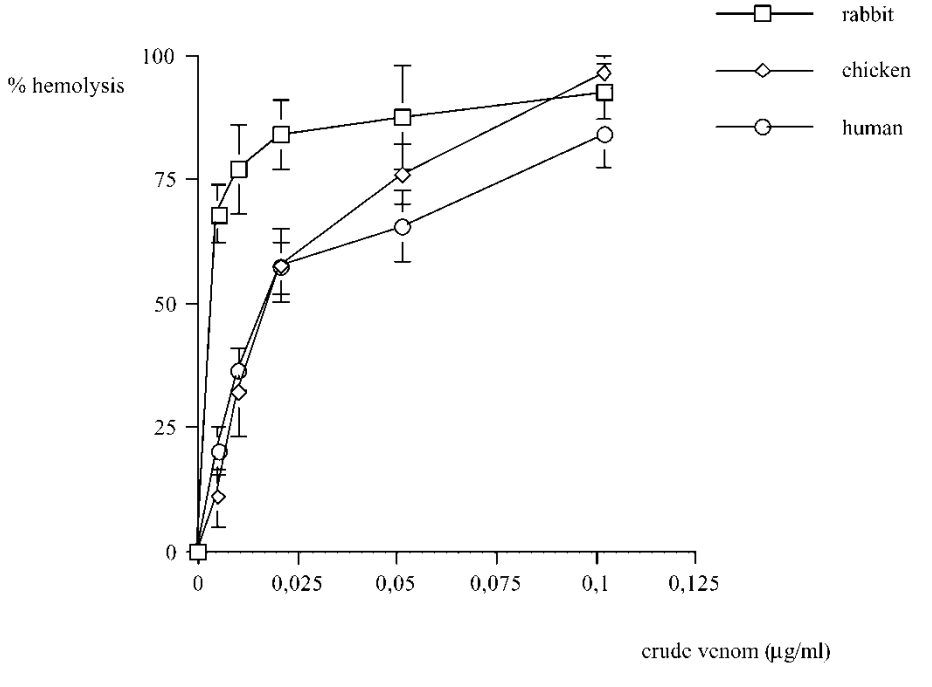

FIGURE 3 Dose–response curve for the hemolytic activity of *A. mutabilis* nematocyst crude extract on human, chicken and rabbit 0.5% RBCs suspension. The diagram includes data from five experiments (Means ± SE).

TABLE I Sensitivity of Erythrocyte Types to *A. mutabilis* Venom Hemolysis.

Erythrocytes sources	*Crude venom (μg/ml) for 50% hemolysis*
Human	0.02
Chicken	0.02
Rabbit	0.005

4 DISCUSSION

Extracts from Cnidarian tissues have been found to contain biologically active compounds since they have also been detected in tissues (Endean and Noble, 1971; Wittle *et al.*, 1974). Such extracts can be obtained either by tissue homogenization or maceration and grinding (Gierer *et al.*, 1972; Malpezzi and Freitas, 1991) and contain active substances deriving not only from nematocysts but also from other cells surrounding the nematocysts themselves. Therefore a suitable method for the isolation of nematocysts must be found so that a special study on the toxinological features typical of capsular fluid, excluding any biological activity of other cells, can be carried out.

In particular toxinological investigations have never been attempted so far on nematocysts from *A. mutabilis*, a species that can be readily collected in the Strait of Messina. Although this species of Anthozoa has been studied for the most part because of availability and high survival rate in aquaria, Schyphozoa specimens as *Pelagia noctiluca*, whose blooms are well known in the Strait of Messina, are also being studied at present in our laboratory.

Apart from the toxicity of *A. pallida*, already studied by Blanquet (1968) and by Grotendorst and Hessinger (2000), a toxinological study of the genus *A. mutabilis* is entirely new.

Amongst other isolation methods the 1 M sodium citrate treatment produces capsules that are both morphologically and functionally intact, as previously described (Blanquet, 1968). Such isolated nematocysts in control conditions were able to discharge on 553 mM NaSCN treatment. Such a discharge method can be adopted as a capsular fluid extraction technique, since NaSCN, at the concentration of 553 mM, apparently does not affect either the lytic power of crude venom on a 5% RBCs suspension or erythrocytes integrity. The hemolytic area is clearly detectable after nematocyst discharge. Capsular fluid from *A. mutabilis* nematocysts induced a progressively expanding lytic area around the evaginated tubules. The lytic area appears to expand from the distal portion of the everted tubule, as observed by Klug *et al.* (1986) on euryteles from *Chrysaora quinquecyrrha.* The same observations have been performed by La Spada *et al.* (unpublished) on nematocysts isolated from *Pelagia noctiluca* and induced to discharge upon RBCs suspension. In this case the opening of a tip could be the cause depending on the type of nematocysts involved. At any rate such induced hemolytic action takes a very long time, since a definite hemolytic area is detected only after at least 1 h of discharge. This is probably because the capsular fluid spreads only slowly over the slide once the nematocyst is triggered.

On the other hand Klug *et al.* (1989) observed that the hemolytic area, induced by capsular fluid from *Hydra attenuata* nematocysts triggered by 10 mM DTE, seems to start all along the tubule surface, suggesting that hemolysins could be released through the wall of the everted tubule. Recent studies (Lotan *et al.*, 1996) support this theory, showing that in nematocyst of *Rhopylema nomadica* toxins are delivered through barbs located along the tubule. The different observations could be explained by the type of nematocysts involved, according to the Mariscal (1974) classification.

In any case, our data show that lytic area does not appear to be created by the flushing away of the erythrocytes, caused by the rapid acceleration of tubule eversion, in fact in this area ghosts are easily detected through a microscope.

When the physical extraction method was used, the *A. mutabilis* nematocysts were sonicated more often *i.e.* 25 times than was the case described by Rottini *et al.* (1995) for the jellyfish *Carybdea marsupialis* nematocysts, in order to obtain crude venom. This could be due to the type of capsules involved, and suggests possible different mechanical characteristics of the nematocyst wall. Our observations would indicate that sonication is a suitable extraction method for capsular fluid since the effectiveness of the venom in inducing hemolysis is in no way affected. In fact treatment of 5% human RBCs suspension on a slide with capsular fluid extracted using this technique from a nematocyst suspension induced hemolysis within 10 min. Such a hemolytic test consists of a qualitative assay which produces an initial characterization of *A. mutabilis* crude venom, that is its hemolytic effect on RBCs from different sources. A dose–response effect can be evaluated by a hemolytic test performed spectrophotometrically.

In particular, our observations show that the sensitivity of rabbit erythrocytes seems to be higher than that of human and chicken RBCs, since a significant difference ($p < 0.001$) in percentage of hemolysis was clearly observed at the lowest doses of crude venom (Fig. 3).

Our data show that relatively small doses of crude venom from *A. mutabilis*, ranging between 0.005 and 0.1 μg/ml, are sufficient to induce a high degree of hemolysis, whereas other authors (Malpezzi and Freitas, 1991; Radwan *et al.*, 2000) observed RBCs lysis at significantly high venom doses up to 75 μg/ml.

The results concerning heat treatment of capsular fluid were obtained using only one human RBCs source, because of availability. In particular capsular fluid from *A. mutabilis* nematocysts seems to be moderately heat labile since its effectiveness is weakened by incubation at 60 °C for longer than 5 min and completely prevented at 70 °C for 5 min. The stability is obviously temperature dependent, since it decreased by raising the temperature. Such crude extracts are most likely composed of relatively short-lived bioactive compounds, so further investigations will be carried out to better characterize the stability. Our results of the thermal stability of *A. mutabilis* crude venom agree with other studies performed on other Cnidarians venoms (Long-Rowe and Burnett, 1994; Rottini *et al.*, 1995), but apparently differ from observations performed by Malpezzi and Freitas (1991), describing the stability loss of the anemone *Bunodosoma caissarum* hemolysin and from data of Galettis and Norton (1990) reporting a notable stability of Tenebrosin-C. Our data suggest that assays for hemolysis, or for other biological tests, should be performed immediately after rupturing the nematocysts, to avoid the crude venom being inactivated or undergoing a partial loss of biological activity.

In conclusion our results show: (i) sonication is a suitable method to extract venom from isolated nematocysts; (ii) crude venom from *A. mutabilis* nematocysts is strongly hemolytic; (iii) rabbit RBCs are more susceptible to *A. mutabilis* crude venom than those of humans and chicken.

Further investigations into electrophoresis, ion effects, pH optimum profile, and the proteases effect on hemolytic activity, are needed in order to better characterize this crude venom.

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