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Angiotensin-degrading serine peptidase: A new chymotrypsin-like activity in the venom of *Bothrops jararaca* partially blocked by the commercial antivenom

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ABSTRACT

Snakebite envenomation is considered a highly relevant public health hazard in South America, having an impact in terms of mortality and morbidity. In Brazil, *Bothrops (sensu latu)* poisoning is responsible for 90% of the snakebites and in patients treated at the Vital Brazil Hospital (Butantan Institute) this index reaches 97.5%. The objective of the present study was to analyze more specifically the ability of the antibothropic antivenom, produced by the Butantan Institute, São Paulo, Brazil, to neutralize metallo-and serine peptidases, known as the major toxins present in *Bothrops jararaca* venom. A set of Fret peptides (Free Ressonance Energy Transfer) was studied using the BjV (*B. jararaca* venom) and site-directed inhibitors PMSF, EDTA and 1,10-phenanthroline. Two substrates were reached to be used as specific tools for studies with metallo peptidases, Abz-FASSAQ-EDDnp, and the serine peptidases, Abz-RPPGFSPFRQ-EDDnp. In disagreement with the literature, the use of both substrates and the antibothropic serum showed a weak neutralization of the serine peptidases present in this venom and a strong neutralization of the metallo peptidases. In order to investigate possible mechanisms of action that have not yet been described for the serine peptidases from the BjV, the present study shows for the first time a new tyrosine-specific chymotrypsin-like and angiotensin-degrading serine peptidase activity, that was partially blocked by the antibothropic serum. In conclusion, the antivenom presented a good neutralization of metallo peptidases but not of serine peptidases, indicating that further studies about serine peptidases immunogenicity are necessary to improve the antibothropic serum.

1. Introduction

Snake venom poisoning is a public health issue for many countries and despite the great difficulty in raising the actual data of these accidents, some studies show that there are about 5.4 million to 5.5 million accidents, more than 400,000 amputations and about 20,000 to 125,000 deaths *per* year worldwide. These numbers surpass several other neglected tropical diseases in occurrence and number of fatalities, such as leishmaniasis, dengue, schistosomiasis, cholera, and Chagas disease (Williams et al., 2010). In addition, snake bites only joined the list of neglected tropical diseases recently, in April 2009, showing that it was not seen as an important public health issue until recently (World Health Organization, 2011).

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The problem of snake venom poisoning is that it exists in the midst of several factors which complicate its solution, such as: profile of the victim; lack of training programs for health staff; underreporting of accidents; improvement in the production, storage and distribution of sera; further studies on quality and safety of serums produced (World Health Organization, 2010).

The most recommended treatment in cases of snakebite accidents is serum therapy. The neutralizing ability is assessed by evaluating the capacity of the antivenom to inhibit the lethal action of the reference venom, *i.e.*, from *Bothrops jararaca*, in a murine model (World Health Organization, 1981). The antivenom produced by the Butantan Institute is prepared by immunization of horses with a mixture of venoms of the species: *Bothrops alternatus* (12.5%), *Bothrops jararacussu* (12.5%), *Bothrops moojeni* (12.5%), *Bothrops neuwiedi* (12.5%) and *B. jararaca* (50%). But in Brazil, there are several species of the *Bothrops* genus *(sensu latu)* which differ widely in composition of venom and with regard to the neutralization of its components, such as metalloproteinase, PLA2 and hyaluronidases (Queiroz et al., 2008). Indeed, the interspecific variation in venom composition and toxicity of Brazilian snakes from the *Bothrops* genus, poses a challenge to the provision of antivenom to be used in accidents caused by any one of the species. The antibothropic serum produced by the Butantan Institute has saved many lives over the years, but it is known that in the case of *Bothrops* poisoning, even with the application of the serum in large quantities, it still does not address the local symptoms of the bite, although it does improve the systemic symptoms (Cardoso et al., 1993). The *Bothrops* genus is widely distributed in the Neotropics, occurring from Mexico to northern Argentina, being absent only in Chile. The *B. jararaca* species occurs from the South of Bahia to northern Argentina and Paraguay, being distributed in Brazil in the states of Minas Gerais, Espírito Santo, Rio de Janeiro, São Paulo, eastern Mato Grosso do Sul, Paraná and Rio Grande do Sul (Gomes and Puorto, 1993). *Bothrops* poisoning is responsible for 90% of the snakebites in Brazil (Ministério da Saúde, 2001) and in patients treated at the Vital Brazil Hospital (Butantan Institute), where the species were identified, this index reaches 97.5% (Ribeiro and Jorge, 1997). Despite the great variety of components present in the venom from the *Bothrops* species, it is known that proteolytic enzymes of serine and metalloproteinase classes are the most relevant toxins in cases of human accidents. Also, results of proteomic analysis performed with the venom of *B. jararaca,* indicate that 51.5% and 14% of components are metallo- and serine peptidases, respectively (Fox and Serrano, 2008).

Snake venom metallo peptidases, also known as SVMPs (Snake Venom Metalloproteinases), act mainly as hemorrhagic factors, degrading proteins such as laminin, fibronectin, collagen type IV and proteoglycans from the endothelial basal membrane (Fox and Serrano, 2005). SVMPs can also module the release of cytokines (Laing and Moura-da-Silva, 2005) and inhibit platelet aggregation (Schattner et al., 2005). Taken together, these two effects, associated with the proteolytic digestion of the basal membrane, are considered to be the major mechanism of SVMP-induced hemorrhage. On the other hand, SVSPs (Snake Venom Serine Proteases) are enzymes which affect the hemostatic system. They act on a variety of components of the coagulation cascade, on the fibrinolytic and kallikrein–kinin systems and on cells to cause an imbalance of the hemostatic system of the prey (Pirkle, 1998).

Taking into account that snake venom poisoning is a public health issue and the major toxins present in the venoms from the *Bothrops* species are SVMPs and SVSPs, the main focus of this study was to verify the blocking potential of the antibothropic serum produced by the Butantan Institute, on the peptidase activities from both classes (metallo peptidases and serine peptidases), using both FRETs and natural biological peptides.

2. Materials and methods

2.1. Reagents

Ethylene diamine tetracetic acid (EDTA), phenylmethanesulfonylfluoride (PMSF), 1,10-phenantroline, angiotensin I (ang I), dynorphin1-13 (dyn A), neurotensin1-13 and bradykinin were purchased from Sigma–Aldrich, acetonitrile from Carlo Erba and trifluoroacetic acid (TFA) from J.T. Baker. FRETs peptides, Abz-FASSAQ-EDDnp (Abz-Metal) and Abz-RPPGFSPFRQ –EDDnp (Abz-Serine), were provided by Prof. Luiz Juliano Neto, from the Department of Biophysics of UNIFESP-EPM. These peptides were synthesized using automated solid-phase synthesis (Hirata et al., 1994).

2.2. Venoms and antivenom

The venom of *B. jararaca* (50 mg), *Bothrops moogeni* (1.0 mg), *B. alternatus* (1.0 mg), *B. jararacussu* (1.0 mg) and *B. neuwiedi* (1.0 mg) were provided by the Herpetology Laboratory from the Butantan Institute, São Paulo, Brazil. The venom of *B. jararaca* was pooled from 2500 specimens and lyophilized. The stock solutions were prepared in PBS buffer, containing 50 mM phosphate and 20 mM NaCl, pH 7.4 at 1.0 mg/mL. The antibothropic serum produced by the hyperimmunization of horses with a pool of venoms from *B. alternatus* (12.5%), *B. jararaca* (50%), *B. jararacussu* (12.5%), *B. moojeni* (12.5%) and *B. neuwiedi* (12.5%) was obtained from the Hyperimmune Plasmas Processing Section, Butantan Institute, São Paulo, Brazil. The antivenom used (batch no. 0506110) had a protein concentration of 1.8 g/dL and each milliliter was able to neutralize 6.61 mg of *B. jararaca* venom (lethality test in mice).

2.3. Determination of protein concentration

This assay was executed according to Smith (1985), using a "BCA Protein Assay" Kit (Pierce Biotechnology, EUA) and serum albumin (BSA – Calbiochem, EUA) as reference in an Elisa reader (Multiskan EX, Labsystems, Finland).

2.4. Enzymatic assays

The peptidase activity assay was conducted in a 7.4 pH PBS buffer (final volume 100 μL) containing 50 mM

phosphate and 20 mM NaCl, using Corning® 96 well plates, and the peptide substrates in a final concentration of 5 μM. The reactions occurred at 37 °C and were initiated by the addition of 1 μL of BjV (2.74 μg/μL with Abz-Metal and 0.18 μg/μL with Abz-Serine). The reactions were monitored (fluorescence at λ_{EM} 420 nm and λ_{Ex} 320 nm) in a fluorescence spectrophotometer (Victor 3™ Perkin–Elmer, Boston, MA, USA), as described by Araujo et al. (2000). Specific peptidase activity was expressed as units of free fluorescence of cleaved substrate *per* minute *per* μg of venom. There was an incubation period of 30 min at room temperature when phenylmethanesulfonylfluoride (1 mM, PMSF) and 1,10-phenantroline (5 mM) where tested. The EDTA (100 mM) was used without pre-incubation time. When necessary, control samples were made in the presence of the same volume of ethanol used in the preparation of inhibitors stock solutions (PMSF and 1,10-phenantroline). The experiments were made in triplicate.

2.5. HPLC analysis of peptides hydrolyzed by Bothrops *spp* venoms

The peptide solutions of angiotensin I (65 μM), dynorphin1-13 (31 μM) neurotensin 1-13 (12 μM) and bradykinin (50 μM) were incubated in 7.4 pH PBS buffer (50 mM phosphate and 20 mM NaCl) with 2.0, 2.5, 5.0 and 3 μL of BjV (2.74 μg/μL) for each substrate, respectively, at 37 °C for 1–4 h, with a pre-incubation period of 30 min at room temperature when tested with EDTA (100 mM), PMSF (1 mM) and 1,10-phenantroline (5 μM). Hydrolysis products were separated by reverse-phase HPLC (Prominence, Shimadzu), collected manually, and submitted to mass spectrometry analysis. The HPLC conditions used for the analytical procedure were 0.1% trifluoroacetic acid (TFA) in water (solvent A), and acetonitrile and solvent A (9:1) as solvent B. The separations were performed at a flow rate of 1 mL/min using a Shim-pack VP-ODS C-18 column (4.6 × 150 mm) and a 20–60% gradient of solvent B over 20 min. In all cases, elution was followed by ultraviolet absorption (214 nm). The scissile bonds in the peptides were determined by mass spectrometry analyses. The peptide fragments were detected by scanning from *m*/*z* 100 to *m*/*z* 1300 using an Esquire 3000 Plus Ion trap Mass Spectrometer with ESI and esquire CONTROL software (Bruker Daltonics, MA, USA). Purified 18O-labeled or unlabeled oxidized W derivatives were dissolved in a mixture of 0.01% formic acid:acetonitrile (1:1) and infused into the mass (direct infusion pump) spectrometer at a flow rate of 240 μL/h. The skimmer voltage of the capillary was 40 kV, the dry gas was kept at 5.0 L/min, and the source temperature was maintained at 300 °C.

After defining the natural peptides that were hydrolyzed by BjV, the ability of the other venoms to hydrolyze angiotensin I (65 μM) was analyzed using 4 μL of each one (*B. alternatus* [5.74 mg/mL], *B. jararacussu* [3.11 mg/mL], *B. moojeni* [0.86 mg/mL] and *B. neuwiedi* [0.11 mg/mL]). The scissile bonds found in angiotensin-I produced by these venoms were deduced by internal standardization of the HPLC system, using the results obtained with *B. jararaca* as reference.

2.6. Antibothropic serum neutralization of the venoms

2.6.1. Fluorimetric assays

The ability of the antibothropic serum to neutralize the venoms proteolytic activities was estimated by incubating it with *Bothrops* spp. venoms. Samples of *Bothrops* venoms were incubated, at room temperature, in the presence and absence of the antibothropic serum. The residual proteolytic activities of the venoms were measured as described above, using both FRETs substrates. The volume of the antibothropic serum and the pre-incubation time for serum neutralization of the proteolytic activities were established by using the *B. jararaca* venom. After establishing the best conditions to neutralize the metallo- and serine peptidases from the *B. jararaca* venom, the other *Bothops* spp venoms were tested (*B. alternatus*, *B. jararacussu*, *B. moojeni and B. neuwiedi*). The venoms were used in volumes of 2.0 μL when the Abz-Metal was utilized as substrate and 0.2 μL for the kinetics with the Abz-Serine (see concentration on 2.5). For the maximum blocking effect of the proteolytic activity, the venoms were incubated with 10 μL of the antibothropic serum for 30 min at room temperature. After this period, 5 μM of each substrate was added and the residual activity was measured as described above. The experiments were made in triplicate.

2.6.2. HPLC assays

The same concentrations of *Bothrops* spp venoms described in the angiotensin-I degrading assays were utilized to determine the neutralizing potential of the commercial serum. Thus, after a pre-incubation time (venoms and antivenom), 65 μM of angiotensin I was added and after 1 h more samples were analyzed by HPLC reverse-phase.

3. Results

3.1. Search for specific substrates for the different classes of proteases

The first step in this study was to find ways to differentiate the major classes of proteolytic enzymes present in the *B. jararaca* venom, the serine peptidases and the metallo peptidases. For this, a set of FRETs substrates was tested and two substrates that are mostly hydrolyzed by each one of these classes were found. The metallo peptidases act mainly on Abz-FASSAQ-EDDnp and the serineproteases on Abz-RPPGFSPFRQ-EDDnp. Table 1 shows that Abz-FASSAQ-EDDnp hydrolysis was totally inhibited by both EDTA and 1,10-phenantroline and, thus, it was named here as Abz-Metal. This was unlike the hydrolysis of Abz-RPPGFSPFRQ-EDDnp peptide, that was strongly inhibited by PMSF (71%), and was thus named as Abz-Serine. It is important to mention that the rate of hydrolysis of Abz-Metal by the BjV is around 18 times lower when compared to that of Abz-Serine (Table 1).

The preference of both protease classes for these substrates was also found in venoms from other species of the *Bothrops* genus that comprise the pool used in the production of the antivenom (Fig. 1), with exception of the Abz-Serine hydrolysis by the *B. neuwiedi* venom which was inhibited by PMSF with lower potency.

Table 1
Inhibition of hydrolysis of the Abz-Metal and Abz-Serine by BjV using classical inhibitors of metallopeptidase (EDTA and 1.10-phenantroline) and serinepeptidase (PMSF).

Specific activity (UF/min/ìg)[a]		
	Abz-Metal	Abz-Serine
Control	544.7	9716.16
EDTA	n.h.[b]	9428.72
1.10-Phenantroline	n.h.	6179.48
PMSF	390.4	2791.78

Assays were carried out in 100 μL of 50 mM phosphate buffer, pH 7.4, containing 20 mM NaCl, at 37 °C, using 5 μM of Abz-peptides and 2.74 μg and 0.18 μg of BjV (for Abz-Metal and Abz-Serine, respectively). The SD of kinetic results in each case was never greater than 5% of the value obtained. The results were a mean of three independent experiments.
[a] UF = Units of fluorescence.
[b] n.h. no hydrolysis detected.

3.2. Antibothropic serum neutralization

The tests were conducted using the maximum dose of BjV neutralization that was found (10 ìL of antibothropic serum), and incubated at room temperature for 30 min using the Abz-peptides. When the venom from *B. jararaca* was used, we observed a great neutralization of proteolytic activity of metallo peptidases that act on the substrate Abz-Metal, reaching levels above 90%, and a poor inhibition of serine peptidases that act on Abz-Serine, with levels below 40% (Table 2). After determining the best condition to neutralize the proteolytic activity of BjV, the same protocol was used to verify the blocking effect of the commercial serum upon the other venoms used to compose the immunization pool: *B. alternatus*, *B. jararacussu*, *B. moojeni and B. neuwiedi*. Fig. 2 shows that the other *Bothrops* spp venoms studied here present the same pattern of activities that were observed when the *B. jararaca* venom was used. The hydrolysis of Abz-Serine by the *B. neuwiedi* venom was not inhibited through the use of the antivenom. The results obtained using *B. jararaca* and *B. moojeni* venoms showed inhibition levels of Abz-Serine hydrolysis of around 35%. The hydrolysis of Abz-Metal by the *B. alternatus* venom was blocked around 70% by the commercial serum. For the other species, the results obtained using the Abz-Metal as substrate always reached levels above 90%. Thus, the use of Abz-Metal and the antivenom reaching nearly 100% of inhibition in return got poor inhibition of serine peptidases that act on Abz-Serine with levels below 50%–0% inhibition (Fig. 2).

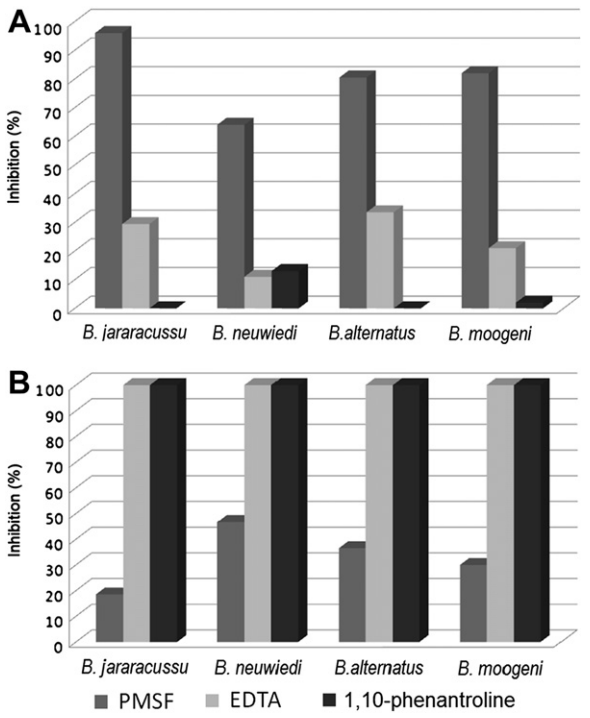

Fig. 1. Hydrolysis of FRETs substrates and the effect of classical inhibitors to serinepeptidases and metallopeptidases. Inhibition effect of PMSF, EDTA and 1,10-phenantroline upon the hydrolysis of Abz-Serine (Panel A) and Abz-Metal (Panel B), by venoms from *B. jararacussu*, *B. neuwiedi*, *B. alternatus* and *B. moojeni*. The experiments were made in triplicate. The SD of kinetic results in each case was never greater than 5% of the value obtained.

3.3. HPLC analysis of natural peptides hydrolyzed by BjV

One of our goals was to find new peptidic substrates that could be hydrolyzed by bothropic venoms and that could explain in greater depth the *Bothrops* venoms mechanism. For this, four human biologically active peptides, possibly related to any physiological change on the victim, were tested. Table 3 shows the rate of hydrolyzes of angiotensin I, dynorphin1-13, neurotensin1-13 and bradykinin, by the *B. jararaca* venom. In this set of putative substrates, only bradykinin was not hydrolyzed by the BjV and a good cleavage of angiotensin I was observed. Dynorphin1-13 was also well hydrolyzed by the *B. jararaca* crude venom, followed by the neurotensin1-13 degradation. Table 3 also shows the cleavage points determined in angiotensin I and dynorphin1-13. As can be observed, angiotensin I presents one cleavage point between the residues Tyr–Ile, that was totally blocked by PMSF and not affected by EDTA or 1,10-phenantroline. Moreover, the commercial serum produced by the Butantan Institute was able to reduce only 44% of the hydrolysis of angiotensin I by BjV.

Dynorphin1-13 presents two scissile bonds, between the residues Arg–Arg and Lys–Leu, that were principally blocked by PMSF (88%) and partially blocked by EDTA

Table 2
Serum neutralization effect upon the proteolytic activity of *Bothrops jararaca* venom using Abz-Metal and Abz-Serine as substrates.

	Specific activity (UF/min/μg)		Inhibition (%)
Substrate	Control	Serum	
Abz-Metal	333.3	22.3	93.3
Abz-Serine	8362.81	5335.5	36.2

Assays were carried out in 100 μL of 50 mM phosphate buffer, pH 7.4, containing 20 mM NaCl, at 37 °C, using 5 μM of Abz-peptides and 2.74 μg and 0.18 μg of BjV (for Abz-Metal and Abz-Serine, respectively). The SD of kinetic results in each case was never greater than 5% of the value obtained. The volume of serum was 10 μL with a pre-incubation of 30 min at room temperature.

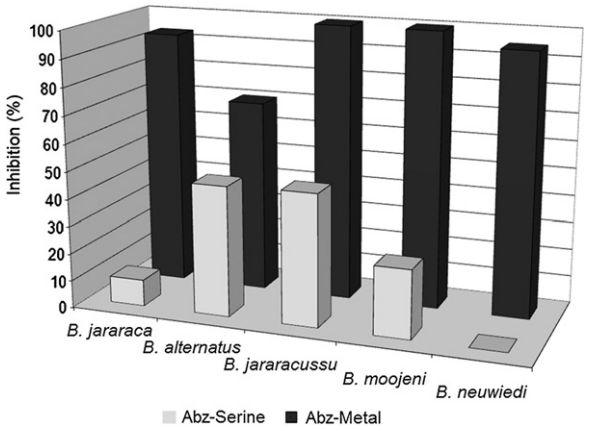

Fig. 2. Inhibition of serinepeptidases and metallopeptidases present in *Bothrops* spp venoms. Blocked effect of the antibothropic serum on hydrolysis of Abz-Serine and Abz-Metal by venoms from *B. jararaca*, *B. jararacussu*, *B. neuwiedi*, *B. alternatus* and *B. moojeni*. The experiments were made in triplicate. The SD of kinetic results in each case was never greater than 5% of the value obtained.

(28%), and 1,10-phenantroline (6%). Table 3 shows that the antibothropic serum was able to block 48% of the hydrolytic activity of the venom on dynorphin A cleavage.

Since the observation of angiotensin I cleavage is mainly due by serine peptidases and partially blocked by the antivenom, we decided to test the other four bothropic venoms used to make the immunization pool.

The results obtained with the venoms used to compose the immunization pool, again showed the presence of a chymotrypsin-like activity in these venoms, although with distinct specific activities (Table 4). The cleavage points were unique between Tyr–Ile bonds (data not shown) and were determined by the internal standardization of the HPLC conditions using the BjV. The blocked effect of the antibothropic serum was different for each venom, showing variations in their composition. The angiotensin-I hydrolyzes by the venom from *B. jararacussu* and *B. jararaca* were only partially blocked by the commercial antivenom (Table 4). In contrast, angiotensin I degradation was fully inhibited by using the antivenom when the venoms from *B. moojeni* and *B. neuwiedi* were used.

Table 3
Hydrolysis of bioactive peptides by *B. jararaca* venom.

	Specific activity (μM/min/μg)					Cleavage site
	Control	EDTA	PMSF	1.10-Phe	Serum	
Angiotensin I	0.18	0.23	n.h.[a]	0.21	0.10	DRVY///IHPFHL
Dyn 1-13	0.058	0.04	0.007	0.054	0.03	YGGFLR// RIRPK//LK
Neurotensin	0.0012	n.d.[b]	n.d.	n.d.	n.d.	n.d.
Bradykinin	n.h.	–	–	–	–	–

The peptide solutions (12–65 μM) were incubated in 7.4 pH PBS buffer containing 50 mM phosphate and 20 mM NaCl with BjV, at 37 °C for 1–4 h, with a pre-incubation period of 30 min at room temperature when tested with EDTA, PMSF and 1.10-phenantroline. The volume of serum was 10 μL with a pre-incubation of 30 min at room temperature. The SD of kinetic results in each case was never greater than 5% of the value obtained. The experiments were made in triplicate.

[a] n.h. – no hydrolysis detected.

[b] n.d. – not determined.

Table 4
Blocked effect of the antibothropic serum on hydrolysis of angiotensin I by the venoms of *B. jararaca*, *B. alternatus*, *B. moojeni*, *B. neuwiedi* and *B. jararacussu*.

Venom	Specific activity (μM/min/μg)		Inhibition (%)
	Control	Serum	
B. jararaca	0.18	0.10	44
B. alternatus	0.046	0.028	61
B. moojeni	0.15	0	100
B. neuwiedi	1.66	0.075	96
B. jararacussu	0.046	0.01	21

Assays were carried out in 500 μL of 50 mM phosphate buffer, pH 7.4, containing 20 mM NaCl, at 37 °C, using 65 μM of angiotensin I. The venoms were used in different concentrations (see materials and methods for details). The SD of kinetic results in each case was never greater than 5% of the value obtained. The volume of serum was 10 μL with a pre-incubation of 30 min at room temperature.

4. Discussion

Although it was proposed that *B. jararaca* and *Bothrops neuwied* should be included in the genus *Bothropoides*, and *B. alternatus* into genus *Rhinocerophis*, there is no clear consensus about the systematics of this group (SBH, 2007). Since human envenomations involving these species are treated with the antibothropic serum, this study still considers these snake venoms as belonging to the genus *Bothrops*.

The objective of the present study was to analyze the ability of the antivenom produced by the Butantan Institute, São Paulo, Brazil, to neutralize *B. jararaca* major venom toxins. A set of FRET peptides (*F*ree *R*essonance *E*nergy *T*ransfer) was studied using the BjV and site-directed inhibitors PMSF, EDTA and 1,10-phenanthroline. The results indicated that two substrates can be used as specific tools for studies with metallo peptidase, Abz-Metal (Abz-FASSAQ-EDDnp), and serine peptidases, Abz-Serine (Abz-RPPGFSPFRQ-EDDnp). The use of both substrates and the antivenom produced by the Butantan Institute showed a weak neutralization of serine peptidases in this venom and a strong neutralization of the metallo peptidases. These results are in disagreement with the literature, since the symptoms attributed to the serine peptidases are considered to be controlled when the antibothropic serum is administered (Cardoso et al., 1993).

Indeed, the antivenom is capable of reducing the systemic effects caused by poisoning from *Bothrops* snakes, but it is not effective to block the local effects observed in accidents with humans (Cardoso et al., 1993). This observation leads us to believe that some of the enzymes present in the snake venom are not neutralized by the antivenom - *i.e.*: that serine peptidases may be related to local effects through the activation of latent forms of human MMP's (Saravia-Otten et al., 2004).

The serine peptidases of snake venoms are classified in clan SA of S1 of the chymotrypsin family (Rawlings et al., 2010). The mammalian trypsin and enzymes present in poisons have similar "fold" and are believed to have evolved from a common ancestor (Itoh et al., 1988). The *B. jararaca* venom contains several serineproteases, and the best characterized are: Bothrops protease A (BPA), recently

described as a specific defibrinogenating agent; KN-Bj is able to release bradykinin from low molecular weight bovine kininogen; TL-BJ, a thrombin-like protease with clotting activity; PA-BJ, an enzyme with activity in aggregating platelet-rich plasma and suspensions of washed platelets; Bothrombin is a serine peptidase which acts by cleavage of fibrinopeptide A without affecting fibrinopeptide B (see Serrano and Maroun as review).

Although the SVSP described above have defined protein substrates, there are no published data indicating possible biologically active peptides as substrates for these enzymes. In fact, the majority of the methods used to screen the proteolytic activities of animal venoms have not considered the possibility of peptidase activities, which could contribute directly or indirectly to the envenomation. Peptidase activity can increase permeability to the venom toxin targets, and produces other peptides with different activities from the parent peptide and destruction of both epitopes MHC class I and II.

The results presented here show that the crude venom of *B. jararaca* was able to cleave angiotensin I, dynorphin1-13 and, to a lesser extent hydrolysis neurotensin1-13. Surprisingly, angiotensin I was well hydrolyzed by the BjV, and the use of 1,10-phenantroline and PMSF clearly indicated that it is a serine protease-like activity. The use of the antibothropic serum showed, again, a flaw in the action of the commercial antivenom to block serine peptidases.

The cleavage point in ang I was determined as Tyr–Ile by mass spectrometric analysis and was the same hydrolysis observed using the venoms from *B. jararacussu*, *B. neuwiedi*, *B. alternatus* and *B. moojeni*. These results are in accordance with other serine peptidases activities reported as being able to hydrolyze ang I between the Y–I bond, like human kallikrein 1-related peptidase 3 (KLK3), best known as "prostate-specific antigen" (PSA) (Andrade et al., 2010) and rat chymase-1 (Sanker et al., 1997). Recently, a serine protease purified from the venom of *Vipera libetina* showed the same angiotensin I scissile bond reported here (Siigur et al., 2010).

The decapeptide ang I (DRVYIHPFHL) is a precursor of the ang II (DRVYIHPF), well-known as an important hypertensive peptide. The most important peptidase responsible for the conversion of ang I to ang II is the Angiotensin Converting Enzyme (ACE, peptidyl dipeptidase A, EC 3.4.15.1) (Skeggs et al., 1956).

In this scenario, it is important to note that a family of peptides with ACE inhibitory activity, the BPPs (Bradykinin-Potentiating Peptides), is present in the venom of *B. jararaca* (Ferreira and Rocha e Silva, 1965; Ferreira et al., 1970; Ondetti et al., 1971). One of then, the BPP-5a, is a molecule that originally inspired the design of current commercial inhibitors of ACE (Ondetti et al., 1977). Since the hypotensive effects observed in accidents with humans are related to the presence of BPPs, the destruction of angiotensin I is another activity in this BjV that leads to *in vivo* hypotension. Moreover, BjV contains a serine peptidase able to release bradykinin from the low molecular weight kininogen (KN-BJ) (Serrano et al., 1998). Apart from the results of angiotensin I hydrolysis, we also show that the bradykinin was totally stable to the action of *B. jararaca* venom and no cleavage could be detected even after a long period of incubation. Taken together, it seems that the venom of *B. jararaca* can be considered an arsenal that leads the victim to hypotensive shock. Most critically, it is possible that these activities, caused by BPPs and serine peptidases, are not blocked by the antibothropic serum. It is important to mention that the antibothropic serum produced by the Butantan Institute presents high specific activity to neutralize the lethal activity of the *Bothrops* venoms, when compared with other commercial antivenoms (Dias da Silva and Tambourgi, 2011).

Taking into account the hydrolyzes on the Tyr–Leu bond in angiotensin I, it is possible to hypothesize that serine proteinase BPA (Bothrops Protease A) could be responsible for this activity, since it is able to cleave the insulin â chain with a Tyr residue in the P1 position (Tyr26-Thr27; Mandelbaum et al., 1967). Besides the same specificity to substrate hydrolyzes, it is important to note a high degree of similarity and identity between the serineproteases amino acid sequences. The least degree of similarity can be observed between KN-Bj × PA-Bj (63.4%) and the most degree of similarity was found between BPA × PA-Bj (71.6%). A recent report showing a serine protease from *Vipera lebetina* venom, capable to cleave angiotensin I, also shows a high degree of similarity (85%) and identity (75%) with BPA, including in the catalytic residues (Fig. 3).

Dynorphin1-13 (dyn A, YGGFLRRIRPKLK) was also hydrolyzed by the crude venom of *B. jararaca*, showing at least two cleavage points (YGGFLR-RIRPK-LK), since the fragment RIRPK was detected by mass spectrometry

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VLCTLP          VIGGDECNINEHRSLVYLYNDSNFQCGGTLINQEWVLSAAHCDMENMEIYLGVHNLSLPN
BPA             VIGGDECNITEHRFLVEIFNSSGLFCGGTLIDQEWVLSAAHCDMRNMRIYLGVHNEGVQH
                *********.*** ** ::*.*.: ******:************.**.******* .: :

VLCTLP          KDQKRRDPKEKFFCLSSKNYTKWDKDIMLIKLNRPVKTSTHIAPLSLPSSPPSVGSVCRI
BPA             ADQQRRFAREKFFCLSSRNYTKWDKDIMLIRLNRPVNNSEHIAPLSLPSNPPSVGSVCRI
                 **:** .:********:************:*****:.* *********.**********

VLCTLP          MGWGTVTSPNETLLDVPHCANININLNYTVCRAASPRLPTQSRTLCAGILQGGIDACKGDS
BPA             MGWGTITSPNATFPDVPHCANINLFNYTVCRGAHAGLPATSRTLCAGVLQGGIDTCGGDS
                *****:**** *: *********::******.* . **: *******:******:* ***

VLCTLP          GGPLICNGQIQGIVSWGNHPCAQPLKPGHYTHVFDYTDWIQSIIAGNTTATCPP
BPA             GGPLICNGTFQGIVSWGGHPCAQPGEPALYTKVFDYLPWIQSIIAGNTTATCPP
                ******** :*******.****** :*. **:****  ****************
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Fig. 3. Primary sequence comparison: VLCTLP (*Vipera lebetina* chymotrypsin-like protease) and BPA (*Bothrops* protease A). The catalytic triads are in bold and underlined, and asterisks (*) indicate identical amino acid residues. The symbols (:) and (.) represent conserved and non conserved substitutions, respectively.

analyses. Unlike angiotensin I, dyn A is hydrolyzed by both classes of proteases, metallo- and serine peptidases, so this activity was partially blocked by the commercial antibothropic serum. The pathophysiological mean of dyn A hydrolyzes is possibly correlated with pain sensation and inflammation (Parik et al., 2010; Luo et al., 2008).

Many factors, including phylogeny, sex, geographic origin, season, age and prey preference, may influence composition of the venoms (Chippaux et al., 1991; Mackessy et al., 2003; Furtado et al., 2006). In addition to these considerations, the genus *Bothrops* shows the greatest diversity when it comes to number of species, morphology and natural history characteristics (Campbell and Lamar, 2004). Given these characteristics, the development of a polyvalent antivenom against accidents involving this genus is an even greater challenge. Thus, the production of better antivenoms should take into consideration the quality of poisons, and what poisons should be used to compose the pool of immunization. Finally, the preclinical efficacy of the antivenom must be carefully evaluated. The inter specimen venom composition may be evidenced by the different levels of chymotrypsin-like activity and by the different potential blockers obtained with the antibothropic serum and the five different *Bothrops* venoms studied in this paper. These venom composition variations may be an important factor to explain the failure of the antibothropic serum and, additionally, three other factors also may be responsible for the overall presented result. The first factor suggests a lack of immunoglobulins acting against serine peptidases present in some venoms and the second factor may be related to the failure of blocking by the antibodies, although they may be present. The third and important factor may be related to degradation of the serine peptidases by the metallo peptidases before the inoculation of horses with the pool of venoms used for the production of antivenom, and this degradation could destroy the epitopes responsible for the production of immunoglobulins. These hypotheses are under investigation in our laboratories through new experiments, with the objective of developing strategies to obtain a more effective antibothropic serum.

The antibothropic serum produced by the Butantan Institute is one of the best in Latin America to reduce mortality by snake poisoning from this genus. However, this study showed a flaw in the action of antibothropic serum to neutralized serine peptidases and, to our knowledge, this is the first report on chymotrypsin-like activity in the venom of *B. jararaca.*

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Ethical statement

The authors guarantee that the works presented in this manuscript follow the rules of ethics and respect the duties of authors presented in the Elsevier's Ethical Guidelines for Journal Publication.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Andrade, D., Assis, D.M., Lima, A.R., Oliveira, J.R., Araujo, M.S., Blaber, S.I., Blaber, M., Juliano, M.A., Juliano, L., 2010. Substrate specificity and inhibition of human kallikrein-related peptidase 3 (KLK3 or PSA) activated with sodium citrate and glycosaminoglycans. Arch. Biochem. Biophys. 498, 74–82.

Araujo, M., Melo, R., Cesari, M., Juliano, M., Juliano, L., Carmona, A., 2000. Peptidase specificity characterization of C- and N-terminal catalytic sites of angiotensin I-converting enzyme. Biochemistry 39, 8519–8525.

Campbell, J.A., Lamar, W.W., 2004. The Venomous Reptiles of the Western Hemisphere. Comstock, Ithaca/London.

Cardoso, J.L.C., Fan, H.W., França, F.O.S., Jorge, M.T., Leite, R.P., Nishioka, S. A., Avila, A., Sano-Martins, I.S., Tomy, S.C., Santoro, M.L., Chudzinski, A. M., Castro, S.C.B., Kamiguti, A.S., Kelen, E.M.A., Hirata, M.H., Mirandola, R.M.S., Theakston, R.D.G., Warrel, D.A., 1993. Randomized comparative trial of three antivenoms in the treatment of envenoming by lance-headed vipers (*Bothrops jararaca*) in São Paulo, Brazil. Q. J. Med. 86, 315–325.

Chippaux, J.P., Williams, V., White, J., 1991. Snake venom variability: methods of study, results and interpretation. Toxicon 29, 1279–1303.

Dias da Silva, W., Tambourgi, D.V., 2011. Comment on "Preclinical assessment of the neutralizing capacity of antivenoms produced in six Latin American countries against medically-relevant Bothrops snake venoms". Toxicon 57, 1109–1110.

Ferreira, S.H., Rocha e Silva, M., 1965. Potentiation of bradykinin and eledoisin by BPF (bradykinin potentiating factor) from *Bothrops jararaca* venom. Experientia 21, 347–349.

Ferreira, S.H., Bartelt, D.C., Greene, L.J., 1970. Isolation of bradykinin potentiating peptides from *Bothrops jararaca* venom. Biochemistry 9, 2583–2593.

Fox, J.W., Serrano, S.M., 2005. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. Toxicon 45, 969–985.

Fox, J.W., Serrano, S.M., 2008. Insights into and speculations about snake venom metalloproteinase (SVMP) synthesis, folding and disulfide bond formation and their contribution to venom complexity. FEBS J. 275, 3016–3030.

Furtado, M.F., Travaglia-Cardoso, S.R., Rocha, M.M., 2006. Sexual dimorphism in venom of Bothrops jararaca (Serpentes: Viperidae). Toxicon 48 (4), 401–410.

Gomes, N., Puorto, G., 1993. Atlas anatômico de *Bothrops jararaca*. Wied, 1824 (Serpenes: Viperidae). Mem. Inst. Butantan 55, 69–100.

Hirata, I.Y., Cezari, M.H.S., Nakaie, C.R., Boschcov, P., Ito, A.S., Juliano, M., Juliano, L., 1994. Internally quenched fluorogenic protease substrates: solid-phase synthesis and fluorescence spectroscopy of peptides containing ortho-aminobenzoyl/dinitrophenyl groups as donor-acceptor pairs. Lett. Pept. Sci. 1, 299–308.

Itoh, N., Tanaka, N., Funakoshi, I., Kawasaki, T., Mihashi, S., Yamashina, I., 1988. Organization of the gene for batroxobin, a thrombin-like snake venom enzyme. Homology with the trypsin/kallikrein gene family. J. Bio. Chem. 263, 7628–7631.

Laing, G.D., Moura-da-Silva, A.M., 2005. Jararhagin and its multiple effects on hemostasis. Toxicon 45, 987–996.

Luo, M.C., Chen, Q., Ossipov, M.H., Rankin, D.R., Porreca, F., Lai, J., 2008. Spinal dynorphin and bradykinin receptors maintain inflammatory hyperalgesia. J. Pain 9, 1096–1105.

Mackessy, S.P., Williams, K., Ashton, K.G., 2003. Ontogenetic variation in venom composition and diet of Crotalus oreganos concolor: a case of venom paedomorphosis? Copeia, 769–782.

Mandelbaum, F.R., Carrilio, M., Henriques, S.B., 1967. Proteolytic Activity of Bothrops protease A on the B Chain of Oxidized Insulin, vol. 132 508–510.

Ministério da Saúde, 2001. Manual de diagnóstico e tratamento de acidentes por animais peçonhentos. Fundação Nacional de Saúde/Coordenação de Controle de Zoonoses e Animais Peçonhentos, CENEPI, Brasília.

Ondetti, M.A., Williams, N.J., Sabo, E.F., Pluscec, J., Weaver, E.R., Kocy, O., 1971. Angiotensin-converting enzyme inhibitors from the venom of *Bothrops jararaca*. Isolation, elucidation of structure and synthesis. Biochemistry 10, 4033–4039.

Ondetti, M.A., Rubin, B., Cushman, D.W., 1977. Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. Science 196, 441–444.

Parikh, D., Hamid, A., Friedman, T.C., Nguyen, K., Tseng, A., Marquez, P., Lutfy, K., 2010. Stress-induced analgesia and endogenous opioid peptides: the importance of stress duration. Eur. J. Pharmacol. 650, 563–567.

Pirkle, H., 1998. Thrombin-like enzymes from snake venoms: an updated inventory. Scientific and Standardization Committee's Registry of Exogenous Hemostatic Factors. Thromb. Haemost. 79, 675–683.

Queiroz, G.P., Pessoa, L.A., Portaro, F.C.V., Furtado, M.F., Tambourgi, D.V., 2008. Interspecific variation in venom composition and toxicity of Brazilian snakes from *Bothrops* genus. Toxicon 52, 842–851.

Rawlings, N.D., Barrett, A.J., Bateman, A., 2010. MEROPS: the peptidase database. Nucleic Acids Res. 38, D227–D233.

Ribeiro, L.A., Jorge, M.T., 1997. Bites by snakes in the genus Bothrops: a series of 3,139 cases. Rev. Soc. Bras. Med. Trop. 30 (6), 475–480.

Sanker, S., Chandrasekharan, U.M., Wilk, D., Glynias, M.J., Karnik, S.S., Husain, A., 1997. Distinct multisite synergistic interactions determine substrate specificities of human chymase and rat chymase-1 for angiotensin II formation and degradation. J. Biol. Chem. 272, 2963–2968.

Saravia-Otten, P., Frisan, T., Thelestam, M., Gutiérrez, J.M., 2004. Membrane independent activation of fibroblast proMMP-2 by snake venom: novel roles for venom proteinases. Toxicon 44, 749–764.

SBH, 2007. Brazilian Reptiles – List of Species. Sociedade Brasileira de Herpetologia.

Schattner, M., Fritzen, M., Ventura J de, S., de Albuquerque Modesto, J.C., Pozner, R.G., Moura-da-Silva, A.M., Chudzinski-Tavassi, A.M., 2005. The snake venom metalloproteases berythractivase and jararhagin activate endothelial cells. Biol. Chem. 386, 369–374.

Serrano, S.M., Maroun, R.C., 2005. Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved. Toxicon 45 (8), 1115–1132.

Serrano, S.M., Hagiwara, Y., Murayama, N., Higuchi, S., Mentele, R., Sampaio, C.A., Camargo, A.C., Fink, E., 1998. Purification and characterization of a kinin-releasing and fibrinogen-clotting serine proteinase (KN-BJ) from the venom of *Bothrops jararaca*, and molecular cloning and sequence analysis of its cDNA. Eur. J. Biochem. 251, 845–853.

Siigur, E., Tõnismägi, K., Trummal, K., Samel, M., Vija, H., Aaspõllu, A., Rönnholm, G., Subbi, J., Kalkkinen, N., Siigur, J., 2010. A new tyrosine-specific chymotrypsin-like and angiotensin-degrading serine proteinase from Vipera lebetina snake venom. Biochimie 93, 321–323.

Skeggs, L.T., Kahn, J.R., Shumway, N.P., 1956. The preparation and function of the hypertensin-converting enzyme. J. Exp. Med. 103, 295–299.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchronic acid. Anal. Biochem. 150, 76–85.

Williams, D., Gutiérrez, J.M., Harison, R., Warrel, D.A., White, J., Winkel, K. D., Fopalakrishnakone, P., 2010. The Global snake bite Initiative: an antidote for snake bite. Lancet 375, 89–91.

World Health Organization, 1981. In: Progress in the Characterization of Venoms and Standardization of Antivenoms. WHO Offset Publication, Geneva, p. 58.

World Health Organization, 2010. In: Guidelines for the Production, Control and Regulation of Antivenom Immunoglobulins. WHO website, Geneva. www.who.int/bloodproducts/snakeantivenoms.

World Health Organization, 2011. In: Neglected Diseases. WHO website. http://www.who.int/neglected_diseases/diseases/en/.