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Study of nematocyst discharge of Physalia physalis and venom composition†

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Abstract: In this work we studied the effects of various chemicals on P physalis nematocysts discharge and the composition of released venom. The exposure of nematocyst to K+ and Na+ induced a massive discharge in a short time of exposition. Conversely, the Ca+2 ions apparently resulted in an inhibitory effect. The electric stimulation was shown to be a reproducible and an effective way to induce nematocysts discharge in a few seconds. The SDS-PAGE profile of the venom proteins released revealed a similar pattern, with a broad MW distribution and wide bands with 40, 25 and 20 kDa. The released venom exhibited proteolytic activities that are inhibited by PMSF and EDTA. The present study provides an understanding on discharge sensation and venom released of P physalis nematocysts and could contribute for future venom proteomics research efforts.

Keywords: Physalia physalis; venom toxins; nematocyst discharge; chemosensation

1. Introduction

Physalia physalis, the Portuguese man-of-war, belonging to the Cnidarian group, order Siphonophores [1], is part of the pelagic species of ocean organisms and is one of the most conspicuous and dangerous in the Atlantic Sea [2]. *P. physalis* have an expanded ectodermal cell that form a characteristic gas floating structure, the pneumatophore, that float on the ocean surface acting as a sail [3]. The wind-guided drift together with a life cycle with seasonal blooms and venom sting tentacles, can have several impacts on ecosystems and human health due the high toxicity of its venom. The tentacles can be up to 30 m long and has a specialized function in the production of nematocysts, containing more than 750,000 stinging cells [3]. The nematocysts are used for prey capture, discharging a sting and delivering venom toxins that cause paralysis in prey fish [4], affecting the nervous system and respiratory centers [5]. Accidental contact of tentacles with human cause a painful sting and can provoke a series of symptoms, ranging from local skin necrosis to neurological disorientation, cardiorespiratory problems and, in rare cases, death [6, 7]. A variety of chemosensory, mechanosensory and endogenous pathways regulate the depolarization of the nematocysts membrane that culminate in the discharge of the sting [8]. Different works shown that specific compounds and ions can evoke depolarizing events inducing the discharge of nematocysts [9, 10, 11, 12, 13, 14, 15]. In *Physalia physalis* some works have been conducted to study the discharge [16], but this organism remains yet poorly understood. In the present work we investigated the effect of different organic solvents, ion solutions and electric stimulation on the nematocyst discharge of *P physalia*. This information can be of capital relevance in a sting management scenario in

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accidental contact with this Cnidaria and, under a biotechnology point of view, can be used to separate organism's biomass from venom of the nematocysts.

2. Material and methods

2.1. Animals and nematocysts assay

The specimens of *P. physalis* were captured in the meddle of Atlantic near the coast of S Miguel Island of Azores Archipelago, during June 2022, brought to the laboratory in tanks with seawater at 16–18°C. Tentacles pieces of 1 cm long were cut from distal part of long tentacle and added to eppendorf tube in a 1 mL seawater. To determine the effects of chemicals on nematocytes, one hundred microliters of each chemical solution was added to the tubes containing tentacle probes. Chemicals tested included: 5% acetic acid solution; ethanol at 70%, 80% and 96%; 0.9% NaCl; 0.3M KCl; and, 0.1M CaCl2. Solutions were tested by incubation, without mechanical induction, during 5-, 15- and 30-min. Sea-water alone was used as control. In a second series of experiments, tentacle pieces were added to a 1 ml electroporation chamber, added 100 µl of saline solutions (0.9% NaCl; 0.3M KCl; and 0.1M CaCl2, respectively) and then stimulated with a single pulse 8v for 3s.

2.2. Count of discharged nematocysts

Tentacle nematocytes were examined under a light microscope at 100× magnification. Digital photos were taken to count the number of discharged nematocytes per field in each treatment. Five tentacle probes were used per each experimental condition. Statistical analyses were performed with one-way analysis of variance (ANOVA) using Graph Pad Prism 5 software.

2.4. Crude venom extracts and enzymatic assay

Extraction of venom according to Carrette and Seymour (2004) [17] using 0.5 mm glass beads in an ice-cold solution, has been adopted. Briefly, 1 cm of tentacle samples was incubated with 20 mM Tris-HCl, pH 7.4, shaken in a mini bead mill 1 min, for 5 times with intermittent cooling on ice. The homogenate was then transferred to a new tube, centrifuged at 10 000 g, 5 min and supernatant was used. Venom extract obtained by homogenization was compared with venom released by challenging with ion solutions 5% of acetic acid and ethanol at 96%, by SDS-PAGE. The samples recovered in ethanol were lyophilized and suspended in 20 mM Tris-HCl, pH 7.4.

For enzymatic assay azocasein substrate at 1% (f/c) in 20 mM Tris-HCl, pH 7.4, was used. The samples were incubated 2 hrs at 37ºC, then the reaction was stopped with 20% TCA (f/c) in ice and centrifuged 10 000g, 5 min. The supernatant was transferred to a 96-well plate, neutralized with 0.5 M NaOH and the absorbance measured at 450 nm. The inhibition of caseinolytic activity was analyzed using EDTA (metalloproteinase inhibitor) and PMSF (a serine proteinase inhibitor).

2.5. SDS-PAGE and Zymogram

SDS-PAGE was performed according to Laemmli's method. Briefly, 40 µg of protein samples were mixed with desaturated loading buffer with β-mercaptoethanol, heated at 95ºC, 5 min, before running on 12% polyacrylamide gel. Low molecular standard was used. The gels were stained with Coomassie R-250. For zymogram, 0.02% of gelatin (f/c) was incorporated in SDS-PAGE gel on non-denaturant conditions. After running the gels, gels were washed with 2.5% triton X-100 and incubated for 2 hrs at 37ºC in 20 mM Tris-HCl, pH 7.4, with 1 mM of CaCl2 and 1mM MgCl. The clear zones of proteolytic activity were reveled after stained with Coomassie blue.

3. Results

3.1. Ion solutions induce nematocyte discharge

Little or no nematocyst discharge was observed in seawater (SW), used in this study as control probes. Exposure to the acetic acid solution induces a sparse but significant discharge of nematocysts 15 min after exposure. Surprisingly, the induction with 70% ethanol did not present significant differences in relation to the SW, but the concentrated solutions (80% and 96% ethanol) induce significant discharges (Figure. 1, Table 1), which increase with exposure time and concentration. Tentacles exposed to NaCl and KCl solutions did not discharge nematocysts up to 15 min of exposure, but after that, substantial discharges were observed, reaching values greater than 90%. In contrast, the CaCl2 solution did not show significant differences in relation to the SW. A massive number of discharged nematocysts were observed when the tentacles were electrically stimulated with a single 3s pulse. To be noticed, the electrical stimulation of tentacles immersed in Ca^{+2} solution induced significantly fewer discharges (P=0.001) than in SW, thus suggesting that Ca^{+2} have an inhibitory effect in nematocysts discharge.

Table 1. Quantitation of discharged nematocysts after a chemo- and electro- stimulation of tentacles at different exposition times. .

Chemical		Induction (min.)	Discharge (%)	Protein (mg/ml)
SW Control		**5**	**4±2 ***	**0.08**
(pH 7.8)		15	8±4 *	0.05
		30	10±2 *	0.05
Ethanol	70%	5	1.5±1 *	0.13
		15	2±2 *	0.38
		30	12±5 *	0.82
	80%	5	1±1 *	0.27
		15	15±4 *	0.82
		30	25±8 **	0.96
	96%	5	5±2 *	0.67
		15	27±6 **	1
		30	60±12 ***	1.71
Ac. Acid 5% (pH 2.5)		5	3±2 *	0.54
		15	20±6 **	0.61
		30	30±8 **	0.64
0.9% NaCl (pH 5.8)		5	3±1 *	0.37
		15	14±8 **	0.29
		30	>90****	2.233
0.3M KCl (pH 6.04)		5	18±4	0.64
		15	80*	1.214
		30	>90****	2.646
0.01M CaCl2 (pH 6.4)		5	2±1 *	0.42
		15	2±1 *	0.57
		30	9±4 *	0.76
SW	Electro-stimul		50±11 ***	0.212
0.9% NaCl			>90 ****	0.47
0.3M KCl			>90 ****	0.59
0.01M CaCl2			50±12 ***	0.17

[#] % of discharge ± standard deviation; >90%; not discriminate the percentage of discharged at this orders of magnitude. * significantly different ($p<0.005$) (ANOVA). .

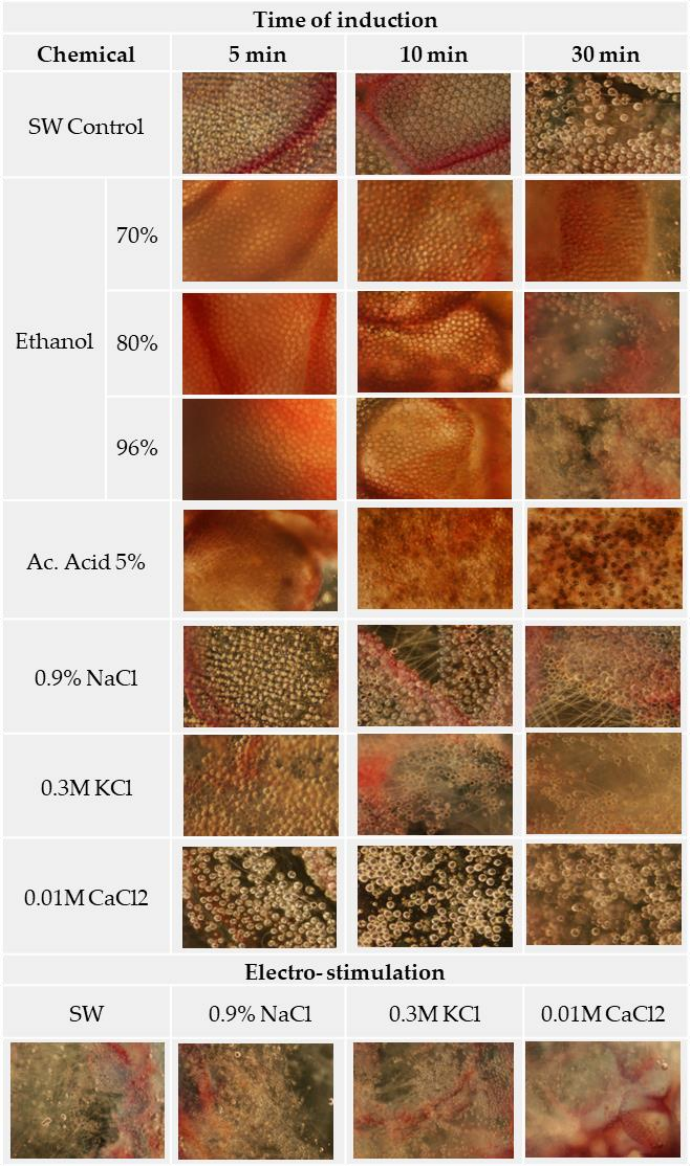

Figure 1. Photomicrographs of jellyfish tentacles after treatment with chemical solutions. *P. physalis* tentacle were exposed to: 5% acetic acid solution; ethanol at 70%, 80% and 96%; saline solutions of 0.9% NaCl, 0.3M KCl, 0.1M CaCl2; and, induced with 8V electrical pulse of 3s. Magnification is 100×. .

3.2. Venom released by discharged nematocysts has proteolytic activity

After chemical stimulation of nematocyst discharge, the venom proteins released to the solutions were compared in terms of quantity, SDS-PAGE profile and enzyme activity. Protein recovered after chemosensing is in line with the percentage of nematocytes discharged. A significantly higher amount of protein was measured in the supernatant of tentacles challenged with Na+ for 30 minutes (2.23 mg/ml) and with K+ for 15 and 30 minutes, 2.21 and 2.6 mg/ml, respectively (Table 1). These supernatants showed proteolytic activity, measured at 97.4 U/mg and 83.6 U/mg in samples induced with Na+ and K+, respectively (Figure 3 A). The proteolytic activity per mg of total protein was significantly higher in venom obtained from the supernatant of discharged nematocysts than in venom obtained from tentacle homogenization, 67.5 U/mg. The proteolytic activity was strongly inhibited by both PMSF and EDTA (Figure 3 B), suggesting the presence of serine- and metallo-proteases. The stimulation of nematocysts with concentrated ethanol solutions allowed the recovery of high protein amounts, 0.96 and 1.71 mg/ ml, but without enzymatic

activity. Nematocysts elicited by electro stimulation, present a high rate of discharge but the protein recovery and the proteolytic activity were low.

The SDS-PAGE profile of the *P physalis* extracts obtained by homogenization with glass beads showed remarkable differences comparing to venom recovered from the supernatant of nematocysts discharge. The greater number of bands identified in the samples obtained by homogenization of the tentacle, possibly are due to a co-extraction of the tissue proteins. Large bands with 40 kDa, 25 kDa and 20 kDa are coincident with those observed on venom obtained by the nematocysts discharged (Figure 2A). The gelatinolytic pattern of venom extract by homogenization and venom recovered by chemosensation with Na^+ and K^+ were quite identical with large digestion bands around 70 kDa, 40 kDa and 25 kDa.

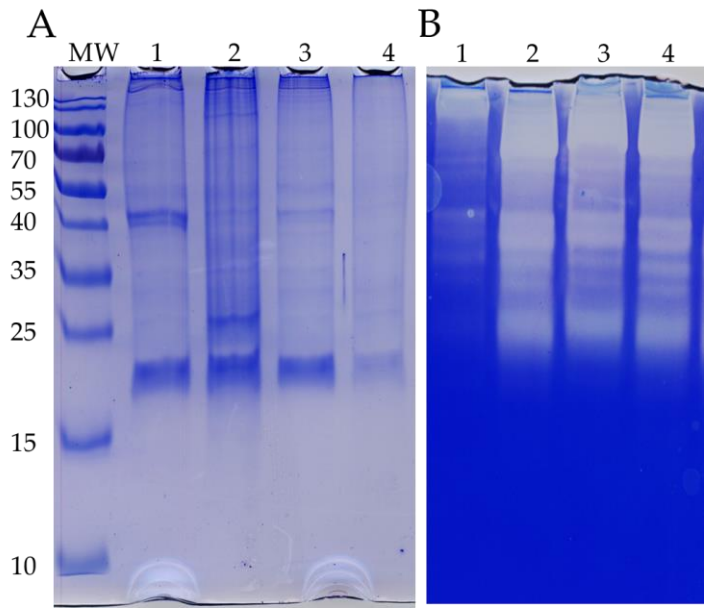

Figure 2. Protein and enzymatic profiles of venom from P physalys. A) SDS-PAGE pattern. B) Gelatin zymogram. Venom samples obtained from extraction with 1, ethanol; 2, homogenization; 3, 0.9% NaCl; 4, 0.3 M KCl.

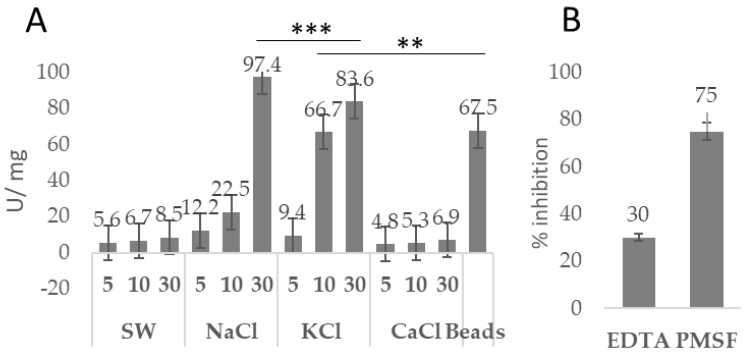

Figure 3. Proteolytic activity of P physalis venom. A) proteolytic activity of venom obtained from the nematocysts challenged with different chemical (SW, NaCl, KCl, CaCl) and venom obtained from tissue homogenization (Bead). B) Inhibitory activity caused by EDTA and PMSF using venom recovered from nematocysts challenge with NaCl. * statistically significant ($p<0.05$) (ANOVA).

4. Conclusions

Jellyfish venom research is a very attractive field due to the presence of various bioactive components that can be used in biotechnology approaches in areas ranging from cosmeceutical to health and to the increasing sting accidents occurring now a days. Nevertheless, information on the venom components, biological activity and pathological mechanisms are still scarce. From biotechnology point of view the present study demonstrated that the use of Na+ and K+ solutions stimulate the discharge of nematocysts from *P physalis* tentacles, thus has great potential for future venom research. This method allowed an accurate extraction of venom proteins in its active form, providing a simple and reproductive approach that circumvents the time and technical limitations associated with mechanical disruption. Based on the results of this study, further research can be conducted to isolate active molecules, like serine and metalloproteases, to further investigate the molecular mechanisms helping the understanding of the pathological symptoms associated with envenomation. On the other hand, the use of ethanol as a chemical stimulus led to efficient trigger discharge of nematocysts and an effective recovery of venom proteins, which could facilitate the identification of venom components by proteomic techniques. Concerning to accidental contacts and health risk to humans, the present research shown that Ca^{2+} seems to have and inhibitory effect of nematocysts discharge. Thus, may provide information for further study first-aid protocol associated to accidental contact with *P. physalis*, inhibiting discharge of adherent nematocysts reducing the impact of the stings.

Author Contributions: DT, design the research experiments, right, performed chemical assays; ZT, and MB performed the assays; LS performed the assays and capture of organisms; NS, revised the manuscript.

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