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Haemolytic activity and characterization of nematocyst venom from *Pelagia noctiluca* (Cnidaria: Scyphozoa)

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Abstract
We investigated the haemolytic capacity of the crude venom extracted from isolated nematocysts of *Pelagia noctiluca* (Cnidaria: Scyphozoa), and evidenced the proteic fractions responsible for this activity. The nematocyst venom was used at various concentrations to evaluate the haemolytic activity and the lysosomal membrane stability of red blood cells of two teleostean species treated with the extract. The nematocyst extract was assayed against erythrocytes of the two teleostean species living in different environments, *Carassius auratus* as a common freshwater species, and *Liza aurata* as a representative of seawater species. Experiments on the haemolytic activity of *P. noctiluca* in the presence of lipid components of erythrocyte membranes showed that sphyngomyelin strongly inhibited this activity. The crude venom was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE and high performance liquid chromatography (HPLC) to detect the proteic composition, and it was found that the active haemolytic components of this venom are distributed in at least four protein fractions. The results of our experiments indicated that *Pelagia noctiluca* venom induces haemolysis and lysosomal membrane destabilisation in both species and that *Carassius auratus* was more susceptible to jellyfish venom than was *Liza aurata*. No significant differences in glutathione (GSH) levels were observed between control and treatments; consequently the toxins do not cause the oxidative stress but likely recognize specific targets (i.e. sphyngomyelin) in the plasmatic membrane of red blood cells.

Keywords: *Crude venom, haemolysis, HPLC analysis, nematocysts,* Pelagia noctiluca

Introduction

The jellyfish are included in the phylum Cnidaria, an ancient group of animals which, in the marine environment, developed several mechanisms related to prey capture and defence. A characteristic cnidarian cell type is the stinging cell nematocyte, mainly found on tentacles and defensive organs. The nematocytes contain the nematocyst organelle which, in turn, is composed of a capsule and an inner hollow tubule in a saline solution, containing toxins that differ in composition between jellyfish species (Cheng et al. 2005; Ramasamy et al. 2005a, 2005b). Nematocysts can discharge in response to a variety of mechanical and chemical stimuli, as well as in response to occasional contact, and they are considered to be the most sophisticated lethal weapons in the animal kingdom (Tardent 1995).

Cnidarian venom is made of a complex mixture of biologically active molecules including 5-hydroxytryptamine, histamine, proteins such as proteases and phospholipases, and small peptides. The nematocyst toxins exert many bioactivities such as haemolytic, cytolytic, clastogenic, enzymatic, cardiotoxic, neurotoxic and insecticidial activities (Mariottini et al. 2002; Li et al. 2005; Yu et al. 2005; Monroy-Estrada et al. 2007; Kang et al. 2009; Birsa et al. 2010). In many cases, cytolytic, haemolytic or neurotoxic effects have been shown by several biological assays, amongst which the haemolytic assay is the most suitable and most used, being highly sensitive and simple to carry out (Marino et al. 2008). However, due to technological difficulties with obtaining and storing venom extracts and their labile nature, the current understanding of cnidarian

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venom is limited (Feng et al. 2010). Studies have been performed on the toxicological features of cnidarians living in the Strait of Messina (Marino et al. 2004a, 2004b, 2006, 2007). In the Strait of Messina the jellyfish *Pelagia noctiluca* is abundant throughout the year, especially in spring and summer when planktonic food is available in greater abundance leading to the transient appearance of large "blooms".

Pelagia noctiluca, a cnidarian of the Class Scyphozoa, Order Semaeostomeae, Family Pelagiidae, is a small pelagic pink jellyfish with a phosphorescent bell measuring 3–12 cm in diameter, and has nematocysts localized in the tentacles, oral arms and upper surface of the bell. The "blooms" of this jellyfish have implications for human health and relapses on economic activities including tourism and fishing. The nematocyst toxins cause various reactions in humans that range from local lesions, vesicles and redness to severe and dangerous complications such as cardio- and neurotoxic effects and Guillain-Barré syndrome (Burnett et al. 1986; Pang & Schwartz 1993; Tibbals 2006; Mariottini & Pane 2010).

This study aims to investigate the activity of the crude venom extracted from isolated nematocysts of *P. noctiluca* on fish red blood cell membranes, by assessing the haemolytic activity and the lysosomal stability, and to study the proteic composition of this venom by HPLC separation and electrophoresis analysis by using rabbit erythrocytes as the best target cells for the related molecular biological assay. Moreover, inhibition experiments on the haemolytic activity of *P. noctiluca* with lipid components of erythrocyte membranes were also performed.

The present study could represent an important contribution towards understanding the biological activities of cnidarian venom and identifying cellular targets for the toxins and, hence, could lead to the discovery and application of new bioactive compounds from natural sources.

Materials and methods

Nematocyst isolation

Specimens of the scyphozoan *P. noctiluca* were collected along the Sicilian coasts of the Strait of Messina. Nematocysts were isolated from marginal tentacles according to Salleo et al. (1983). Briefly, oral arms were excised from each specimen and placed in cold distilled water (4°C) to allow osmotic lysis of the nematocytes and the delivery of the organoids. The obtained suspension was repeatedly filtered by means of plankton nets (40-, 60- and 100-μm mesh) and centrifuged (refrigerated centrifuge Eppendorf, 1700 *g* for 5 min) to discard debris and reduce the volume for venom extraction. The nematocysts, once isolated, were stored at –20°C until used.

Venom extraction

Samples containing 90–100 nematocysts/μL were resuspended in a solution [145 mM sodium chloride (NaCl), 10 mM phosphate, pH 7.4, osmotic pressure 300 mOsm/kg $_{H2O}$] compatible with the physiology of used erythrocytes, and sonicated on ice with a Sonoplus (70 MHz, 20 s, 30 times) to extract the capsular fluid. The crude extract was then separated from crushed nematocysts by centrifugation (refrigerated centrifuge Eppendorf, 1700 *g* for 10 min) and driven to the biological assay. Protein concentration of the venom was determined by the method of Bradford (1976), compared with the bovine serum albumin (BSA) protein concentration standards.

Fish blood samples

Fish blood samples were collected from five specimens of both freshwater goldfish *Carassius auratus* and seawater golden grey mullet *Liza aurata*. Blood samples (1.5 mL) were drawn from the caudal vein into heparinized vials and immediately analyzed for morphological observation, haemolysis assay and glutathione (GSH) measurement. For the lysosomal stability, blood samples (0.5 mL) were collected using a syringe containing 1 mL of physiological saline solution.

Morphological analysis

Morphological analysis of *C. auratus* and *L. aurata* erythrocytes was carried out by smearing heparinized whole blood on a glass slide. Blood samples of both species were incubated with various concentrations of crude extract for 90 min. The slides were air-dried overnight and then fixed in absolute methanol for 20 min before staining with 10% Giemsa solution for 15 min. Blood smears were observed by the Axio Imager Z1 (Zeiss) light microscope with a 63 × oil immersion objective.

Haemolysis assay

Haemolytic activity of *P. noctiluca* venom was tested on the erythrocytes of *C. auratus* and *L. aurata*. To obtain a pure suspension of erythrocytes, the whole blood (500 μL) was rinsed in phosphate

buffered saline (PBS, pH 7.4), and then centrifuged at 1500 *g* for 5 min at 4°C. The rabbit erythrocytes (supplied by Istituto Zooprofilattico della Sicilia) were washed three times with PBS, and centrifuged at 500 *g* for 10 min at 4°C. The supernatant was removed by gentle aspiration and the above process was repeated two times. The erythrocytes were finally resuspended in PBS to make a 1% suspension for the haemolysis assay. For this assay, the crude venom with a protein concentration of 40 μg/mL was added to the suspension of fish red blood cells at concentrations of 0.4, 4, 8, 20, 28 and 40 μg/mL. The venom-erythrocyte mixtures were incubated at room temperature for 30 min and then centrifuged at 1500 *g* for 5 min at 4°C. The venom haemolytic activity was expressed as percentage of the absorbance of the supernatants determined at 545 nm by using a spectrophotometer (UV mini Shimadzu) to measure the amount of haemolysis. Positive control (100% haemolysis) and negative control (0% haemolysis) were also determined by incubating erythrocytes with 1% Triton X-100 in PBS and PBS alone, respectively.

Phospholipid inhibitory effect on haemolytic activity

Sphingomyelin, phosphatidylserine and phosphatidylethanolamine were dissolved in TBS to obtain a concentration of 25.0 and 250.0 μg/mL in the reaction mixture. These inhibitors were relatively insoluble in TBS. For these compounds, stock solutions were sonicated (Branson, Model B15, Danbury, CT) at 4°C for 20 s, and centrifuged at 27000 *g* for 30 min (Tong & Kuksis 1986).

The inhibitors, used at various concentrations, were mixed with the erythrocyte suspensions, and after 20 min of preincubation were added to the venom for the cytotoxic assays. Control lysis was measured as previously described.

Lysosomal stability of fish red blood cells

The lysosomal stability of fish red blood cells was assessed using the neutral red retention (NRR) assay as described by Lowe et al. (1995), with some modifications. For both *C. auratus* and *L. aurata*, six tubes containing 200 μL of the blood/physiological saline mixture were prepared, and then the crude venom was added at concentrations of 0.4, 4, 8, 20, 28 and 40 μg/mL, respectively. The mixtures were incubated for 30 min, and afterwards 40 μL of each sample was pipetted onto the centre of a microscope slide, and incubated for 15 min in a lightproof humidity chamber at 4°C to allow the cells to adhere. The excess solution was then gently tipped off and 40 μL of neutral red dye working solution (10 μL into 2 mL physiological saline from a stock solution of 20 mg NR in 1 mL of dimethyl sulfoxide) was added to the cell layer. After 15 min of incubation, a coverslip was placed on top, and the slides examined using light microscopy (40 ×). The retention of the dye within the lysosomes in the cells was recorded every 15 min for the first hour, and then at 30-min intervals until over 50% of the cells demonstrated leakage of the neutral red dye from the lysosomes, for a total period of 180 min.

Glutathione (GSH) measurement in erythrocytes exposed to crude venom

GSH was measured following the method of Beutler (1975) with a slight modification. To 0.1 mL of fish whole blood, crude venom (90–100 nematocysts/μL) was added at 0.4, 4, 8, 20, 28 and 40 μg/mL concentration. A mix of 0.2 mL was precipitated with 30% tricloroacetic acid (TCA) (1:3 v/v, blood:acid) and centrifuged at 4500 *g* for 5 min, and 0.5 mL of this supernatant was added to 2.0 mL of 0.3 M Na_2HPO_4 (sodium phosphate dibasic) solution and 0.25 ml DTNB (5,5-dithiobis-2-nitrobenzoic acid) was then added to this solution. Reduced GSH was measured as the difference in the absorbance values of samples in the presence and absence of DTNB at 412 nm. The GSH value was calculated as μmol GSH/g haemoglobin.

Haemoglobin concentration (Hb) was determined in a blood sample with the same concentrations of the crude venom used for GSH measurement, based on the cyanometahemoglobin formation using Drabkin's solution (Van Kampen & Zijlstra 1961), with spectrophotometric absorbance at 540 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis analysis was performed in the Mini-Protean II Dual-Slab Cell (Bio Rad). Preparation of gels, samples, and electrophoresis were performed according to the conditions described by Laemmli (1970). Samples were denatured by boiling in loading buffer [25 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS Base) 192 mM glycine, 1% w/v sodium dodecyl sulfate (SDS), pH 8.3] containing β-mercaptoethanol, before they were placed on the gel. Proteins were stained (Brilliant Blue R G250 0.1%, 40% methanol, acetic acid 1%) and destained with acetic acid 0.1%, 0.4% methanol, distilled water. The gels were calibrated with standard protein molecular weight (Sigma-Aldrich).

HPLC size exclusion chromatography

P. noctiluca nematocyst extract was subjected to size exclusion chromatography using BioSuite 250, 10 μm SEC, 7.5 × 300 mm column (Waters) on a liquid chromatography HPLC system (Shimadzu Scientific Instruments, SSI, North America). Column was washed with TBS (NaCl 150 mM, TRIS HCl 10 mM, pH 7.4).

An injection volume of 200 μL was used at a flow rate of 1 mL/min for 30 min. The chromatogram was recorded with a dual UV detector at 280 nm and 220 nm (mAU) in TBS.

The eluate corresponding to each peak was collected in 1 mL/min fractions. The collected fractions were concentrated by centrifugation at 500 *g* with micro-concentrators (3K Omega Centrifugal Devices Nanosep), and the final concentrated samples were stored at –80°C until use.

Statistical analysis

The results are expressed as a mean ± standard deviation. Data were analysed by analysis of variance (ANOVA) and considered statistically significant at $p < 0.05$.

Results

Morphological observation

The effect of the crude extract on the erythrocytes of both species was investigated by morphological observation using light microscopy. The erythrocytes treated with venom (Figure 1) show evident changes with respect to those of the control (Figure 1A, B), both in *C. auratus* and *L. aurata*. The results reported in Figure 1 indicate progressive modifications of the cytoplasm in *C. auratus* erythrocytes, which exhibits marked vacuolization after incubation with the lowest dose of the venom (Figure 1C), whereas higher concentrations lead to cell lysis (Figure 1E, G). In erythrocytes of *L. aurata*, modification of the membrane is observed with the lowest concentration of the venom (Figure 1D), while intense vacuolization appears after incubation with higher doses (Figure 1F, H).

In both species treated with increasing concentrations of jellyfish venom, marked cell lysis was observed with aggregation and subsequent precipitation of the erythrocyte membranes, which may affect the number of cells present in a slide and then successive statistical analysis. The cells damaged by the venom have been counted and expressed as percentages ($p < 0.0001$; Figure 2A).

Haemolytic activity

P. noctiluca crude venom was assessed for its haemolytic activity using the erythrocytes of two species of fish. All the data were obtained using aliquots of the same venom extract. The venom showed concentration-dependent haemolytic activities in both species tested. The results were reported in Figure 2B. The venom caused 50% haemolysis in *C. auratus* erythrocytes at the lowest employed concentration, equal to 4 μg/mL, whereas in *L. aurata* the 50% haemolysis was displayed at a venom concentration of about 28 μg/mL. Statistical significant differences were observed between the two species of fish tested at each venom concentration employed ($p < 0.0001$). The activity against rabbit erythrocytes was almost 20 times higher than activity against fish erythrocytes. The gel stained with Coomassie Brilliant blue showed a pattern of total protein in the nematocysts extract. Many bands were recorded with different molecular weights ranging from more than 100 to less than 20 kDa, from which six are clearly defined (105, 64, 49, 45, 29, 20 kDa) (Figure 2C).

Lysosomal stability

The results of the NRR assay measured at various times on erythrocytes of the two species of fish are shown in Figure 2D. A destabilization of the lysosomal membranes in *C. auratus* was evidenced after 150 min at 8 μg/mL of venom, after 90 min at 20 μg/mL of venom, after 60 min at 28 μg/mL and after 30 min at the highest concentration of the toxin. In *L. aurata*, the destabilization of 50% of lysosomal membranes was observed after 90 min solely at the highest concentration of venom. There was a statistically significant difference between the two species of fish ($p < 0.0001$).

Haemoglobin concentration and GSH levels

No significant differences were found in the haemoglobin concentration or GSH levels between erythrocytes of the control and those treated at the various toxin concentrations of the two species investigated. The data are shown in Figure 2E.

HPLC active fraction analyses

The crude extract of *Pelagia noctiluca* was fractioned by molecular weight exclusion chromatography column (Figure 3). The analysis of the extract showed one major first high peak obtained about 5 min after entering the sample corresponding to high

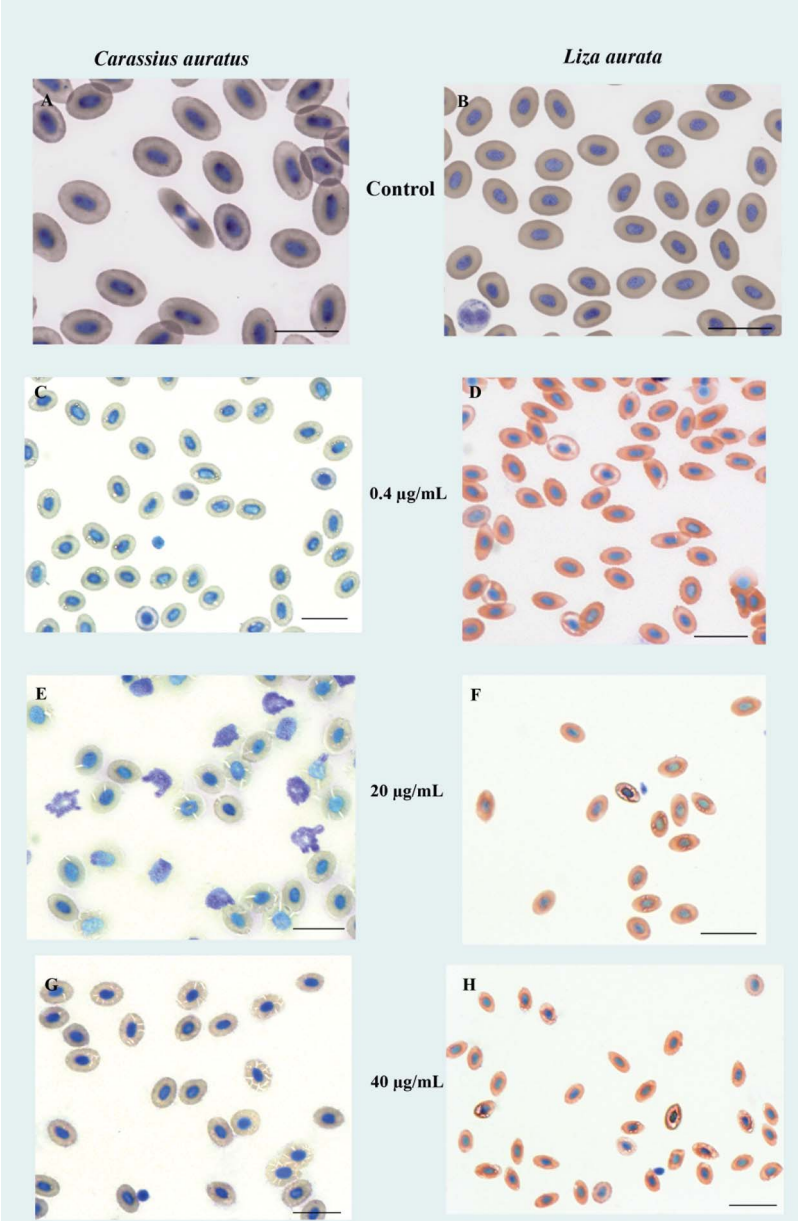

Figure 1. Erythrocytes stained with Giemsa and observed with Zeiss AxioImager Z1 light microscope. Untreated erythrocytes of *Carassius auratus* and *Liza aurata* (**A** and **B**, respectively). Erythrocytes treated with 0.4 (**C** and **D**), 20 (**E** and **F**) and 40 (**G** and **H**) µg/mL of *Pelagia noctiluca* venom and observed after 90 min of incubation. Scale bar: 20 µm.

molecular weight proteins, and two small peaks at higher elution volume. From all the collected fractions, only the fractions corresponding to 1, 3, 6 and 7 peaks were found to exhibit haemolytic activity indicated by flashes (Figure 3B) against rabbit erythrocytes. Compared to the standards used (Figure 3A), these fractions ranged from very different molecular weights.

The curves of Fractions 3 and 6 shown in Figure 3C, obtained by plotting the sample dilutions vs. haemolitic activity, are sigmoidal in shape showing a rapid decrease at 5 µg/mL. By contrast, the unfractionated extract and Fractions 1 and 7 showed a slower decrease in haemolitic activity with respect to the sample dilutions.

Discussion

Haemolytic activity has been described on a variety of cnidarian venoms against erythrocytes of many different species, suggesting that jellyfish venom can have highly variable haemolytic activity among

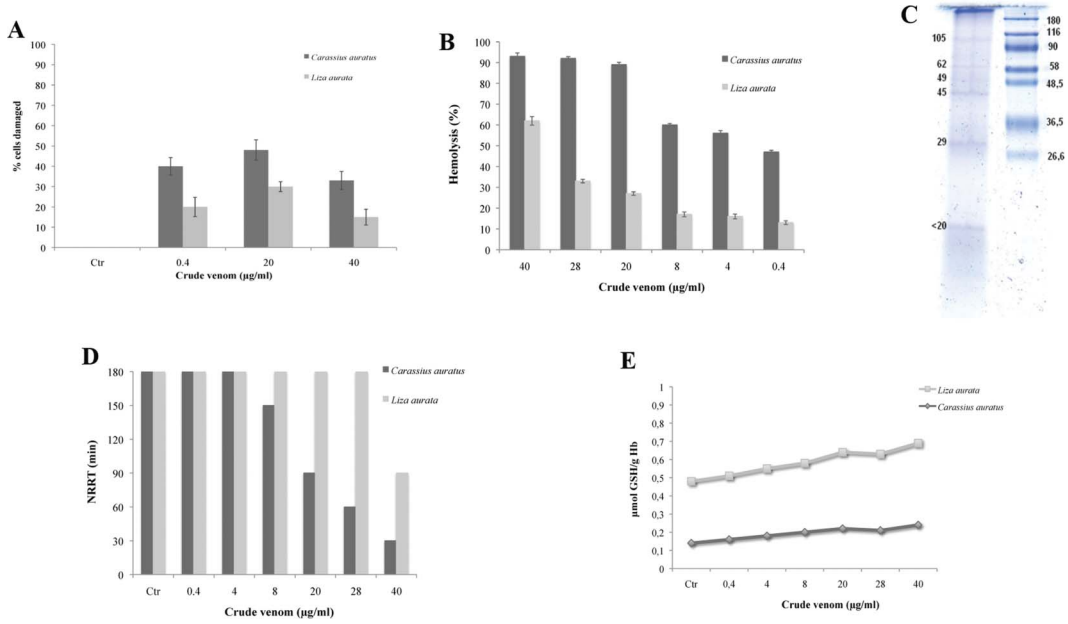

Figure 2. Percentage of cells damaged by venom, haemolytic activity and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *Pelagia noctiluca* crude venom, neutral red retention time (NRRT) test and glutathione (GSH) concentration in erythrocytes exposed to crude venom. **A**) Percentage of cells damaged after 90 min of treatment with different concentrations of venom. **B**) Haemolytic activity of *P. noctiluca* crude venom. Erythrocytes were re-suspended in phosphate buffered saline (PBS) to make a 1% solution and then incubated with various concentrations of venom for 30 min at 37°C. The samples were centrifuged at 1500 *g* for 5 min at 4°C and the absorbance of the supernatants was determined at 545 nm. The results are expressed as mean ± SD. **C**) Coomassie stained SDS-PAGE gel of *P. noctiluca* nematocyst crude extract. Lane 1: High mass marker with sizes in kDa; Lane 2: Stained bands ranging from 180 to less than 20 kDa molecular weight showed various proteic components with different electrophoretic mobility. **D**) NRRT of erythrocytes from fish exposed to various concentrations of crude venom. The retention of the dye within the lysosomes of the cells was recorded at 30-min intervals until over 50% of the cells demonstrated leakage of the neutral red dye from the lysosomes, for a total period of 180 min. **E)** Reduced GSH concentration, expressed in mmol GSH/g Hb, in erythrocytes of *Carassius auratus* and *Liza aurata.*

species (Rottini et al. 1995; Torres et al. 2001; Kang et al. 2009). In the present study, we tested the haemolytic activity of crude venom from isolated nematocysts of *P. noctiluca* on erythrocytes of *Carassius auratus* and *Liza aurata*, two teleostean fish. For *C. auratus* the venom caused 50% haemolysis already at a concentration 0.4 µg/mL, whereas in *L. aurata* erythrocytes showed 50% haemolysis at a concentration equal to 28 µg/mL. Because the mechanisms of action of the toxins include membrane fragility, in this study the effect of venom upon lysosomal membrane stability was also assessed. From our observations a destabilization of the lysosomal membrane in *C. auratus* was evident from 8 µg/mL of venom up to the highest concentration. On the contrary, in *L. aurata* destabilization was observed solely with 40 µg/mL of jellyfish venom. Our findings on freshwater and marine fish erythrocytes, as well as lysosomal membranes, showed differential responses to jellyfish crude venom, with *C. auratus* being more sensitive compared with the other species considered in this study.

The toxins can lyse cells directly or even make cells more susceptible to damage by hydrolyzing membrane lipids, so that, as a general feature, venoms can be divided into two categories: one including toxins with a direct lytic action upon erythrocytes, and the other including toxins leading to lipid peroxidation and then to lysis, as reported by several authors (Monroy-Estrada et al. 2007). It is known that oxidative damage to the red blood cell membrane leads to a reduction in membrane fluidity and an increase in membrane permeability (Marino et al. 2008). The most potent non-enzymatic antioxidant system used by fish is GSH, with Vitamin E (Storey 1996; Droge 2002). Thus, in this study the levels of GSH were assessed in order to examine the possibility of oxidative damage caused by the venom, but our results show no significant differences between the erythrocytes of the control and those treated with toxin. This suggests that haemolysis under *P. noctiluca* crude venom is not due to free radical formation. On the other hand, the presence of specific targets for the toxin on red blood cell membranes can be supposed, but this hypothesis needs further study.

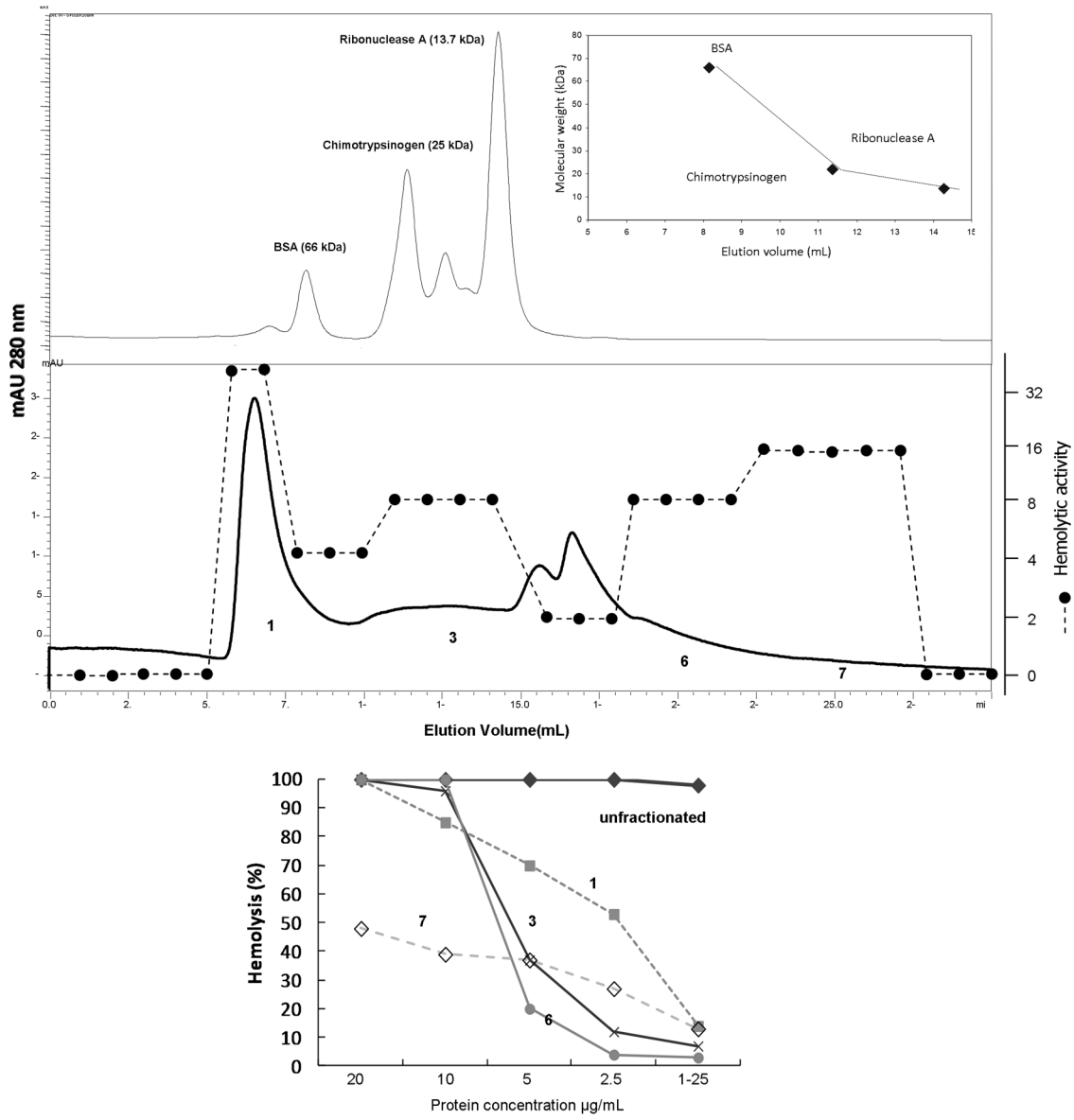

Figure 3. Separation of high pressure liquid chromatography (HPLC) on molecular weight exclusion column of *Pelagia noctiluca* tentacle extracted fractions and haemolytic activity assays. **A**) HPLC profiles of bovine serum albumin (BSA), chimotrypsinogen and ribonuclease used as standards separated on a molecular weight exclusion column BioSuite 250 (10 microns; Waters). **B**) Separation of *P. noctiluca* tentacle extract. The fractions were assayed for haemolytic activity. The active fractions 1, 3, 6 and 7 were indicated by flashes. **C**) Haemolytic activity of unfractionated tentacle extract and isolated fractions against rabbit erythrocytes. Samples were incubated at various dilutions with 1% erythrocyte suspension for 1 h at 37°C. After centrifugation (1500 *g*, 5 min, 4°C) the absorbance of the supernatants was determined at 545 nm and expressed as haemolysis percentage.

The present study contributes to characterizing the crude venom from nematocysts of *P. noctiluca*. It has been proposed that phospholipids are involved in the regulation of some cytotoxic mechanisms, and these findings are based on cytotoxic inhibition experiments. Of interest is the finding that among the phospholipids tested only sphingomyelin inhibits in a dose-dependent manner (Figure 4). Although we are not able to explain the lytic mechanism, we postulate that one of the toxins interacting with this lipid causes changes in erythrocyte membrane permeability, leading to lysis.

The protein components of crude venom were separated by SDS-PAGE, and numerous proteinic fractions with molecular weights between $\leq$ 20 and 100 kDa were identified. By HPLC, with molecular exclusion chromatography, only the fractions corresponding to 1, 3, 6 and 7 peaks were found to exhibit haemolytic activity. The activity vs. sample dilutions of Fractions 3 and 6 are sigmoidal in shape compared

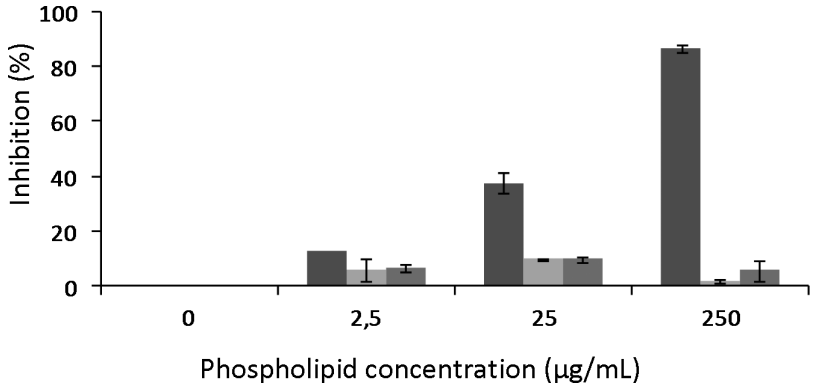

Figure 4. Phospholipid inhibitory effect on the haemolytic activity of *Pelagia noctiluca* tentacle extract. Inhibitory effect of sphingomyelin (■), phosphatidylserine (□), phosphatidylethanolamine (▩) (2.5 µg/mL – 25 µg/mL – 250 µg/mL) on haemolytic activity of *P. noctiluca* tentacles extract against rabbit erythrocytes. Samples were incubated with 1% erythrocyte suspension for 1 h at 37°C, after centrifugation (the absorbance of the supernatants was determined at 545 nm). The results ($n = 5$) are expressed as mean ± SD.

to Fractions 1 and 7 that showed a slower decrease. This result could indicate the existence of at least two different mechanisms of action from these different proteic fractions.

In summary, our results showed that the crude venom from isolated nematocysts of *P. noctiluca* has strong haemolytic activities, which could be attributed to the separated proteinic fractions. The data obtained indicate a greater resistance in *Liza aurata* than in *Carassius auratus*, and it is feasible to hypothesize adaptive mechanisms developed by marine Teleostei. Thus, further studies aimed at identifying and characterizing each obtained protein fraction should be carried out, in order to highlight potential targets. The lipid-proteic layer interaction suggests that the single lytic action is an unrestricted, relatively simple mechanism involving target membrane lipids, which may also be important in the expression of Cnidaria toxins, a process that has endured in the evolutionary development of biotoxins. Therefore, these data could lead to the identification of novel bioactive components potentially useful for the development of pharmacological and therapeutic agents.

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