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Hemostatic properties of Venezuelan *Bothrops* snake venoms with special reference to *Bothrops isabelae* venom

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ABSTRACT

In Venezuela, *Bothrops* snakes are responsible for more than 80% of all recorded snakebites. This study focuses on the biological and hemostatic characteristics of *Bothrops isabelae* venom along with its comparative characteristics with two other closely related *Bothrops* venoms, *Bothrops atrox* and *Bothrops colombiensis*. Electrophoretic profiles of crude *B. isabelae* venom showed protein bands between 14 and 100 kDa with the majority in the range of 14–31 kDa. The molecular exclusion chromatographic profile of this venom contains five fractions (F1–F5). Amidolytic activity evaluation evidenced strong thrombin-like followed by kallikrein-like activities in crude venom and in fractions F1 and F2. The fibrinogenolytic activity of *B. isabelae* venom at a ratio of 100:1 (fibrinogen/venom) induced a degradation of Aα and Bβ chains at 15 min and 2 h, respectively. At a ratio of 100:10, a total degradation of Aα and Bβ chains at 5 min and of γ chains at 24 h was apparent. This current study evidences one of rarely reported for *Bothrops* venoms, which resembles the physiologic effect of plasmin. *B. isabelae* venom as well as F2 and F3 fractions, contain fibrinolytic activity on fibrin plate of 36, 23.5 and 9.45 $mm^2/\mu g$, respectively using 25 μg of protein. Crude venom and F1 fraction showed gelatinolytic activity. Comparative analysis amongst Venezuelan bothropoid venoms, evidenced that the LD_{50} of *B. isabelae* (5.9 mg/kg) was similar to *B. atrox*-Puerto Ayacucho 1 (6.1 mg/kg) and *B. colombiensis*-Caucagua (5.8 mg/kg). *B. isabelae* venom showed minor hemorrhagic activity, whereas *B. atrox*-Parguasa (Bolivar state) was the most hemorrhagic. In this study, a relative high thrombin-like activity was observed in *B. colombiensis* venoms (502–568 mUA/min/mg), and a relative high factor Xa-like activity was found in *B. atrox* venoms (126–294 mUA/min/mg). Fibrinolytic activity evaluated with 10 μg protein, showed that *B. isabelae* venom contained higher specific activity (50 $mm^2/\mu g$) than *B. colombiensis* and *B. atrox* venoms, which should encourage the isolation of these fibrinolytic molecules to improve the quality of immunotherapy.

Abbreviations: Fg, Human fibrinogen; DTT, Dithiothreitol; TEMED, Tetramethylethylenediamine; EDTA-Na_2, Ethylenediaminetetraacetic acid disodium; PMSF, Phenylmethylsulphonyl fluoride; SDS, Sodium dodecyl sulfate; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LD_{50}, Lethal dose fifty; kDa, kilo Daltons; MHD, Minimal hemorrhagic dose; PBS, Phosphate-buffered saline.

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1. Introduction

Snake venoms are a complex mixture of components, which produce damage to the organic system of an envenomated prey. Many of these toxic components are biologically active proteins that function to kill or immobilize prey as well as assist in the digestion process (Braud et al., 2000; Mackessy et al., 2003). Many of these toxins that interfere with hemostasis are enzymes, such as L-amino acid oxidases, phosphodiesterases, serine- and metalloproteases, phospholipases A2 and nucleotidases while others, such as disintegrins and C-type lectins, have no enzymatic activity. The active components from snake venoms that interfere with hemostasis may affect many steps either nonspecifically by proteolytic degradation, or selectively by activating or inhibiting specific factors involved in coagulation, platelet aggregation and fibrinolysis (Markland, 1998; Arocha-Piñango et al., 2000; Kini, 2005; White, 2005; Swenson and Markland, 2005).

Bothropoid snake envenomations may cause local tissue damage in the form of bleeding, swelling, myonecrosis, severe pain and systemic effects such as the release of pharmacologically active substances (histamine, serotonin and bradykinin) and alterations to the hemostatic system, which can lead to death (Gutierrez, 1995; Rodríguez-Acosta et al., 2000; Núñez et al., 2009). In Central and South America, most ophidic accidents are produced by Viperidae of whom 90% are caused by snakes of the *Bothrops* genus (Duque et al., 2007). In Venezuela, *Bothrops* are widely distributed across the country (Kornacker, 1999) and are responsible, such as described in Brazil and Central America, for more than 80% of all recorded snakebites (Rengifo and Rodríguez-Acosta, 2004). Hemostatic disorders constitute the main manifestation of *Bothrops* accidents. The general symptoms are coagulopathy, urinary, oral, nasal and digestive bleeding. Acute renal failure and shock could also occur (Kamiguti and Cardoso, 1989; Rodríguez-Acosta et al., 1995).

Although the mtDNA of *Bothrops isabelae* has been studied and compared to other *Bothrops* species (Salomão et al., 1999), the biological characteristics of the venom have not been studied. *B. isabelae* is broadly distributed in the Andean range piedmont in Venezuela (Táchira, Merida, Trujillo and Barinas states) (Sandner-Montilla, 1979), and its envenomation is characterized by significant local tissue damage, including hemorrhage, necrosis and edema with alterations to the hemostatic system; however, its envenomation incidence is unknown in Venezuela (Rengifo and Rodríguez-Acosta, 2004). A solid understanding of the composition of the toxins found in *Bothrops* venoms is not only relevant to the medical, toxinology and pharmacology fields but also for snake systematic studies. The knowledge of the inter-species geographic variability will likely have an influence in the understanding of snake venom toxins and in selecting specimens from various geographical locations for the production of more effective antivenoms (Gutierrez et al., 2009).

The main intent of this study was to focus on the biological and hemostatic characteristics of *B. isabelae* venom along with its comparative characteristics with two other closely related *Bothrops* venoms, *Bothrops atrox* and *Bothrops colombiensis* (Salazar et al., 2007; Girón et al., 2008) from Venezuela (Fig. 1). This information gives insight on the toxic potential and the presence of biomedical relevant molecules found in *B. isabelae* venom that may have therapeutic potential in strokes and heart attacks as well as in antivenom therapy.

Fig. 1. Geographical distribution of the current study of Venezuelan *Bothrops*.

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2. Materials and methods

2.1. Reagents

Superose 12 10/300 GE (GE Healthcare, USA), human fibrinogen (which contained fibronectin and plasminogen as contaminants, batch N° 18), human plasmin and two chains tissue-type plasminogen activator (American Diagnostica, Stanford, USA) and human fibrinogen (Hyphen Biomed, batch N° 071113C). Chromogenic substrates (Chromogenix, Milan, Italy), bromophenol blue, β-mercaptoethanol and ammonium persulfate (Merck, Darmstadt, Germany). Bovine albumin, Brilliant Blue Coomassie R-250, ammonium acetate, sodium dodecyl sulfate (SDS), tricine, dithiothreitol (DTT), tetramethylethylenediamine (TEMED), aprotinin, benzamidine, ethylenediaminetetraacetic acid Disodium (EDTA-Na_2), phenylmethylsulphonyl fluoride (PMSF), gelatin from porcine skin type A, bovine thrombin, rabbit anti-fibronectin, goat anti-rabbit IgG (peroxidase conjugated). Other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Albino Swiss, male mice (NIH strain) from 18 to 22 g were obtained from the National Institute of Hygiene "Rafael Rangel", Caracas, Venezuela. The mice were kept at a temperature of 22–24°C, with a relative humidity of 45–70%, and a 12-h light/dark cycle (lights on at 07.00 h). The mice were acclimated for at least 1 week before beginning each experiment and received water and food *ad libitum*. The Animal House authorities' surveillance reports established that mice were free of known pathogenic bacteria, viruses, mycoplasmas, and parasites.

2.3. Venoms

B. isabelae venom was obtained from adult snakes captured in Valera, Bolívar and Sucre municipalities, Trujillo state, Venezuela. *B. colombiensis* venoms were obtained from adult snakes found in close geographical locations of Caucagua and El Guapo towns, (Miranda state), Venezuela, and *B. atrox* venoms were obtained from adult snakes captured in different geographical locations: Parguasa (Bolívar state); Puerto Ayacucho 1, Serranía del Cuao and Puerto Ayacucho 2 (Amazon state), Venezuela. The snakes are currently housed in the Institute of Tropical Medicine's Serpentarium of the Universidad Central de Venezuela. Each locality was represented by a pool of venom from at least three different specimens. The venoms were obtained by extracting once from each animal into sterile Petri dishes. The venoms were lyophilized, divided into 30 mg samples and kept at −80 °C until use.

2.4. Ethical statement

All the experimental events concerning the use of live animals were done by specialized personnel. The Venezuelan pertinent regulations as well as institutional guidelines, according to protocols approved by the Tropical Medicine Institute of the Universidad Central de Venezuela, and the norms obtained from the guidelines for the care and use of laboratory animals, published by the US National Institute of Health (NIH, 1985) were followed.

2.5. Protein concentration determination

The protein concentrations of crude venoms and fractions were measured by the method of Lowry et al. (1951).

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed following Laemmli (1970) or Schagger and von Jagow (1987) methods, using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, California, USA). Protein bands were visualized with Coomassie R-250 Blue. Molecular mass estimations were determined using commercial standard proteins (Invitrogen, SeeBlue Plus2 Pre-Stained Markers, USA).

2.7. Molecular exclusion chromatography

B. isabelae venom was run preparatively using molecular exclusion chromatography on a Superose 12 10/300 GE column. The column was equilibrated at 4 °C with 50 mM ammonium acetate buffer pH 6.9. Venom samples were resuspended in the equilibrating buffer (10 mg/100 μL) and infused into the column. The elution of venom fractions was run with the same buffer used for equilibrating at a flow rate of 0.5 mL/min. Venom protein fractions were detected at 280 nm.

2.8. Lethal activity

Lethality of crude venom was determined by intraperitoneal injections into mice and the LD_{50} value was calculated according to the method of Spearman-Karber (1964). Six groups containing five mice were injected (0.2 mL) intraperitoneal with different venom concentrations. The venom was diluted in phosphate-buffered saline solution (PBS). An equivalent volume of PBS was injected as a negative control group. The animals were observed for 48 h after post injections in order to calculate LD_{50}.

2.9. Hemorrhagic activity

Hemorrhagic activity was determined by the Gutierrez et al. (1985) method. One hundred microliters of variable amounts of venom that was diluted in steril saline solution, were intradermally injected into the abdominal skin of NIH Swiss albino mice (groups of five animals/dose). The skins were removed 2 h post injections, and the diameters of the hemorrhagic spots were measured. A minimal hemorrhagic dose (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.

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2.10. Amidolytic activity

Amidolytic activity of *B. isabelae* venom and fractions was measured by a micromethod standardized by Guerrero and Arocha-Piñango (1992). Briefly, in 96-well polystyrene plates, a mixture of 80 μL of the recommended buffer for each substrate, 10 μL of the venom or fractions (0.1 mg/mL, 0.5 mg/mL or 1 mg/mL) and 10 μL of substrate (final concentration) (0.60 mM S-2238, 0.80 mM S-2222, 0.80 mM S-2251 and 0.16 mM S-2444) were placed in each well. After incubation at 37 °C for 15 min, the absorbance at 405 nm was measured. Specific activity was calculated as mUA/min/mg.

2.11. Fibrinogenolytic activity

The effects of *B. isabelae* venom and fractions on fibrinogen were evaluated following the method of Salazar et al. (2007). Briefly, fibrinogen (Fg) was incubated with venom at 37 °C at different fibrinogen:venom (Fg:venom) ratios and at different time intervals. Fifty microliter aliquots of the Fg:venom solution were mixed with an equivalent volume of denaturing solution (DTT 2% and SDS 2%). To examine detailed cleavage of Fg chains, they were run on a 9% SDS-PAGE, using the Tris–Tricine-system (Schagger and von Jagow, 1987). The assay was also carried out with the presence of serine proteinase (10 mM benzamidine, 100 IU/mL aprotinin and 10 mM PMSF) and metalloproteinase (10 mM EDTA) inhibitors.

2.12. Fibrinolytic activity

Fibrinolytic activity of *B. isabelae* venom and fractions were studied by the fibrin plate method as described by Marsh and Arocha-Piñango (1972). Briefly, fibrin plates were settled using 3-cm diameter Petri dishes with 1.5 mL of a 0.1% plasminogen-rich fibrinogen (10% plasminogen as contaminant) in 5 mM imidazol saline buffer, pH 7.4, was allowed to clot by adding 75 μL of bovine thrombin (10 IU/mL, in 0.025 M $CaCl_2$). The mixture was incubated at room temperature for 30 min and then 10 μL (1–50 μg) of each sample were applied over the fibrin. After 24 h incubation at 37 °C, the diameters of the fibrin hydrolysis (lysed areas) were measured. The activity was then recorded as lysis area consisting of the greatest and least diameter, which could be measured at right angles, and this was expressed in mm^2. Specific fibrinolytic activity ($mm^2/\mu g$) was calculated dividing the lysed area (mm^2) by the given protein dose (μg). 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 protease (10 mM benzamidine, 100 IU/mL aprotinin and 10 mM PMSF) and metalloproteinase (10 mM EDTA) inhibitors.

2.13. Gelatinolytic zymogram

To determine the gelatinolytic activity of *B. isabelae* venom and its fractions, a gelatin zymography was performed (Da Silveira et al., 2002). Venom proteins and fractions 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 the 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:v) solution and then destained with the same solution. The presence of gelatinolytic activity was defined as clear bands on a homogeneous dark background.

2.14. Fibronectin identification

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

2.15. Statistical analysis

All experiments, with the exception of the LD_{50} were repeated three times. Results were expressed as the mean ± standard deviation, and analyzed using the two-tailed Student's *t* test for samples with equal variances. Differences were statistically significant if p was less than 0.05.

3. Results

3.1. SDS-PAGE analysis of B. isabelae venom

Fig. 2 shows the electrophoretic profiles of crude *B. isabelae*, *B. colombiensis* and *B. atrox* venoms under reduced conditions. Many venom proteins were observed between 14 and 100 kDa with the majority between 14 and 34 kDa in *B. isabelae*.

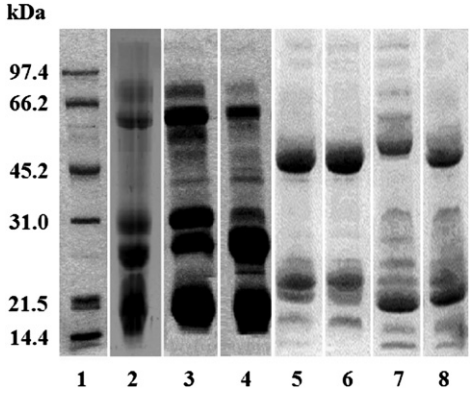

Fig. 2. Comparative analysis of crude *B. isabelae*, *B. colombiensis* and *B. atrox* venoms by 12% SDS-PAGE. 1) Molecular weight markers; 2) *B. isabelae*; 3) *B. colombiensis* (El Guapo); 4) *B. colombiensis* (Caucagua); 5) *B. atrox* (Parguasa); 6) *B. atrox* (Puerto Ayacucho 1); 7) *B. atrox* (Serranía del Cuao); 8) *B. atrox* (Puerto Ayacucho 2). The gels were stained with Brilliant Blue Coomassie R-250.

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3.2. Venom chromatographic profiles of B. isabelae venom

The molecular exclusion chromatographic profile of *B. isabelae* venom is shown in Fig. 3. Five fractions were collected (F1–F5) with elution times of 26, 30, 34, 38 and 45 min. These results indicate the existence of protein components with molecular masses higher than 60 kDa, as well as proteins of intermediate molecular masses between 20 and 60 kDa, and peptides of very low molecular mass.

3.3. Lethal activity of B. isabelae venom

Lethal activity of *B. isabelae* crude venom expressed as LD_{50} was 5.9 mg/kg (Table 1).

3.4. Hemorrhagic activity of B. isabelae venom

The minimal hemorrhagic dose (MHD) of *B. isabelae* crude venom was 11.5 ± 1.2 μg (Table 1).

3.5. Amidolytic activity of B. isabelae venom and fractions

The study of *B. isabelae* crude venom by the amidolytic method showed a high thrombin-like activity of 132 mUA/min/mg ($p < 0.05$) followed by a kallikrein-like activity of 125 mUA/min/mg. Other tested substrates, S-2222 (factor Xa), S-2444 (urokinase) and S-2251 (plasmin), revealed very low activities (Table 2). The molecular exclusion fractions F1 and F2 demonstrated high thrombin (226.5 and 95.8 mUA/min/mg, respectively) and kallikrein-like (160.1 and 105.4 mUA/min/mg, respectively) activities. Fraction F1 was more active ($p < 0.05$) in relation to both of these activities, even when compared to crude venom.

3.6. Fibrinogenolytic activity of B. isabelae venom and fractions

Fig. 4 shows the fibrinogenolytic activity of crude *B. isabelae* venom. In presence of venom, at a Fg:venom ratio of 100:1 (Fig. 4A), the degradation of the Aα and Bβ chains was observed without apparent alteration to the γ chains, even after 24 h of incubation. The degradation of the Aα chains began at 15 min and it was completed at 30 min. In contrast, the degradation of the Bβ chains began at 2 h and was completed at 4 h. Additionally, fibronectin (the highest molecular weight band demonstrated by immunoblotting assay) degradation was observed at 24 h. This adhesive protein is a contaminant found in the commercial "purified" fibrinogen used in this assay. At a higher Fg:venom ratio (100:10) (Fig. 4B), the results evidenced a total degradation of the Aα and Bβ chains at 5 min. Additionally, at 24 h, γ chains degradation was also observed. The fibrinogen lot (Hyphen Biomed) that was used in this experiment did not contain fibronectin as a contaminant.

Fibrinogenolytic activity of *B. isabelae* chromatographic fractions is shown in Fig. 5. Fraction F2 and F3 at a ratio 100:5 degraded Aα and Bβ chains at 5 min; in contrast, γ chains were degraded by F2 and F3 at 24 h and 60 min, respectively (Fig. 5A). The fibrinogenolytic activity present in F2 and F3 were also evaluated in presence of proteases inhibitors. Metalloproteinase inhibitors were able to inhibit this activity; however, the serine proteinase inhibitors did not (Fig. 5B).

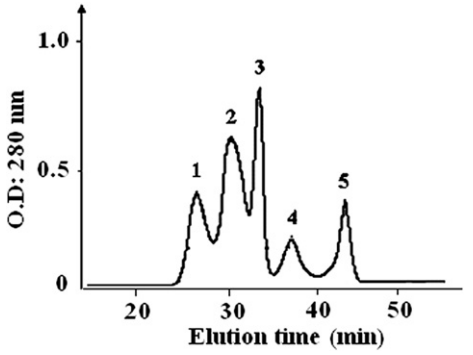

Fig. 3. Molecular exclusion chromatographic profile of *B. isabelae* crude venom. The venom was run on a Superose 12 10/300 GE (10 mg/100 μL) eluted with 50 mM ammonium acetate buffer, pH 6.9 at a rate of 0.5 mL/min. Proteins were monitored at 280 nm.

3.7. Fibrinolytic activity of B. isabelae venom and fractions

The results on fibrin plate in presence of plasminogen evidenced that *B. isabelae* venom at 10 and at 25 μg had specific activities of 50 and 36 $mm^2/\mu g$, respectively (Tables 1 and 2). Fractions 2 and 3 had specific activities of 23.5 and 9.5 $mm^2/\mu g$, respectively, while the other fractions had no activity (Table 2). Metalloproteinase inhibitors inactivated crude venom and fractions for fibrinolytic activity. The serine proteinase inhibitors partially inhibited this activity (data not shown). Standards of plasmin and tct-PA showed a lysis area of 200 and 5280 $mm^2/\mu g$, respectively.

3.8. Gelatinolytic activity of B. isabelae venom and fractions

Fig. 6 shows an acrylamide-gelatin gel zymography of *B. isabelae* venom and its fractions. The results demonstrated that the venom and F1 presented gelatinolytic activity evidenced by clear bands with relative molecular masses of ~116, 66 and 45 kDa. F2 and F3 had low levels of activity at the high molecular weight area. In contrast, F4 and F5 did not present gelatinolytic activity.

3.9. Comparative study of activities among the different Bothrops venoms

The venom of *B. isabelae* was compared to both *B. atrox* and *B. colombiensis* venoms. The *B. atrox* and *B. colombiensis* venoms have been analyzed in previous studies (Salazar et al., 2007; Girón et al., 2008). The comparative biochemical studies amongst Venezuelan *Bothrops* venoms showed several variations existing in composition and concentration. The electrophoretic profiles evidenced less differences between *B. isabelae* and *B. colombiensis* venoms both showing protein bands with molecular masses between 14 and 100 kDa (Fig. 2). In contrast, *B. atrox* venoms showed a very characteristic protein group

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Table 1
Comparison of Venezuelan *Bothrops* venom activities.

Venoms and locations	Activities					
	LD_{50} mg/kg	MHD μg	Procoagulant (mUA/min/mg)		Fibrinolytic	
			Thrombin (S-2238)	Factor Xa (S-2222)	Kallikrein (S-2302)	Fibrin Plate Pg + Lysis area mm^2 (10 μg)
B. isabelae						
Trujillo	5.9	11.5 ± 1.2	132 ± 10.1	22 ± 3.0	125 ± 9.4	500 ± 39 [c]
B. atrox[a]						
Parguasa	4.0	2.7 ± 0.4	438 ± 78[c]	294 ± 29[c]	25 ± 7	100 ± 11
Puerto Ayacucho 1	6.1	5.2 ± 0.7	190 ± 37	126 ± 17	48 ± 8	257 ± 41
Puerto Ayacucho 2	7.9	5.9 ± 0.8	473 ± 85[c]	177 ± 20	27 ± 5	121 ± 11
Serranía del Cuao	8.3	4.2 ± 0.5	383 ± 85[c]	235 ± 39[c]	28 ± 2	51 ± 7
B. colombiensis[b]						
El Guapo	11.6	5.6 ± 0.8	568 ± 22 [c]	116 ± 19	1126 ± 51[c]	168 ± 32
Caucagua	5.8	14.3 ± 1.7	502 ± 19[c]	56 ± 10	446 ± 24[c]	306 ± 55

[a] : Salazar et al., 2007.
[b] : Girón et al., 2008.
[c] Statistical differences between groups.

between 45 and 60 kDa, which was not observed or was very weak in the other two species.

The venoms from different Venezuelan locations had LD_{50}s ranging from 4.0 to 11.6 mg/kg. The LD_{50} for *B. isabelae* was 5.9 mg/kg (Table 1).

The MHDs for the venoms ranged from 2.7 to 14.3 μg. The MHD of *B. isabelae* crude venom was 11.5 μg, which fell in the upper range.

The procoagulant and fibrinolytic activities were the hemostatic parameters studied. The procoagulant activities evaluated by the amidolytic method evidenced thrombin-like activity, followed by factor Xa-like activity. The comparative analysis among the diverse *Bothrops* venoms showed higher thrombin-like activities in *B. colombiensis* (502–568 mUA/min/mg) and higher factor Xa-like activities in *B. atrox* venoms (126–294 mUA/min/mg) (Table 1).

The fibrinolytic activity evaluated by the amidolytic method demonstrated significant kallikrein-like activities in all venoms, with those of *B. colombiensis* being the most active (446–1126 mUA/min/mg). On the other hand, the activity on fibrin plate in presence of plasminogen was higher in *B. isabelae* venom (50 mm^2/μg) (Table 1).

4. Discussion

The comparative biochemical studies amongst Venezuelan *Bothrops* venoms indicate that several variations exist in composition and concentration between them, which could be related with many variables such as ecological interactions, sex, genetics, age, among others (Chippaux et al., 1991; Daltry et al., 1996; Pifano and Rodriguez-Acosta, 1996; Saldarriaga et al., 2003; Menezes et al., 2006; Queiroz et al., 2008; Lanari et al., 2010).

In previous studies, the biological and biochemical characteristics of *B. colombiensis* and *B. atrox* venoms from specimens captured in different Venezuelan locations (Salazar et al., 2007; Girón et al., 2008) evidenced several geographical variations in chromatographic and electrophoretic profiles, as well as lethal, hemorrhagic and hemostatic activities.

This work compares a venom pool from three *B. isabelae* snakes specimens collected in three different locations of Trujillo state, Venezuela with venom pools from *B. atrox* and *B. colombiensis* snakes previously reported (Salazar et al., 2007; Girón et al., 2008). In these studies, the animals' age, weight, inoculation routes and experimental conditions were comparable.

Table 2
Fibrinolytic and coagulant activities found in *B. isabelae* crude venom and fractions.

Samples	Activities					
	Amidolytic (mUA/min/mg)					Specific Fibrinolytic Activity mm^2/μg[a]
	Thrombin (S-2238)	Factor Xa (S-2222)	Kallikrein (S-2302)	Urokinase (S-2244)	Plasmin (S-2251)	
Crude venom	132.0 ± 14.0	20.5 ± 6.7	124.5 ± 32.0	13.4 ± 5.8	7.7 ± 2.7	36 ± 3.5
Fractions						
1	226.5 ± 10.5[b]	34.4 ± 12.8[b]	160.1 ± 29.1[b]	14.0 ± 7.4	8.5 ± 3.5	0
2	95.8 ± 11.0	20.8 ± 10.2[b]	105.4 ± 22.2[b]	17.4 ± 6.6	13.4 ± 6.2	23.5 ± 2.8[b]
3	7.3 ± 1.8	2.7 ± 0.9	4.1 ± 1.8	2.9 ± 0.8	1.7 ± 0.4	9.5 ± 1. 0
4	7.1 ± 1.3	2.7 ± 1.1	8.7 ± 3.0	2.5 ± 0.1	2.5 ± 1.3	0
5	3.2 ± 1.2	2.9 ± 1.0	3.1 ± 1.4	1.1 ± 0.2	2.9 ± 2.1	0

[a] Statistical difference between crude venom and fractions.
[b] Specific fibrinolytic activity (mm^2/μg) was calculated by dividing the lysis area (mm^2) by the total protein dose tested (μg). In this experiment the fibrinolytic activity was evaluated using 25 μg of protein.

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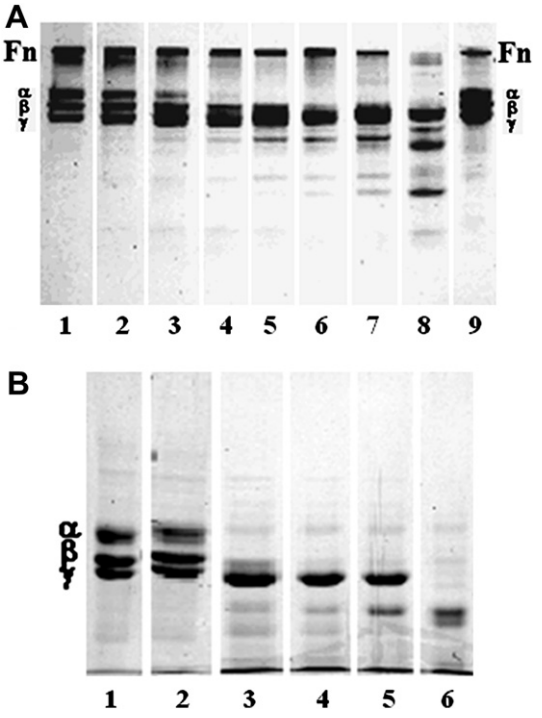

Fig. 4. Fibrinogenolytic activity of *B. isabelae* crude venom. Samples were run under reducing conditions on a 9% SDS-PAGE. **A**) Fibrinogen:venom at a 100:1 ratio, after various incubation times at 37 °C. Lanes: 1) Fg control: 0 min; 2 to 8) Fg + venom at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, and 24 h, respectively 9) Fg control: 24 h. Fn represents fibronectin contaminant. **B**) fibrinogen:venom at a 100:10 ratio, after various incubation times at 37 °C. Lanes: 1) Fg control: 0 min; 2) Fg control: 24 h; 3 to 6) Fg + venom at 5 min 30 min, 1 h, and 24 h, respectively. The gels were stained with Brilliant Blue Coomassie R-250.

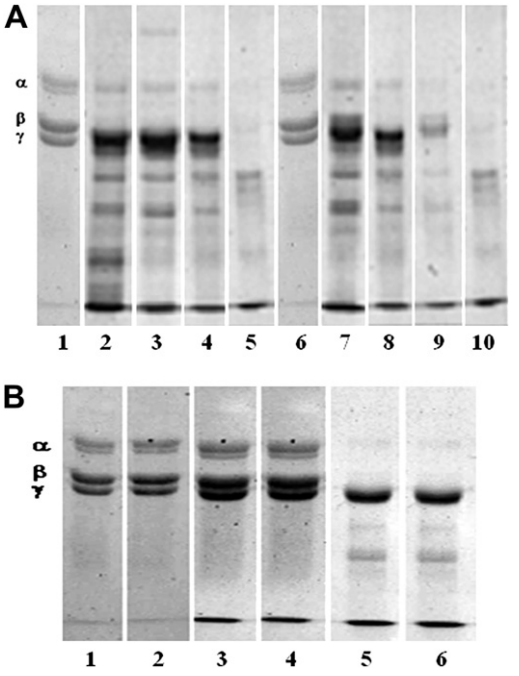

Fig. 5. Fibrinogenolytic activity of *B. isabelae* molecular exclusion fractions F2 and F3 at a 100:5 fibrinogen:venom ratio, at 37 °C and 1 h incubation time. Samples were run under reducing conditions on a 9% SDS-PAGE. **A)** Different incubation times: Lanes: 1) Fg control: 0 min; 2 to 5) Fg + F2 at 5 min, 30 min, 1 h, and 24 h, respectively; 6) Fg control: 24 h; 7 to 10) Fg + F3 at 5 min, 30 min, 1 h, and 24 h, respectively **B)** Activity in presence or absence of protease inhibitors: Lanes: 1) Fg control at 0 min; 2) Fg control at 1 h; 3) Fg + (F2 + EDTA); 4) Fg + (F3 + EDTA); 5) Fg + (F2 + serine-protease inhibitors); 6) Fg + (F3 + serine-protease inhibitors). The gels were stained with Brilliant Blue Coomassie R-250.

The electrophoretic profile of *B. isabelae* venom revealed protein bands with molecular masses between 14 and 100 kDa (Fig. 2), corresponding to the range in which the main components of *Bothrops* spp. venoms are found and most likely equivalent to metalloproteinases (40–64 kDa), serine proteinases (20–40 kDa) and phospholipases A2 (13–16 kDa) (Moura-da-Silva et al., 1990; Kini, 2005). Comparative analysis evidenced that the *B. isabelae* venom profile most closely resembled those of *B. colombiensis* venoms (Girón et al., 2008).

The toxic activities observed in the different venoms are found in Table 1. The present study demonstrated that the LD_{50} of *B. isabelae* (5.9 mg/kg) was similar to *B. atrox*-Puerto Ayacucho 1 (6.1 mg/kg) and *B. colombiensis*-Caucagua (5.8 mg/kg), *B. colombiensis*-El Guapo showed the lowest activity (11.6 mg/kg).

Hemorrhagic activity is caused by metallo- and serine proteinases. These enzymes promote hemorrhage by degrading extracellular matrix proteins such as laminin, nidogen, fibronectin, collagen type IV (constituents of vessel walls), and proteoglycans from the endothelial basal membrane, facilitating the diffusion of venom components across the membranes (Fox and Serrano, 2005). The comparative analysis of hemorrhagic activity expressed as MHDs indicated that *B. isabelae* venom is less potent (11.5 μg) than all *B. atrox* venoms (2.7–5.9 μg) and more potent than *B. colombiensis*-Caucagua venom (14.3 μg) (Table 1).

This study revealed lethal and hemorrhagic differences amongst the Venezuelan bothropoid venoms amid geographical regions, with no significant inter-species differences. These venoms mainly induce hemostatic and extracellular matrix alterations. It would be important to carry out an extensive study with a higher number of

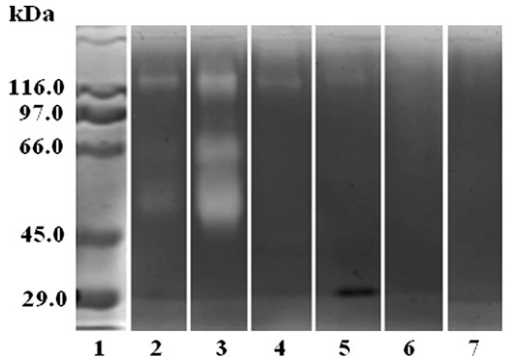

Fig. 6. Zymographic-gelatin of *B. isabelae* venom and molecular exclusion fractions. The white areas represents gelatinolytic activity. Lanes: 1) high molecular weight markers; 2) crude venom; 3) F1; 4) F2; 5) F3; 6) F4; 7) F5. Each lane contains 40 μg of protein.

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snakes obtained from various geographical locations to establish the maximum, intermediate and minimal activity values for each region. In addition, there is a need to study the venom proteomics for each species in order to establish qualitative and quantitative variations of many of these venom proteins involved in the alteration of the hemostasis pathways.

The complex biological effects of the snake venoms may be explained by different molecules with diverse actions. *Bothrops* species contain numerous proteins that induces various hemostatic alterations involving platelet, coagulation and fibrinolysis systems in addition to tissue integrity, which in envenoming victims are manifested as local tissue necrosis and bleeding syndrome (Calvete et al., 2009a,b; Gutierrez et al., 2009). Snake venoms contain proteases that can act on the blood coagulation cascade, either by specific proteolytic activation or by nonspecific degradation of blood factors. The coagulant activity is due to enzymes that act on plasma procoagulant components, such as factor IX, factor X, prothrombin activators, or thrombin-like enzymes. The crude venom of *B. isabelae* and its molecular exclusion fractions F1 and F2 expressed significant thrombin-like and factor Xa-like activities (Table 2). Comparative analysis among bothropoid venoms demonstrated that the observed thrombin-like activity was higher in *B. colombiensis*-El Guapo venom (568 ± 22 mUA/min/mg, $p < 0.001$). The factor Xa-like activity was greater in the *B. atrox*-Parguasa venom (294 ± 29 mUA/min/mg, $p < 0.001$). *B. isabelae* venom was the least active in relation to these activities (132 ± 10.1 and 22 ± 3 mUA/min/mg, respectively). This coagulant activity is involved in intravascular disseminated coagulation problems that increase hemorrhagic episodes (Kamiguti et al., 1986; Kamiguti and Cardoso, 1989).

In addition to coagulant actions, fibrino(geno)lytic activity caused by snake venoms complicates a patient's clinical picture. Many snake venom proteases rapidly hydrolyze the Aα chains of fibrinogen (metalloproteinases), others hydrolyze the Bβ chains (serine proteinases), and they rarely act on the γ chains (Swenson and Markland, 2005; Markland, 1998). The fibrinogenolytic activity evaluated by electrophoresis indicated that *B. isabelae* venom, at a ratio of 100:1, induced a rapid degradation of Aα chains (15 min) and a slow degradation of Bβ chains (2 h), without apparent alteration of the γ chains even after 24 h. However, at a ratio of 100:10 a total degradation of all chains was observed after 24 h with crude venom and fractions F2 and F3 (Fig. 5). These results suggest that *B. isabelae* venom possibly contains two different types of fibrinogenases, one (or more) that preferentially degraded the Aα chains and other(s) that degraded all three chains, or it could be the same enzyme with different kinetic activities, these possibilities will be further studied. This current study evidences one of rarely reported actions for *Bothrops* venoms, which resembles the physiologic effect of plasmin that acts on fibrinogen, degrading all three chains (Aα, Bβ and γ). This uncommon effect has been reported in *Bothrops moojeni* and *Agkistrodon acutus* venoms (Serrano et al., 1993; Xiuxia et al., 2001).

B. isabelae venom also degraded the fibronectin contaminant in the "purified" fibrinogen, demonstrating the effect on extracellular matrix components, which explains the hemorrhagic consequences caused by this venom. The neutralization of fibrinogenolytic and fibronectinolytic activities in both crude venom and fractions by metal chelators evidenced that metalloproteinases were involved in this activity, which is similar to that observed with *B. colombiensis* venoms and their active fractions (Girón et al., 2008).

The fibrinolytic activity observed in snake venoms may be due to either the direct action of plasminogen activators release from endothelial cells or to proteolytic enzymes with direct action on fibrin/fibrinogen (Braud et al., 2000; Zhang et al., 1995; Markland, 1998; Swenson and Markland, 2005; Bello et al., 2006; Salazar et al., 2007). *B. isabelae* crude venom and its fractions F2 and F3 presented fibrinolytic activity on fibrin plates. With crude *B. isabelae* venom, the fibrinolytic activity on fibrin plates using 10 μg of proteins was of 500 mm^2 (Table 1), which results in a specific activity of 50 $mm^2/\mu g$; however at 25 μg, the venom showed a specific activity of 36 $mm^2/\mu g$ (Table 2). This indicated the presence of possible fibrinolytic inhibitors. Similar results were observed in *B. atrox* venoms (Salazar et al., 2007), demonstrating the profound effects that *Bothrops* venoms cause on the hemostatic system of victims, which would depend not only on the quantity of venom, age and sex of the snake but also on the balance between activators and inhibitors present in the venom. The exploration of components isolated from various venoms is currently in progress in order to provide other hemostatic alternatives based on plasmin inhibition, which would allow the discovery of new antifibrinolytic components to be used in diseases associated with injuries, surgeries, and coagulation disorders (Masci et al., 2000; Eaton, 2008; Brazón et al., 2009).

The fibrinolytic activity in *B. isabelae* crude venom and their chromatographic fractions was abolished by a metal chelator (EDTA) suggesting that these enzymes belong to the family of metalloproteinases. Some fibrinolytic metalloproteinases, such as FII that do not induce hemorrhage, have been isolated from snake venoms. FII is a P-I class metalloproteinase isolated from *A. acutus* snake venom (Lou et al., 2005). These proteinases are of great interest since they may have clinical relevance as thrombolytic agents.

Fibrinolytic activity by the fibrin plate revealed that *B. isabelae* venom contained higher activity than the other two types of venoms compared in this work. This fibrin plate activity in *B. isabelae* venom could be due to higher concentrations of metalloproteinases, while *B. colombiensis* venoms possess higher concentrations of serine proteinases.

Analysis of amidolytic activity evidenced the presence of serine proteinases with kallikrein, plasmin and urokinase-like activities. The kallikrein-like amidolytic activity was more significant. When comparing the kallikrein-like activities in the *Bothrops* venoms, *B. colombiensis* venom contained a higher amount of these serine proteinases (Table 1). The high kallikrein-like amidolytic activity could be producing an effect on the contact system, activating factor XII, prekallikrein or kinin systems.

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Physiologically, it has been shown that the serine protease, kallikrein, presents fibrinolytic activity mainly mediated by activation of the pro-urokinase and to a lesser degree (1%) by direct activation of plasminogen (Colman, 2006). This activity has been observed in other snake venoms, especially those of the Viperidae family. After envenomation, kallikrein-like proteins are responsible for local pain, augmented capillary permeability, systemic hypotension and contraction of smooth muscle (Weinberg et al., 2004a,b).

Degradation of tissue substrates is an important feature of snake venom proteolytic enzymes. Gelatin has been utilized as an instrument to illustrate both metalloproteinases and serine proteinases activities (Shannon et al., 1989; Serrano et al., 1993). The gelatinolytic activity on a SDS gel was observed in *B. isabelae* crude venom and its molecular exclusion fraction F1, with activity in the areas between 46 and 105 kDa. This activity was neutralized by metal chelants, indicating the presence of metalloproteinases.

Proteomic studies carried out by Calvete et al. (2009a,b) and Gutierrez et al. (2009), compared *Bothrops* venoms from several species captured in very distant geographic localities in Central and South America. Data presented in their study reinforce the existence of a significant range of variations in the composition and activities of *Bothrops* species venoms. These variations influence the clinical manifestations as well as response to antivenom therapy (de Roodt et al., 2006). For instance, physicians from Trujillo state, Venezuela (personal communication) have observed that snake envenomations caused by *B. isabelae* have required higher doses of antivenom to alleviate clinical complications as compared to other Venezuelan *Bothrops* envenomations.

Our comparative study demonstrated that *B. isabelae* venom presented the highest fibrinolytic activity and the lowest hemorrhagic activity suggesting that this venom may have the potential to degrade blood clots without causing hemorrhagic complications. The differences observed in the bothropoid venoms evaluated in this paper propose the use of crude venoms to isolate molecules with prominent fibrinolytic activity that can be utilized in antivenom preparation which can help reinforce immunotherapy.

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Conflict of interest

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

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