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DESCRIPTION OF HUTMANNIN-1, A NEW PIII-METALLOPROTEASE FROM THE VENOM OF THE NEOTROPICAL LANSBERG'S HOGNOSE VIPER *(Porthidium lansbergii hutmanni)* WITH FIBRINO(GENO)LYTIC AND HAEMORRHAGIC ACTIVITIES

DESCRIPCIÓN DE HUTMANNIN-1, UNA NUEVA METALOPROTEASA PIII DEL VENENO DE LA SERPIENTE NEOTROPICAL MAPANARE DE LANSBERG *(Porthidium lansbergii hutmanni)* CON ACTIVIDADES FIBRINO (GENO) LÍTICAS Y HEMORRÁGICAS

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ABSTRACT

The objective was to characterise hutmannin-1 (hut-1), a new ~ 62 kDa P-III-class metalloprotease from *Porthidium lansbergii hutmanni* (*P.l.h*) (Margarita Island, Venezuela). To characterise this protein, the crude venom of *P.l.h* was fractionated by size exclusion chromatography, anion exchange chromatography and High Performance Liquid Chromatography (HPLC). Hutmannin-1 was identified by MALDI-TOF/TOF mass spectrometry, and the venom was analysed by SDS-PAGE. The lethality, minimum haemorrhagic dose (MHD), effect of temperature on the activity, procoagulant activity on human plasma, and anticoagulant, defibrinating, gelatinolytic fibrinolytic, and fibrinogenolytic and platelet aggregation activities of hut-1 were determined. Antigenic recognition assays were performed on *P.l.h* crude venom and hut-1 by a venezuelan polyvalent anti-ophidic serum (PAOS) Hut-1 had strong fibrinogenolytic and moderate fibrinolytic activity. These activities and the haemorrhagic activity of hut-1 were completely inhibited by EDTA. P.l.h crude venom had potent anticoagulant activity on recalcified plasma and inhibited the platelet aggregation induced by thrombin, ADP, collagen and ristocetin. In contrast, the anticoagulant, coagulant and platelet aggregation inhibition of hut-1 were not observed with any of the agonists. This result suggests that other proteins in the crude venom, markedly impact platelet functions and/or coagulation factors. Commercial venezuelan antivenin showed limited ability to neutralise the haemorrhagic activity of hut-1.

Key words: Anticoagulant; antivenin; coagulation; haemostasis; haemorrhage; snake venom

RESUMEN

El objetivo de este trabajo fue caracterizar hutmannin-1 (hut-1), una nueva metaloproteasa de clase P-III de ~ 62 kDa de la serpiente *Porthidium lansbergii hutmanni* (*P.l.h*) (Isla Margarita, Venezuela). Para caracterizar esta proteína, el veneno crudo de *P.l.h* se fraccionó mediante cromatografía de exclusión molecular, cromatografía de intercambio aniónico y cromatografía líquida del alto rendimiento (HPLC). Hutmannin-1 se identificó por espectrometría de masas MALDI-TOF / TOF, y el veneno se analizó por SDS-PAGE. Se determinó la letalidad, la dosis hemorrágica mínima (MHD), el efecto de la temperatura sobre la actividad, la actividad procoagulante en el plasma humano y las actividades anticoagulantes, desfibrinantes, fibrinolíticos gelatinolíticos y fibrinogenolíticos y de agregación plaquetaria de hut-1. Se realizaron ensayos de reconocimiento antigénico en *P.l.h* veneno crudo y hut-1 mediante suero anti-ofídico polivalente (PAOS) venezolano. Hut-1 tuvo una fuerte actividad fibrinolítica y fibrinolítica moderada. Estas actividades y la actividad hemorrágica de hut-1 fueron completamente inhibidas por ethylenediaminetetraacetic acid (EDTA). El veneno crudo de *P.l.h* tuvo una potente actividad anticoagulante en plasma recalcificado e inhibió la agregación plaquetaria inducida por trombina, ADP, colágeno y ristocetina. En contraste, la inhibición de la agregación plaquetaria, actividad anticoagulante y coagulante de hut-1 no se observó con ninguno de los agonistas. Este resultado sugiere que otras proteínas en el veneno crudo, afectan notablemente las funciones plaquetarias y / o los factores de coagulación. El antiveneno comercial venezolano mostró una capacidad limitada para neutralizar la actividad hemorrágica de la hut-1.

Palabras clave: Anticoagulante; antitoxina; coagulación; hemostasia; hemorragia; veneno de serpiente

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INTRODUCTION

Snake bite is a significant work-related and countryside menace in the tropical and sub-tropical Countries. Precise statistics of the occurrence of snakebite and its morbidity and mortality throughout different geographical areas does not exist; nevertheless, it is sure that it is higher than what is officially reported. Clinical and toxinologically, description of snake envenomations are considered into haemotoxic, neurotoxic, and myotoxic pathological conditions.

Haemorrhagic signs are the characteristic symptoms associated with Viperidae snake bites. This activity has been attributed to haemorrhagic enzymes, usually metalloproteases. Several studies have investigated the haemostatic effects of Viperidae snake venoms and their isolated protein components [10, 16, 37, 40, 41]. Proteolytic enzymes and myotoxins are the central components in the venom of members of the Viperidae family, among which snake venom metalloproteases (SVMPs) induce symptoms such as haemorrhages [36, 45]. These proteases interact with different targets to regulate haemostasis or with important tissues associated with vital physiological functions in prey and predators, causing the most palpable effect: haemorrhages [3]. The different actions of these proteases involve different targets, such as the activation of coagulation factors [43], fibrinogen [14], and the endothelial extracellular matrix of capillary vessels [21].

The taxonomic classification of species in the *Porthidium* genus has been controversial over the past several years. Species of this genus were previously included in the literature as members of the *Bothrops* genus [29] however, based on taxonomic criteria and molecular studies, phylogenetic relationships have been established among Viperidae family members in the *Porthidium*, *Atropoides* and *Cerrophidion* genera in the so-called “Central American Lineage», which is widely distributed in Central America [2, 6, 23]. In this lineage, the *Porthidium* genus is the only genus found in Venezuela [4, 11, 44]; this lineage forms a paraphyletic group of «South American Lineage» genera and comprises *Bothrocophias, Bothrops* and *Bothriopsis* [4, 11, 31].

The main objective of this work was the purification and the biological-biochemical characterisation of a haemorrhagic component found in the venom of the snake *Porthidium lansbergii hutmanni* (*P.l.h*).

MATERIALS AND METHODS

Reagents

The next materials were used for electrophoresis: reagents (Bio-Rad, USA) and immobilized pH gradient (IPG) strips, pH 3-10, 11 centimetres (cm) (Bio-Rad, USA). The following materials were used for haemostasis: human fibrinogen (Sigma, MO, USA) and bovine thrombin (Sigma, MO, USA). These materials were used for immunoblotting: equine peroxidase-coupled-equine IgG antibody (Santa Cruz Biotechnology, CA, USA); nitrocellulose membrane (Bio-Rad, USA); and SuperSignal West Pico® chemiluminescence development kit (ThermoScientific, USA). These materials were used for MALDI-TOF/TOF: α-cyano-4-hydroxycinnamic acid matrix (α-CHCA) (Sigma, MO, USA); ACN; trifluoroacetic acid (TFA); and diethyl ether (Sigma, MO, USA). The next materials were used for LC-MS/MS: OFFGEL RoomTemp HighRes® Kit (Agilent Technologies, USA); IPG strips, pH 3-10, 24 cm strips (GE Healthcare, USA); swine trypsin (PROMEGA); electro spray calibrant solution 63606; and Calibration Tune Mix ESI (Sigma-Fluka, USA). Working solutions were composed of reagents of high purity (≥98%, Merck and Riedel de Haen, Germany). Polyvalent antiophidic serum (PAOS) was obtained from Biotecfar C.A., Caracas, Venezuela.

Software

Prism® (GraphPad, Software) [51] was used for statistical analyses. For one dimension gels analysis and two-dimensional gels electrophoresis analysis QuantityOne® (Bio-Rad, USA)(54) and PDQuest® (Bio-Rad) [48], respectively, were used. For the MALDI/TOF experiment, the Compass 1.2 SR1 for Flex Analysis (BrukerDaltonics)[30} software was employed.

The liquid chromatography (LC)-MS/MS analysis was done with the Compass 1.2 SR1 program for Microtof/Maxis® (Bruker-Daltonics).

Experimental animals

The animals were purchased from the National Institute of Hygiene “Rafael Rangel” (Caracas, Venezuela) animal facility. The mice were kept in cages at room temperature with 12 hours (h) of natural light and *ad libitum* water and food until experimentation. Male mice (*Mus musculus*) of the National Institute of Health (NIH) strain weighing 20 to 22 grams (g) and 25 to 27 g were used to determine lethality and haemorrhagic activity, respectively.

Venom

The pooled *P.l.h* venom was obtained by manually milking 11 adult specimens of both sexes that were captured at 3 metres above sea level (m.a.s.l) in the flat regions of Margarita Island, Nueva Esparta State (Venezuela),The animals were captured in Macanao peninsula, geographically located at Longitude: “O64°16’59.99” and Latitude: “N11°1’0.01”). The area these specimens originate from has a climate favourable to xerophytes; the climate is influenced by northeast trade winds (“vientos aliseos”), the tropical floor as an average annual temperature of 28°C and an annual rainfall less than 800 millimetres (mm), and the vegetation is very similar to the Venezuelan coastal inland vegetation. The majority of this area comprises flat terrain, and common vegetation is the arborescent cacti group known as “Cardonal”, which is mainly characterised by columnar cacti (“cardones”) and spiny Mimosaceae with a squat appearance (“cujíes”). The prevailing vegetation near

the seashore is these “Cardonales”; however, spine bushes or “Espinares” (“cujíes”) are common inland [38].

On the other hand, the *Bothrops colombiensis* venom originated from the pooled venom of 12 adult specimens of both sexes, which were captured in different Venezuelan regions. All snakes were maintained in captivity in the Serpentarium of the Pharmacy School Faculty, Universidad Central de Venezuela (Caracas, Venezuela). Once obtained, the venom was crystallized under vacuum in a desiccator (Pyrex ®, 2.4L Small Knob Top Desiccator Corning, USA) containing $CaCl_2$ as a desiccant and maintained at 4°C (Frigidaire FGVU21F8QF Vertical Freezer, USA) until use.

Fractionation of *P.I.h* crude venom by gel filtration

The fractionation of the *P.I.h* crude venom was initiated with a Sephadex® G-100 molecular sieve chromatography column (90 x 2.5 centimetres (cm) following the method by Grillo and Scannone [18]. Venom samples were dissolved in 5 millilitres (mL) of mobile phase, composed of 0.2 Molar (M) ammonium acetate buffer at pH 6.8 (four runs were performed, for a total of 1000 milligrams (mg of venom). Protein elution was achieved by mobile phase at a flow rate of 7 mL/h. The eluates were monitored at 280 nanometres (nm). Fractions were lyophilised, weighed, and stored at -20°C (Frigidaire FGVU21F8QF Vertical Freezer, USA) until used to evaluate haemorrhagic action. To determine which of the fractions (FI to FIV) obtained from *P.I.h* venom by molecular exclusion had the highest haemorrhagic activity, a single dose of 1 microgram (µg) in 0.1 mL of the FI and FII fractions diluted in 0.85% saline solution was inoculated in experimental animals, and four animals were used per group. The haemorrhagic area was established as described for the determination of the Minimum Haemorrhagic Dose (MHD). FI had the highest activity and was selected for further purification with anionic exchange chromatography.

Anion exchange chromatography

The fractionation was carried out in an automated work station (Bio-rad, USA) by means of an anion exchange column [1] in several stages. Briefly, FI was dissolved in a 20 millimolar (mM) acetic acid/sodium acetate buffer at pH 5.4 (“A” solution). Then, a gradient between solution “A” and an elution buffer solution “B” (20 mM acetic acid/sodium acetate, pH 3.0) was established, with the percentage of the “B” solution increasing by 10% at each step. Finally, the proteins that failed to be eluted under these conditions were subjected to a linear gradient of NaCl at a final concentration of 2 M.

Throughout this process, the flow rate of the mobile phase was 1 mL/minute (min), and protein detection was carried out at 280 nm. A total of 20 runs (100 mg) were performed to obtain a batch of each fraction. Each individual batch was concentrated and desalted by centrifugation at 2,500 x G), using concentrator tubes, with a 3 kilodaltons (kDa) cut-off, until 90% of the volume was reduced. Then, the samples were resuspended in deionised water. This process was repeated three times for each fraction until an aqueous solution with a neutral pH was obtained at an appropriate protein concentration. The fractions obtained at the end of this process were stored at -20 °C (Frigidaire FGVU21F8QF Vertical Freezer, USA) until use.

Selection of the anion exchange fraction of interest

A single dose of 1 µg of protein from each ion exchange fraction was intradermically injected into experimental animals, and four animals were used per group. The fraction with the highest haemorrhagic activity, protein content, and purity as evidenced by one-dimensional electrophoresis was selected for toxicological characterization and proteomic identification.

Protein determination

The protein content in the fractions obtained by anion exchange chromatography was determined [32] using bovine serum albumin as a standard for the calibration curve.

High-performance liquid chromatography of hutmannin-1

The purified fraction (100 µg) was dissolved in 200 µL of 1% TFA in deionised water and subjected to a reverse phase C-18 column on an High Performance Liquid Chromatography (HPLC) (Waters Alliance,) instrument.

A linear gradient from 0 to 100% acetonitrile (ACN) in 0.1% TFA was established over one h at a flow rate of 1 mL/min. The eluates were detected at 280 nm. The appearance of a single, acute and symmetric peak was considered the purity criterion of the component named hut-1.

Identification of hutmannin-1 with MALDI-TOF/TOF mass spectrometry

The identification of hut-1 was carried out at the Toxicology Laboratory, Department of Physiology and Pharmacodynamics, Oswaldo Cruz Institute, Rio de Janeiro, Brazil. For this method, band fragments from SDS-PAGE were treated with 65 mM DL-Dithiothreitol (DTT) (Sigma-Aldrich, USA) for 30 min at 56°C to reduce the protein disulphide bonds, and then the samples were subjected to alkylation with 100 µL of 200 mM iodoacetamide for 30 min. Later, the gel fragments were decolourised with 50% ACN in 25 mM ammonium bicarbonate at pH 8.0, dehydrated with 200 µL of ACN and trypsinised with 15 µL of a (20 ng/µL) trypsin solution, prepared in 40 mM ammonium bicarbonate. The obtained peptides were analysed by MALDI-TOF/TOF mass spectrometry [14, 39]. The mass spectrum was obtained, and *de novo* sequencing of the analysed peptides was carried out. The obtained sequence was compared with the protein sequences deposited in the NCBI (National Center for Biotechnology Information, USA) with the BLAST program.

Assessment of FI and hutmannin-1 haemorrhagic potency

at each purification step

The performance of FI and hut-1 was defined as the per-cent increase in haemorrhagic potency at each purification step.

SDS-PAGE analysis of venom

SDS-PAGE (12% gel) was carried out [28]. Briefly, samples were dissolved at a concentration of 5 µg/µL in a protease inhibitor cocktail composed of 4-(2-aminoethyl)-benzene-sulphonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin and disodium

EthylendiamineTetraacetic acid (EDTA), and then the samples were diluted to the optimum concentration for visualisation in 0.5 M Tris-HCl buffer at pH 6.8, 10% SDS, 1% glycerol and 0.02% bromophenol blue. For hut-1, a sample under reduced conditions was also prepared. Once loaded in their respective gel wells, the samples were run at 100 V for approximately 120 min. Afterward, the appropriate gels were selected for Blue Silver staining [5], which has a sensitivity of 1 ng per band. Next, the gels were washed with deionised water to remove excess dye and digitised. Each experiment was performed in triplicate.

Hutmannin-1 lethality

Venom lethality (deaths and signs of toxicity) was determined in mice intravenously injected with 50 µg or 25 µg of hut-1 samples, corresponding to doses of 2.5 mg/kg and 1.25 mg/kg, respectively. These doses were selected on basis of the lethality of the crude venom and FI. The animals were autopsied, and the macroscopic observations of the haemorrhages were performed.

Determination of the minimum haemorrhagic dose (MHD)

To determine the MHD of the *P.l.h* hut-1, a modified method [27] was used. Serial doses of hut-1 in the range of 0.0088 µg to 0.044 µg were intradermally injected into the depilated backs of mice (*Mus musculus*). The mice were sacrificed, and the skin was removed after 2 h. The diameter of the haemorrhage on the skin was measured, and the MHD was defined as the amount of venom protein required to induce a 10 mm haemorrhage.

With the experimental data, a dose response graph was constructed. Linear regression analysis was performed to estimate the MHD from the equation line with Prism® (GraphPad). This procedure was repeated in triplicate, and the mean and standard deviation of the MHD were calculated.

Effect of temperature on hutmannin-1 activity

Hutmanin-1 was prepared in 0.1 mL of 0.85% saline solution such that the amount of protein corresponded to 10x MHD. From this solution, 2 mL aliquots were incubated for 30 min at different temperatures: 40, 50, 60, 70, 80, and 90°C. After the incubation period, 0.1 mL of each sample was injected into groups of four mice. The diameter of the produced haemorrhagic area was calculated as indicated for the determination of the MHD. The per-cent haemorrhagic activity was calculated relative to the activity of the control, which was a sample incubated for 30 min at 30°C.

Determination of procoagulant activity on human plasma

The ability of *P.l.h* crude venom and hut-1 to induce blood coagulation was determined through the physical observation of clot formation [47] Briefly, different venom or fraction dilutions were prepared in a coagulation solution composed of 0.02 M phosphate-saline buffered solution (PBS) at pH 7.4. Aliquots of 50 µL of crude venom and hut-1 dilutions were added to 200 µL of citrated human plasma from healthy laboratory donors. In the case of crude venom, concentrations ranging from 0.1 µg to 100 µg per 50 µL were used. For hut-1, the concentration ranged from 5 to 40 µg. The coagulation time was recorded, and the samples that induced plasma coagulation in less than 30 min were considered procoagulants.

Four replicates were carried out for each dilution. Additionally, four tubes of the coagulation control solution without *P.l.h* crude venom were prepared. A sample 5 µg of *B. colombiensis* venom and 200 µL of plasma as a positive control was also carried out, in which the coagulation time should not exceed 60 seconds (sec).

Anticoagulant activity determination

In addition to the previous experiment, whether the *P.l.h* crude venom or hut-1 inhibited or promoted plasma coagulation when recalcified was determined [13]. The procedure consisted of making dilutions of venom or hut-1 preparation in coagulation solution to contain the required dose in 50 µL of solution. With crude venom, doses ranging from 0.1 µg to 100 µg were used. For hut-1 doses from 5 µg to 40 µg, 50 µL of each dilution was added to 200 µL of citrated plasma and incubated at 37°C for 10 min. During this interval, it was observed if plasma coagulation occurred. If not, 100 µL of 1 M $CaCl_2$ was supplemented to the tube and again incubated, recording the coagulation time for another 30 min. Four replicates were made for each trial. The experimental control consisted of 50 µL of 0.85% saline solution and incubated with plasma in the absence of venom or hut-1.

Determination of defibrinating activity

The ability of venom or fractions to degrade fibrinogen *in vivo* was assessed [13]. Briefly, groups of five (20-22 g) mice were intravenously injected with different dilutions of *P.l.h* crude venom (7.5 µg to 120 µg) or a single dose of 25 µg of hut-1, prepared in 0.2 mL 0.85% saline solution. One hour after the injection, blood was drawn from the axillary plexus of each experimental animal under anaesthesia. Samples were stored in glass tubes for two h at room temperature, and clot formation was observed. The minimal defibrinating dose (MDD) was defined as the minimum amount of venom that induced incoagulability in all inoculated mice. In addition, a control group was inoculated with 0.85% saline solution and developed a firm clot one h after collection.

Determination of the fibrinogenolytic activity of *P.I.h* crude venom and hutmannin-1

To evaluate the proteolytic activity of *P.I.h* crude venom and hut-1 on the α, β, and γ chains of fibrinogen, we proceeded according to a modified protocol [34]. In brief, a stock solution of purified human fibrinogen was prepared at a concentration of 2 mg/mL in a 0.1 M Tris-HCl buffer solution at pH 7.4. Different concentrations of *P.I.h* crude venom (0.03 µg to 2 µg) and hut-1 (0.5 µg to 32 µg) were incubated at 37°C for 30 min with pre-prepared aliquots of fibrinogen solution (50 µL/100 µg). After incubation, each sample was diluted 1:1 with reducing solution containing 0.5 M Tris at pH 6.8, 10% SDS, 1% glycerol, 0.02% bromophenol blue, and 3% 2-β-mercaptoethanol. Then, the samples were placed in a water bath (Whip Mix, WPM-05350, USA) at 100°C for 5 min. An aliquot of 15 µL of each sample was electrophoresed as indicated. The degradation of different fibrinogen chains was observed. A fibrinogen sample was run under the same conditions in the absence of *P.I.h* crude venom or hut-1.

Determination of fibrinogenolytic activity as a function of time

After establishing the lowest amount of crude venom (0.25 µg) and hut-1 (1 µg) capable of completely degrading the α chain of human fibrinogen under the conditions described above, the appropriate concentrations of venom and hut-1 were incubated with 100 µg of fibrinogen at 37°C for the following incubation periods: 30 seconds, 1 min, 5 min, 15 min, 30 min, 60 min, 3 h and 24 h. Subsequently, to determine the fibrinogenolytic activity, these samples were electrophoresed (SDS-PAGE) as indicated and compared to the electrophoretic pattern of a control sample consisting of the corresponding dose of crude venom or fraction, incubated with 100 µg of fibrinogen and immediately subjected to the reducing action of the reducing solution (time 0).

Effect of protease inhibitors on fibrinogenolytic activity

Constant amounts of *P.I.h* crude venom (1 µg) and hut-1 (2 µg) were incubated at 37°C for 30 min with 100 µg of human fibrinogen in a 0.02 M Tris-HCl buffer solution at pH 7.5. To evaluate the fibrinogenolytic serine protease activity, 2 mM benzamidine was added to the incubation mixture, whereas to evaluate the metalloprotease activity, EDTA was added to the samples. As controls, samples without protease inhibitors were used. After the incubation period, the samples were evaluated by SDS-PAGE as indicated to determine fibrinogenolytic activity.

Effect of the pH on fibrinogenolytic activity

A constant dose of hut-1 (50 µL/3 µg) was prepared in the following buffer solutions at different pH values: citric acid/0.1 MNa2HPO4 (pH 3, pH 4, pH 5 and pH 6) and 0.1 M Tris-HCl (pH 7, pH 8, pH 9 and pH 10). The mixtures were incubated at 37°C for 30 min with (50 µL/100 µg) human fibrinogen, and then SDS-PAGE was performed as indicated to determine fibrinogenolytic activity.

Determination of fibrinolytic activity

The ability of *P.I.h* crude venom and hut-1 to degrade fibrin was determined [34]. Briefly, 1.5 mL of 0.1% fibrinogen solution in imidazole-buffered 0.85% saline solution at pH 7.4 was added to Petri dishes (3 cm). Then, 75 µL of 10 U/mL bovine thrombin containing 0.025 M $CaCl_2$ was added to form a uniform fibrin layer. Afterward, 10 µL (1 µg/µL) of crude venom or 10 µL of (1 µg/µL) hut-1 in 0.85% saline solution was placed in the centre of the fibrin layer and incubated for 24 h at 37°C. After the incubation period, the diameter of the lysis area on the fibrin surface was determined. Fibrinolytic activity was expressed as the diameter (mm^2) of the lysis area per microgram of venom or fraction.

Determination of proteolytic activity on gelatine

The modified methodology proposed by Terra *et al.* [46] was followed for this step. Discontinuous 12.5% polyacrylamide gels copolymerised with 1% gelatine were run. Then, 3 mL of each sample of *P.I.h* crude venom and hut-1 at a concentration of 2 µg/µL in 0.5 M Tris buffer at pH 6.8, 10% SDS, 1% glycerol and 0.02% bromophenol blue, was added to the gel. In addition, as a positive control, 1 µL (2 µg/µL) of *B. colombiensis* venom was added to the gel. After electrophoresis, the gel was equilibrated in a 2.5% Triton X-100 solution, stirring for 1 h at room temperature, washed with double distilled water (two washes of 10 min each) and incubated at 37°C for 18 h in a buffer solution of 20 mM Tris-HCl at pH 7.4, 150 mM NaCl, and 5 mM CaCl2. The proteolytic activity on gelatine was evidenced by the zones of degradation in the gel, which were observed as translucent areas after Coomassie R-250 blue staining. [5].

Determination of the effects of *P.I.h* crude venom and hutmannin-1 on platelet aggregation

The effects of venom and hut-1 on platelet aggregation were assessed by the turbidimetry method [7]. Briefly, blood was obtained from healthy laboratory donors and centrifuged at 190 x G and 20°C for 15 min to obtain platelet-rich plasma (PRP). After counting platelets, an aliquot was subjected to a second centrifugation at 1700 x G for 15 min to obtain the platelet-poor plasma (PPP). The plasma used during the trials consisted of the PRP at a concentration of $1x10^5$ platelets/mL adjusted with the PPP.

Each measurement was obtained in an aggregometer (Crono-Log®560, USA), and aliquot suspensions of 500 µL were placed under agitation at 37°C in a silicon cuvette during the determination. A total of 10 µL of different dilutions of crude venom (0.6 µg to 16 µg), prepared in normal saline solution, were added to each sample. For hut-1, an amount corresponding to five times the IC_{50} of crude venom value was used. After 4 min, the aggregation agonists ADP (10 µM), ristocetin (1.25 mg/mL), collagen (8 µg/

mL) and thrombin (1 U/mL) were added. The aggregation curve was recorded over 8 min for all assays. As a reaction control, the agonists were placed on the platelet suspension without venom and instead adding 10 µL of 0.85% saline solution. In the case of crude venom, a dose response curve was prepared with the obtained results, and IC_{50} of each agonist was determined. The IC_{50} was defined as the amount of venom capable of reducing platelet aggregation by 50% with respect to the control.

Neutralisation and antigenic recognition assays of *P.l.h* crude venom and hutmannin-1 by the polyvalent anti-**ophidic serum**

Neutralisation of haemorrhagic activity

The antivenom used in the neutralisation experiments was PAOS, produced by the Biotechnology Centre of the Faculty of Pharmacy of the Universidad Central de Venezuela (Biotecfar C. A), lot L-162. PAOS consists of $F(ab)_2$ fragments of hyperimmune immunoglobulins obtained from horses. *(Equus ferus caballus)* The species used for the immunisation were *Crotalus durissus cumanensis, Crotalus vegrandis, Crotalus pifanorum, Crotalus ruruima, Bothrops atrox, B. colombiensis, Bothrops venezuelensis,* and *P.l.h*.

The capacity of PAOS neutralising the haemorrhages induced by *P.l.h* crude venom, FI and hut-1 was determined. Briefly, crude venom, F1 or hut-1 was combined with different antivenom dilutions, using as a reference value of the titre declared by the PAOS manufacturer for *Bothrops* genus (1 mL of PAOS must neutralise the activity of 2 mg of *Bothrops* venom).

The neutralising test was prepared to obtain 10 MHD in 0.1 mL of the mixture and different venom/antivenom proportions of crude venom, F1 or hut-1. The mixtures were incubated for 30 min at 37°C and centrifuged at 2,500 x G for 10 min to eliminate the antigen-antibody complexes that formed.

The experimental animals were assigned to groups of five mice each. Each mouse in the groups was injected with 0.1 mL of the appropriate venom/antivenom mixture.

Additionally, there was a venom control group challenged with the crude venom or fraction and a serum control group that received the highest PAOS dose used in the experiments. Two h after the injection, the haemorrhagic lesion as described for the MHD was evaluated. The per-cent reduction in the haemorrhagic lesion diameter induced by each dose was calculated with respect to the control. The ED_{50} was defined as the amount of PAOS capable of reducing the diameter of the haemorrhagic lesion by 50%.

Immunoblotting assays

The PAOS reactivity against the epitopes present in the *P.l.h* crude venom and hut-1 were evaluated using western immunoblotting. In this determination, the selected gel was incubated for 10 min in transfer solution (50 mM Tris-HCl at pH 8.0, containing 380 mM glycine, 0.1% SDS and 20% methanol). Then, the gel was placed in a transfer chamber, allowing the proteins to pass from the polyacrylamide matrix to a nitrocellulose membrane. This process was carried out at 180 milliampers (mA) for 2 h.

After the transfer, the nitrocellulose membrane was blocked for 2 h at room temperature with a 0.2 M PBS solution at pH 7.0, with 5% (w/v) skimmed milk and 0.1% (w/v) Tween 20. Later, three washes were performed for 5 min, each with a solution of 0.05% (w/v) Tween 20 and 0.2 M PBS at pH 7.0. The membrane was incubated again at room temperature for another 90 min, with PAOS diluted to 1:3000 in blocking solution. After the incubation period, the membrane was washed three times with the washing solution for five min each wash. Immediately after the washes, the secondary antibody anti-equine IgG (coupled to horseradish peroxidase) diluted 1:7000 in blocking solution was added. Then, the membrane was incubated at room temperature for another 90 min and washed, as indicated. The electrophoretic bands recognised by PAOS were visualised using a chemiluminescence development kit, and the image was analysed.

Effect of protease inhibitors on the haemorrhagic activity of hutmannin-1

The effect of protease inhibitors on the haemorrhagic activity of hut-1 was tested using EDTA, a metalloprotease activity inhibitor, and benzamidine, a trypsin or trypsin-like inhibitor. In each case, the proteases were preincubated with the corresponding inhibitor at 37°C for 30 min.

Six experimental groups, consisting of four mice each, were used. Each mouse was inoculated with dose of hut-1 that corresponded to 5 times the MHD. The first group received hut-1 preincubated with 2 mM EDTA, the second group was inoculated with hut-1 preincubated with benzamidine, and the third group received a dose of hut-1 preincubated in 0.85% saline solution, representing the haemorrhage control group. The remaining groups were inoculated with the following vehicles: 0.85% saline NaCl, 2mM EDTA and 2 mM benzamidine. Each animal was injected and the haemorrhagic area was determined as indicated to determine MHD. The arithmetic mean was calculated for the results of each group. The per-cent reduction induced by the protease inhibitors was calculated by considering the diameter of the haemorrhagic area for the crude venom control group as 100%.

Statistical analysis

The MHD and neutralisation were analysed by linear regression. The data were expressed as the mean ± standard deviation. To determine differences between experimental groups (three replicates of each condition), one-way ANOVA with Dunnett's post hoc test was used to compare the experimental conditions to the control conditions. Results with an error probability <0.01 were

considered significant. Analysis was completed using SPSS version 2.0 [35]

RESULTS AND DISCUSSION

The majority of snake venoms exert their actions on almost all tissues, and their pharmacological activities are determined by several biologically active fractions [21]. The most significant components of these bioactive fractions are SVMPs, the major components of the venom produced by the *Porthidium* genus [23, 29]. SVMPs are enzymes considered to have the highest haemorrhagic potential among the components of Viperidae snake venoms. These proteins are capable of degrading extracellular matrix proteins such as laminin, nidogen, fibronectin, type IV collagen (constituents of vessel walls) and proteoglycans in the endothelial basement membrane, which promotes the diffusion of venom through the membranes and weakens the capillary structure. Together with the hydrostatic pressure generated inside the blood vessel, SVMPs can produce blood extravasation [9]. Other toxic activities attributed to these enzymes include fibrinogenolytic activity, prothrombin and Factor X activation, apoptosis induction, platelet aggregation inhibition, proinflammatory activity, and inactivation of serine protease blood inhibitors [26, 33]. Most Viperidae snake venoms alter blood coagulation, but there are a few venoms, such as those produced by *Bothriechis lateralis* and *Porthidium nasutum*, that do not alter blood coagulation; however, these venoms do induce haemorrhages due to other protease activities [19] According to the current analysis of *P.l.h* crude venom, only high concentrations of F1 showed procoagulant activity, indicating that the proportion of proteins with procoagulant activity is low in this venom.

Other researchers [25, 37] have reported that mammalian experimental models with disrupted platelet aggregation did not present abnormalities in coagulation tests, despite showing signs of systemic haemorrhaging.

In the size exclusion chromatography, four protein peaks from the *P.l.h* venom were obtained (data not shown). Two predominant fractions were isolated (FI and FII) and used for further purification. These fractions presented the largest areas in the chromatogram. FI (1 µg) produced the largest haemorrhagic area (22.29 ± 2.79 mm) on mouse skin and was selected for the next purification stage using anion exchange chromatography. FII at this dose did not produce any haemorrhagic lesions, and it was discarded.

After passing FI through an anion exchange chromatographic column, seven well-defined peaks were obtained [FIG.1]. A total of 20 runs were carried out, and the fractions (named according to the percentage of buffer "B" composing the mobile phase) were grouped and homogenised as follows: first, a fraction called F0% (tubes 5 and 6), whose protein content did not interact with the negatively charged resin. Second, a series of fractions were bound to the resin with different affinities, according to their isoelectric

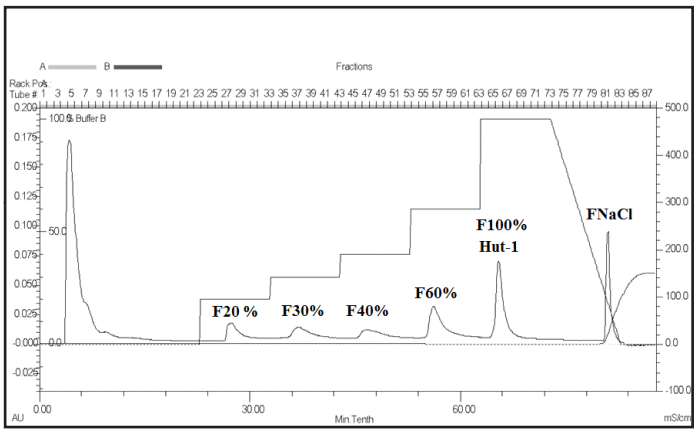

FIGURE 1. PURIFICATION OF HUT-1 BY ANION EXCHANGE CHROMATOGRAPHY. Fraction I obtained from the crude venom of *P. lansbergii hutmanni* was applied to a Q1 column (BioRad, USA). Elution was performed by establishing a pH step gradient with buffer pH 3 in five steps (20, 30, 40, 60 and 100%) during first 73 min of the run (showed on X axis), following with a NaCl 2M gradient, evidenced by an increasing of conductivity value expressed in mS/cm. The fraction with the highest haemorrhagic activity was called hut-1 and was selected to check its homogeneity by means of HPLC.

Mice were intradermally injected with 1 µg of the protein from the anion exchange fractions, and the haemorrhagic lesions that resulted from these injections are shown in TABLE I. The fractions F40%, F60%, F100% and FNaCl had a strong haemorrhagic action. The fraction F100% was selected for further biochemical and toxicological characterisation because it has the highest haemorrhagic activity.

TABLE I
HAEMORRHAGIC LESIONS PRODUCED BY ANION EXCHANGE FRACTIONS

Anion exchange fraction	Haemorrhagic lesion (10 mm diameter) (µg protein/fraction)
F0%	NH
F20%	NH
F30%	NH
F40%	20.7 ± 0.5µg
F60%	20.9 ± 0.6µg
F100%	22.5 ± 0.3µg
FNaCl	17.8 ± 0.8µg

NH: No haemorrhages were observed.

It was possible to purify a 62 kDa protein called hut-1 using a combination of size exclusion chromatography and anion exchange chromatography. Hut-1 showed a single protein peak that eluted

during the acetonitrile (ACN) gradient under high-performance liquid chromatography (HPLC). As shown in FIG.2, this peak was symmetric, and no major peaks corresponding to protein sample contaminants were observed. This protein was composed of a single polypeptide chain, as evidenced by treatment with the reducing agent B-mercaptoethanol. Hut-1 was identified as a member of the SVMP family, with homology to class P-III of the zinc-dependent metalloprotease domain family based on the molecular mass determined by tandem mass spectrometry (MS/MS). The toxic action of hut-1 demonstrated that, similar to other SVMPs [45], its main toxicological targets were haemostasis components.

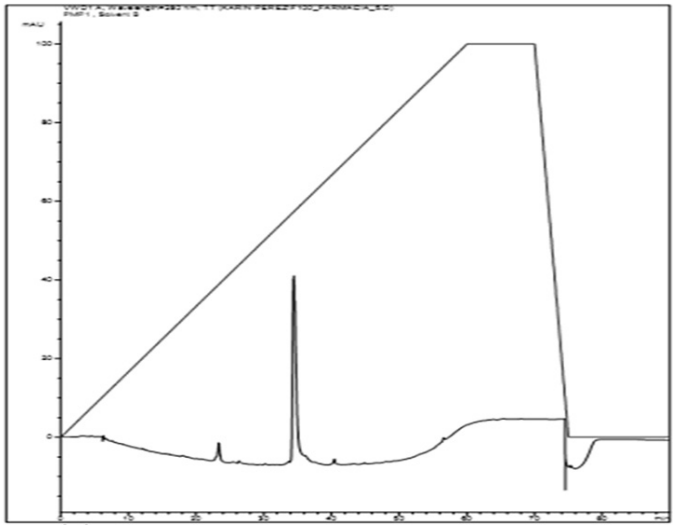

FIGURE 2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF HUT-1. Hutmannin-1 (100 µg) was dissolved in 0.1% trifluoroacetic acid (TFA) and run in a C-18 reverse phase column (HPLC) with a linear gradient from 0 to 100% acetonitrile (ACN), in one h at a flow rate of 1mL/min.

Exploring the lethality of *P.l.h* FI and hutmannin-1 was obtained that the LD_{50} of FI (1.45 ± 0.16 mg/kg) was lower than that of the crude venom of *P.l.h* (2.51 ± 0.16 mg/kg), showing that this fraction possessed the majority of the toxic components in the venom. Hut-1 showed high toxicity. It was not feasible to determine the LD_{50} of hut-1 because it was not possible to obtain enough sample for this test; however, when doses as low as 2.63 mg/kg and 1.32 mg/kg were tested, all the animals died within 24 h after the injection, which indicated that the hut-1 LD_{50} was below 1.32 mg/kg. Before the animals injected with both doses of hut-1 died, they all presented motor incoordination, flaccid paralysis in the anterior and posterior limbs, cyanosis, respiratory insufficiency, tachycardia and haematemesis. Autopsies of mice revealed atrial thrombosis and massive pulmonary and hepatic haemorrhages, indicating the high toxicity of hut-1 (data not shown).

The acute toxicity of hut-1 was found to be more potent than those of FI and crude venom. This toxicity is notable when comparing hut-1 with a PI class metalloprotease (Porthidin-1), previously isolated from *P.l.h* venom [15], which was found to be non-lethal in experimental mice intravenously injected with a dose of 6 mg/kg.

The results obtained in each purification step are shown in TABLE II. Here, the performance and efficiency of the purification process of hutmannin-1 (hut-1) were determined, and the minimum haemorrhagic dose (MHD, µg) was obtained at each purification step.

The MHD of hut-1 was 83 times higher than that of crude venom, showing that hut-1 is one of the most powerful haemorrhagic toxins described to date; this conclusion becomes evident when hut-1 is compared with similar toxins from the venoms of various Viperidae snakes [49]. Moreover, the autopsies of mice treated with hut-1 showed extensive systemic haemorrhages. The macroscopic study of the lung tissue of treated mice revealed profuse haemorrhages in the lung, and these lesions rapidly appeared 15 min after injection. Observations of the structural changes that occur following exposure to *P.l.h* crude venom [49] revealed the appearance of erythrocytes and glomerular kidney oedema, as well as the detachment from the basement membrane and plasma membrane rupture of endothelial cell. This result could demonstrate the possible systemic haemorrhagic activity of hut-1, the haemorrhagic fraction from this venom. Previous studies that investigated other metalloproteases from *Bothrops* snakes, such as jararagin, have shown that similar pulmonary haemorrhages occur in mice [8, 36]. The MHD value is shown in TABLE II. At nanogram (0.0088 to 0.044 µg) doses, hut-1 presented a high haemorrhagic capacity, resulting in bleeding skin lesions in the experimental animals (data not shown).

TABLE II

PERFORMANCE AND EFFICIENCY OF THE PURIFICATION PROCESS OF HUT-1. THE MHD ACTIVITY (µG) RESULTS OBTAINED AT EACH PURIFICATION STEP

Sample	Amount obtained	MHD activity (µg)	Purification factor
Crude venom	1000 mg	1.475	1
Fraction I	697.2 mg	0.102	14.5
Hutmannin-1	39 mg	0.021	69.9

Hut-1 maintained its haemorrhagic activity when incubated for 30 min at 40°C, but haemorrhagic activity of hut-1 decreased by approximately 20% after incubation at 50°C for the same period of time. After exposure to temperatures equal to or higher than 60°C, hut-1 completely lost its haemorrhagic capacity [FIG. 3A]. This temperature restriction is similar to the temperature restriction of other viperid metalloproteases, such as uracoin-1 [1], and elapid metalloproteases, such as EpyHTI and EcoHTI, which presented a 50% reduction in haemorrhagic activity at temperatures close to 50°C and completely lost activity at 70°C [49].

The haemorrhagic activity of hut-1 was completely abolished by preincubation with 2 mM EDTA, a metal chelator, whereas preincubation with 2 mM benzamidine, a trypsin or trypsin-like inhibitor, did not induce significant differences from untreated hut-1 (n=4, FIG. 3B), demonstrating the dependence of the activity of hut-1 on divalent ions, as previously reported for a large variety of metalloproteases [7,15,16,19, 36, 46].

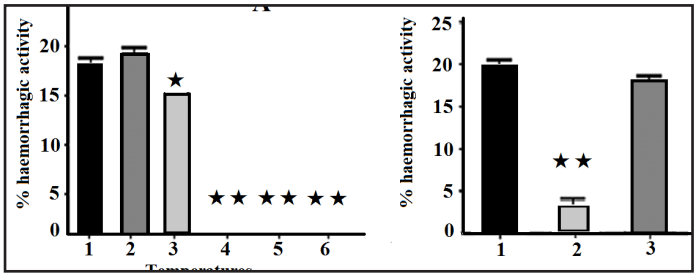

FIGURE 3. EFFECT OF TEMPERATURE AND PROTEASE INHIBITORS ON THE HAEMORRHAGIC ACTIVITY OF HUT-1. (A) 1: Control; 2: 40ºC; 3: 50ºC; 4: 60ºC; 5: 70ºC; 6: 80ºC. The bars represent the standard error. Dunett test one-way ANOVA analysis = * α <0.05 ** α <0.01. **(B)** 1) control; 2) Hut-1 + 2 mM EDTA; 3) Hut-1 + 2mM benzamidine. The bars represent the standard error. Dunett test one-way ANOVA analysis = ** α <0.01

The haemorrhagic potential of high class P-III metalloproteases has been attributed to two main facts: (1) the inability of α2-macroglobulin to inhibit these toxins and (2) the presence of domains with disintegrin-like activity that are rich in cysteine residues and specifically degrade extracellular matrix components, especially type IV collagen [22].

The identification hutmannin-1 by tandem mass spectrometry showed that the fragmentations with matrix assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF) resulted in two main signals: m/z 3182.95 and 2197.84. The defragmentation of these peptides and their subsequent *de novo* sequencing led to the identification of two peptide sequences, NLLVAVTMAHELGHNL (m/z: 3182.95) and VECETGECC (m/z: 2197.84), which are sequences of zinc-metalloprotease domains found in SVMPs.

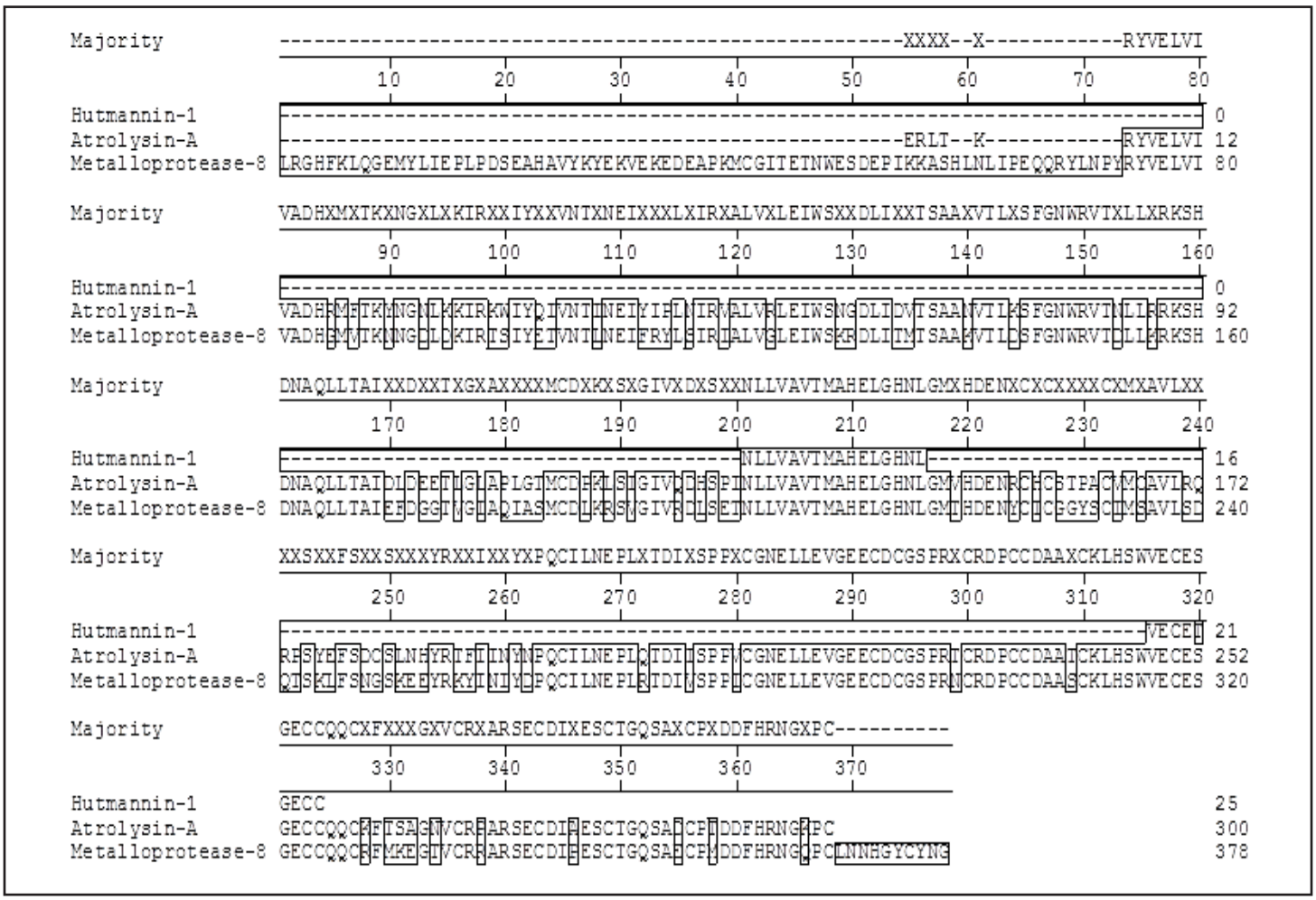

FIGURE 4. NCBI/BLAST search comparison of arrangements *de novo* peptide NLLVAVTMAHELGHNL and VECETGECC sequenced from MS/MS spectrometry, of Hutmannin-1 to partial amino acid sequences of two snake venom metalloproteases, atrolysin-A 17 and metalloprotease-8 [18]. The sequence similarities are shown in the figure

After analysing these sequences with the BLASTp program, hut-1 was identified as an SVMP, showing similarity with adamalisin-1 (*Crotalus adamanteus*), atrolysin-A (*Crotalus atrox*) (E-value 9e-07) [23], metalloprotease-8 (*C. adamanteus*) (E-value 5e-07) [41], and an additional 43 SVMPs [24], all with E-value estimates that are considered significant (≤1e -04). The similarity of the sequences obtained with the sequences of these proteins is shown (FIG. 4).

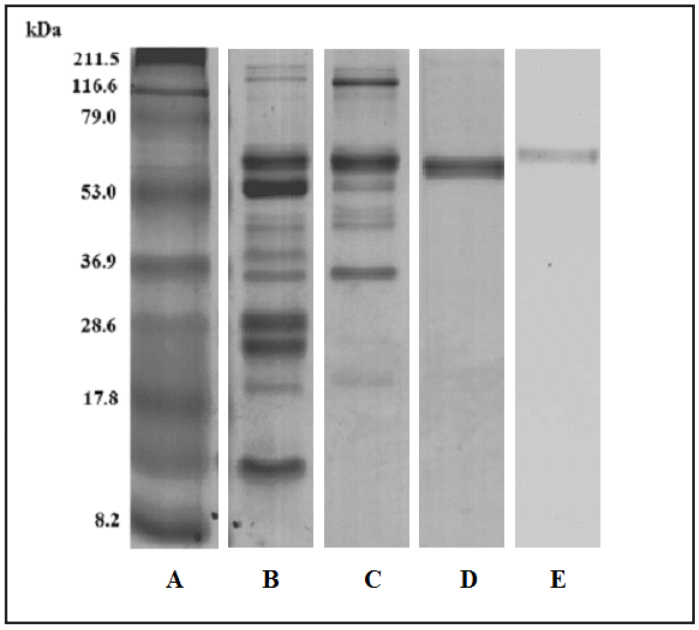

FIGURE 5. *Porthidium l. hutmanni* SDS-PAGE. A) molecular weight markers; B) crude venom; C) FI; D) hutmannin-1 under native conditions; E) hutmannin-1 under reduction conditions. All samples were run with 25µg of protein.

After hut-1 was reacted with the reducing agent 2-β-mercaptoethanol and subsequently underwent electrophoresis, a single band with a slightly higher molecular weight than the unreduced protein (~ 69 kDa) was observed. This result suggested that hut-1 is composed of a single polypeptide chain.

In the procoagulant activity of FI and hutmannin-1 was demonstrated that FI of the *P.l.h* venom dose-dependently induced the coagulation of human plasma, as measured by physical clot formation; all the tested FI doses were markedly more effective than 5 µg of *B. colombiensis* venom, in which the plasma coagulation time was 40 sec (data not shown). Hut-1 did not show plasma procoagulant activity during 30 min of incubation (data not shown). On the other hand, Hut-1 did not show anticoagulant activity on human plasma. Compared to the control, hut-1 did not significantly affect the recalcified plasma coagulation times at the highest doses tested 200 µg (data not shown). The experimental results indicated that mice treated with FI or hut-1 maintained their coagulant capacity 2 h after the intravenous injection of each fraction. This result shows that FI and hut-1 lack defibrinating activity.

In the fibrinogenolytic activity assay, *P.l.hutmanni* crude venom degraded the fibrinogen Aα chain at the concentration of 0.5 µg venom/100 µg fibrinogen (data not shown). Moreover, hut-1 proved to have potent proteolytic action on the fibrinogen α chain at the same hut-1/fibrinogen ratio of 0.5 µg/100 µg. However, it was not easy to determine whether the degradation of the A chain was complete at this ratio; at the 0.5 µg/100 µg ratio, Coomassie blue staining revealed that the hut-1 band was located at the

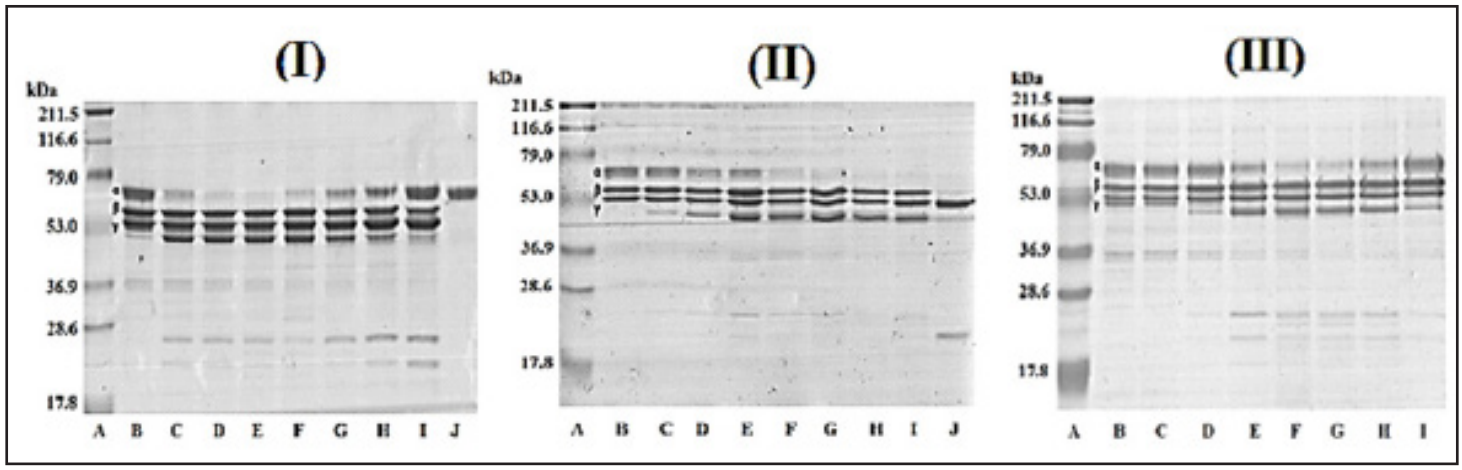

FIGURE 6. (I) FIBRINOGENOLYTIC ACTIVITY OF HUT-1/FIBRINOGEN (µG/µG). A) molecular weights; B) 0 µg/100µg; C) 0.5µg/100µg; D) 1.0µg/100µg; E) 2.0µg/100µg; F) 4.0µg/100µg; G) 8.0µg/100µg; H) 16.0µg/100µg; I) 32.0µg/100µg; J) hut-1 (32µg) in the absence of fibrinogen. **(II) FIBRINOGENOLYTIC HUT-1 EFFECT AS A TIME FUNCTION.** A) molecular weight markers; B) Control (time 0); C) incubation for 30 sec; D) incubation for 1 min; E) incubation for 5 min; F) incubation for 15 min; G) incubation for 30 min; H) incubation for 1 h; I) incubation for 3 h; J) incubation for 24 h. **(III) EFFECT OF PH FOR THE ACTIVITY OF HUT-1 ON FIBRINOGEN AT 37°C INCUBATED FOR 30 MIN.** A) molecular weight markers; B) pH 3; C) pH 4; D) pH 5; E) pH 6; F) pH 7; G) pH8; H) pH 9; I) pH 10

same position as the Aα chain gel due to the similar molecular weights of these proteins. The degradation of the B or y chain was not observed at any of the tested doses [FIG. 6I]. The effect of hut-1 on the fibrinogen α chain was incubation time-dependent. After incubating 1 µg of hut-1 with 100 µg of fibrinogen for different time intervals, the degradation of the fibrinogen α chain was observed. After 30 min of incubation, the fibrinogen Aα chain began to degrade, and it was undetectable after 3 h of incubation. After 24 h, the Bβ chain was completely degraded [FIG. 6II].

Hut-1 lacks the procoagulant activity of other P-III class metalloproteases, such as the Factor V activator RVV-X and the prothrombin activators ecarin and carinactivase-1 [26], and the defibrinating activity associated with the exacerbated activation of some *Porthidium* venom coagulation factors, such as Porthidin-1 [14]. Additionally, the fraction from which hut-1 was isolated FI showed a procoagulant effect at high concentrations, suggesting the presence of other low potency procoagulant toxins. The effect of hut-1 on human fibrinogen was similar to that of crude venom on human fibrinogen, indicating the strong degradation power of hut-1 on the α subunit of fibrinogen. In addition, the β chain was completely degraded after 24 h of incubation. The optimum pH of this activity was between 7 and 9, similar to that of colombienases 1 and 2, as previously reported [17] [FIG.6III].

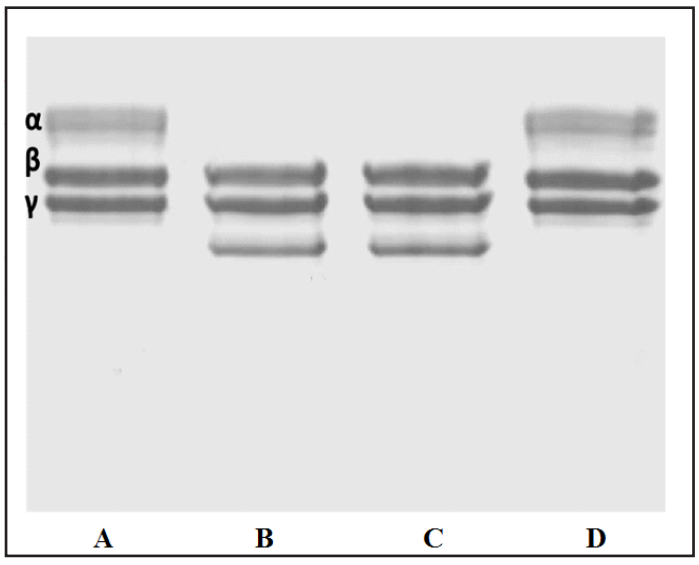

FIGURE 7. EFFECT OF PROTEASE INHIBITORS ON THE FIBRINOGENOLYTIC ACTIVITY OF HUT-1. A) Fibrinogen (Fb) (100µg); B) Fb + hut-1 (100 µg+ 32 µg) ; C) Fb + hut-1 (100 µg+ 32 µg) + 2mM benzamidine ; D) Fb + hut- 1 (100 µg+ 32 µg) + EDTA 2mM.

Alternatively, the degradation of fibrin mesh induced by hut-1 proved to be less effective than that induced by the zymogen. The lysis area obtained in the fibrin plates was 3.5 times

smaller than that obtained with crude venom, wh

ich suggests that toxins other than hut-1 were involved in the fibrinolytic activity of *P.l.h* venom [FIG.8]. Hut-1 showed more activity against the fibrinogen molecule than against the polymerised fibrin mesh.

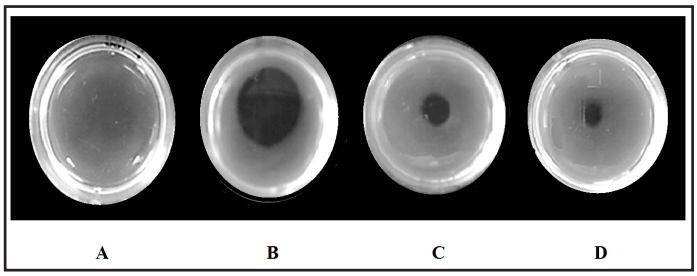

FIGURE 8. FIBRINOLYTIC ACTIVITY OF HUT-1. A) Negative control PBS; B) Positive control *B. colombiensis* crude venom; C) *P.l.hutmanni* crude venom; D) Hut-1.

No proteolytic activity of hut-1 on the hydrolysed collagen that forms gelatine was observed [FIG.9].

Similarly, several authors have correlated gelatinolytic activity with the haemorrhagic action of SVMPs [8,39,41,42]; however, some P-III metalloproteases, such as alsophinase [50] and VLH2 [20], have high fibrinogenolytic and haemorrhagic activity but have not been shown to have gelatinolytic activity [12] [FIG.9]. In the case of hut-1, it was necessary to evaluate this activity by using different forms of colparticularly type IV collagen, as collagen is one of the main toxicological targets for these enzymes. However, when the hut-1 precursor FI was tested, two lysis areas corresponding to ~ 37 and 27 kDa were clearly evident, but no activity was observed in the region corresponding to the molecular weight of hut-1.

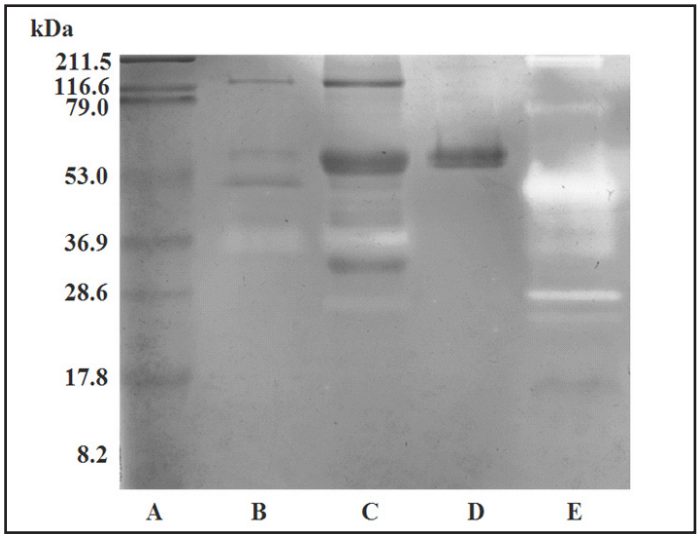

FIGURE 9. GELATINOLYTIC ACTIVITY OF FRACTIONS OF *P.L.H* . A) molecular weight markers; B) 6 µg of *P.l.h* crude venom; C) 6 µg of *P.l.h* FI; D) 6 µg of hut-1; E) 2 µg of *B. colombiensis* crude venom.

In the platelet aggregation assay, hut-1 did not inhibit platelet aggregation in response to the tested agonists (collagen, ADP, ristocetin). This result contrasts with the potent inhibitory action of *P.l.h* crude venom, suggesting that other proteins in this venom induce platelet aggregation [52]

Compared to the titre, which is the volume of serum necessary to neutralize the concentration of bothropic venom [47], as determined by the manufacturer, PAOS had low efficacy at neutralising the haemorrhages induced by the *P.l.h* crude venom, nearly four times the amount recommended by the manufacturer was required. PAOS was even less effective against hut.1 than the crude venom; a concentration 11 times higher than the recommended by the manufacturer was required to neutralise the activity of hut-1 (1 mL of PAOS per 2 mg of venom).

To correlate these results with the antigenic recognition evaluation by Western blot analysis, the antivenom exhibited limited antigenic recognition of some protein bands, specifically, 54, 45 and 30 kDa [FIG.10]. These molecular weights are within the range reported for PII and PI metalloproteases, such as porthidin-1, a 23 kDa haemorrhagic metalloprotease that was previously

isolated from this venom [14]. These observations could indicate that additional metalloproteases exist in the *P.l.h* venom that are not antigenically recognised by the immunoglobulins present in the antivenom and, therefore, are not neutralised. These results markedly contrast the results obtained for hut-1, which was antigenically recognised by PAOS [FIG.10]; however, PAOS did not effectively neutralise the haemorrhagic activity of hut-1, which could be a consequence of the antigen binding at a site that does not corresponding to the activity of hut-1. The ecological area of the specimens whose venom were used corresponds, as detailed in materials and methods, to the Macanao peninsula. It is known that the manufacturer of antivenoms (Biotecfar CA) uses a pool of venoms from specimens that are randomly collected throughout the island, it is also known that venoms have intra-species variability [42] and that geographic variability surely influences the synergistic strategies of predominant toxins components of snake venoms [53].

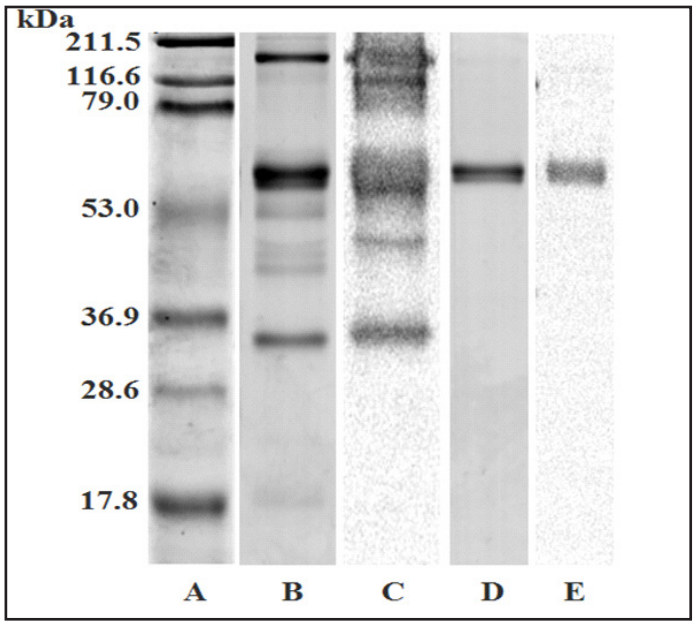

FIGURE 10. WESTERN BLOT PA INTERACTION AGAINST *P.L.H* FI AND HUT-1. A) molecular weight markers; B) SDS-PAGE *P.l.h* FI (10 µg); C) Western blot PA against *P.l.h* FI; D) SDS-PAGE hut-1 (10 µg); E) Western blot PA against hut-1.

CONCLUSIONS

P. lansbergii hutmanni is an epidemiologically important venomous snake species located on Margarita Island (Venezuela). Here, it has been shown that the *P.l.h* venom lacked the marked *in vitro* procoagulant activity characteristic of bothropic venoms, which could have implications in the diagnosis of envenomations considered to be from bothropic snakes. The *P.l.h* crude venom showed very high haemorrhagic and anticoagulant activities, and hut-1, an ~ 62 kDa enzyme classified as a P-III metalloprotease, was identified in this venom. Additionally, hut-1 had a strong fibrinogenolytic and moderate fibrinolytic action and did not exhibit anticoagulant activity. The antivenom PAOS was not able to effectively neutralise the haemorrhagic activity of crude venom, but PAOS did neutralise hut-1; therefore, treatment with this antivenom could have reduced efficacy in the treatment of envenomation by *P.l.h*.

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Ethical statement

Qualified staff arranged all the experimental methods relating to the use of live animals. These methods were approved by the Institute of Anatomy Ethical Committee of the Universidad Central de Venezuela on 7 March 2018 under assurance number 07-03-18 and followed the norms obtained from the Guidelines for the Care and Use of Laboratory Animals, published by the US National Institute of Health (1985). The research questions asked, the technical methods chosen, and the conclusions reached are exclusively responsibility of the authors.

Conflicts of Interest

The authors declare no conflict of interest.

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