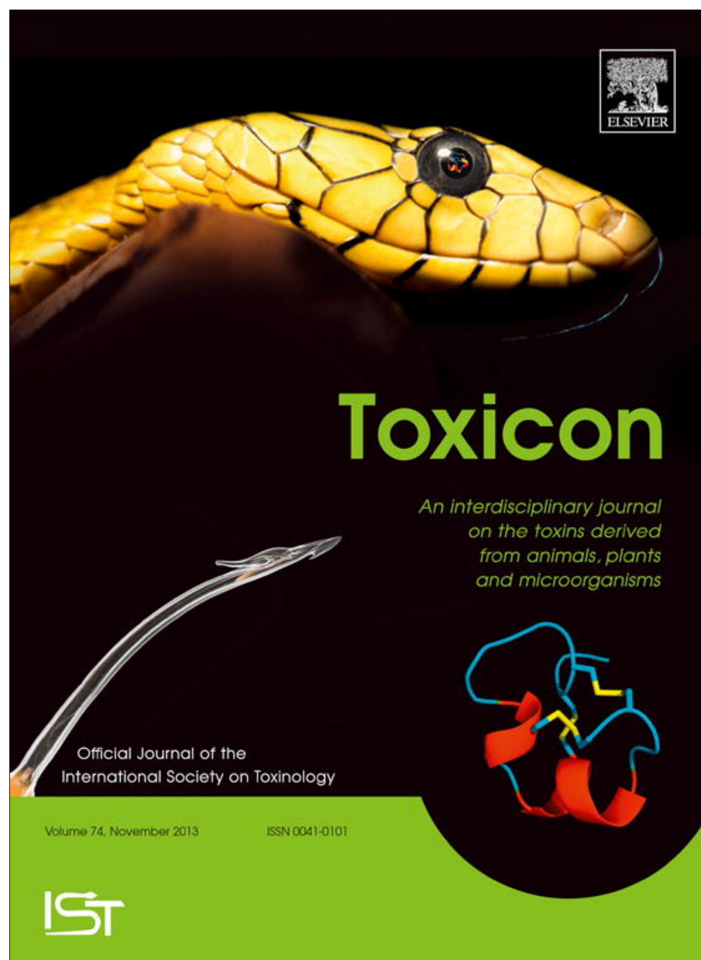
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/authorsrights

Author's personal copy

Toxicon 74 (2013) 116–126

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Functional characterization of fibrinolytic metalloproteinases (colombienases) isolated from *Bothrops colombiensis* venom

María E. Girón [a], Belsy Guerrero [b], Ana María Salazar [b], Elda E. Sánchez [c], Marco Alvarez [d], Alexis Rodríguez-Acosta [a,b,*]

[a] Laboratorio de Inmunoquímica y Ultraestructura, Instituto Anatómico de la Universidad Central de Venezuela, Caracas, Venezuela
[b] Laboratorio de Fisiopatología, Centro de Medicina Experimental, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela
[c] Department of Chemistry and the National Natural Toxins Research Center, Texas A&M University-Kingsville, Kingsville, TX 78363, USA
[d] Sección de Microscopia Electrónica, Instituto Anatómico de la Universidad Central de Venezuela, Caracas, Venezuela

ARTICLE INFO

Article history:
Received 26 February 2013
Received in revised form 28 May 2013
Accepted 30 July 2013
Available online 17 August 2013

Keywords:
Bothrops colombiensis
Colombienases
Fibrinolytic
Hanging drop cardiac myoblast
Metalloproteinases

ABSTRACT

Researchers trying to improve the safety and efficacy of fibrinolytic therapy have been isolating fibrinolytic enzymes from snake venoms. Two fibrinolytic enzymes, colombienases 1 and 2, with significant activity have been isolated from the venom of *Bothrops colombiensis*.

Methods: The colombienases were characterized for various biological activities which include hemorrhagic, fibrinogenolytic, proteolytic, hemolytic, edematogenic and cytotoxic.

Results: Colombienases directly acted on fibrin by degrading fibrinogen Aα and Bβ chains without activating the fibrinolytic system (plasminogen/plasmin), additionally colombienase-2 degraded fibrinogen γ chains as well as the fibronectin molecule. Laminin and type IV collagen were colombienases resistant. Gelatin–zymogram activity was present in *B. colombiensis* venom (BcV) bands between 64 and 148 kDa. All activities were abolished by metalloproteinases inhibitors. Both enzymes lacked hemorrhagic, hemolytic, cytotoxic, plasminogen activator and coagulant activities.

Conclusions: Both colombienases had direct fibrino(geno)lytic activity without other toxic side effects including *in vivo* hemorrhaging, which could be promising in terms of therapeutic potential as thrombolytic agents.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

At the present time, diseases of the cardiovascular system such as acute myocardial infarction, cerebral stroke, coronary heart disease, hypertension and atherosclerosis are the most important causes of the human mortality around the world. Thrombosis is probably the most common symptom among cardiovascular diseases (Yuan et al., 2012). The hemostatic system is designed to maintain blood in its fluid stage by inhibiting blood coagulation and platelet aggregation and promoting fibrinolysis. Thrombosis may occur if the hemostatic stimulus is unregulated, either because the capacity of inhibitory pathways is impaired or, more commonly because the capacity of the natural anticoagulant mechanism is overwhelmed by the intensity of the stimulus (Colman et al., 2006). The final consequence of coagulation is the deposition of fibrin polymers to fibrin clot. The enzymatic reactions that drive these pathways are mainly serine proteases (Rau et al., 2007); so far, more than 100 snake venoms are known to affect the hemostatic system and/or fibrinolytic by various

* Corresponding author. Laboratorio de Inmunoquímica y Ultraestructura, Instituto Anatómico de la Universidad Central de Venezuela, Caracas 1041, Venezuela. Tel.: +58 4168192537.
E-mail address: rodriguezacosta1946@yahoo.es (A. Rodríguez-Acosta).

0041-0101/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2013.07.025

Author's personal copy

mechanisms (Markland, 1998; Kini, 2005). Studying these mechanisms of action is of vast interest for diverse reasons, among them are the understanding and the interpreting of the clinical pictures and thus helping with the therapeutics, and exploring its therapeutic potential as possible thrombolytic agents, anticoagulants, antiplatelet amongst others will be useful for diagnostic purposes.

These drugs include the first-generation agents such as streptokinase (SK) and two-chain urokinase-type plasminogen activator (tcu-PA or urokinase); the second-generation drugs, namely recombinant-tissue-type plasminogen activator (rt-PA), recombinant single-chain urokinase (rscu-PA or prourokinase), anisoylated plasminogen-SK activator complex (APSAC); and the third generation compounds derived from t-PA such as TNK-tPA; recombinant deletion mutants of t-PA (reteplase and lanoplase) and scu-PA; conjugates of plasminogen activators with monoclonal antibodies against fibrin, platelets or thrombomodulin. Therefore, the search remains open for a thrombolytic agent able to overcome resistance to clot lysis, accelerate recanalization, avoiding reocclusion and reduction of bleeding side-effects. In light of this, much more attention must be focused on the development of new types of fibrinolytic enzymes; for instance, enzymes isolated from snake venoms (Guerrero et al., 2001; Liang et al., 2005; Moura-da-Silva et al., 2011; Girón et al., 2013; Jonebring et al., 2012).

Colombienases fibrinolytic enzymes (CFe) were isolated from *Bothrops colombiensis* venom (BcV). The *B. colombiensis* is Venezuelan's most significant snake since it is involved in 85% of the ophidic accidents in the country. Colombienases, already described by our group (Girón et al., 2013) can degrade fibrin directly and proficiently. Additionally, they did not have toxic action and other side effects, such as hemorrhages. Colombienases 1 and 2 contained high fibrinolytic activity. In the current paper, the *in vitro* proteolytic activities of these colombienases were investigated. The study and development of CFe has importance for treating thrombo-embolic diseases (Abou-Chebl, 2011; Suzuki and Urano, 2011).

2. Materials and methods

2.1. Reagents

Delta 1525, 1525 binary HPLC pump, 2487 dual λ absorbance detector, and Waters Breeze software were obtained from Waters™ Corp. (Milford, MA, USA). Dialysis tubing was from Thermo Scientific, and the YM10 Amicon membranes from Amicon. Human fibrinogen was from Aniara Corp. (Mason, OH, USA). Sepharose Heparin and Phenyl Sepharose were from Pharmacia Biotechnology (Uppsala, Sweden). Molecular mass standards for SDS-PAGE were from Bio-Rad (Bio Rad Laboratories Ltd. California, USA). Sepharose–Gelatine gel, aprotinin, bovine thrombin, 1,10-phenantroline, benzamidine/HCl, ethylene glycol-bis-*N*-tetraacetic acid (EGTA), thermolysin, ethylene diamine tetraacetic acid (EDTA), dithiothreitol (DTT), *p*-nitrophenyl phosphate, anti-human fibronectin, goat anti-rabbit IgG conjugated to horseradish peroxidase, 3,3′-dimethyloxacarbocyanine ($DiOC_1$), agar, agarose, laminin from Engelbreth–Holm–Swarm murine sarcoma basement membrane, collagen type IV from human placenta basal membranes, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The Polyvalent snake antivenom (SAOP) was obtained from the Faculty of Pharmacy of the Universidad Central de Venezuela.

2.2. Venom

Ten adults *B. colombiensis* snakes from Caucagua, Miranda state, Venezuela were used to obtain the venom used in this study. The snakes are currently housed in the Serpentarium of the Tropical Medicine Institute of the Universidad Central of Venezuela. Venom was extracted by allowing the snake to bite into Para-film stretched over a disposable plastic cup. Each sample was centrifuged ($500 \times g$ for 10 min), filtered through a 0.45 μm, lyophilized and stored at −80 °C.

2.3. Animals

Male Swiss mice (NIH strain), weighing from 18 to 20 g were purchased from the National Institute of Hygiene "Rafael Rangel" Animal Facility (Caracas, Venezuela).

Chicken embryos (Shaver Red Sex-Link hens) at 27, 35 and 37 development stages (Hamburger and Hamilton, 1992), corresponding to 5, 8 and 11 days of incubation respectively, were obtained from the "Mary Poultry Farm" located in San Antonio de los Altos, Miranda State, Venezuela.

2.4. Ethical statement

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

2.5. Protein quantification

Protein concentration was tested by Lowry et al. (1951) method, using BSA as standard and spectrophotometrically assuming that 1 unit of absorbance at 280 nm corresponds to 1 mg protein/mL (Simonian and Smith, 2006).

2.6. SDS-polyacrylamide gel electrophoresis

Protein samples were electrophoresed on polyacrylamide gels using Laemmli (1970) or Schägger and von Jagow (1987) systems.

2.7. Purification of colombienases

B. colombiensis metalloproteinases were isolated using cation exchange chromatography as the initial step,

Author's personal copy

followed by hydrophobic interaction and molecular exclusion chromatographies (Girón et al., 2013).

2.8. Lethality (LD_{50})

Five groups of five mice for each sample were located in mice cages and inspected during the experiments. *B. colombiensis* venom (BcV) and colombienases were dissolved in 0.85% saline at the highest test dose per mouse. The lethal toxicity was determined by injecting 0.2 mL of BcV and colombienases at varying dosages (0.175–2.5 μg) into the tail veins of 18–20 g female BALB/c mice. Saline controls were used. The LD_{50} was calculated by Spearman-Kärber method (1978) after the 48 h experimental period. In these animals the potential bleeding effect was determined by dissection of the animal and evaluating the different organs to macroscopically determine the presence of hemorrhage or fibrin formation.

2.9. Hemorrhagic activity

Hemorrhagic activity for BcV and fractions was assayed by Gutiérrez et al. (1985) method. One hundred microliters of variable amounts (μg) of venom and fractions were intradermally injected into the abdominal skin of NRMI mice. After 2 h, the mice were sacrificed 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 that causes a 10-mm hemorrhagic spot. Saline solution was used as a negative control.

2.10. Fibrinolytic activity

Fibrinolytic activity of colombienases was studied by the fibrin plate method as described by Marsh and Arocha-Piñango (1972).

2.11. Fibrinogenolytic activity

Fibrinogenolytic activity of colombienases was assayed by Salazar et al. (2007) method. Fibrinogen in 0.05 M Tris–HCl, pH 7.4 was incubated with sample at different ratios at 37 °C and at different time periods. Fifty microliter aliquots of the Fg:venom solution were mixed with an equivalent volume of denaturing solution (2% DTT and 2% SDS). To test the fibrinogen chains, the samples were run on a 10% SDS-PAGE, using the Tris–Tricine system under reduced conditions (Schägger and von Jagow, 1987). This activity was also carried out in presence of protease inhibitors by incubating venom or colombienases for 30 min at 37 °C with a mixture of inhibitors. Serine proteinase inhibitors were employed as a mixture of 10 mmol/L benzamidine/HCl, and 100 IU/mL aprotinine (final concentrations). Metalloproteinase inhibitors were used as a mixture of 10 mmol EGTA-Na and 10 mmol EDTA-Na (final concentrations) (Girón et al., 2008).

2.12. Plasminogen activation

Plasminogen activator activity in colombienases was determined by a modification of Mussoni et al. (1979) method, measuring amidolytic activity of the plasmin generated on S-2251 substrate (Girón et al., 2013).

2.13. Fibronectin purification

Human fibronectin was purified by Vuento and Vaheri (1979) modified method. In brief, human citrated plasma was filter through nylon cloth and 10 mmol benzamidin/HCl and 1 mmol phenylmethanesulfonyl fluoride were added. The vitamin K-dependent factors were adsorbed using 1 mol/L $BaCl_2$ (80 ml/L plasma); the supernatant was dialyzed against 0.05 mol/L Tris–HCl, pH 7.5 buffer (TB), and then applied to a Sepharose–Gelatine column (2.8×20 cm^2) formerly equilibrated with the same buffer (flow rate, 1.5 mL/min). The column was initially washed with 1 mol/L NaCl and later with 0.2 mol/L arginine in TB. Elution of fibronectin was run with 1 mol/L arginine in the same buffer. The eluted fractions were pooled and dialyzed against TB and applied to a Sepharose–Arginine column (2.5×10 cm^2) (flow rate, 0.5 mL/min). The column was washed with TB and the fibronectin was eluted with 0.1 mol/L NaCl in the same buffer. The eluted fractions were concentrated by ultrafiltration with an Amicon YM-100 membrane and stored at −80 °C until used. Western blotting recognized fibronectin in each pool and fibronectin was visualized with a rabbit anti-fibronectin IgG at a dilution of 1:1000 followed by a goat anti rabbit IgG, peroxidase conjugated at a dilution of 1:1000. The protein concentration was determined by assuming an extinction coefficient at 1 mg/mL and 280 nm of 1.28. Purity was evaluated by 7.5% SDS-PAGE under reduced conditions using the Laemmli system.

2.14. Degradation of fibronectin

Purified fibronectin was incubated with BcV and colombienases, at a 1 μg venom/100 μg fibronectin ratio, for 2 and 24 h at 37 °C. Fibronectin degradation was visualized by SDS-PAGE on a 7.5% gel under reduced conditions. This fibronectin pre-treated with colombienases was used to evaluate its functional activities. Changes in the molecules were visualized by electrophoresis (SDS-PAGE) under reduced conditions using the Laemmli system.

2.15. Effect of venom on extracellular matrix proteins (ECM)

The effects of BcV and colombienases on ECM proteins were tested using purified laminin and collagen type IV. For the analysis of laminin incubation was at 37 °C, and for collagen type IV was at 25 °C, using a 100:2 ratio. Incubation times were 2 and 24 h, and the reactions were stopped by placing the mixture in ice bath and adding 2 μL of 20% glycerol bromophenol blue and 3 μL of 2% DTT 2%-SDS (Guerrero et al., 2010). Changes in the molecules were visualized by electrophoresis (SDS-PAGE) under reduced conditions using the Laemmli system.

2.16. Zymogram analysis of gelatinolytic activity

To study the colombienases gelatinolytic activity, gelatin–zymography was performed as previously

Author's personal copy

described by Guerrero et al. (2010). Briefly, proteins were diluted in SDS sample buffer without reducing agents and electrophoresed on 12% SDS–polyacrylamide gels co-polymerized with 3 mg/mL of gelatin. Next, the gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS and then incubated in a zymography buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$) at 37 °C for 18 h. After incubation, gels were stained with Coomassie blue for 2 h and then distained. The presence of gelatinolytic activity was recognized as clear bands on a uniform blue background.

2.17. Indirect hemolytic activity

The indirect hemolytic activity was tested according to Theakston and Reid (1983) method. This test used a medium consisting of a solidified mixture of agar, blood and egg-yolk in Petri dishes. Then 20 μL of BcV or colombienase-1 and colombienase-2 at concentrations of 25 and 50 μg/20 μL were place in 3 mm holes. The plates were incubated in a humid chamber at 37 °C for 24 h and the diameters of the hemolysis halos of each dose were measured. Saline solution was used as negative control and crude venoms of *B. colombiensis* and *Crotalus durissus cumanensis* as positive controls. Each experiment was performed in triplicate.

2.18. Edema-forming activity

The edema activity of BcV and colombienases was tested by Yamakawa et al. (1976) method. For the determination of minimum edema-forming dose (MED), five sample doses were prepared using isotonic saline solution as diluent. Dilutions of each BcV and colombienases solutions (0.05 mL) were injected in the right plantar pad of four mice ($n = 4$). The left plantar pads of the mice were injected with 0.05 mL of isotonic saline solution as controls.

One hour after the injections, the mice were sacrificed with CO_2, next both injected limbs at the tarsal joint were sectioned and weighed using an analytical balance. The results were expressed as MED, which is defined as the amount of venom that induces, after 1 h, 30% edema with respect to the limb control. For the determination of edema-forming activity from colombienase-1 and colombienase-2, the MED was calculated using BcV (3 μg/50 μL) as reference (Girón et al., 2011).

2.19. Recognition of colombienases by polyvalent antivenom

The detection of specific antibodies against colombienases was carried out using the double immunodiffusion technique (Ouchterlony, 1958). The antivenom (SAOP) was developed at the Faculty of Pharmacy of the Universidad Central de Venezuela and neutralizes at least 2 mg of *B. colombiensis* venom per mL. Slides were pretreated with 1% agarose and then covered with 3.5 mL of 2% agarose. Once the gel solidified, equidistant wells were drilled and distributed by making one central hole and six peripheral. In the central hole, 10 μL of SAOP was placed. In each peripheral well, 10 μL of colombienases at dilutions of 26.1, 13.05 and 6.53 μg/well (colombienase-1) and 17.0, 8.5 and 4.25 μg/well (colombienase-2) were placed and incubated at 37 °C for 24 h. Once the precipitation band was detected, the plate was washed with 0.9% saline and stained with Coomassie Blue.

2.20. Cardiac myoblasts hanging drop

Hanging-drop cultures on glass coverslips were prepared from 5 days old chick embryo heart fragments. The explants were grown in thin chicken plasma dilute 1:2 with Tyrode solution which contained a small amount of extract from 8 day old chicken embryos (Alvarez and Barasa, 1996). Only primary cultures were employed; in general they were 24 h old. The cultures were incubated at 38 °C and before the experiment they were examined with the microscope and only those that showed a well developed zone of outgrowth were selected The culture selected were divided into 2 groups: control and treated. The estimation of LC_{50} was carried out by selecting the cells according to their morphological aspects that included collapsed or rounded cells, for which the proportion of cellular death was determined (in terms of percentage of response of all or nothing), depending on the concentration of BcV. The cardiac myoblasts monolayers were treated at different dilutions of BcV (20, 10, 5, 2.5 and 1.25 μg/100 μL) and incubated for 1 h at 37 °C. Acute toxicity test allowed the determination of the LC_{50}, which represents the venom amount necessary, in direct contact with culture myoblasts for 1 h exposure at 37 °C, to induce 50% of cell death. Based on the concentration–response curve, the calculated LC_{50} was 40.38 μg/mL, with a 37.67 μg/mL sub-lethal range and a supra-lethal of 43.11 μg/mL.

2.21. Cellular damage of BcV and colombienases

The cardiac myoblast monolayers obtained by hanging-drop and selected for treatment were placed in direct contact with BcV or colombienases (50 μg/100 μL). Once the treatment was finished, they were incubated in DiOC1 fluorochrome (Jacobberger et al., 1984; Poot, 1998) for 1 min and washed three times with Tyrode solution and observed for living cells in an inverted fluorescence microscope (Olympus IX71) equipped with a thermal plate at 37 °C and a Blue excitation (λ_{exc} Blue) filter. Images were captured with a DP71CCD camera and processed with Adobe Photoshop CS3.

The cellular response to acute exposure with venom is of quental type, all or nothing, cell death being the final expression of damage. The different concentrations effect of BcV on the cell groups of cardiac myoblasts hanging-drop primary culture was evaluated by determining the proportion, in terms of the percentage, of cells with rounded shape associated with cell death.

2.22. Statistical analysis

Data are expressed as mean ± SD, using $n = 3$. Means and standard deviations were calculated using Microsoft Excel. In case of differences between the experimental groups, comparisons were performed by one-way ANOVA test using the Tukey's multiple comparison (GraftPad).

Author's personal copy

3. Results

3.1. Colombienases

Colombienases 1 and 2 with fibrinogenolytic activity were isolated from BcV using a Mono S HR 10/10 cation exchange column followed by Phenyl–Sepharose hydrophobic interaction and finally a Bio-Silect 125-5 or 10/300GL Superose 12 exclusion chromatography columns. The purity criterion was assessed by a 12% SDS-PAGE under reduced conditions and confirmed by Symmetry® C-18 phase reverse chromatography (Girón et al., 2013). Electrophoresis of *B. colombiensis* venom showed bands between 14 and 70 kDa. The fractions F1, F2 and F3, obtained from cation exchange chromatography, showed bands around 25 kDa. The silver nitrate stain revealed additional bands between 14 and 6 kDa (data not shown). Protein separation continued with the isolation of active metalloproteinases with the highest degree of purity. Colombienase-1 and colombienase-2 showed a single homogeneous band of 25 kDa (Fig. 1).

3.2. Hemorrhagic and fibrinogenolytic activities

The hemorrhagic and fibrinolytic activities of BcV, active fractions and colombienase-1 and colombienase-2 are shown in Table 1. The BcV presented 250 mm^2/50 µg and 30.6 mm^2/µg of hemorrhagic and fibrinolytic activities, respectively.

The cation exchange F2 fraction proved to be higher in fibrinolytic (70.2 mm^2/µg) activity but with less hemorrhagic activity (18 mm^2/50 µg). Colombienases 1 and 2 isolated from F2 fraction did not have hemorrhagic activity at a dose of 50 µg, with fibrinolytic activities similar in fibrin plates in presence or absence of plasminogen. Both colombienases had no plasminogen activation activity.

Table 1
Hemorrhagic and fibrinolytic activities of BcV, fractions and colombienases.

B. colombiensis Venom/Fractions	Hemorrhagic Activity mm^2/50 µg	Fibrinolytic Activity Pg^+ mm^2/µg
BcV	250	30.6
Fractions		
Cationic interchange		
F1	28	9.5
F2	18	70.2
F3	229	64.4
Hydrophobic Interaction (F2)		
F2-A	–	85.7
F2-B	–	131.3
Molecular Exclusion		
Colombienase-1	–	94.2
Colombienase-2	–	211.6

Pg^+: in presence of Plasminogen.
– Negative activity.

Colombienase-1 at 100:0.5, 100:0.1 and 100:5 (fibrinogen:colombienase) ratios induced rapid degradation of the fibrinogen Aα chains (30 s), which was complete at 15 min, without apparent degradation of γ chains (data not shown). Colombienase-2, at 100:1 ratio, additionally induced γ chains degradation observed at 120 min (Fig. 2). Fibrinogen treated with BcV and/or colombienases at 100:1 ratio, after 4 h, lost the coagulant activity by thrombin. The results also showed that the metal chelants (EDTA, EGTA, or 1,10-phenanthroline) and DTT completely blocked this activity (data not shown).

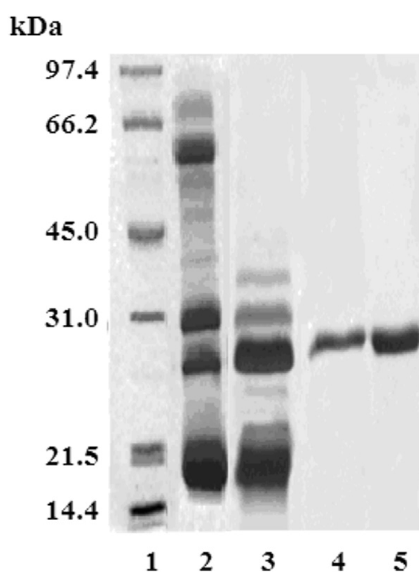


Fig. 1. SDS-PAGE under reduced conditions on a 12% Gel): 1) Molecular weight markers; 2) BcV; 3) Cationic Interchange F2 fraction; 4) colombienase-1; 5) colombienase-2.

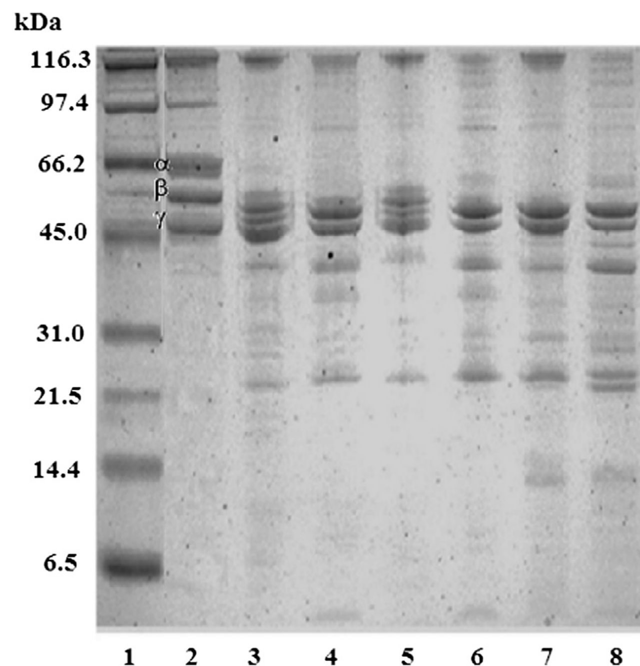


Fig. 2. Fibrinogenolytic activity of colombienase-2 at a 100:1 ratio and 24 °C. SDS-PAGE under reduced conditions on a 10% Gel. 1) Molecular weight markers; 2) Control fibrinogen (Fg) chains; 3 and 4) Fg + colombienase for 5 and 120 min incubation time.

Author's personal copy

3.3. Activity on extracellular matrix proteins and basal membranes

3.3.1. Collagen

Under the experimental conditions tested, no effect of BcV or colombienases on collagen type IV molecule from human placenta basal membranes was observed. The collagen control showed the same pattern as the samples incubated with colombienases-1 (Fig. 3). The BcV and colombienase-2 showed a pattern similar to the one obtained with colombienase-1 (results not shown).

3.3.2. Fibronectin

Fibronectin control, purified from human plasma, showed a band of ~210 kDa. Colombienase-1 at a 100:1 ratio (Fn:enzyme), at 37 °C and 2 h of incubation, induced the disappearance of high molecular weight bands, generating products between 148 and 65 kDa molecular weights. After 24 h incubation, the corresponding band of molecular weight between 98 and 36 kDa were intensified (Fig. 4). Colombienase-2 induced a similar effect (results not shown).

3.3.3. Laminin

Colombienases, under experimental conditions, at 100:2 and 100:5 protein:venom ratios and 37 °C for 2 and 24 h incubation did not induce changes in electrophoretic migration of laminin chains (data not shown).

3.4. Gelatin zymography

Fig. 5 shows an acrylamide–gelatin gel zymography of BcV and colombienases. The results demonstrated that BcV presented gelatinolytic activity evidenced by clear bands with relative molecular masses between ~148 and 98 kDa and also near ~22 kDa. Colombienase-2 presented an active band at ~22 kDa. In contrast, colombienase-1 showed a weak ~22 kDa band (Fig. 5).

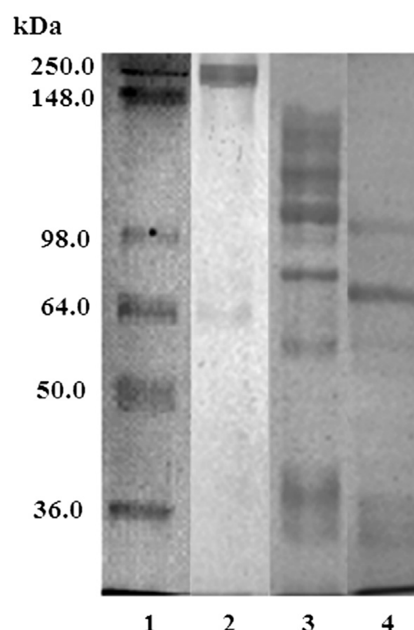


Fig. 4. Effect of colombienase-1 on fibronectin at a 100:1 ratio and 37 °C. SDS-PAGE, under reduced conditions on a 7.5% Gel. 1) Molecular weight markers; 2) Fibronectin control; 3 and 4) Fibronectin + colombienase at 2 and 24 h incubation time.

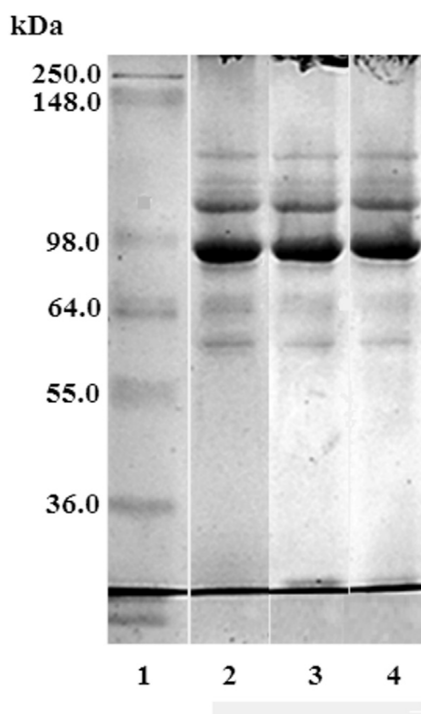


Fig. 3. Effect of colombienase-1 on human type IV collagen at a 100:2 ratio and 25 °C. SDS-PAGE under reduced conditions on a 5–10% gradient gel. 1) Molecular weight markers; 2) Collagen control; 3 and 4) Collagen + colombienase at 2 and 24 h incubation time.

3.5. Edematogenic activity of BcV and colombienases

A significant edema developed in the footpad the mice after a subcutaneous injection of BcV (3 μg/mouse) (Girón

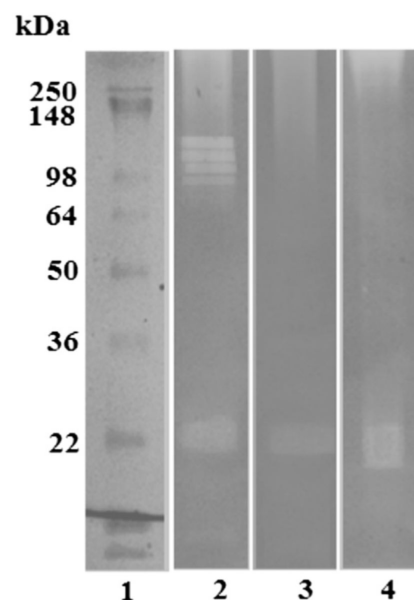


Fig. 5. Acrylamide-gelatin gel zymography of *Bothrops colombiensis* venom (BcV) and colombienases. 1) Molecular weight markers; 2) BcV (5 μg); 3) colombienase-1 (10 μg); 4) colombienase-2 (5 μg).

Author's personal copy

et al., 2013). A restrained edema was also observed with colombienase-1 and colombienase-2 (3.9 μg/mouse) inducing 12.66% and 8.45% MEDs, respectively (Table 2).

3.6. Hemolytic activity

B. colombiensis venom showed a 176.71 mm^2/50 μg hemolytic area. Treatment with 2 mM EDTA abolished the hemolytic activity. Incubation of colombienases did not develop hemolytic activity compared with the positive control (*C. durissus cumanensis* venom: 192 mm^2/50 μg) (Table 2).

3.7. Colombienases recognition by polyvalent antivenom

Via a double Immunodiffusion method, colombienase-2 did not react with polyvalent antivenom (SAOP). In contrast, colombienase-1 showed a precipitation band with the three evaluated doses (Fig. 6).

3.8. B. colombiensis *venom and colombienases toxic effects on monolayers of cardiac myoblasts cultures*

The myoblasts treated with BcV and colombienases cultures showed diverse changes. It highlights the orthodox form filamentous mitochondria, with intense fluorescence in contrast to the loss of this conformation in cardiac myoblasts treated with venom. In terms of the monolayer, loss of cell adhesion to the substrate, irregular monolayer areas, as well as cell rounding in suspension were observed. In contrast, the monolayer control and remained adhered to the substrate and maintained a continuity of cells.

3.9. Acute toxicity

The changes induced by BcV were evaluated by light microscopy, as well as through fluorescent probes, specifically through the type $DiOC_1$ lipophylic fluorochrome cationic, which allows studying the damage at a subcellular level. The results obtained with BcV showed a cell structure collapsed, with intense diffuse fluorescence, mitochondrial and perinuclear areas lacking structural definition. In contrast, the population of cardiac myoblasts controls, as well as those treated with colombienase-1 and colombienase-2, showed intense fluorescence; highlighting the conventional structure of mitochondria (shaped like elongated canes) throughout the cytoplasm and in the peri-nuclear area. The most prominent nuclear alterations were condensation and fragmentation of nuclear material.

Table 2
Hemolytic and Edema-forming activities induced by BcV and colombienases.

Venom/fractions	Edema-forming activity (% edema)	Hemolytic activity (lysis areas in mm^2)
BcV	30.0	176.7
Colombienase-1	12.7	0
Colombienase-2	8.5	0

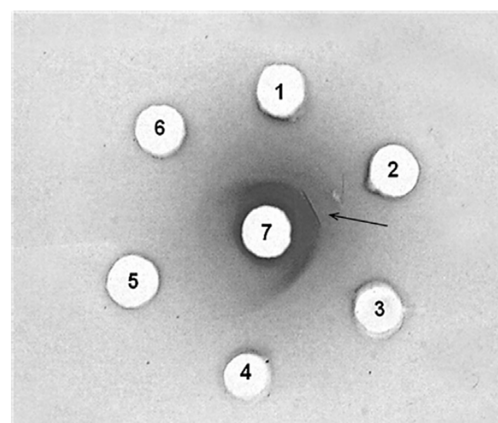


Fig. 6. Immunodiffusion of colombienases and SAOP antivenom on a 2% agarose gel. 1–3) Colombienase-1 at 26, 13 and 6.5 μg/well; 4–6) colombienase-2 at 17, 8.5 and 4.25 μg/well; 7) 10 μL of SAOP. The samples were incubated for 24 h incubation at 37 °C.

4. Discussion

To date, many investigators have been trying to improve the safety and efficacy of fibrinolytic therapy through the isolation of fibrinolytic enzymes from different snake venoms (Maruyama et al., 1993; Sugiki et al., 1995; Cintra et al., 2012). Amid these, a good deal of consideration has been dedicated to fibrinolytic enzymes isolated from *Bothrops* snake venoms (De Roodt et al., 2003; Girón et al., 2008; Gomes et al., 2011).

In the current work, several experiments were carried out to test the colombienases 1 and 2 (non-hemorrhagic metalloproteinases from *B. colombiensis* snake venom) *in vitro* fibrinolytic effects (Girón et al., 2013). The results showed that colombienases directly act on the fibrin polymer, without the fibrinolytic system activation. Both colombienases can promote anti-coagulant effects, given that they degraded the Aα and Bβ chains of fibrinogen; additionally colombienase-2 degrade γ chains, as well as α-polymers, dimers γ-γ and α and β chains of fibrin (Girón et al., 2013). There are few descriptions of fibrinogenases that degrade the fibrinogen γ chains. Various authors (Liang et al., 2005) reported venom fractions degrading the γ chains, such as FII(a) (*Agkistrodon acutus*) fraction, which degrades the γ chains. Serrano et al. (1993) evaluated three proteases isolated from *Bothrops moojeni* venom, two serine proteases and one metalloproteinase that reduced the three fibrinogen chains. Recently, Rodríguez-Acosta et al. (2010) reported the γ chain degradation with *Bothrops isabelae* crude venom and its fibrinogenolytic fractions.

Results related to colombienases fibrinogenolytic activity show that they act as α-fibrinogenases. Most of the α-fibrinogenases are metalloproteinases, which may or may not present proteolytic and/or hemorrhagic activities on basal membranes proteins (Gay et al., 2005; Sánchez and Swenson, 2007). Since they do not activate plasminogen because their action is directed on fibrinogen, they may induce few systemic alterations. Laminin and type IV collagen were resistant to the actions of colombienases. This finding could explain the absence of colombienases'

Author's personal copy

hemorrhagic activity on mice. The dose used (50 μg) did not have hemorrhagic effects subcutaneously and did not elicited a hemorrhagic syndrome in the mice.

Colombienases lacked hemorrhagic activity, and their fibrino (geno) lytic activity is inactivated by metalloproteinases inhibitors; they can be classified as P-Ib type. The absence of hemorrhagic activity may be due to a lower affinity by components of extracellular matrix, or its structure since metalloproteinases P-Ib class do not have disintegrin and cysteine rich domains. They present only the metalloproteinase domain, characterized by the HEXXHXXGXXH consensus sequence, which seem to determine their union to collagen and other extracellular matrix components (Clissa et al., 2006).

Fibronectin is a protein which interacts with coagulation and fibrinolysis components and thus regulates processes such as platelet aggregation and adhesion, tissue remodeling and fibrinolysis mediated plasminogen activators (Lucena et al., 2007). Both colombienases hydrolyze fibronectin molecule generating different degradation fragments. This effect has been reported for snake venoms containing metalloproteinases, such as *Bothrops atrox*: atroxlysin (Sánchez et al., 2010); *Bothrops leucurus*: leucurolysin-A and leucurolysin-B, (Sánchez and Swenson, 2007; Bello et al., 2006); *B neuwiedi*: neuwiedasa (Rodrigues et al., 2000); *Bothrops asper*: BaH4 (Franceschi et al., 2000); as well as in disintegrins, such as colombistatin from *B. colombiensis* (Sánchez et al., 2009) and cumanastatin from *C. durissus cumanensis* (Da Silva et al., 2009), among others.

Gelatinolytic activity was evaluated by zymography with BcV having several high molecular weight bands (<148–>64 kDa) that may correspond to P-III class metalloproteinases and another band was detected at ~22 kDa region, which was also evident with colombienase-2. On the contrary, colombienase-1 presented a weak gelatinolytic activity in this experimental condition. This activity was EDTA inhibited, corroborating its metalloproteinase nature. Similar results have been reported by Terra et al. (2009), comparing *B. lanceolatus* gelatinolytic activity from this class P-I metalloproteinases-rich venom and *Bothrops jararaca* rich in class P-III metalloproteinases, finding an increased gelatinolytic activity in the latter.

Our results suggest that colombienases have little relevance in the edema produced by *B. colombiensis* ophidic accidents (Rodríguez-Acosta et al., 2000). The edema in bothropic envenomation pathogenesis has been associated to the phospholipases (PLA_2) myotoxic activity acting on the smooth muscle of the lymph vessels, inducing contraction and irreversible cellular damage (Mora et al., 2008). However, with *B. jararaca* venom, it has been established that the metalloproteinases play a major role in inflammatory reactions than the serino proteases or phospholipases (Zychar et al., 2010). Yet, BjussuMP-II, a metalloproteinase isolated from *Bothrops jararacussu*, presented a low edema-forming activity with respect to crude venom (Marcussi et al., 2007). Neuwiedasa, a 22 kDa metalloproteinase isolated from *Bothrops neuwiedi* venom free of hemorrhagic activity, contributes to local damage inducing edema, inflammatory infiltrate, myotoxicity and degradation of extracellular matrix components (Rodrigues et al., 2001). BaP1, class PI metalloproteinase from *B. asper* venom, induces edema, myonecrosis and moderate bleeding, with leukocytes polymorph nuclear and macrophages infiltrate (Rucavado et al., 2002; Moreira et al., 2012). Porthidin-1, class P-I hemorrhagic metalloproteinase isolated from *Porthidium lansbergii hutmanni*, degrades fibronectin, gelatin, casein, fibrinogen, fibrin and induces mice foot-pad edema (Girón et al., 2011).

Results from the present study showed that the colombienase-1 is recognized by the polyvalent antivenom (SAOP). In contrast, colombienase-2 was not recognized by this antivenom suggesting that this protein presents low antigenic determinants or that their presence in BcV has a low percentage, unable to induce an antibody response. Now that the involvement of colombienases in fibrinogenolytic activity is known, it would be desirable to incorporate them in the inoculum used in immunization protocols for antivenom production, which may ensure better antivenom effectiveness.

Table 3 provides the assayed characteristics of both colombienases in the current work. These biological activities confer therapeutic potential for these enzymes since they contain a high fibrinogenolytic activity independent of plasminogen activation and devoid of hemorrhagic activity as well as other activities. These metalloproteinases might be explored as thrombolytic agents given that they dissolve the fibrin clot or prevent its formation.

Enzymes showing diverse activities present in bothropics venoms may act on a wide spectrum of organs and tissues causing multiple local and systemic effects, such as severe muscle and tissue necrosis, vascular endothelial damage and coagulation disorders (Queiroz et al., 1984; Milani et al., 1997). In order to evaluate the therapeutic potential of the colombienases and elucidate if they have no cytotoxic potential and/or necrotoxic activities, a hanging drop cellular model was used in this study. At the cellular and sub-cellular levels, changes were easily

Table 3
General characteristics of colombienases

Activity	Colombienase-1	Colombienase-2
Fibrinolytic (fibrin plate)	94.16 $mm^2/\mu g$	211.57 $mm^2/\mu g$
Fibrin degradation (SDS-PAGE)	Positive	Positive
Fibrinogen degradation	Chains Aα fast degradation Bβ slow degradation. γ not altered	Chains Aα fast degradation. Bβ slow degradation. γ low degradation.
Plasminogen activation	Negative	Negative
Gelatinolytic	Positive (weak)	Positive
Proteolytic under:		
Fibronectin	Positive	Positive
Collagen type IV	Negative	Negative
Laminin	Negative	Negative
Hemorrhagic on skin	Negative	Negative
Edematizing	Weak	Weak
Coagulant	Negative	Negative
Hemolytic	Negative	Negative
Platelet aggregation effects	Negative	Negative
Immunological recognition	Positive	Negative

Author's personal copy

visualized. Fluorescent probes allow organelle labeling with $DiOC_1$ fluorochrome cationic lipophilic.

Under VBi action, a reduction of cell adhesion to the substrate, increase in intercellular spaces, and cellular organization loss were observed, which compromised cell viability, with a decrease and condensation of cytoplasm, cell rounded appearance, presence of filamentous structures, as well as changes in mitochondrial pattern. The most prominent nuclear alterations were condensation and fragmentation of nuclear material; these observed morphological changes have been associated with cell death by apoptosis. In comparison, colombienases (50 μg/mL) did not induce significant changes in LC_{50}.

Myoblast response to colombienase-1 and colombienase-2 exposure was similar to the control without treatment, showing intense brightness of the emitted fluorescence, highlighting the conventional structure of mitochondria, shaped like cones elongated throughout the cytoplasm and in the peri-nuclear area. These results indicate that the colombienases, highly fibrinogenolytic, do not seem involved in the BcV cytotoxicity. The class P-I metalloproteinases lack of disintegrin domains and rich-in-cysteine appear to be important for the induction of apoptosis (Tanjoni et al., 2005). Other authors, using canine renal epithelial cell monolayers, have shown similar results with *B. moojen*, *Bothrops alternatus* and *Lachesis muta muta* venoms (Collares-Buzato et al., 2002; Nascimento et al., 2004; Damico et al., 2007).

The potential therapeutic activity of the colombienases is similar to other described snake venom molecules such as kistomin, a P-I class metalloproteinase, which has antithrombotic activity both *in vitro* and *in vivo* (Hsu et al., 2007). Girón et al. (2011) described Porthidin-1 from *P. lansbergii hutmanni*, which was two times more specific for fibrinolytic activity than the crude venom. They assumed that these proteinases have clinical relevance as thrombolytic agents. Teklemariam et al. (2011) proposed that the GST-disintegrin-like snake venom peptide designated as acocostatin, from *Agkistrodon contortrix contortrix* was capable of inducing apoptosis of Human Umbilical Vein Endothelial Cells and HeLa cells; it also prevented migration of SK-Mel-28 cells having therapeutic value in the treatment of cancer and other hemostatic diseases. With the development of the peptide synthesis industry and extraction of synthetic products, using known and fibrinolytic aminoacid sequences of toxins, there could be important clinical use of these colombienases as fibrinolytic agents.

Ethical statement

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

Acknowledgments

The authors thank Luis F. Navarrete, Zoila Carvajal and Amparo Gil San Juan for their technical assistance. This work was funded by the Science and Technology Foundation (FONACIT, Grant PG-2005000400), Venezuela; CDCH de la UCV (Grant: PG 09-8760-2013); the Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela and the National Natural Toxins Research Center at Texas A&M University-Kingsville, Kingsville, Texas USA: NCRR/Viper Resource Center P40 OD01960-10.

Conflict of interest

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

References

Abou-Chebl, A., 2011. Intra-arterial therapy for acute ischemic stroke. Neurotherapeutics 8, 400–413.

Alvarez, M., Barasa, A., 1996. Efecto de d-carnitina sobre mioblastos cardiacos en cultivo primario. Arch. Venez. Farm. Terap. 15, 37–42.

Bello, C.A., Hermogenes, A.L., Magalhaes, A., Veiga, S.S., Gremski, L.H., Richardson, M., Sanchez, E.F., 2006. Isolation and biochemical characterization of a fibrinolytic proteinase from *Bothrops leucurus* (white-tailed jararaca) snake venom. Biochimie 88, 189–200.

Cintra, A.C., De Toni, L.G., Sartim, M.A., Franco, J.J., Caetano, R.C., Murakami, M.T., Sampaio, S.V., 2012. Batroxase, a new metalloproteinase from *B. atrox* snake venom with strong fibrinolytic activity. Toxicon 60, 70–82.

Clissa, P.B., Lopes-Ferreira, M., Della-Casa, M.S., Farsky, S.H., Moura-da-Silva, A.M., 2006. Importance of jararhagin disintegrin-like and cysteine-rich domains in the early events of local inflammatory response. Toxicon 47, 591–596.

Collares-Buzato, C.B., Le Sueur, L.P., Cruz-Hofling, M.A., 2002. Impairment of the cell-to-matrix adhesion and cytotoxicity induced by *Bothrops moojeni* snake venom in cultured renal tubular epithelia. Toxicol. Appl. Pharmacol. 181, 124–132.

Colman, R.W., Clowes, A.W., George, J.N., Goldhaber, S.Z., Marder, V.J., 2006. Overview of hemostasis. In: . In: Colman, R.W., Clowes, A.W., Goldhaber, S.Z., Marder, V.J., George, J.N. (Eds.), Hemostasis and Thrombosis: Basic Principles and Clinical Practice, fifth ed., Cap. 1. Editorial Lippincott Williams & Wilkins, pp. 3–16.

Da Silva, M., Lucena, S., Aguilar, I., Rodríguez-Acosta, A., Salazar, A.M., Sánchez, E.E., Girón, M.E., Carvajal, Z., Arocha-Piñango, C.L., Guerrero, B., 2009. Anti-platelet effect of cumanastatin 1, a disintegrin isolated from venom of South American Crotalus rattlesnake. Thromb. Res. 123, 731–739.

Damico, D.C., Nascimento, J.M., Lomonte, B., Ponce-Soto, L.A., Joazeiro, P.P., Novello, J.C., Marangoni, S., Collares-Buzato, C.B., 2007. Cytotoxicity of *Lachesis muta muta* snake (bushmaster) venom and its purified basic phospholipase A2 (LmTX-I) in cultured cells. Toxicon 49, 678–692.

De Roodt, A.R., Litwin, S., Vidal, J.C., 2003. Hemorrhagic activity of *Bothrops* venoms determined by two different methods and relationship with proteolytic activity on gelatin and lethality. Toxicon 41, 949–958.

Franceschi, A., Rucavado, A., Mora, N., Gutiérrez, J.M., 2000. Purification and characterization of BaH4, a hemorrhagic metalloproteinase from the venom of the snake *Bothrops asper*. Toxicon 38, 63–77.

Gay, C.C., Leiva, L.C., Maruñak, S., Teibler, P., Acosta de Pérez, O., 2005. Proteolytic, edematogenic and myotoxic activities of a hemorrhagic metalloproteinase isolated from *Bothrops alternatus* venom. Toxicon 46, 546–554.

Girón, M.E., Salazar, A.M., Aguilar, I., Pérez, J.C., Sánchez, E.E., Arocha-Piñango, C.L., Rodríguez-Acosta, A., Guerrero, B., 2008. Hemorrhagic, coagulant and fibrino(geno)lytic activities of crude venom and fractions from mapanare (*Bothrops colombiensis*) snakes. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 147, 113–121.

Girón, M.E., Estrella, A., Sánchez, E.E., Galán, J., Tao, W.A., Guerrero, B., Salazar, A.M., Rodríguez-Acosta, A., 2011. Purification and characterization of a metalloproteinase, Porthidin-1, from the venom of Lansberg's hog-nosed pitvipers (*Porthidium lansbergii hutmanni*). Toxicon 57, 608–618.

Author's personal copy

Girón, M.E., Rodríguez-Acosta, A., Salazar, A.M., Sánchez, E.E., Galán, J., Ibarra, C., Guerrero, B., 2013. Isolation and characterization of two new non-hemorrhagic metalloproteinases with fibrinogenolytic activity from the mapanare (*Bothrops colombiensis*) venom. Arch. Toxicol. 87, 197–208.

Gomes, M.S., de Queiroz, M.R., Mamede, C.C., Mendes, M.M., Hamaguchi, A., Homsi-Brandeburgo, M.I., Sousa, M.V., Aquino, E.N., Castro, M.S., de Oliveira, F., Rodrigues, V.M., 2011. Purification and functional characterization of a new metalloproteinase (BleucMP) from *Bothrops leucurus* snake venom. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 290–300.

Guerrero, B., Arocha-Piñango, C.L., Pinto, M.A., Müller, C.A., Gil San Juan, A., Amorim, S., Perales, J., 2001. Thrombolytic effect of lonomin V in a rabbit jugular vein thrombosis model. Blood Coagulation Fibrinolysis 12, 521–529.

Guerrero, B., Finol, H.J., Reyes-Lugo, M., Salazar, A.M., Sánchez, E.E., Estrella, A., Roschman-González, A., Ibarra, C., Salvi, I., Rodríguez-Acosta, A., 2010. Activities against hemostatic proteins and adrenal gland ultrastructural changes caused by the brown widow spider *Latrodectus geometricus* (Araneae:Theridiidae) venom. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 151, 113–121.

Gutiérrez, J.M., Gené, J.A., Rojas, G., Cerdas, L., 1985. Neutralización of proteolytic and hemorrhagic activities of Costa Rican snake venoms by polyvalent antivenom. Toxicon 23, 887–893.

Hamburger, V., Hamilton, H.L., 1992. A series of normal stages in the development of the chick embryo. 1951. Dev. Dyn. 195, 231–272.

Hsu, C.C., Wu, W.B., Chang, Y.H., Kuo, H.L., Huang, T.F., 2007. Antithrombotic effect of a protein-type I class snake venom metalloproteinase, kistomin, is mediated by affecting glycoprotein Ib-von Willebrand factor interaction. Mol. Pharmacol. 72, 984–992.

Jacobberger, W.J., Honan, P.K., Hare, J.D., 1984. Flow cytometric analysis of blood cells stained with the cyanine $DiOC_1$ [3]: reticulocyte quantification. Cytometry 5, 589–600.

Jonebring, A., Lange, U., Bucha, E., Deinum, J., Elg, M., Lövgren, A., 2012. Expression and characterization of recombinant ecarin. Protein J. 31, 353–358.

Kini, R.M., 2005. Serine proteases affecting blood coagulation and fibrinolysis from snake venoms. Pathophysiol. Haemost. Thromb. 34, 200–204.

Laemmli, U., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. Nature 227, 608–609.

Liang, X.X., Zhou, Y.N., Chen, J.S., Qiu, P.X., Chen, H.Z., Sun, H.H., Wu, Y.P., Yan, G.M., 2005. Enzymological characterization of FII(a), a fibrinolytic enzyme from *Agkistrodon acutus* venom. Acta Pharmacol. Sin. 26, 1474–1478.

Lowry, O.H., Rosembrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lucena, S., Arocha-Piñango, C.L., Guerrero, B., 2007. Fibronectina. Estructura y funciones asociadas a la hemostasia. Invest. Clin. 48, 249–262.

Marcussi, S., Bernardes, C.P., Santos-Filho, N.A., Mazzi, M.V., Oliveira, C.Z., Izidoro, L.F., Fuly, A.L., Magro, A.J., Braz, A.S., Fontes, M.R., Giglio, J.R., Soares, A.M., 2007. Molecular and functional characterization of a new non-hemorrhagic metalloprotease from *Bothrops jararacussu* snake venom with antiplatelet activity. Peptides 28, 2328–2339.

Markland, F.S., 1998. Snake venoms and the hemostatic system. Toxicon 36, 1749–1800.

Marsh, N.A., Arocha-Piñango, C.L., 1972. Evaluation of the fibrin plate method for estimating plasminogen activator. Thromb. Diathes. Haemorrh. 28, 75–88.

Maruyama, M., Tanigawa, M., Sugiki, M., Yoshida, E., Mihara, H., 1993. Purification and characterization of low molecular weight fibrinolytic/hemorrhagic enzymes from snake *(Bothrops jararaca)* venom. Enzyme. Protein 47, 124–135.

Milani Jr., R., Jorge, M.T., de Campos, F.P., Martins, F.P., Bousso, A., Cardoso, J.L., Ribeiro, L.A., Fan, H.W., França, F.O., Sano-Martins, I.S., Cardoso, D.I., de Fernandez, C., Fernandes, J.C., Aldred, V.L., Sandoval, M.P., Puorto, G., Theakston, R.D., Warrell, D.A., 1997. Snake bites by the jararacu-u (*Bothrops jararacussu*): clinicopathological clinicopathological studies of 29 proven cases in Sao Paulo State, Brazil. Q. J. Med. 90, 323–334.

Mora, J., Mora, R., Lomonte, B., Gutiérrez, J.M., 2008. Effects of *Bothrops asper* snake venom on lymphatic vessels: insights into a hidden aspect of envenomation. PLoS. Negl. Trop. Dis. 2, e318.

Moreira, V., Dos-Santos, M.C., Nascimento, N.G., da Silva, H.B., Fernandes, C.M., D'Império Lima, M.R., Teixeira, C., 2012. Local inflammatory events induced by *Bothrops atrox* snake venom and the release of distinct classes of inflammatory mediators. Toxicon 60, 12–20.

Moura-da-Silva, A.M., Furlan, M.S., Caporrino, M.C., Grego, K.F., Portes-Junior, J.A., Clissa, P.B., Valente, R.H., Magalhães, G.S., 2011. Diversity of metalloproteinases in *Bothrops neuwiedi* snake venom transcripts: evidences for recombination between different classes of SVMPs. BMC. Genet. 12, 94.

Mussoni, L., Raczka, E., Chmielewska, J., Donati, M.B., Latallo, Z.S., 1979. Plasminogen assay in rabbit, rat and mouse plasma using the chromogenic substrates S-2251. Thromb. Res. 15, 341–349.

Nascimento, J.M., Collares-Buzato, C.B., Hyslop, S., 2004. Cytotoxic action of *Bothrops alternatus* snake venom in cultured MDCK cells. In: Abstract from Eighth Symposium of the Pan American Section of the International Society on Toxinology. Angra dos Reis, Rio de Janeiro, Brazil, p. 129.

NIH, 1985. Principles of Laboratory Animal Care, vol. 85. National Institute of Health, USA, pp. 1–112.

Ouchterlony, O., 1958. Diffusion-in-gel methods for immunological analysis. Prog. Allergy 5, 1–78.

Poot, M., 1998. Staining of mitochondria. In: Cell Biology: a Laboratory Handbook. Academy Press, pp. 513–517.

Queiroz, L.S., Neto, H.S., Rodrigues-Simioni, L., Prado-Franceschi, J., 1984. Muscle necrosis and regeneration after envenomation by *Bothrops jararacussu* snake venom. Toxicon 22, 339–346.

Rau, J.C., Beaulieu, L.M., Huntington, J.A., Church, F.C., 2007. Serpins in thrombosis, hemostasis and fibrinolysis. J. Thromb. Haemostasis (Suppl 1), 102–115.

Rodrigues, V.M., Soares, A.M., Guerra-Sá, R., Rodrigues, V., Fontes, M.R., Giglio, J.R., 2000. Structural and functional characterization of neuwiedase, a nonhemorrhagic fibrin(ogen)olytic metalloprotease from *Bothrops neuwiedi* snake venom. Arch. Biochem. Biophys. 381, 213–224.

Rodrigues, V.M., Soares, A.M., Andrião-Escarso, S.H., Franceschi, A.M., Rucavado, A., Gutiérrez, J.M., Giglio, J.R., 2001. Pathological alterations induced by neuwiedase, a metalloproteinase isolated from *Bothrops neuwiedi* snake venom. Biochimie 83, 471–479.

Rodríguez-Acosta, A., Uzcátegui, W., Azuaje, R., Aguilar, I., Girón, M.E., 2000. A clinical and epidemiological analysis of accidental bites by snakes of the genus *Bothrops* in Venezuela. Rev. Cubana Med. Trop. 52, 90–94.

Rodríguez-Acosta, A., Sánchez, E.E., Márquez, A., Carvajal, Z., Salazar, A.M., Girón, M.E., Estrella, A., Gil, A., Guerrero, B., 2010. Hemostatic properties of Venezuelan *Bothrops* snake venoms with special reference to *Bothrops isabelae* venom. Toxicon 56, 926–935.

Rucavado, A., Escalante, T., Teixeira, C.F., Fernándes, C.M., Diaz, C., Gutiérrez, J.M., 2002. Increments in cytokines and matrix metalloproteinases in skeletal muscle after injection of tissue-damaging toxins from the venom of the snake *Bothrops asper*. Mediators Inflamm. 11, 121–128.

Salazar, A.M., Rodríguez-Acosta, A., Girón, M.E., Aguilar, I., Guerrero, B., 2007. A comparative analysis of the clotting and fibrinolytic activities of the mapanare (*Bothrops atrox*) snake venom from different geographical areas in Venezuela. Thromb. Res. 120, 95–104.

Sánchez, E.F., Swenson, S., 2007. Proteases from South American snake venoms affecting fibrinolysis. Curr. Pharm. Anal. 3, 147–157.

Sánchez, E.E., Rodríguez-Acosta, A., Palomar, R., Lucena, S.E., Bashir, S., Soto, J.G., Pérez, J.C., 2009. Colombistatin: a disintegrin isolated from the venom of the South American snake (*Bothrops colombiensis*) that effectively inhibits platelet aggregation and SK-Mel-28 cell adhesion. Arch. Toxicol. 83, 271–279.

Schägger, H., von Jagow, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368–379.

Serrano, S.M., Sampaio, C.A., Mandelbaum, F.R., 1993. Basic proteinases from *Bothrops moojeni* (caissaca) venom–II. Isolation of the metalloproteinase MPB. Comparison of the proteolytic activity on natural substrates by MPB, MSP 1 and MSP 2. Toxicon 31, 483–492.

Simonian, M.H., Smith, J.A., 2006. Spectrophotometric and colorimetric determination of protein concentration. Curr. Protoc. Mol. Biol. (Chapter 10): Unit 10.1A.

Spearman-Kärber, F., 1978. Alternative methods of analysis for quantal responses. In: Statistical Method in Biological Assay. Finney, D.J. Charles Griffin & Co. Ltd., Londres.

Sugiki, M., Maruyama, M., Yoshida, E., Mihara, H., Kamiguti, A.S., Theakston, D.G., 1995. Enhancement of plasma fibrinolysis *in vitro* by jararhagin, the main haemorrhagic metalloproteinase in *Bothrops jararaca* venom. Toxicon 33, 1605–1617.

Suzuki, Y., Urano, T., 2011. Novel situations of endothelial injury in stroke-mechanisms of stroke and strategy of drug development: novel mechanism of the expression and amplification of cell surface-associated fibrinolytic activity demonstrated by real-time imaging analysis. J. Pharmacol. Sci. 116, 19–24.

Teklemariam, T., Seoane, A.I., Ramos, C.J., Sánchez, E.E., Lucena, S.E., Perez, J.C., Mandal, S.A., Soto, J.G., 2011. Functional analysis of a

Author's personal copy

recombinant PIII-SVMP, GST-acocostatin; an apoptotic inducer of HUVEC and HeLa, but not SK-Mel-28 cells. Toxicon 57, 646–656.
Terra, R.M., Pinto, A.F., Guimarães, J.A., Fox, J.W., 2009. Proteomic profiling of snake venom metalloproteinases (SVMPs): insights into venom induced pathology. Toxicon 54, 836–844.
Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterization of snake venoms. Bull. W. H. O. 61, 949–956.
Vuento, M., Vaheri, A., 1979. Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. Biochem. J. 183, 331–337.
Yamakawa, M., Nozaki, Z., Hokama, 1976. Fractionation of sakishima–habu (*Trimeresurus elegans*) venom and lethal hemorrhagic and edema forming activity of the fractions. In: Ohsaka, A., Hayashi, K., Sawai, Y. (Eds.), Animal, Plant and Microbial Toxins, vol. 1. Plenum Press, New York, p. 97.
Yuan, J., Yang, J., Zhuang, Z., Yang, Y., Lin, L., Wang, S., 2012. Thrombolytic effects of Douchi fibrinolytic enzyme from *Bacillus subtilis* LD-8547 *in vitro* and *in vivo*. BMC Biotechnol. 12, 36.
Zychar, B.C., Dale, C.S., Demarchi, D.S., Gonçalves, L.R., 2010. Contribution of metalloproteases, serine proteases and phospholipases A2 to the inflammatory reaction induced by *Bothrops jararaca* crude venom in mice. Toxicon 55, 227–234.