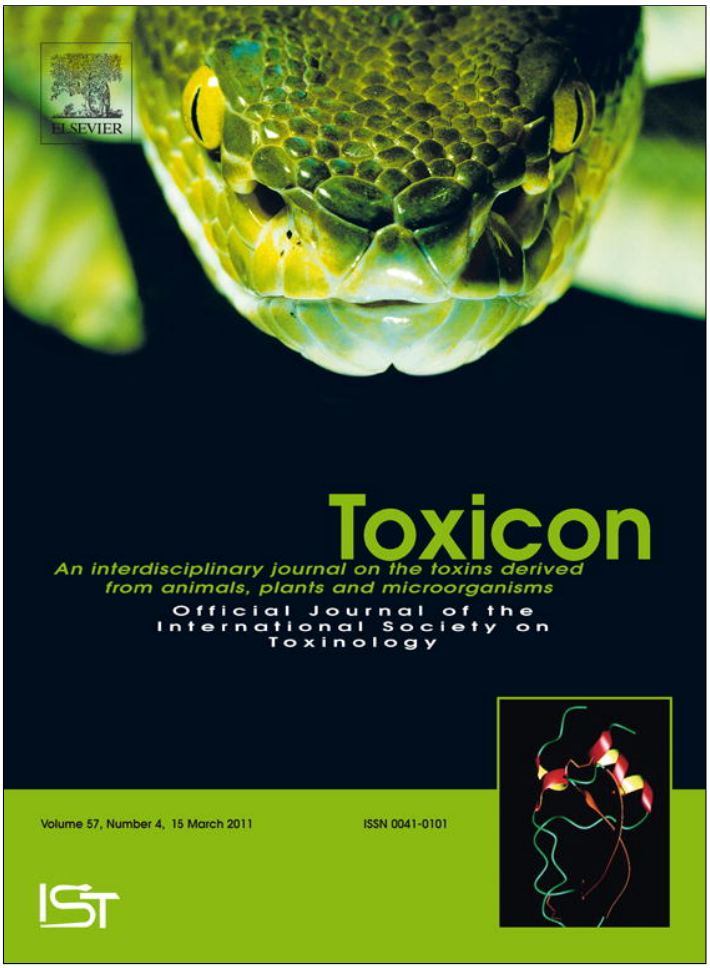

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Clinical report

Purification and characterization of a metalloproteinase, Porthidin-1, from the venom of Lansberg's hog-nosed pitvipers (*Porthidium lansbergii hutmanni*)

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ABSTRACT

Porthidium lansbergii hutmanni is a small pit viper found on Margarita Island, Venezuela. Local tissue damage is one of the most obvious characteristics of *P. l. hutmanni* envenomation, which can lead to diverse pathological effects, such as hemorrhage, edema, blistering, necrosis, lymphatic vessel damage and degradation of extracellular matrix. Metalloproteinases are one of the major components in venoms responsible for these effects. To date, very little is known or has been reported on *P. l. hutmanni* venom. Crude *P. l. hutmanni* venom had a LD_{50} of 2.5 mg/kg and was considered very hemorrhagic (minimal hemorrhagic dose [MHD]: 0.98 μg) when compared to other hemorrhagic (*Bothrops*) venoms in Venezuela. Crude *P. l. hutmanni* venom also inhibited ADP-induced platelet aggregation. A metalloproteinase, Porthidin-1, from this venom was isolated by three chromatography steps (Sephadex G100, Superose 12 HR10/30 and Bioscale Q2). Porthidin-1 falls in the SVMP P-I class having a molecular weight of 23 kDa, verified by both SDS-PAGE and mass spectrometry. High-resolution mass spectrometry and a database search identified a peptide from Porthidin-1 (YNGDLDK) belonging to the SVMP family of proteins. Porthidin-1 contained hemorrhagic, fibrino(geno)lytic, caseinolytic and gelatinolytic activities, and these activities were capable of being neutralized by metalloproteinase inhibitors but not serine proteinase inhibitors. The peptide YNGDLDK shared similarities with five venom proteins with a BLAST e-value of <1. This work details the biochemical and pathophysiological effects that can result from envenomations, and highlights the importance and significance for characterizing unknown or poorly documented venoms from different geographical regions.

1. Introduction

The genus *Porthidium* consist of several species dispersed from southern Mexico and Central America to northern and northwestern South America, including Colombia, Ecuador, and Venezuela (Campbell and Lamar, 1989). Venezuela contains three of these species, which include the *Porthidium lansbergii hutmanni*, *Porthidium lansbergii lansbergii* and *Porthidium lansbergii rozei*, which can be found in the North and West inlands (Lancini, 1979). Lansberg's hog-nosed pit viper (*P. l. hutmanni*), one of the smallest venomous snakes in the world, is found in xerophytic semi-arid forest and piedmonts in Margarita Island, Venezuela. *Porthidium l. hutmanni* is known to be responsible for

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ophidic accidents in Margarita Island, Venezuela, and its venom produces local and systemic hemorrhage, and edema (Rengifo and Rodríguez-Acosta, 2004).

Porthidium venoms have been poorly studied; therefore, purification and characterization of specific hemorrhagic toxins in these venoms have not been previously published. Although in a recent study (Pineda et al., 2008), *P. l. hutmanni* crude venom was analyzed and its hemorrhagic activity was inhibited by *Didelphis marsupialis* serum and serum fraction. In another study, venom fractions of *P. l. hutmanni* were able to inhibit platelet aggregation (Lopez-Johnston et al., 2007a, 2007b).

To our knowledge, this study is the first to report the purification and characterization of a 23 kDa metalloproteinase, Porthidin-1, from the venom of *P. l. hutmanni* containing hemorrhagic, fibrin(ogen)olytic, gelatinase, edematogenic, anticoagulant and caseinolytic activities.

2. Materials and methods

2.1. Reagents

Sephadex-G100, Bioscale Q2 column and molecular mass protein standards were obtained from Bio-Rad (Hercules, CA, USA). The Superose 12 HR10/30 size exclusion (SE) high-performance liquid chromatography column was obtained from GE Healthcare (Piscataway, NJ, USA). Coomassie Brilliant Blue R-250, Benzamidine/HCL, 1-10 phenantroline, phenylmethylsulfonyl fluoride (PMSF), ethylene glycol-bis-N,N,N',N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid and other chemicals and solvents were obtained from Sigma (St. Louis, MO, USA). Bovine alpha thrombin and plasmin were from American Diagnostica Inc. (Greenwich, CT, USA). ADP, collagen and thrombin were purchased from Chrono-log (Havertown, PA, USA). HYPHEN-BioMed Human Fibrinogen was purchased from Aniara (Mason, OH, USA). Molecular mass standards for SDS-PAGE were acquired from Invitrogen (Carlsbad, CA, USA).

2.2. Venom

Porthidium l. hutmanni specimens were captured near Juan Griego town, Margarita Island, Venezuela and maintained at the Serpentarium of the Tropical Medicine Institute of the Universidad Central de Venezuela. Venom was collected in 50 mL test-tubes covered with Parafilm® while submerged in ice. The venom was then frozen at −80 °C overnight, lyophilized and maintained at - 80 °C until further used.

2.3. Animals

Outbreed, male, NIH Swiss albino mice weighing 18–22 g were used for all studies. The mice were obtained from the central animal house of the Instituto Nacional de Higiene "Rafael Rangel", Caracas, Venezuela.

2.4. Ethical statement

Expert personnel prepared all the experimental events relating to the use of live animals according to the Venezuelan pertinent regulations and institutional guidelines for the care and use of laboratory animals. These guidelines were published by the US National Institute of Health (NIH 1985) and approved by the Institute of Anatomy of the Universidad Central de Venezuela.

2.5. Determination of protein concentration

Protein concentration was determined by the method of Lowry et al. (1951) or by absorbance at 280 nm, standardized with bovine serum albumin.

2.6. Porthidin-1 purification

Porthidium l. hutmanni crude venom was run using a semi-preparative procedure by molecular exclusion chromatography on a Sephadex G100 column equilibrated with 50 mM ammonium acetate buffer pH 6.9. Venom samples (350 mg/4 mL) were dissolved in the equilibrating buffer and injected into the column. The elution was carried out with the same buffer at 0.3 mL/min and monitored at 280 nm. The eluting peaks were assayed for hemorrhagic activity.

Ten milligrams of hemorrhagic fraction B (FB) were diluted in 100 μL of 50 mM ammonium acetate buffer, pH 6.9 and injected into Superose 12 HR10/30 size exclusion - HPLC column. Elution of proteins was monitored at 280 nm. The eluting peaks were also tested for hemorrhagic activity, and Peak 4 (P4) was chosen for further analysis due to its higher hemorrhagic activity. Finally, 1.25 mg/200 μL of P4 in 50 mM Tris–HCl, pH 7.0 were fractionated at 4 °C on anion exchange Bioscale Q2 column equilibrated with 50 mM Tris–HCl buffer, pH 7.0. Two fractions were eluted at 1.5 mL/min with 0–1 M NaCl gradient dissolved in the same buffer. The eluted fractions were dialyzed against water at 4 °C and lyophilized. The hemorrhagic activity was detected only in fraction II (FII), named Porthidin-1.

2.7. Gel electrophoresis (SDS-PAGE) of P. l. hutmanni crude venom and fractions

Crude venom and fractions were run on a 12.5% Tricine-SDS-PAGE (Schägger and von Jagow, 1987) under reduced conditions using a SureXCell system (Invitrogen) at 125 V for 90 min. The gel was stained with Comassie brilliant Blue R-250 for 1 h.

2.8. Lethal dose 50 (LD_{50})

Five groups containing 5 mice weighing 18–22 g were used to determine the LD_{50} for both crude venom and Porthidin-1. The mice were intravenously injected with 200 μL of serially diluted sample ranging from 20 to 320 μg. Deaths were recorded during a 48-h period. The LD_{50} was calculated by the Spearman-Karber (1978).

2.9. Minimal hemorrhagic dose (MHD)

The method of Gutiérrez et al. (1985) was used to determine the minimal hemorrhagic dose (MHD). A series of doses were prepared for *P. l. hutmanni* crude venom

(from 0.625 to 20 μg protein) and Porthidin-1 (from 2 to 32 μg protein). One hundred microliters of each dose was injected intracutaneously into the abdominal skin of male NIH Swiss albino mice. The mice were sacrificed with CO_2 after 3 h, and the internal skin surface was observed for hemorrhage. A caliper was used to measure the hemorrhagic diameter on the skin and the MHD determined. A MHD was taken as the end point and defined as the amount of venom resulting in a mean diameter of 10 mm considering both perpendicular major diameters of the hemorrhagic spot. Saline solution was utilized as negative control.

Inhibition of hemorrhagic activity was measured by incubating Porthidin-1 in 50 mM Tris–HCl buffer pH 8.2 containing metalloproteinase inhibitors (10 mM EDTA and 10 mM EGTA-Na) or serine proteinase inhibitor (10 mM benzamidine, and 10 mM PMSF) for 30 min at 37 °C. Aliquots of 100 μL containing 2 MHD ($11.12 \times 2 = 22.24$ μg) of Porthidin-1 previously treated with inhibitors were injected intradermally into the abdominal skin of three mice (Girón et al., 2008). Hemorrhagic activity was determined and measured as described above.

2.10. Temperature effect on Porthidin-1 hemorrhagic activity

Samples of Porthidin-1 (11.12 μg/0.1 mL) in 50 mM Tris–HCl buffer, pH 8.2 were maintained for 15 min at 4, 25, 37 and 56 °C. The samples were cooled in an ice bath and the hemorrhagic activity was determined as described above.

2.11. Edematogenic activity

Mouse paw edema was performed in conformity with the Yamakawa et al. (1976) method. Edematogenic activity was measured by injecting four male mice (18–22 g) subcutaneously in the sub-plantar region of their right paw with 10–30 μg/50 μL of Porthidin-1. The edematogenic activity was also tested with 0.8–3.2 μg/50 μL *P.l. hutmanni* crude venom. Their left paw was injected with saline as a control. After 1 h, the mice were sacrificed by cervical dislocation and both paws were removed at the ankle joints and weighed individually. The increase in weight due to edema was designed as the edema ratio, which equals the weight of edematous foot × 100/weight of the control foot. Minimum edematogenic dose was determined as the amount of protein causing an edema ratio of 30%.

2.12. Platelet aggregation activity

Platelet aggregation was estimated through turbidimetry by means of a dual channel Chrono-log model 560 CA aggregometer (Havertown, PA, USA). Platelet-rich plasma (PRP) was prepared by mixing fresh blood sample with trisodium citrate solution (3.8%, w/v) in a volume ratio of 9:1, followed by centrifugation at 190 g, 24 °C for 20 min to sediment leukocytes and erythrocytes. The platelet count was adjusted to 3.0×10^5 platelets/μL with platelet-poor plasma. Four hundred ninety microliters of citrated PRP were pre-incubated at 37 °C with a stir bar in a silicone treated glass cuvette. Then, 10 μL (from 2 to 20 μg) of crude venom or Porthidin-1 in Tyrode's buffer were added 4 min prior to the addition of the platelet aggregation inducer. Aggregation was induced by adding 5 μL of ADP (final concentration of 10 μM) and the modifications by light transmittance were continuously recorded for 8 min. The maximum platelet aggregation obtained after addition of inducer in the absence of crude venom or fractions was taken as 100% aggregation (control). The percent inhibition was calculated by comparing light transmittance obtained in presence of crude venom or Porthidin-1 against the control sample. The IC_{50} value was calculated from a dose dependent curve that was achieved from at least five different inhibitor concentrations using the software program Graph Pad Prism.

2.13. Coagulant activity

Coagulant activity of Porthidin-1 was tested by the Austen and Rhymes (1975) method and a modification of Salazar et al. (2007). Briefly, 0.1 mL of 0.3% purified fibrinogen solution in Tris–HCl buffer, pH 7.4 (coagulation buffer) or human plasma was incubated in a borosilicate tube at 37 °C for 1 min. Then, 0.1 mL venom sample (diluted in coagulation buffer at 0.05 and 0.5 mg/mL) or 0.1 mL thrombin solution (0.5–15 IU/mL) and 0.1 mL of coagulation buffer were added. The solution was manually mixed thoroughly in a 37 °C water bath and the clotting time recorded when the appearance of a clot was visually detected. The assays were carried out four times and the mean clotting time calculated.

2.14. Fibrinogenolytic activity

The effect of Porthidin-1 on fibrinogen was evaluated following the Salazar et al. (2007) method. Briefly, fibrinogen: Porthidin-1 ratios of 100:1 were incubated at 37 °C for 5, 30 min, 1 h, 2, 4 and 24 h. Fibrinogen chains were visualized by SDS-PAGE on a 10% tricine gel under reduced conditions (Schägger and von Jagow, 1987). The assay was also carried out in the presence of serine proteinase (10 mM benzamidine, and 10 mM PMSF), metalloproteinase (10 mM EDTA and 10 mM EGTA-Na) or cysteine proteinase (10 mM iodoacetic acid) inhibitors.

2.15. Fibronectin identification

Fibronectin in each purified fibrinogen batch was identified by western blot using a Mini-Trans-Blot (Bio-RAD Laboratories Ltd). Fibronectin was detected with a rabbit anti-fibronectin IgG at a 1/1000 dilution, followed by a peroxidase conjugated goat anti-rabbit IgG at a 1/1000 dilution (Lucena et al., 2006).

2.16. Fibrinolytic activity

Fibrinolytic activity of crude venom or Porthidin-1 was studied by the fibrin plate method as described by Marsh and Arocha-Piñango (1972). Briefly, fibrin plates (3 cm Petri dishes) were set by adding 75 μL bovine thrombin (10 IU/mL, in 0.025 M $CaCl_2$) to 1.5 mL of a fibrinogen plasminogen free in imidazol saline buffer, pH 7.4. The mixture was incubated at room temperature for 30 min. Then, 10 μL of crude venom or Porthidin-1 was added over

the fibrin film. After 24 h incubation at 37 °C, the lysis degree was then recorded by calculating the lysis area as the product of both perpendicular major diameters, which could be measured at right angles. The results were expressed as the lysed area diameter per μg of protein ($mm^2/\mu g$). Human plasmin (2 μg) and two chains tissue-type plasminogen activator (0.1 μg) were used as positive controls. The assay was also carried out in the presence of serine proteinase (10 mM benzamidine, 100 IU/mL aprotinin and 10 mM PMSF) and metalloproteinase (10 mM EDTA) inhibitors.

2.17. Gelatinase activity

a) Gelatin zymography: To determine the gelatinolytic activity of *P.l. hutmanni* crude venom and Porthidin-1, a gelatin zymography was carried out according to the method of Da Silveira et al. (2002). Crude venom and Porthidin-1 were diluted in SDS sample buffer under non-reducing conditions and run on 9% SDS–polyacrylamide gels co-polymerized with 1 mg/mL of gelatin. After electrophoresis, the gels were washed two times for 30 min in 2.5% Triton X-100 to eliminate the SDS and then incubated in the zymography incubation buffer (0.05 M Tris–HCl, pH 7.3, 0.2 M NaCl, 0.001 M $CaCl_2$, 0.001 M $MgCl_2$) at 37 °C for 18 h. The gels were stained with 0.25% Brilliant Blue Coomassie R-250 in acetic acid:ethanol:water (7:30:63, v/v) solution and then distained with the same solution. The presence of gelatinolytic activity was defined as clear bands on a dark background.

b) X-ray film: A modified method by Lemoine et al. (2004) was also used to test the gelatinase activity of crude venom and Porthidin-1. An X-ray film (Kodak X-OMAT) was rinsed with distilled water and incubated at 37 °C for 45 min. After incubation, the film was completely dried and 20 μL of serial diluted crude venom or Porthidin-1 (starting at 50 μg protein) was placed on the X-ray scientific imaging film containing a gelatin coating. The X-ray film was incubated for 2 h at 37 °C in a humid incubator. Hydrolysis of gelatin was determined by washing the film with distilled water and observing a clear area. Serial dilutions were performed to determine the minimum amount of venom required to cause a clear spot on the film. The titer was defined as the reciprocal of the highest dilution that caused a clear spot on the film. The specific gelatinase activity was calculated by dividing the titer by the amount of protein (μg) applied on the film. The assay was repeated 3 times.

2.18. Caseinolytic activity

Specific cleavage on casein (Gay et al., 2005) by Porthidin-1 was demonstrated by electrophoresis on 12.5% polyacrylamide gels (Laemmli, 1970). Casein (5 mg/mL) was heated at 100 °C for 15 min in 0.1 M Tris–HCl buffer (pH 8.0). Then, 5 μL of casein solution was incubated with Porthidin-1 at 37 °C for 30 min at different ratios (casein:Porthidin-1): 100:1, 100:5, 100:10. The samples were run under reduced conditions by SDS-PAGE. For inhibition analysis, Porthidin-1 was incubated in presence of 20 mM EDTA or 20 mM benzamidine for 1 h at 37 °C prior to casein incubation (Fig. 5).

2.19. Mass spectrometry analysis of Porthidin-1

The proteins samples were dried in Vacufuge Eppendorf 5301 for 30 min at 30 °C and resuspended in 10 μL of 0.1% TFA/50% ACN, desalted using Zip Tip_{C18} (Millipore ZTC18S096). Then 0.5 μL of α-HCCA (Alpha-cyano-4-Hydroxycinnamic Acid) matrix was spotted on an MTP AnchorChip target plate 600/384 TF (Bruker Daltonics), 0.5 μL of sample was added to the matrix. Mass analysis was performed with Flex Control software on the AUTOFLEX II-TOF/TOF Mass spectrometer (Bruker Daltonics) in positive reflectron mode using external standards: (Insulin Bovine, I-5500 Sigma) and (Lysozyme Chicken egg, L-6876 Sigma) in order to determine the monoisotopic mass of the intact protein.

2.20. Amino acid sequence analysis (nanoFlow LC-MS/MS)

Protein identification and sequencing analysis was done according to Salazar et al. (2009). Additional protein analysis were done using BLAST (NCBI/BLAST, USA) search.

3. Results

3.1. Porthidin-1 purification

Porthidin-1, a hemorrhagic protein from *P.l. hutmanni* venom, was purified by three chromatographic steps. Size exclusion chromatography of the crude venom on Sephadex-G100 column generated 4 peaks (FA to FD) (Fig. 1A). Size exclusion chromatography of FB fraction on a Superose 12 HR10/30 size column produced seven peaks (F1 to F7) (Fig.1B). The F4 fraction was then re-chromatographed on an anion exchange Bioscale Q2 column generating two peaks (FI and FII). The hemorrhagic activity was detected only in FII, named Porthidin-1 (Fig. 3A). From 350 mg of protein present in the crude venom, 4.17 mg of Porthidin-1 were obtained, which represented 1.19% total of the initial protein.

3.2. Gel electrophoresis (SDS-PAGE) of P. l. hutmanni venom

The 12.5% SDS-PAGE of *P.l. hutmanni* crude venom revealed ~15 major protein bands with 2 bands located at the 90–95 kDa ranges, 2 bands between 65 and 55 kDa, 4 bands between 45 and 34 kDa, 3 bands between 30 and 22 kDa, and 2 bands below 14.4 kDa (Fig. 2, line 2). The electrophoretic gels from the fractions with hemorrhagic activity that were used in the Porthidin-1 purification are shown in Fig. 2 (Lanes: 3–6). A group of proteins with molecular masses ranging from 110 to 22 kDa were located in the Superose fraction F4 containing the highest hemorrhagic specific activity. Finally, the hemorrhagic fraction eluted from the Bioscale Q2 column (FII), now named as Porthidin-1, migrated as a homogeneous single band (Mr ~23 kDa) in an SDS-PAGE under reducing and non-reducing conditions (Fig. 2, lanes 5 and 6).

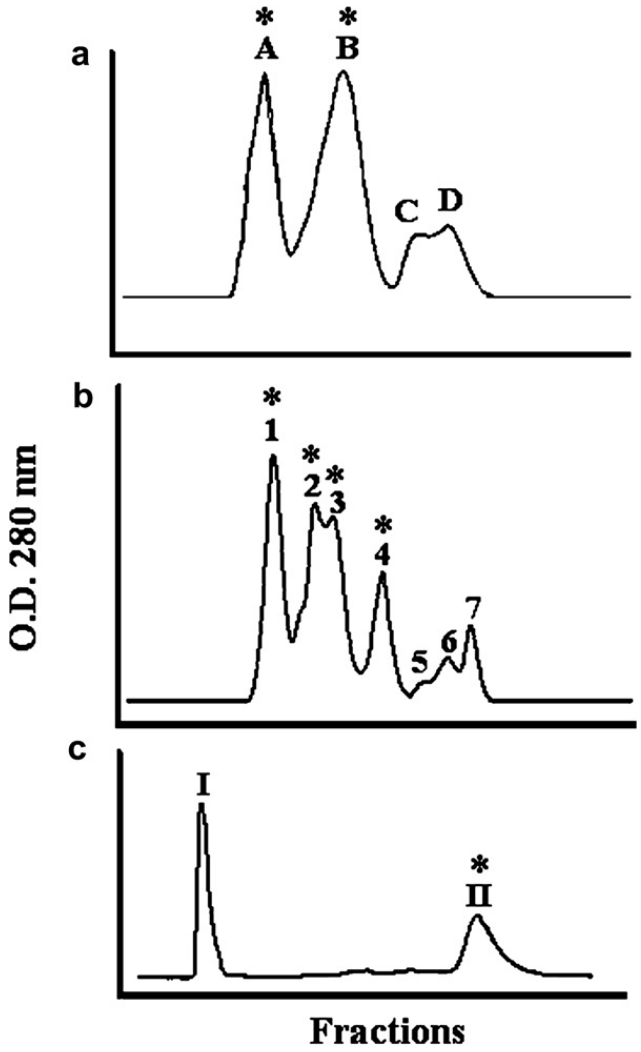

Fig. 1. A) Porthidin-1 purification scheme. a) *Porthidium l. hutmanni* crude venom (350 mg/100 μL) in 50 mM ammonium acetate buffer, pH 6.9 was chromatographed on a Sephadex-G100 molecular exclusion column; b) Hemorrhagic fraction (FB) was further separated on a Superose 12 10/300 column (10 mg/200 μL)(equilibrated and eluted with the same buffer); c) Fraction F4 (Fig. 1b) (1.25 mg/200 μL) was finally fractionated by anionic exchange chromatography on a Bioscale Q2 column equilibrated with 50 mM Tris–HCl buffer, pH 7.0 and eluted with a 0–1 M NaCl gradient in the same buffer. The asterisks (*) represent the hemorrhagic fractions. Fraction FII was denoted as Porthidin-1.

3.3. Lethal dose 50 (LD_{50})

Porthidin-1 was not lethal to mice at a maximum dose of 6.0 mg/kg, while crude venom had an LD_{50} of 2.5 mg/kg (Table 1).

3.4. Minimal hemorrhagic dose (MHD)

The MHD for crude venom was 0.98 μg/mouse. Among the chromatographic fractions obtained in the course of Porthidin-1 isolation, fractions FB, F4 and FII contained hemorrhagic activity. The anion exchange chromatography profile (Fig. 1C) produced 2 peaks, with only FII (Porthidin-1) having hemorrhagic activity, with a MHD of 11.12 μg/mouse (Table 1).

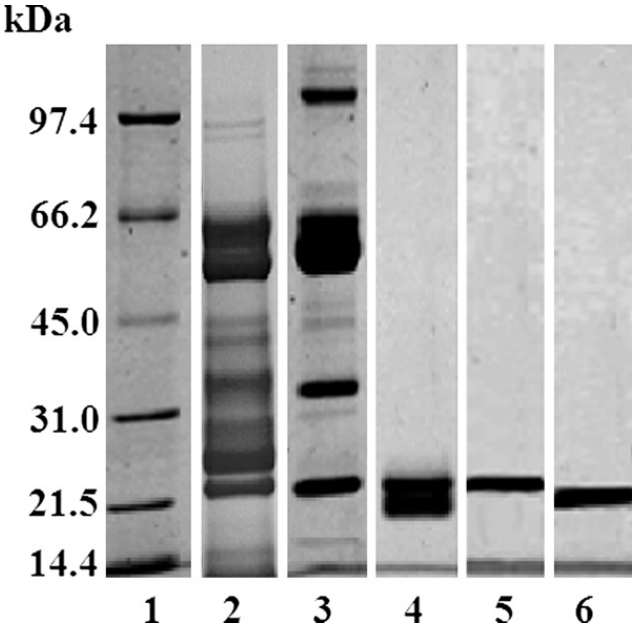

Fig. 2. Tricine-SDS-PAGE of crude *P. l. hutmanni* venom and fractions. A total of 40 μg of reduced venom was run on a 12.5% SDS Tricine gel at 125 V for 90 min. Lanes: 1) Molecular weight markers; 2) Crude *P. l. hutmanni* venom; 3) Fraction FB (Sephadex-G100); 4) Fraction F4 (Superose 12 HR10/30); 5) Fraction FII non-reduced (Bioscale Q2); 6) Fraction FII reduced (Bioscale Q2). The gel was stained with Comassie brilliant Blue R-250.

3.5. Temperature effect on the hemorrhagic activity of Porthidin-1

The hemorrhagic activity of Porthidin-1 after incubation at 4, 25, 37 and 56 °C for 15 min showed 51 (49% inhibition), 100 (no inhibition), 100 (no inhibition) and 0% (100% inhibition) hemorrhagic activity, respectively.

3.6. Edematogenic activity of Porthidin-1

A prominent edema developed in the footpad after a subcutaneous injection of crude venom and Porthidin-1,

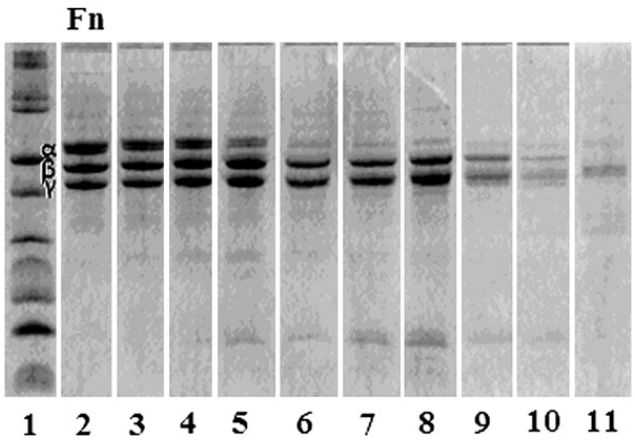

Fig. 3. Fibrinogenolytic activity of Porthidin-1. fibrinogen: Porthidin-1 (100:1) samples incubated at 37 °C at various times were run under reducing conditions on a 10% SDS Tricine gel. Lanes: 1) Molecular weight markers; 2) Fg control (contaminated with fibronectin (Fn)); 3–11) Fg + Porthidin-1 at 30 s, 1, 5, 15, 30, 60 min, 2, 4 and 24 h, respectively. The gel was stained with Comassie brilliant Blue R-250.

Table 1
Activities present in several snake venoms and Porthidin-1.

Sample	MED (μg)	Inhibitions of Platelet aggregation IC_{50} (μg/mL)	LD_{50}(mg/kg)	MHD (μg)	Fibrinolytic[a] activity (mm^2μg)	Reference
B. atrox[b]	N/A	N/A	4.0–8.3	2.5–5.6	51–100	Salazar et al., 2007
B. colombiensis[b]	N/A	N/A	5.8–11.6	5.3–13.8	14–29	Girón et al., 2008
C. d. cumanensis[b]	N/A	N/A	0.18–0.86	None-4.1	3.4–49.2	Aguilar et al., 2007
C. vegrandis	N/A	N/A	0.26	12	NA	Aguilar et al., 2001
B. isabelae	N/A	N/A	5.9	11.5	50	Rodríguez-Acosta et al., 2010
P. l. hutmanni	1.03	2.25	2.5	0.98	6.8	This work
Porthidin-1	17.36	No activity	NL	11.12	13.23	This work

NL: Not lethal.
$n = 3$.
MED: minimal edematogenic dose.
NA: Not available.
[a] Activity was measured at 24 h in presence of plasminogen.
[b] The values used for comparison were based on a range of activities for these venoms.

and a minimum edematogenic dose (30%) was reached with 1.03 and 17.36 μg, respectively (Table 1).

3.7. Platelet aggregation activity

Porthidium l. hutmanni crude venom inhibited ADP-induced platelet aggregation with an IC_{50} of 2.25 μg/mL, while 20 μg/mL inhibited 100%. In contrast, Porthidin-1 up to 20 μg/mL did not inhibit ADP – induced platelet aggregation (Table 1).

3.8. Coagulant activity on plasma and fibrinogen

Porthidin-1 (50 μg) was not able to coagulate plasma or fibrinogen after 6 min of incubation time.

3.9. Fibrinogenolytic activity

Fig. 3 shows the fibrinogenolytic activity of Porthidin-1. In presence of Porthidin-1, at a Fg: Porthidin-1 ratio of 100:1, whole degradation of the Aα chains as well a light degradation of the Bβ chains was observed. No apparent alteration to the γ chains, even after 24 h of incubation, was detected. The degradation of the Aα chains occurred between 5 to 15 min. In contrast, the degradation of the Bβ chains was between 2 to 4 h. This fibrinogenolytic activity was completely inhibited by metalloproteinase and cysteine-proteinase inhibitors (CPI), while the serine proteinase inhibitor had no effect (data not shown). Coagulant activity of fibrinogen (pre-treated with crude venom [100:1]) by thrombin resulted in the formation of fine fibrin threads after 280 s. Additionally, fibronectin (the highest molecular weight band demonstrated by immunoblotting assay) degradation was only observed at 24 h. This adhesive protein is a contaminant found in the commercial "purified" fibrinogen used in this assay.

3.10. Fibrinolytic activity

The results on fibrin plate, in presence or absence of plasminogen, evidenced that *P. l. hutmanni* crude venom at 10 μg present a fibrinolytic activity of 68.1 and 66.8 mm^2, respectively. Porthidin-1 at same doses had an activity of 132.3 mm^2 (Table 1). These results evidenced that the fibrinolytic specific activity of Porthidin-1 is two-fold higher than the crude venom. Metalloproteinase inhibitors inactivated crude venom and Porthidin-1 fibrinolytic activity while the serine proteinase inhibitor did not. Standards of plasmin and tct-PA showed a lysis area of 200 and 5280 mm^2/μg, respectively.

3.11. Gelatinase activity

a) Gelatin zymography:

Fig. 4 shows an acrylamide-gelatin gel zymography of *P. l. hutmanni* crude venom and Porthidin-1. The results demonstrated that the crude venom (lane 1) presented gelatinolytic activity evidenced by clear bands with relative molecular masses of ~23, 31 and 45 kDa. Porthidin-1 presented an active band at ~23 kDa (lane 2).

b) X-ray film:

The minimal dilution capable of obtaining a clear area on the X-ray film was 1/16 for both crude venom and Porthidin-1, which corresponded to 5.12 U/μg of gelatinase activity.

3.12. Caseinolytic activity

Porthidin-1 hydrolyzed bovine casein at different concentrations (Fig. 5). Hydrolysis of casein was dose dependent (lanes 3 to 5). The α_{S1}, β and κ chains of casein were degraded when ratios of Porthidin-1 were of 100:5 and 100:10 (Fig.5). Porthidin-1 had no caseinolytic activity when treated with EDTA, but was not affected with benzamidine (data not shown).

3.13. Mass spectrometry analysis of Porthidin-1

The molecular mass of Porthidin-1 as estimated by mass analysis (MALDI-TOF/TOF) was 23,151.48 Da (Fig. 6), and was unaffected when reduced, demonstrating Porthidin-1 as a single polypeptide chain.

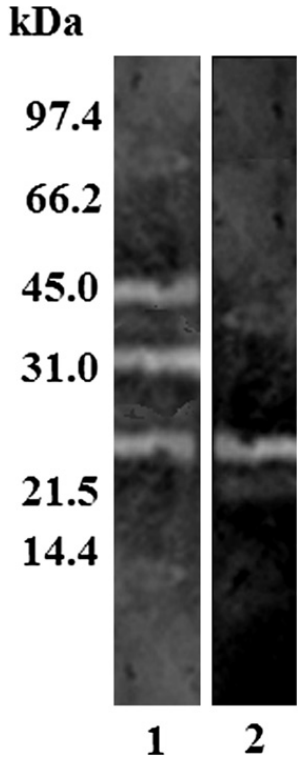

Fig. 4. Gelatinolytic activities of *P.l. hutmanni* crude venom and Porthidin-1. Samples containing 20 μg of crude venom (lane 1) and 10 μg of Porthidin-1 (lane 2) were applied to a 9% zymogram SDS-PAGE co-polymerized with gelatin. The clear areas indicate the location of gelatinolytic activities.

3.14. Amino acid sequence analysis (LC-MS/MS) (Porthidin-1)

Protein sequencing (LC-MS/MS) determination of the Porthidin-1 identified 8 peptides YNGDLDK, FSSSSGYGGGSSR, DYGDIDK, KDTEFAQQVLNK, ALEESNYELEGK, TNAENEFVTIK, RFQSTDVAEEVYTR and MEGDIDK. Database search against the NCBI/BLAST identified the YNGDLDK peptide having an e-value of <1 with five venom molecules consisting of the chain A of the metalloproteinase from venom of *Agkistrodon acutus;* three metalloproteinases: acutolysin e2, acurhagin and the acutolysin-E (*Deinagkistrodon acutus*) and with the zinc metalloproteinase-disintegrin, bilitoxin-1 (*A. bilineatus*). The other amino acid peptides had some sequence similarities with snake venom molecules but were not significant. No other venom proteins were identified in the mass spectrometry analysis. Due to the incomplete genome sequence and/or species variation, several but not all precursor ions were detected. These two limitations have been shown to reduce peptide and protein identifications in venom-related proteomic analysis (Calvete et al., 2009; Patiño et al., 2010) (Figs. 7 and 8).

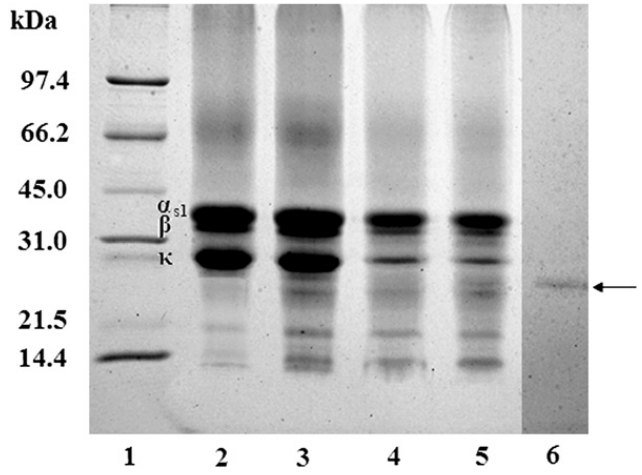

Fig. 5. SDS-PAGE analysis of bovine casein after digestion by Porthidin-1. Lanes: 1) low range molecular weight markers; 2) bovine casein control (25 μg); 3–5) bovine casein with Porthidin-1 at 100:1, 100:5, 100:10 ratios, respectively; 6) Porthidin-1 control (3 μg) (arrow). The samples were run under reducing conditions on a 12.5% gel. The gel was stained with Coomassie Brilliant Blue R-250. α_{s1}, β and κ represent the casein chains.

4. Discussion

The venomous pitvipers, *Porthidium lansbergii* are found in Central and South America (Campbell and Lamar, 1989), and not much is known of their venom activities or venom composition. *Porthidium l. hutmanni* are found on the island of Margarita, Venezuela and according to medical personnel (personal communication), envenomations by *P. l. hutmanni* can be neutralized with the Venezuelan polyvalent (anti-*Bothrops*/anti-*Crotalus*) antivenom. Since *P. l. hutmanni* venom was not used in the preparation of this antivenom, its venom molecules must have immunological similarity with Venezuelan *Bothrops* and *Crotalus* venom toxins. It is not uncommon that antivenoms can neutralize venoms that were not used in their preparation (Wisniewski et al., 2003; Sánchez and Rodríguez-Acosta, 2008).

Local tissue damage is one of the most obvious characteristics of snake envenoming, involving complex pathological effects, such as hemorrhage, edema, blistering, necrosis, lymphatic vessel damage and degradation of extracellular matrix (Gutiérrez et al., 2009). Two of the major components in venoms responsible for these effects are phospholipases A_2 (PLA_2s) (Fernández et al., 2010) and metalloproteinases (Gutiérrez et al., 2009). The metalloproteinases cause local hemorrhage, extracellular matrix degradation, blistering and necrosis (Fox and Serrano, 2009).

This current work evaluates the lethal and proteolytic activities of crude *P. l. hutmanni* venom and its purified metalloproteinase, Porthidin-1. The activities of *P. l. hutmanni* crude venom were compared to other medically significant Venezuelan venoms (Table 1). Even though a recent study by Pineda et al. (2008) reported *P. l. hutmanni* venom to be less potent in its lethal and hemorrhagic activities than the venom used in this work, it is not uncommon to encounter activity variation in venoms within the same species (Glenn et al., 1983; Salazar et al., 2007, 2009; Girón et al., 2008). The lethality of *P. l. hutmanni* venom reported in this current study was more potent than the *Bothrops* venoms; however, the lethal activities for the *Crotalus* venoms were significantly more potent due in part to neurotoxins present in these South American *Crotalus* venoms (Aguilar et al., 2001, 2007). Since the venom of *P. l. hutmanni* has not been extensively studied, it is not certain if this venom contains neurotoxins as well, which could contribute to the LD_{50} being more potent than those of the *Bothrops* venoms.

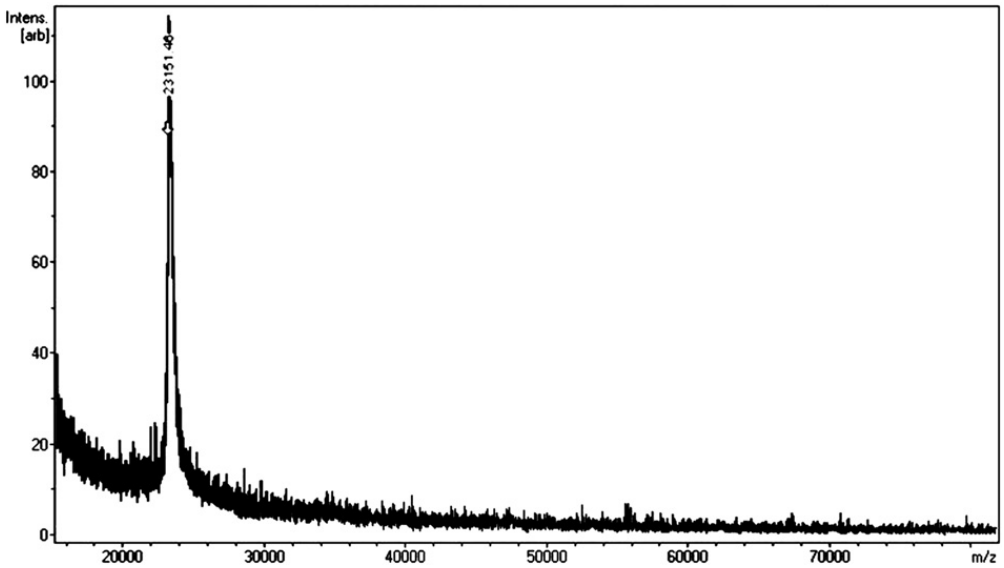

Fig. 6. MALDI-TOF/TOF mass spectrum analysis of Porthidin-1. Mass spectrometry was utilized to establish the molecular weight and sequence of the purified Porthidin-1. Analysis was carried out on the AUTOFLEX II-TOF/TOF Mass Spectrometer, Bruker Daltonics. The instrument was operated in linear mode using external standards in order to determine the monoisotopic mass of the intact protein.

On the other hand, the strong hemorrhagic activity (MHD: 0.98 μg/mouse) present in *P. l. hutmanni* venom could very well contribute to the lethal potency. Although Porthidin-1 is a low molecular weight hemorrhagin, the SDS-PAGE of the crude venom shows (Fig. 2, lane 2) various venom molecules at ranges in which the high molecular weight metalloproteinases reside (Mackessy, 2008). These P-III classes of metalloproteinases tend to be more hemorrhagic than the P-I and P-II classes (Fox and Serrano, 2009). These enzymes produce hemorrhage by altering extracellular matrix proteins such as laminin, nidogen, fibronectin, collagen type IV (constituents of vessel walls), and proteoglycans from the endothelial basal membrane, helping the diffusion of venom across the membranes (Fox and Serrano, 2009). The comparative analysis of hemorrhagic activity expressed as MHDs indicated that *P.l. hutmanni* crude venom is more potent (0.98 μg/mouse) than all *B. atrox* venoms (2.5–5.6 μg) (Salazar et al., 2007), and also more potent than *B. colombiensis* venom (5.3–13.8 μg) (Girón et al., 2008) (Table 1).

In the purification scheme to obtain the Porthidin-1, three chromatographic steps were run, beginning with a Sephadex-G100 exclusion chromatography in which the crude venom generating two active fractions: FA and FB, with a hemorrhagic activity of 1.47 and 11.74, respectively. In the search of a hemorrhagic protein with a high degree of purity, FB displaying the highest specific activity was run on the Superose column, which generated 4 hemorrhagic fractions of which F4 was the most active (12.87 μg/mouse). This fraction generated Porthidin-1 on the BioScale Q2 column having a MHD of 11.12 μg/mouse that in comparison to crude venom was less active, which could be explained by the presence of others metalloproteinases existing in the crude venom.

The hemorrhagic activity of Porthidin-1 was stable at 4 and 37 °C, but at 57 °C this activity was completely abolished. In 1994, Ownby et al., 1994 reviewed 28 diverse snake venoms for the presence of hemorrhagic activity after heating the venoms. It was established that some snake venoms retained hemorrhagic activity after heat exposure above 100 °C for 5 min, signifying that a number of hemorrhagic toxins may be comparatively heat-stable and preserve significant activity. The inactivation of activity due to heat is characteristic of metalloproteinases because serine proteinases, particularly serine fibrinogenolytic enzymes, are quite resistant to inactivation by extreme heat and pH (Swenson et al., 2005). There is a general conformity that resistance to denaturation is due to covalently bound carbohydrates (Mahar et al., 1987; Siigur et al., 1991).

Edema is produced by the action of inflammatory substances, including PLA_2, liberated or produced during envenoming, which provoke an increase in permeability of

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                                  170       180       190       200       210       220       230       240
ChainA_A.acutus   ---------------------------------YDPFKYVETVFVVDKAMVTKYNGDLDKIKTKMYEAANNMNEMYRYMF
Acutolysin_e2     DEAPKMCGVTQKWKSYEPIKKVSQLNLIPEQQIYDPFKYVETVFVVDKAMVTKYNGDLDKIKTKMYEAANNMNEMYRYMF
Acurhagin         DEAPKMCGVTQKWKSYEPIKKISQLNLIPEQQIYDPFKYVETVVVVDKAMVTKYNGDLDKIKTKMYEAANNMNEMYRYMF
Acutolysin_e      DEAPKMCGVTQKWKSYEPIKKISQLNLIPEQQIYDPFKYVETVVVVDKAMVTKYNGDLDKIITKMYEAANNMNEMYRYMF
Bilitoxin-1       ---------------------------------QRYNPYKYIELFLVVDNRMVTKYNGDLDKIKTRIYELVNILNEIYRPLY
Porthidin-1       ------------------------------------------------------YNGDLDK
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Fig. 7. NCBI/BLAST search comparison of the amino acid peptide,YNGDLDK, of Porthidin-1 to partial amino acid sequences of five snake venom metalloproteinases. Chain A (accession #: 3HDBA), Acutolysin e2 (accession #: ACT33415.1), Acurhagin (accession #: AAS57937.1), Acutolysin-e (accession #: Q9W6M5.1), Bilitoxin-1 (accession #: P0C6E3.1). The boxed amino acids represent the consensus sequences.

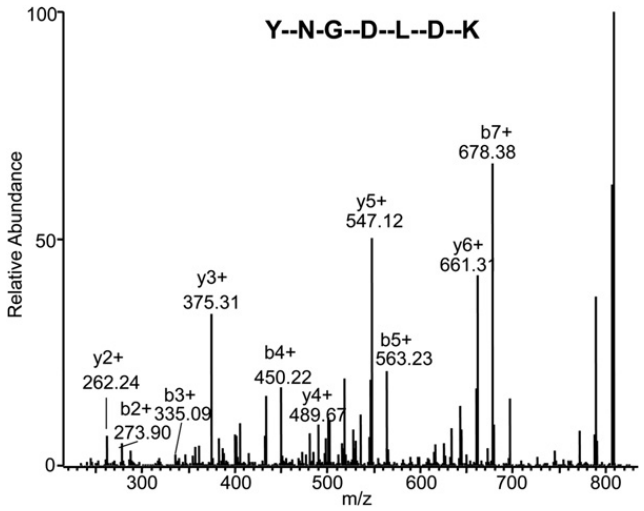

Fig. 8. Collision-induced dissociation ion spectra of peptide YNGDLDK *m/z* 824.378, which was used to identify Porthidin-1 as a PI-SVMP. The fragment b and y ions are labeled.

microvessels. Under the influence of toxins, the lymphatic system moves fluids from the blood vessels into the cellular interspaces producing mild to severe edema around the snakebite (Teixeira et al., 2003; Petretski et al., 2000; Soares and Giglio, 2003). In this current study, local edema and signs of pain emerged immediately after injecting Porthidin-1 into the footpads of mice.

Edema is usually associated in bothropic envenomation to myotoxic phospholipase A_2s (PLA_2s) action on the smooth muscle of the lymphatic vessels inducing contraction and irreversible cell damage leading to local edema (Mora et al., 2008). However, in terms of metalloproteinases, the P-I leucurolisin-a (leuc-a), a non hemorrhagic metalloproteinase from *B. leucurus*, increased blood capillary permeability inducing prominent edema which contributed largely to the local effect observed after *B. leucurus* envenomation (Gremski et al., 2007). In contrast, the P-I BaP1 metalloproteinase from *B. asper* injected into mouse gastrocnemius muscle induced moderate bleeding, myonecrosis and edema (Rucavado et al., 1995), as well as inflammatory infiltration composed mainly of leukocytes polymorphonuclear and macrophages accompanied by increased serum levels of cytokines IL-1β and IL-6 (Rucavado et al., 2002).

The contribution of platelets in hemostasis and thrombosis has been widely recognized. When a vein or artery is injured, platelets stick to the damage surface. The adherent platelets, once activated, discharge biologically active constituents and aggregates. Aggregation is mediated by agonists, such as ADP, epinephrine, thrombin, platelet-activating factors, or collagen (Da Silva et al., 2009). In the current work, crude *P. l. hutmanni* venom produced inhibition of ADP-induced platelet aggregation with an IC_{50} of 2.25 μg/mL. However, Porthidin-1 did not have this activity. As it is known, other proteinases present in *P. l. hutmanni* crude venom can have several inhibitory activities on platelet aggregation (Lopez-Johnston et al., 2007a).

Snake venoms also contain proteinases such as factor IX, factor X, prothrombin activators, or thrombin-like enzymes, which have been described to act on the blood coagulation cascade (Rodriguez-Acosta et al., 2010). Porthidin-1 did not present coagulant activity. In light of these results, the presence of fibrinogenases could be masking this activity.

Fibrinolytic and fibrinogenolytic activities are caused by both metalloproteinases and serine proteinases that differ in their mode of action (Swenson et al., 2005). Compared to the *Bothrops* venoms, *P. l. hutmanni* venom did not display very strong fibrinolytic activity. However, Porthidin-1 was two times more specific for fibrinolytic activity than the crude venom (Table 1). This fibrinolytic activity suggests that this venom contains metallo- or serine proteinases that act directly on fibrin. These proteinases are of great interest since they may have clinical relevance as thrombolytic agents.

The results also demonstrated that the Porthidin-1 is an α-fibrinogenase since it degraded the fibrinogen Aα chains (Swenson et al., 2005). Porthidin-1 induced a rapid degradation of Aα chains (15 min) and a slower degradation of Bβ chains (4 h). The γ chains were unaffected. To date there have been only a few reports of fibrin(ogen)olytic snake venom enzymes that act on the γ chains (Daoud and Tu, 1986; Gasmi et al., 1991; Rodríguez-Acosta et al., 2010).

Porthidin-1 also degraded the fibronectin contaminant in the "purified" fibrinogen, showing the effect on extracellular matrix components, which should explain the hemorrhagic consequences caused by this venom. The neutralization, by metal chelators, of fibrinogenolytic and fibronectinolytic activities in both crude venom and fractions confirmed that metalloproteinases were implicated in this activity, which was similar to that observed in other Viperidae venoms and their active fractions (Girón et al., 2008). Comparable examples of metalloproteinases with fibrinogenolytic and hemorrhagic activities such as Porthidin-1 have been demonstrated in other snake venoms such as *Bothrops jararaca* (Maruyama et al., 1992), *Bothrops asper* (Gutiérrez et al., 1995), *Crotalus vegrandis* (Aguilar et al., 2001) and *Philodryas patagoniensis* (Peichoto et al., 2007).

Gelatin has also been used as a substrate to demonstrate both metalloproteinase and serine proteinase activities (Shannon et al., 1989; Serrano et al., 1993; De Roodt et al., 2003). The proteolytic activity on gelatin seems to be related to hemorrhagic activity (De Roodt et al., 2003). Gelatin zymography is likely to prove useful for assessing the function of venom zinc metalloproteinases and serine proteinases on collagen. The gelatinolytic activity for *P. l. hutmanni* crude venom was observed on a zymography gel in the protein bands of 23, 31, and 45 kDa (Fig. 4, lane 1). The 23 kDa protein band corresponds to Porthidin-1 (Fig. 4, lane 2). This activity was neutralized by metal chelants, indicative of the presence of metalloproteinases. The gelatinolytic activity could represent, to a lower degree, the necrotic potential of the venom. Physicians from Margarita Island, Venezuela (personal communication) have observed that snake envenomations caused by *P.l. hutmanni* produce clinical necrosis in patients, manifested by a progressive deep necrosis more characteristic of a myolysis in respect to the skin and the aponeurosis.

Porthidin-1 had activity on casein and was inhibited by metalloproteinase inhibitors. Most hemorrhagic toxins that have been isolated and characterized from snake venoms

are determined to be metalloproteinases with caseinolytic activity (Bjarnason and Fox, 1994; Chakrabarty et al., 2000; Peichoto et al., 2007). Porhidin-1 slightly degraded the 3 casein chains showing a weak proteolytic activity on these protein chains.

The snake venom metalloproteinases (SVMP) have been recently reclassified as P-I, P-II and P-III with each classification having subclasses to accommodate structures produced by random proteolytic processing as well as by dimerization (Fox and Serrano, 2009). The previously P-IV class of metalloproteinases has been included into the P-III class since no P-IV mRNA transcript has been observed to date. The SVMP, Porthidin-1, isolated from *P. l. hutmanni* venom falls in the P-I class of SVMP having a molecular weight of 23 kDa, verified by both SDS-PAGE and mass spectrometry, and hemorrhagic activity capable of being neutralized by metalloproteinase inhibitors but not serine proteinase inhibitors. Porthidin-1 can be compared to the 23 kDa leucurolysin-a (leuc-a) metalloendopeptidase reported for *B. leucurus* (Bello et al., 2006) and mutalysin II, a 22.5 kDa zinc endopeptidase, isolated from *Lachesis muta muta* snake venom (Souza et al., 2001).

The sequence information by LC-MS/MS provided eight peptides for Porthidin-1 and was compared to several proteinases isolated from snake venoms, for which primary structure information is available on NCBI. The sequence YNGDLDK was the most noteworthy peptide with an e-value < 1, producing significant alignments with chain A of the metalloproteinase from venom of *Agkistrodon acutus*, with three metalloproteinases: acutolysin e2, acurhagin, and acutolysin-E (*Deinagkistrodon acutus*), and with the zinc metalloproteinase-disintegrin, bilitoxin-1 (*A. bilineatus*). These proteins were zinc metalloproteinases from snake venom and possess hemorrhagic activity. The unsequenced snake genome of *P.l. hutmanni,* probably restricted further sequence identification from purified Porthidin-1, which is a known limit for snake venom studies, recognized by other groups (Galán et al., 2008; Calvete et al., 2009).

In conclusion, the venom of *P. l. hutmanni* has been further studied and a snake venom metalloproteinase, Porthidin-1, was isolated for the first time from the venom. Its hemorrhagic activity, molecular weight (23 kDa), and its inhibition by EDTA suggest it is a P-I class snake venom metalloproteinase. Even though encounters with this snake species are uncommon (Personal communication, Margarita Island physicians), envenomation can pose serious pathophysiological effects comparable, if not more serious, to some of the most medically significant venomous snakes in Venezuela. *P. l. hutmanni* venom activity on platelet aggregation and proteolytic effects on extracellular matrix components, fibrinogen chains, gelatin and fibronectin demonstrated its possible association to the hemorrhagic syndromes that are produced when envenomated by this snake. Further studies to identify these factors could shed additional light on hemostatic modifications due to envenomation.

Conflict of interest

The authors declare that there are no conflicts of interest concerned with this work.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.toxicon.2011.01.003.

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