Proteolytic and hemolytic activity in the venom of the lionfish *Pterois volitans*, an invasive species of Cuban sea coasts

Actividad hemolítica y proteolítica en el veneno del Pez León *Pterois volitans*, especie invasora de las costas cubanas

Lenia Manso, Uris Ros, Gilberto Valdés, Maday Alonso del Rivero, María E. Lanio y Carlos Álvarez*

Centro de Estudio de Proteínas, Facultad de Biología, Universidad de La Habana

* Autor para correspondencia: calvarez@fbio.uh.cu

ABSTRACT

The Indo-Pacific lionfish *Pterois volitans* (Family Scorpaenidae) is becoming rapidly established in Cuban sea waters. Due to its potential adverse effects on human health and the scarce information on venom composition, the main purpose of this communication is to report a partial biochemical characterization of its venom. The crude venom extract of lionfish *Pterois volitans* contents abundant proteins with a molecular weight range between 40 up to 100 kDa and possesses gelatinolytic activity in a wide range of pHs, been maximal at pHs between 7-9 as well as hemolytic activity which is exerted on rabbit but not on human erythrocytes. Remarkably, both hemolytic and gelatinolytic activities were abolished after heat treatment (60°C, ~15 min) suggesting the proteinaceous nature of the active entity(ies). Furthermore, the molecule(s) responsible for the gelatinolytic activity in the crude extract was(were) not able to hydrolyze Suc-(Ala)_2-Phe-Phe-pNA , BAPA , BAEE, Leu-pNa, Ala-pNa, AAFP, AAFR, Gly-Pro-pNa, classical chromogenic substrates for serine-, cysteine-, and metalloexopeptidases. The proteolytic activity corresponding to the most two active components with molecular weight lower than 45 kDa were inhibited by EDTA, PMSF, and DTT. Altogether the results obtained indicate that the venom of lionfish contains thermolabile protein(s) with high molecular weight, and exhibits hemolytic and proteolytic activities. Furthermore, the proteolytic activity profile and the effect of proteinase inhibitors together with the molecular weight of the proteases suggest the presence of matrix metalloendoproteases in the lionfish venom.

Keywords: venomous fish, hemolytic activity, proteolytic activity, lionfish, invasive species

Recibido: 2015-02-06
Aceptado: 2015-08-04
RESUMEN
El pez león, *Pterois volitans* (Familia: Scorpaenidae), oriundo del océano Indo-Pacífico, se ha establecido en las costas de Cuba. Debido a su potencial efecto adverso en la salud humana y la escasa información que existe sobre la composición de su veneno, el propósito fundamental de esta comunicación es informar los resultados de la caracterización bioquímica parcial del veneno. El extracto crudo del veneno del pez león contiene abundantes proteínas con pesos moleculares entre 40 y hasta 180 kDa y posee actividad gelatinolítica en un amplio rango de valores de pH siendo máxima a pH entre 7 y 9 así como actividad hemolítica (AH) en eritrocitos de conejo. Dicho extracto carece de AH en eritrocitos humanos. Es de destacar que ambas actividades son abolidas ante el tratamiento con calor (60°C, ~15 min) lo que sugiere la naturaleza proteica de la(s) entidad(es) involucrada(s). Además, la(s) molécula(s) responsable(s) de la actividad gelatinolítica en el veneno crudo no fueron capaces de hidrolizar Suc-(Ala)₂-Pro- Phe- pNA , BAPA , Leu-pNa, Ala-pNa, AAFP, AAFR, Gly-Pro-pNa, sustratos cromogénicos clásicos para serino-, cisteíno- y metaloexo-peptidásas. La actividad proteolítica de los dos componentes más activos de peso molecular menor de 45 kDa resultó inhibida por EDTA, PMSF y DTT. En conjunto, los resultados obtenidos indican que el extracto del veneno del pez león contiene proteínas termolábulas de alta masa molecular y exhibe actividad hemolítica y proteolítica. El perfil de actividad proteolítica obtenido y el efecto de los inhibidores de proteasas, así como los pesos moleculares de las proteasas, sugiere la presencia de metaloenzima proteolíticas en el veneno del pez león.

**Palabras clave:** peces venenosos, actividad hemolítica, actividad proteolítica, pez león, especie invasora

**INTRODUCTION**

The species *Pterois volitans*, commonly known as lionfish, belong to the *Scorpaenidae* family and is naturally found in Indo-Pacific tropical sea waters. Since 2008, Chevalier et al. (2008) have reported the increasing presence of the lionfish *Pterois volitans* in Cuban waters where this species has become an extensive invader with unpredictable impact on marine ecosystems (Schofield, 2010). Besides, this fish possesses venomous glands situated in the base of the dorsal, pelvic, and anal spines. Accidents caused by lionfish envenomation are usually not lethal but produce edema, intense pain, necrosis at the site of puncture, neuromuscular and profound cardiovascular affections commonly associated with the protein components of this venom (Church and Hodgson, 2002; Gomes et al., 2009).

In contrast with terrestrial animals, venomous fishes have been by far less studied. In particular, in lionfish venom only a gelatinolytic protease of about 45 kDa (Balasubashini et al. 2006 a), a proapoptotic peptide of around 7.6kDa (Balasubashini et al. 2006 b) as well as a cytolytic protein of approximately 160 kDa deduced from a nucleotide sequence (Kiriake and Shiomi, 2011) have been reported. In order to obtain insight into the proteinaceous composition of the venom produced by the Cuban coast invader *Pterois volitans* here we describe its protein profile as well as its proteolytic (PA) and hemolytic activity (HA).

**MATERIAL AND METHODS**

Lionfish specimens were collected along the coast of Havana, and the venom was prepared according to Church and Hodgson (2002). Briefly, fishes were killed by cooling and the venomous spines removed and stored in 10 % glycerol solution at -80°C until processed. The spines were thawed and ground in a chilled mortar in 10% glycerol and centrifuged at 7000 g for 15 min at 4°C, the supernatant filtered through fiberglass and its protein concentration determined by Bradford (1976). Aliquots were stored at
HEMOLYTIC AND PROTEOLYTIC ACTIVITY IN LIONFISH VENOM

LENIA MANSO ET AL.

-80°C until use. The molecular weight profile of protein lionfish venom was examined by SDS-PAGE performed according to Laemmli (1970). The proteolytic activity of lionfish crude extract was analyzed by zymography using 10% SDS-PAGE containing 0.1% copolymerized gelatin as substrate, according to Heussen and Dowdle (1980). The effect of pH on the proteolytic activity was determined using the same method (Heussen and Dowdle, 1980). Briefly, gels were incubated overnight at room temperature (22 ± 2°C) in the following buffers: 20 mM sodium acetate (pH 3, 4 and 5), 20 mM sodium citrate (pH 6), 20 mM Tris-HCl (7 and 8), and 20 mM glycine (9 and 10). To identify the mechanistic class of the gelatinolytic proteases in the crude venom, adequate amounts (1 mM) of classical inhibitors: TLCK (for trypsin-like serine-proteases), PMSF (for trypsin-chymotrypsin-like serine-proteases), and E64 (for cysteine-proteases), from Sigma-Aldrich (St Louis, USA) were preincubated with venom during 10 minutes. The residual gelatinolytic activity of the extract-inhibitor mixture was evaluated as previously described, by zymography at pH 8 in 20 mM Tris-HCl where the activity of crude extract was optimal.

We also evaluated the effect of the following compounds on the crude venom gelatinolytic activity: Ca²⁺ (5 mM), Pefabloc (5 mM), PMSF (5 mM), DTT (5 mM), EDTA (5 mM) and ortophenantroline (1 mM). They were included in the buffer in which the zymogram was incubated (20 mM Tris-HCl, pH 8). In addition we evaluated the specificity of cleavage of gelatinases in Pterois volitans extract using the following chromogenic substrates: Suc-(Ala)₂-Pro-Phe-pNA, BAPA, BAEE, Leu-pNa, Ala-pNa, AAFP, AAFR, Gly-Pro-pNa, from Sigma-Aldrich (St Louis, USA) which recognize different types of proteases (Reytor et al., 2011), see table 1 for details. Hemolytic activity (HA) against human and rabbit erythrocytes was evaluated turbidimetrically at 600 nm at room temperature (22 ± 2°C) as previously described (Martinez et al., 2001). In short, erythrocyte suspensions were prepared using pooled fresh red blood cells, washed and resuspended in physiological TrisHCl-buffered saline (TBS, 145 mM NaCl, 10 mM Tris–HCl, pH 7.4). The cell suspension was diluted to an absorbance of 0.1 at 600 nm. The crude venom was two-fold serially diluted in saline buffer and the reaction was started by adding the same volume of cell suspension to each well (200 µl final volume).

RESULTS AND DISCUSSION

The protein composition of the crude venom as evaluated by SDS PAGE showed numerous protein bands with molecular weight ranging from 40 to 100 kDa (figure 1A). These molecular characteristics are in agreement with those reviewed by Gomes et al., (2009) for the crude-venom of other fishes from the Scorpaenidae family, to which the lionfish belongs. Upon incubation with β-ME inter-chain disulfide bonds were reduced as revealed by the disappearance of the 66 and 104 kDa bands, reinforcement of the 45 kDa molecular specie(s), and appearance of some other bands in the electrophoregram (figure 1A). This result evinces, as described for the venom of other fishes of the Scorpaenidae family, the presence of multimeric proteins stabilized by covalent interactions (Garnier et al., 1995; Karmakar et al., 2004; Poh et al., 1991). On the other hand, the venom extract was heated at different temperatures and centrifuged (1000 g, 15 min) in order to eliminate those proteins denatured as a consequence of the heat treatment. The loss of bands as visualized by SDS-PAGE, insofar
as the incubation temperature increases, indicates that lionfish venom comprises thermolabile proteins that become denatured and insolubilized by incubation at 60°C during 15 min (figure 1B). Similarly, it has been described the presence of thermolabile proteins for other fishes in the Scorpaenidae family (Gomes et al., 2009). As the best of our knowledge, this is the first report of the protein profile of the crude venom of lionfish and the presence of thermolabile and multichain proteins stabilized by disulfide bonds in the venom of this species.

Lionfish produces one of the most potent and toxic fish venoms so far described (Haddad Jr. et al., 2004). This venom exerts cardiovascular, neuromuscular, and cytotoxic effects that have been associated with the presence of several proteinaceous toxins and other active components as acetylcholine or noradrenaline and even pore-forming toxins (Church and Hodgson, 2002); however, the presence of pore-forming activity has not been experimentally demonstrated since venom did not promote conductance increase when evaluated in planar lipid membranes (Cohen and Olek, 1989). The venom of Pterois volitans contains gelatinolytic proteases with different molecular masses (around 45 and 60 kDa) that show activity from pH 3-10, being largest at pH 7-9 (figure 2A). This result is in contrast with that obtained by Balasubashini et al., 2006 a who reported the presence of a single protein band with proteolytic activity in the venom of lionfish. Remarkably, the activity found in this work was abolished after heat treatment (60°C, 15 min) suggesting the proteinaceous nature of the active entity(ies), (figure 2B). This is the first report of a thermolabile proteolytic activity in this venom.

The entity(ies) responsible for the gelatinolytic activity in the crude extract was/were not able to hydrolyze classical serine, cysteine, or metalloexopeptidases chromogenic substrates (table 1). The proteolytic activity corresponding to the two active components with molecular weight lower than 45 kDa were inhibited by EDTA, PMSF, and DTT; additionally, these two bands were reinforced when Ca was added to the incubation medium. These bands can be not attributed to serine or aspartic proteases, since proteins from these two latter classes are characterized by a lower molecular weight (around ~ 20-30 kDa) (MEROPS database). Particularly, the maximal gelatinolytic activity was found between pH 7-9 that would preclude the contribution of aspartic proteases whose optimum pH are typical in the acid pH range. Summarizing: i. the lack of proteolytic activity against the classical chromogenic substrates, ii. the gelatinolytic activity inhibition by EDTA and DTT (Guo-Ping et al. 2010), iii. the reinforcement of the proteolytic activity bands by Ca (Kupai et al., 2010), as well as iv. the molecular weight banding pattern observed in the zymography, similar to others reported for gelatinolytic activity, suggest the presence of matrix metalloendoproteases in this venom (Guo-Ping et al., 2010). Work is in progress in our laboratory in order to get more insights into the proteases present in lionfish venom.

In order to assess the activity of the venom, the loss of turbidity of a red cell suspension was quantitatively related to the crude venom HA, which was expressed as a function of the extract protein concentration (figure. 2C). The crude venom extract caused rabbit erythrocytes lysis at protein concentrations in the range 2.5 to 30 µg.mL⁻¹ in a dose-dependent way. The
HA of lionfish venom was compared with the HA of the total body extract obtained from the sea anemone *Stichodactyla helianthus* which contains two pore-forming toxins with high HA (in the nM concentration range) that have been well characterized by our laboratory (Alvarez et al., 2009; Lanio et al., 2001). The HA of lionfish venom lied below the positive control of hemolysis induced by *Stichodactyla helianthus* total body extract for concentrations lower than 20 µg.mL⁻¹.

Furthermore, the lionfish venom extract was unable to promote lysis of human erythrocytes (Figure 2C). This result is in agreement with those reported by Shiomi et al., (1989) who demonstrated that *Pterois volitans* venom’s HA is highly specific against rabbit erythrocytes. Since most fish venoms lack phospholipase activity, their hemolytic action has been postulated to be preceded by binding of the lytic component to a protein receptor on the surface of erythrocytes.


Chhatwal, I., F. Dreyer (1992) Biological properties of crude venom extract from the greater weaver fish *Trachinussdraco*. Toxicon 30:77-85.


MEROPS, the peptidase database: http://merops.sanger.ac.uk/


