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Inhibition of collagen, and thrombin-induced platelet aggregation by Lansberg's hognose pit viper (*Porthidium lansbergii hutmanni*) venom

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Abstract The *Porthidium* genus is represented by the *P. lansbergii rozei* and *P. lansbergii hutmanni* (*Plh*) subspecies in Venezuela. The venom components of these have been little studied, probably due to the low incidence of reported accidents, although acute and serious local effects such as invasive edema and disseminated ecchymosis are present during human envenonation. The aim of this work was to characterize the in vitro effects of crude *P. l. hutmanni* venom, and its fractions, on platelet aggregation triggered by two physiologic agonists: thrombin and collagen. The effects of thrombin and collagen were observed on a platelet-rich plasma (PRP) solution (3×10^5 platelets/μL) using serial dilutions of *P. l. hutmanni* venom (0.625–40 μg). The crude venom was fractionated by anionic exchange chromatography and two peaks obtained. Crude venom and both fractions were highly inhibitory on platelet aggregation mediated by the two agonists. The anti-aggregating dose (AD_{50}) for both agonists was determined. PRP collagen-triggered aggregation was most inhibited by the crude venom (AD_{50} = 0.67 μg) when compared with PRP thrombin-triggered aggregation (AD_{50} = 4.92 μg). Collagen-induced aggregation was more intensely inhibited by venom than thrombin-induced aggregation. In conclusion, to specify the inhibition mechanisms involved for each of the active components in the venom from these subspecies, we must characterize and purify the inhibitors of aggregation from *P. l. hutmanni* venom, with the purpose of suggesting new pharmacological substances to be incorporated into the therapeutic arsenal to treat hemostatic pathologies related to high levels of platelet aggregation.

Keywords Platelet aggregation · *Porthidium lansbergii hutmanni* · Snake venom · Thrombin · Collagen

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Introduction

Snakes are the most prodigious source of toxins that could be useful in human therapeutics. Their venoms are complex mixtures of polypeptides and proteins that induce a variety of pharmacological activities that provide a useful tool for the understanding of many molecular events in normal physiologic processes [1]. It is well known that venoms from the Viperidae (Crotalinae) family cause severe pharmacological effects, such as platelet aggregation dysfunction [2–6].

The *Bothrops* and *Crotalus* genera are responsible for the highest number of severe human snakebites in Venezuela. Accidents produced by the *Lachesis*, *Porthidium*, *Bothriechis*, and *Bothriopsis* genera from the Viperidae family also occur [7]. However, due to their geographical location they are poorly documented.

The *Porthidium* genus is represented by the *P. lansbergii rozei* and *P. lansbergii hutmanni* subspecies in Venezuela [7]. The venom components of these have been little studied, probably due to the low incidence of reported accidents, although acute and serious local effects such as

invasive edema and disseminated ecchymosis are present during envenonation [8–10].

The aim of the present investigation was to describe the effects of crude *Porthidium lansbergii hutmanni* (*Plh*) venom, and its fractions, on platelet aggregation mediated by two of the main physiologic ligands: collagen and thrombin.

Materials and methods

Porthidium lansbergii hutmanni venom

A total of 12 mature females and males *P. l. hutmanni* snakes were captured in Juan Griego and Porlamar, Margarita Island, Venezuela. The snakes were maintained in the serpentarium of the Laboratorio de Investigaciones in the Facultad de Farmacia of the Universidad Central de Venezuela. Venom extractions took place every four - weeks. The extracted venom was pooled and dehydrated in vacuum desiccators, maintained with calcium chloride at 4°C until total crystallization.

Blood samples

Blood samples were collected in plastic tubes with 3.8% sodium citrate (1:10 citrate/blood) from healthy donors who came to the municipal blood bank of Caracas. Patients were chosen according to the blood bank norms and signed a consent form.

Platelet-rich plasma

Platelet-rich plasma (PRP) was obtained by centrifugation (90–120*g* for 15 min) of blood samples using a Sorval RC3 centrifuge. The PRP fractions were carefully collected and pooled in a clean dry silicon-coated tube.

Platelet-poor plasma

The pellet (cellular body) obtained from PRP after centrifugation was recentrifuged at 3,000 rpm for 10 min, and the cell-free plasma was dissolved in cold 9% saline (pH 7.4), collected and identified as platelet-poor plasma (PPP).

PRP quantification and final platelet concentration adjustment

The quantification of the pooled PRP platelets was carried out using a Coulter model T-890. The final PRP concentration was adjusted to 3×10^8 platelets/mL by diluting with previously pooled PPP.

Effects of crude *Porthidium lansbergii hutmanni* venom and its fractions on platelet aggregation

An aggregometer (CHRONOLOG, USA) at 37°C was used for the determination of the effects of snake venom on platelet aggregation mediated by two agonists: thrombin at (5 μL: [0.15 U/560 μL], SIGMA, USA) and collagen (3 μL: [2 μM/563 μL], HORM, USA).

The interpretation of the responses was semiquantitative, using as reference the dose-response graphs obtained without venom samples ($n = 3$). Each test cell contained 440 μL of PRP (3×10^8 cells/ mL), 50 μL at several (1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 μg/μL) CV*Plh* dilutions, suspended in PBS, and a volume of agonist (collagen = 8 μg/553 μL and thrombin = 15 U/565 μL) to a specified final concentration. The control consisted of 50 μL of PBS without venom sample.

Tests were performed in a silicon-coated aggregometer cell as follows. To 440 μL (3×10^8 cells/mL) of PRP, venom sample solution (1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 μg/μL) was added and allowed to stand for 60 s. Immediately after, the selected agonist was added to trigger the reaction. The aggregation curve was measured for 5 min. Changes in platelet aggregation were determined by comparing the different samples of venom against the controls. The graph scale was calibrated by using the control average to represent 100% platelet aggregation.

Partial purification of crude venom by ion exchange chromatography

CV*Plh* was fractionated by ion exchange chromatography using a Biologic (Biorad, USA) work station in an anionic exchange Mono-Q1 (Biorad, USA) column. The mobile phase consisted of a solution of 50 mM Tris–HCl, pH 8.3 with 0.5 M KCl and another without 0.5 M KCl, to create the saline gradient. One milliliter (20 mg/mL) solution of crystallized CV*Plh* dissolved in 50 mM Tris–HCl, pH 8.3 buffer solutions was processed each time. After applying the sample, the first peak was allowed to elute with the non-saline buffer. The potassium chloride gradient was applied up to 0.5 M to complete elution. Both peaks were dialyzed in distilled water for 48 h at 4°C using a 2 kDa cut-off membrane (Sigma, USA). The peaks were lyophilized and stored at 4°C until use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the Laemmli method [11] using a MINI-PROTEAN II (BioRad, USA) chamber. A 12.5% gel under non-reducing conditions was

used. Molecular-weight markers (Bio-Rad) were run in parallel and gels stained with Commassie blue R-250.

Determination of protein concentration

Protein concentrations of tested venoms by the Lowry method [12] were measured using bovine serum albumin as standard protein.

Results

CV*Plh* effect on the platelet aggregation in vitro

CV*Plh* presented a clear (dose-dependent) inhibitory capacity of platelet aggregation on PRP for all tested agonists. Table 1 shows the anti-aggregation dose 50 (AD_{50}) of CV*Plh*, which is defined as the quantity of venom (μg) that was able to reduce by 50% the amplitude of the aggregation measurement compared with the control (100%) during the 5 min after the mediator's addition. Each of the obtained registers, either controls or in the presence of the venom samples, showed particular characteristics depending on the agonist used.

The CV*Plh* AD_{50} value is calculated based on the control measurement (0.0043 μg) and corresponds to the lowest inhibitory dose compared to that obtained with the other agonists. This value is presented as a theoretical performance indicator because in practice it was only possible to reach a minimum of 84% inhibition, with the lowest doses of *Plh*. The measurement of the PRP mediated by 3 μL collagen (2 μM/563 μL) aggregation in the presence or absence of CV*Plh* is shown in Fig. 1. The CV*Plh* showed a clear (dose-dependent) inhibition of platelet aggregation mediated by collagen, which was directly proportional to the dose from 0.625 μg (47.9 ± 27.7%) to total inhibition with 10 μg, in all cases.

The measurement of the PRP mediated by 15 μL thrombin (0.15 U/560 μL) aggregation in the presence or absence of CV*Plh* is shown in Fig. 2. The AD_{50} CV*Plh* value calculated from the baseline control was 4.92 μg.

Ionic interchange chromatography

Figure 3 showed two well-defined *Plh* venom peaks obtained with a Mono-Q1 (Biorad, USA) chromatography column. Peak I eluted before the saline gradient initiated, while peak II eluted during the saline gradient phase.

The total elution volume from each peak was measured, and the total sample recovery was based on the optical density readings at 280 nm (1 absorbance unit is equivalent to 1 mg/mL of total protein). The approximate protein recovery was 45 mg (45%) for peak I and 42 mg (42%) for peak II, with respect to the total amount of CV*Plh* originally processed (approximately 106 mg). Each peak was then immediately dialyzed, lyophilized, and stored at 4°C until its use.

Electrophoretic profiles of CV*Plh* and peaks I and II

Figure 4 showed the electrophoretic elution profiles of CV*Plh* and both peaks obtained by ion interchange chromatography. CV*Plh* showed 14 molecular-weight (MW) bands from 7.6 to 206 kDa. Peak I showed 15 bands, located in the same range of MW as CV*Plh*, without the 37.4 and 7.6 kDa bands seen in CV*Plh*. Peak II showed a smaller number of bands (nine) with molecular weights ranging from 7.6 to 79 kDa. The 37.4 and 7.6 kDa bands absent in peak I were clearly observed in peak II. Additionally, a group of six common bands were observed in both peaks.

Determination of the protein concentration of CV*Plh*, and peaks I and II

CV*Plh* presented 90% protein with respect to the total weight of crystallized venom. In contrast, peaks I and II had 71.3% and 72.0% proteins, respectively.

The effects of peaks I and II on platelet aggregation mediated by thrombin and collagen

Once it was determined that the agonist with greatest sensitivity to the inhibitory activity of CV*Plh* was

Table 1 Anti-aggregation dose 50 (AD_{50}) of crude *P. l. hutmanni* venom and peak I and II fractions

Species	AD_{50}	Mechanism	References
Vipera russelli	–	–	Kini et al. [13]
Echis carinatus	13.8 μg/mL	PLA_2–acid enzymatic	Kemparaju et al. [14]
Heloderma horridum	8 μg/mL	PLA_2–acid	Huang and Chiang [15]
Pseudechis papuanus	–	Vt (PLA_2)	Kamiguti et al. [16]
Crude venom *Plh*	0.0043 μg	ND	–
Peak I/mono-Q1 *Plh* venom	3.2 μg	ND	–
Peak II/mono-Q1 *Plh* venom	0.0130 μg	ND	–

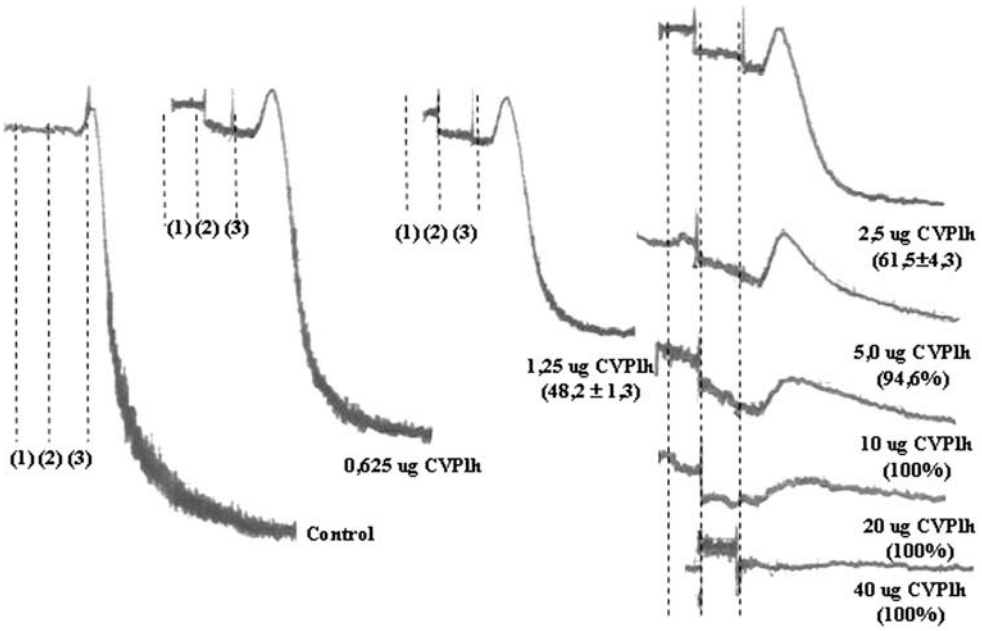

Fig. 1 Curves of platelet aggregation mediated by collagen (8 μg/553 μL) and inhibited by crude *P. l. hutmanni* venom: (1) baseline, (2) venom sample, (3) agonist

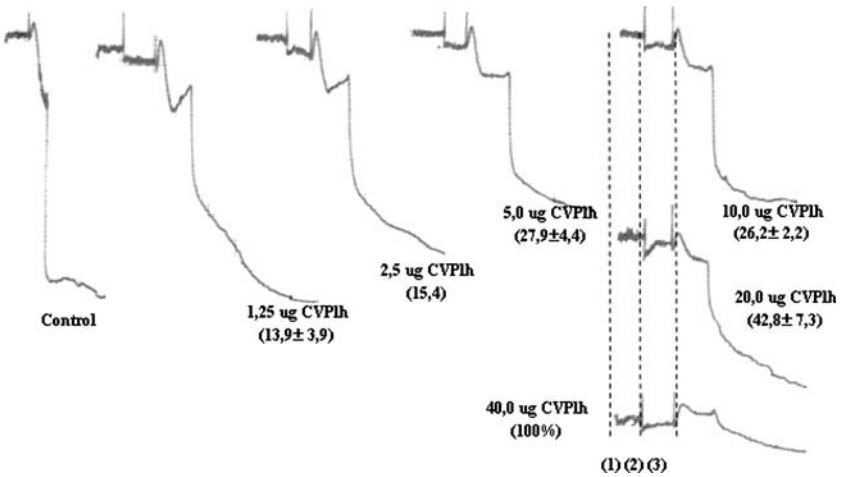

Fig. 2 Curves of platelet aggregation mediated by thrombin (15 U/565 μL) and inhibited by crude *P. l. hutmanni* venom: (1) baseline, (2) venom sample, (3) agonist

collagen, which showed the lowest AD_{50} value, the AD_{50} values from peaks I and II were estimated. Thrombin inhibitory effects were only proven with a single sample of each peak.

Figure 5A shows the effect of peak I/mono-Q1 *Plh* venom on platelet aggregation mediated by the collagen (3 l [2 M]/563 l). The effect of the peak I/mono-Q1 *Plh* venom at a dose of 40 μg was complete inhibition of aggregation, observed as a change in shape, returning the measurements to their baseline level after 5 min of the assay. The dose of 0.625 μg, equivalent to the DA_{50} CV*Plh* in the presence of this mediator, produced a total lack of inhibition, with equivalent measurements to the control curves.

Figure 5B shows measurements of platelet aggregation mediated by collagen in the presence of the pre-incubated PRP with the peak II/mono-Q1 *Plh* venom. The curves do not show differences in the double-wave knee feature, with a continuous slope until the beginning of the stabilization of the measurement. The effect of the peak II/mono-Q1 *Plh* venom at a dose of 40 μg was complete inhibition of aggregation. When adding the aliquot of peak II/mono-Q1 *Plh* venom to the PRP with agitation, changes (measured as an increase in the turbidity reading) in the baseline measurements, which continued during the minute of incubation and then stabilized, were observed. Once the agonists were added, the measurement remained stable until the end of the 5 min assay. In contrast, the aliquots that contained a weight equivalent to the peak II DA_{50} CV*Plh* value did not inhibit aggregation mediated by collagen.

Figure 6A shows the effect on platelet aggregation produced by the peak I/mono-Q1 *Plh* venom and thrombin. The curves for aggregation mediated by thrombin (0.15 U)

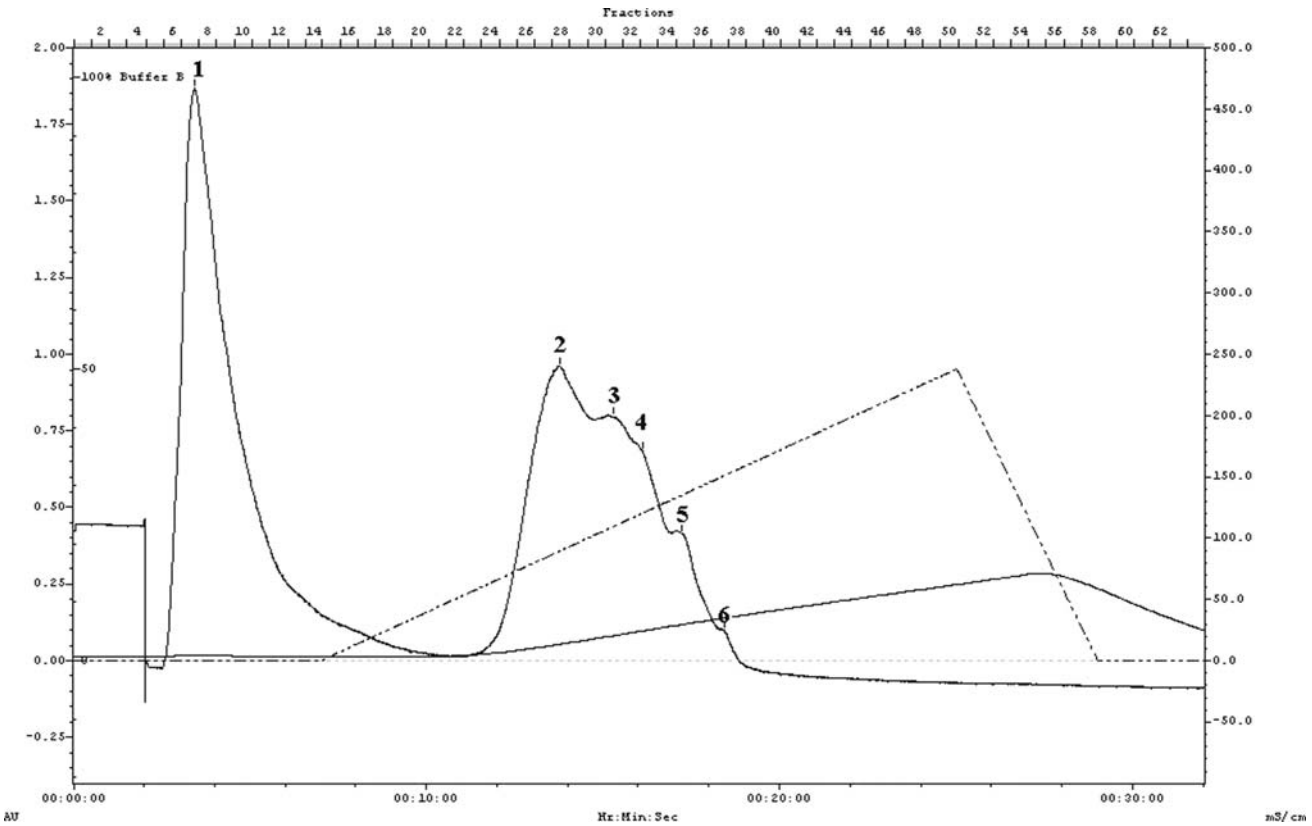

Fig. 3 CV*Plh* elution profile obtained by ion exchange chromatography on a Mono-Q1 (Biorad) column, using Tris-HCl (50 mm) pH 8.3 with a saline gradient of KCl (0–0.5 M)

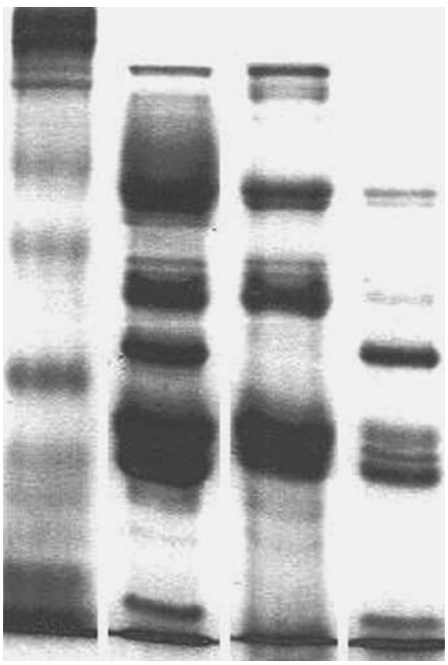

Fig. 4 SDS-PAGE of crude *P. l. hutmanni* venom and peaks I and II from ion exchange chromatography under non-reducing conditions

were characterized by an immediate drop immediate after adding the mediator, reaching a minimum after approximately 1 min. In general, their characteristics were similar to those described above.

Figure 6B shows the effect on platelet aggregation produced by the peak II/mono-Q1 *Plh* venom and thrombin (0.15 U/565 μL). Incubating 40 μg of peak II/mono-Q1 *Plh* venom with PRP, a clear inhibition of aggregation was observed (95.7%). Once the agonist doses were added at the end of the minute of preincubation, the measurements showed a turbidity increase, corresponding to the form-change phase, which continued until the beginning of the first wave feature; another feature in the turbidity reading (a small peak) was then observed before a final decrease, reaching a constant slope at the end of the 5 min assay.

However, the aliquot that contained the weight equivalent of peak II at the DA_{50} CV*Plh* (5 μg) value showed some degree of aggregation inhibition (24.8 ± 11.1%) mediated by thrombin. The effect of the peak I/mono-Q1 *Plh* venom at a dose of 40 μg was complete aggregation inhibition, observed as a very slow change in shape, returning the measurements to their baseline level after 5 min of the assay. The 0.625 μg dose, equivalent to the DA_{50} of CV*Plh* in the presence of this mediator, produced

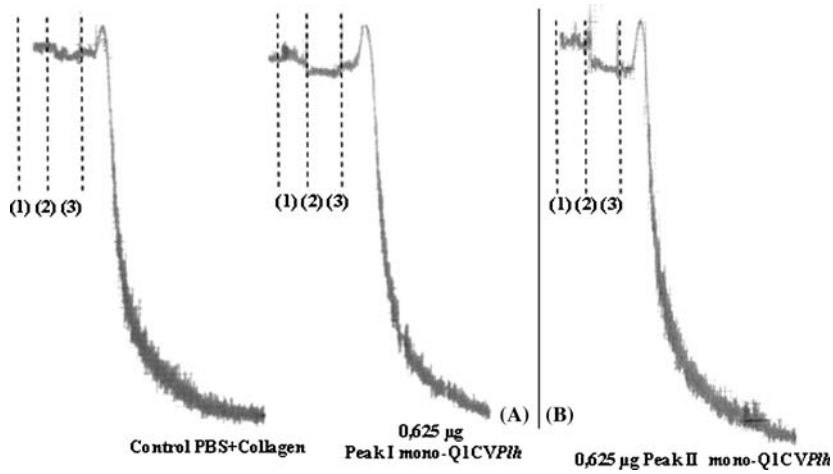

Fig. 5 Curves of platelet aggregation mediated by collagen (8 µg/553 µL) and inhibited by peaks I (**A**) and II (**B**) from crude *P. l. hutmanni* venom: (1) baseline, (2) venom sample, (3) agonist

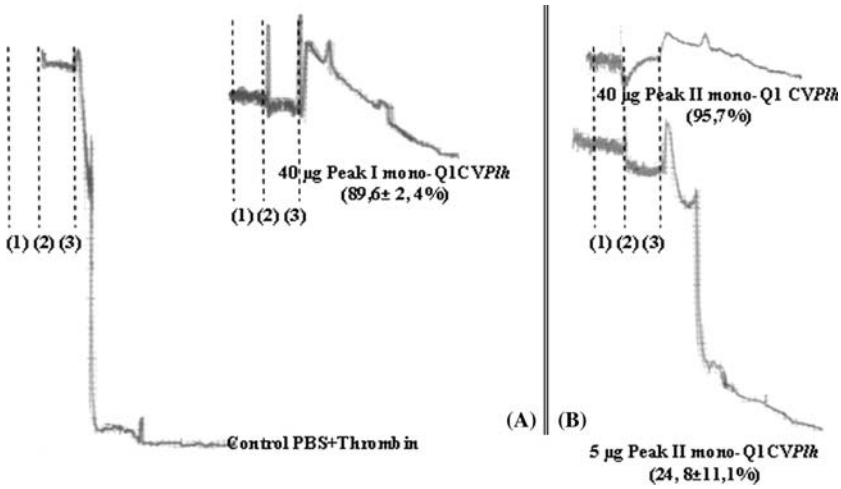

Fig. 6 Curves for platelet aggregation mediated by thrombin (15 U/565 µL) and inhibited by peaks I (**A**) and II (**B**) from crude *P. l. hutmanni* venom

a total lack of inhibition, with equivalent measurements to the control curves.

Discussion

The values and comparisons between time scales and measurements described are qualitative descriptions to indicate the presence of an observed phenomenon.

The impact of snake venoms on the hemostatic system and their ability to alter platelet functions is well known [17]. For instance, snake venoms are an excellent source of phospholipases and envenonation by a variety of snakes leads to platelet aggregation inhibition, resulting in hemorrhagic disorders [18]. A number of these enzymes have been isolated and shown to be potent inhibitors of platelet aggregation [13, 14, 19]. However, the molecular basis for these inhibitory effects has remained imprecise. There are other snake venom proteins, such as disintegrins, ADpases and fibrinogenases, which inhibit platelet aggregation via clearly defined mechanisms.

In contrast, collagen leads to platelet activation and aggregation through GPIIb/IIIa cell surface exposure, alpha granules, release of dense bodies and thromboxane synthesis [20]. Another agonist is thrombin, regarded as one of the strongest platelet aggregation agonists [21, 22], which acts through enzymatic activity on fibrinogen and other platelet surface receptors [22]. In the current work, the effects of CV*Plh* and peaks I and II on platelet aggregation (PRP) mediated by both collagen and thrombin agonists showed a clear inhibition of platelet function. This activity can be explained: (1) by means of blocking of the receptor by the venom or modification of their structural conformation, and (2) by means of the interaction of the venom with the mediator linked to this receptor or its structural modification. CV*Plh* inhibited in a dose-dependent way platelet aggregation mediated by collagen at all the tested doses, with higher efficiency for this agonist than for thrombin. We can classify this response as single phase [16], characterized by the clear decrease in the width of the first wave and the total inhibition of the second aggregation wave during the tested time period.

Here, it was showed that the inhibitory effect of CV*Plh* and peaks I and II of the *Plh* venom on platelet aggregation mediated by collagen proceeds as other authors [19] have previously reported for proteins (5́-nucleotidase) isolated from another snake venoms. CV*Plh* also produces intense local hemorrhages caused by the metalloproteases present in this venom [23], such as that reported by Uma and Veerabasappa [24] for the Zn^{2+}-dependent metaloprotease participation, which causes profuse hemorrhages in the event of Viperidae snake envenonation, due to the synergetic effect of the inhibition of platelet aggregation and the degradation of basal membrane components.

Moreover, CV*Plh* is highly myotoxic and rich in phospholipases [23]. Among these enzymes could be one responsible for the inhibition of platelet aggregation mediated by collagen, for instance phospholipase A2 from *Agkistrodon rhodostoma* snake venom [6], which has been described as being able to inhibit platelet aggregation in PRP mediated by collagen.

Experiments in our laboratory (work in process) have showed that CV*Plh* impedes the agonistic activity of ADP on platelet aggregation mediated by collagen. Ouyang and Huang [25] reported the inhibitory effect of an ADPase from *Agkistrodon acutus* snake venom on platelet aggregation mediated by collagen on PRP. Others [26] have identified platelet aggregation on PRP mediated by collagen-inhibitory protein purified from *B. atrox*, *B. jararaca*, and *Agkistrodon halys blomhoffii* South American snake venoms belonging to the Viperidae family, the same family as *P. l. hutmanni*.

Discussing the effects on platelet aggregation mediated by thrombin, some authors [25] have reported the inhibitory effect of two fibrinogenases (fraction I and IX) from the *Agkistrodon acutus* snake venom on platelet aggregation mediated by thrombin on PRP. CV*Plh* and its fractions inhibit platelet aggregation mediated by thrombin on PRP in a dose-dependent way. Other authors [6] have referred a protein in the venom of the Malaysian snake *Agkistrodon rhodostoma* that is able to inhibit platelet aggregation mediated by thrombin on PRP.

Fuly et al. [5] described the isolation of a PLA_2 from the venom of the Brazilian snake *Lachesis muta* (LM-PLA2), which discreetly inhibited the platelet aggregation mediated by thrombin, in washed platelets treated with lysophosphatidylcholine from phosphatidylcholine incubated with LM-PLA2. As explained above CV*Plh* is myotoxic and rich in phospholipases [23]. Some of these enzymes could account for the inhibition of platelet aggregation mediated by thrombin.

Another possibility is the discovery of disintegrins in *P. l. hutmanni* venom since many of these have been isolated from Viperidae venom [27]. For instance, Liu et al. [28] describe the inhibitory effect of a disintegrin (crotavirin), isolated from *Crotalus viridis* snake venom, which inhibited platelet aggregation mediated by thrombin.

The results of this study indicate for the first time that *Plh* venom possesses potent inhibitors of platelet aggregation evaluated using in vitro assays. The CV*Plh* possesses, at least, two inhibitors of platelet aggregation with opposed charges to the fixed working pH (pH 8.3) during the first step of separation of its components by anionic exchange chromatography (peaks I and II/mono-Q1 *Plh* venom) with positive and negative charges, respectively. The inhibitory power of CV*Plh* is therefore a consequence of the synergetic effect of the action of the inhibitors present in peaks I and II/mono-Q1 of the *Plh* venom. The pro-aggregating effect most strongly inhibited by CV*Plh* was mediated by the collagen, followed by the thrombin.

Characterization of CV*Plh* and peaks I and II/mono-Q1 is in progress, with the purpose of demonstrating the presence of enzymes that may be responsible for the inhibition of platelet aggregation. These may be valuable anti-thrombotic agents and potential pharmacological substances to treat hemostatic pathologies related to high levels of platelet aggregation.

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References

1. Boquet P (1992) History of snake venom research. Handb Exp Pharmacol 52:3–14
2. Andrews R, Berndt M (2000) Snake venom modulators of platelet adhesion receptors and their ligands. Toxicon 38:775–791
3. Bryan J, Dangelmaier C, Selak M (1991) Identification of 50 kDa snake venom protein which specifically inhibit platelet aggregation on collagen. FEBS 283:307–310
4. Fujimura Y, Ikeda Y, Miura S et al (1995) Isolation and characterization of jararaca GPIb-BP, a snake venom antagonist specific to platelet glycoprotein Ib. Thromb Haemost 74:743–750
5. Fuly A, Machado O, Alves E, Carlini C (1997) Mechanism of inhibitory action on platelet activation of a phospholipase A_2 isolated from *Lachesis muta* (Bushmaster) snake venom. Thromb Haemost 78:1372–1380
6. Huang T, Wu Y, Ouyang C (1987) Characterization of a potent platelet aggregation inhibitor from *Agkistrodon rhodostoma* snake venom. Biochim Biophys Acta 925:248–257
7. Fuentes O, Rodríguez-Acosta A (1997) Sobre los generos *Bothriechis*, *Bothriopsis*, *Bothrops* y *Porthidium* (Serpentes: Crotalidae) existentes en Venezuela. Claves para su identificacion de interes biomedico. Acta Biol Ven 17:31–38
8. Otero R, Osorio R, Valderrama R, Giraldo C (1992) Efectos Farmacológicos y Enzimáticos de los Venenos de las Serpientes de Antioquia y Choco (Colombia). Toxicon 30:611–620
9. Otero R, Gutiérrez J, Rojas G et al (1999) A randomized blinded clinical trial of two antivenoms, prepared by caprilic acid or ammonium sulphate fraction of IgG, in *Bothrops* and *Porthidium*

snake bites in Colombia: correlation between safety and biochemical characteristics of antivenoms. Toxicon 37:895–908
10. Otero R, Fonnegra R, Jiménez S et al (2000) Snakebites and ethnobotany in the northwest region of Colombia. J Ethnopharmacol 71:493–504
11. Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680–685
12. Lowry OH, Rosebrough NJ, Farr AL, Randal JH (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
13. Kini M, Evans H (1990) Effects of snake venom proteins on blood platelet. Toxicon 28:1387–1422
14. Kemparaju K, Krishnakanth T, Gowda T (1999) Purification and characterization of a platelet aggregation inhibitor acidic phospholipase A_2 from Indian saw-scaled viper (*Echis carinatus*). Toxicon 37:1659–1671
15. Huang T, Chiang H (1994) Effect on human platelet aggregation of phospholipase A_2 purified from *Heloderma horridum* (beaded lizard) venom. Biochim Biophys Acta 1211:61–68
16. Kamiguti A, Laing G, Lowe G, Zuzel M, Warrell D, Theakston R (1994) Biological properties of the venom of the Papuan black snake (*Pseudechis papuanus*): presence of a phospholipase A_2 platelet inhibitor. Toxicon 32:915–925
17. Satish S, Tejaswini J, Krishnakantha TP, Gowda TV (2004) Purification of a class B1 platelet aggregation inhibitor phospholipase A2 from Indian cobra (*Naja naja*). Biochimie 86:203–210
18. Colman RB, Hirsh J, Mader VJ, Salzman EW (1982) Hemostasis and thrombosis. Philadelphia, PA: Lippincott
19. Ouyang C, Huang T (1983) Potent platelet aggregation inhibitor from *Trimeresurus gramineus* snake venom. Biochem Biophys Acta 757:332–341
20. Watson S (1999) Collagen receptor signaling in platelets and megakaryocytes. Thromb Haemost 82:365–376
21. Roberts H, Monroe D III, Hoffman M (2001) Williams, hematology, 6th edn. McGraw-Hill, Inc, USA
22. Parise L, Smyth S, Coller B (2001) Williams, hematology, 6th edn. McGraw-Hill, Inc, USA
23. Navarrete LF, Vargas AM, Scannone H, Rodríguez-Acosta A (2004) Actividades hemorrágicas y necrosantes de los venenos de *Porthidium l. hutmanni y P. l. rozei* de dos regiones de Venezuela. IX Jornades Herpetologiques Catalanes. *Soc Catalana DÖHerpetol*. Barcelona, España
24. Uma B, Veerabasappa G (2000) Molecular mechanism of lung hemorrhage induction by VRV-PL-VIIIa from Russel's viper (*Vipera russelli*) venom. Toxicon 38:1129–1147
25. Ouyang C, Huang T (1986) Platelet aggregation inhibitors from *Agkistrodon acutus* snake venom. Toxicon 34:1099–1106
26. Smith JB, Dangelmaier C, Selak M (1991) Identification of 50 kDa snake venom protein which specifically inhibit platelet aggregation on collagen. FEBS 283:307–310
27. Soto JG, White SA, Reyes SR, Regalado R, Sanchez EE, Perez JC (2007) Molecular evolution of PIII-SVMP and RGD disintegrin genes from the genus *Crotalus*. Gene 389:66–72
28. Liu C, Peng H, Huang T (1995) Crotavirin, a potent platelet aggregation inhibitor purified from the venom of the snake *Crotalus viridis*. Toxicon 33:1289–1298