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Identification of hyaluronidase and phospholipase B in *Lachesis muta rhombeata* venom

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Abstract

Hyaluronidases contribute to local and systemic damages after envenoming, since they act as spreading factors cleaving the hyaluronan presents in the connective tissues of the victim, facilitating the diffusion of venom components. Although hyaluronidases are ubiquitous in snake venoms, they still have not been detected in transcriptomic analysis of the *Lachesis* venom gland and neither in the proteome of its venom performed previously. This work purified a hyaluronidase from *Lachesis muta rhombeata* venom whose molecular mass was estimated by SDS-PAGE to be 60 kDa. The hyaluronidase was more active at pH 6 and 37 °C when salt concentration was kept constant and more active in the presence of 0.15 M monovalent ions when the pH was kept at 6. Venom was fractionated by reversed-phase liquid chromatography (RPLC). Edman sequencing after RPLC failed to detect hyaluronidase, but identified a new serine proteinase isoform. The hyaluronidase was identified by mass spectrometry analysis of the protein bands in SDS-PAGE. Additionally, phospholipase B was identified for the first time in *Lachesis* genus venom. The discovery of new bioactive molecules might contribute to the design of novel drugs and biotechnology products as well as to development of more effective treatments against the envenoming.

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6.CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Abstract

Keywords

Bushmaster; snake venom; spreading factor; hyaluronidase; serine proteinase; phospholipase B

1. INTRODUCTION

Snakes belonging to *Lachesis* genus inhabit tropical forested areas in South and Central America. They are popularly known as “bushmasters” and are called “surucucu” in Brazil. These snakes are the only neo-tropical pit viper that lay eggs and are the longest venomous snakes in America, reaching, in some cases, up to 3 m in length (but usually range from 2 to 2.5 m) (Jorge et al., 1997; Madrigal et al., 2012; Málague and França, 2003; Sanz et al., 2008).

Lachesis genus is subdivided into four species: *L. stenophrys* (Caribbean coast of Central America), *L. melanocephala* (black-headed bushmaster – Costa Rica and Panama), *L. acrochorda* (Panama, Colombia and Ecuador) and *L. muta* (South America bushmaster – Colombia, Venezuela, Trinidad, Guyana, Suriname, French Guyana, Ecuador, Peru, Bolivia and Brazil), which comprehend the subspecies *L. m. muta* and *L. m. rhombeata*. The last one is endemic in The Brazilian Atlantic forest from Ceará to Rio de Janeiro states (Madrigal et al., 2012; Otero et al., 1998; Zamudio and Greene, 1997).

Human envenoming by *Lachesis* is not very frequent, but these snakes can inject a large quantity of venom (200–400 mg) in the victim (Málague and França, 2003). This envenoming is characterized by intense local pain, haemorrhage, oedema, necrosis in the bite’s site, coagulopathies, abdominal pain, nausea, bradycardia, hypotension, renal failure and even shock (Damico et al., 2005; Jorge et al., 1997; Otero et al., 1998).

Snake venoms are very complex mixtures that include several peptides and proteins (da Silva Cunha et al., 2011; Li et al., 2004; Warrell, 2010), such as enzymes (e.g. serine proteinases, Zn²⁺-metalloproteinases, L-amino acid oxidases, hyaluronidases and phospholipases A₂), and non-enzymatic components (e. g. C-type lectins, natriuretic peptides, myotoxins, cysteine-rich secretory protein -CRISP-, bradykinin potentiating peptides and nerve and vascular growth factors). Some of these components disrupt vital physiological processes of preys (Ciscotto et al., 2011; Sanz et al., 2008; Tucker and Miletich, 2010) or act as defence against predators (Ciscotto et al., 2011; Kardong, 1982;

Tucker and Miletich, 2010). Venom composition reflects not only evolutionary differentiation of phylogenetic lineages (Escoubas et al., 2008; Gutiérrez, 2011), but also distinct developmental stages, dietary behaviour and habitat variation (Ciscotto et al., 2011; Da Rocha and Furtado, 2005; Madrigal et al., 2012). As an example, adults *L. stenophrys* present higher abundance of snake venom metalloproteinases (SVMPs) and Gal-lectin than juvenile specimens, whereas serine proteinases and vasoactive peptides are lower in adult specimens than in juvenile ones (Madrigal et al., 2012).

Among the enzymatic compounds, hyaluronidases are noteworthy by the hydrolysis of hyaluronan, the major glycosaminoglycan from connective tissue, facilitating the diffusion of other toxins present in venoms (Bordon et al., 2012; Cevallos et al., 1992; Tu and Hendon, 1983). These enzymes are expressed in low amounts in snake venoms (Fox, 2013), which hinders its purification and identification.

The identification of new molecules in venom proteomes can open perspectives to the design of new drugs for therapeutic purposes, such as treating cancer and homeostasis disorders (Escoubas et al., 2008; Junqueira-de-Azevedo et al., 2006) and to enable the production of better antidotes against envenoming (Calvete, 2011a, b, 2013; Gutiérrez, 2011; Mendoza et al., 2009). In this work, we report the identification, purification and enzymatic characterization of a new hyaluronidase and the presence of phospholipase B and a new serine proteinase in *Lachesis muta rhombeata* venom (LmrV).

2. MATERIAL AND METHODS

2.1. Venom

Lachesis muta rhombeata venom (LmrV) was obtained from one single adult specimen, desiccated and stored at -20°C until use. The specimen is from Ilhéus (Bahia, Northeast of Brazil, $14^{\circ} 47' 20''$ S, $39^{\circ} 02' 58''$ W), but it was kept at the Serpenterium Bosque da Saúde in Americana (São Paulo state, Southeast of Brazil, $22^{\circ} 44' 21''$ S, $47^{\circ} 19' 53''$ W) with the Ibama register number 647.998.

2.2. Isolation of hyaluronidase

The first step of fractionation of LmrV was performed with slight modifications of our previously published method (Bregge-Silva et al., 2012). Desiccated LmrV (22 mg) was dispersed in 500 μL of 0.05 M sodium acetate buffer (pH 6) centrifuged at 13,400 $\times g$, 4°C , for 10 min, and filtered on a HiPrep 16/60 Sephacryl S-100 HR column (1.6×60 cm, GE Healthcare, Sweden). Fractions were eluted at a flow rate of 0.5 mL/min. The fraction named S3S4 (in which was detected hyaluronidase activity) was loaded on a DEAE Sepharose Fast Flow column (1×40 cm, GE Healthcare) previously equilibrated with 0.05 M sodium acetate buffer (pH 6). Fractions were eluted using a step concentration gradient from 0 to 1.0 M NaCl in the same buffer, at a flow rate of 0.5 mL/min. The fraction D2 was loaded on a HiTrap Heparin HP column (1.6×2.5 cm, 5 mL, GE Healthcare) and the hyaluronidase was eluted using a linear concentration gradient from 0 to 1 M NaCl in the same buffer, at a flow rate of 1.5 mL/min. The fraction H7 obtained from D2 was collected and the purity was checked by reversed-phase chromatography using a Grace C4 column

(4.6 × 250 mm, Vydac, USA) eluted with a step concentration gradient from 0 to 100% of solvent B (60% ACN, 0.1% TFA), at a flow rate of 0.7 mL/min. All purification procedures were monitored at 280 nm by FPLC Äkta Purifier UPC-10 system (GE Healthcare). The chromatographic profile and the protein percentage of the eluted fractions of the venom (considering the area under the absorbance peak) were obtained using the software Unicorn 5.20 (GE Healthcare).

2.3. SDS-PAGE

LmrV (20 µg) was analysed by SDS-PAGE (13.5%) under reducing conditions (β-mercaptoethanol and heating) according to the Laemmli method (Laemmli, 1970). The gel was stained with PlusOne Coomassie Blue PhastGel™ R-350 (GE Healthcare) and bands obtained were submitted to mass spectrometry analysis. A low range (6.5 – 66 kDa) molecular mass marker (Sigma-Aldrich, USA) was also loaded on the gel and the mass of hyaluronidase was estimated based on the migration pattern in the gel.

Additionally, SDS-PAGE (10%) containing hyaluronan (0.4 mg/mL) in gel matrix was carried out according to Cevallos et al. (1992). Part of the gel was stained with PlusOne Coomassie Blue PhastGel™ R-350 (GE Healthcare) and the remaining with Stains-all (Sigma Chemical Co.) to detect hyaluronidase activity.

2.4. Mass spectrometry analysis

Protein bands of interest were excised from the Coomassie-stained SDS-PAGE, destained with a solution of 0.1 M ammonium bicarbonate (pH 8):methanol (50:50, v:v), dehydrated with 200 µL of ACN and dried for 30 min in a Savant SPD111V SpeedVac Concentrator (Thermo Scientific, USA). Subsequently, the proteins were reduced with 100 µL of 0.02 M dithiothreitol (DTT) at 57 °C for 1 h and alkylated with 100 µL of 0.05 M iodoacetamide (IAD) for 45 min at room temperature in the dark. Alkylated bands were dehydrated and dried again (as described above) and digested with 200 ng of modified trypsin (Promega, USA) at 25 °C, for 15 h with shaking. Tryptic peptides for each band were extracted as described by Cotto-Rios et al. (2012) and analysed by LC-MS/MS using an Easy-nLC 1000 (Thermo Scientific) coupled to an Orbitrap Elite™ Mass Spectrometer (Thermo Scientific). High resolution full MS spectra were acquired with a resolution of 60,000 (at m/z 400), and an automatic gain control (AGC) target of 1e6. Subsequently, after each full MS scan twenty data-dependent HCD MS/MS were acquired with a resolution of 15,000 (at m/z 400), AGC target of 5e4, normalized collision energy of 35, and isolation window of ±2 Da. The data were searched against two databases downloaded from UniProt (UniProt Consortium, 2015) using the error tolerant search engine Byonic™ v2.3.5 (Protein Metrics; Bern, Kil and Becker, 2012) in combination with manual *de novo* sequencing. The first database was downloaded using 'hyaluronidase' and 'snake' and the second one using 'phospholipase B' and 'venom' as keywords.

2.5. Hyaluronidase assay

Hyaluronidase activity was performed turbidimetrically (Pukrittayakamee et al., 1988) and adapted to a microplate. LmrV (1 mg) was dissolved in deionized water (1mL) and centrifuged at 13,400 × g, 4 °C, for 10 min. The supernatant (10 µL) and hyaluronan were

incubated for 15 min at different pH levels (4 – 8) and temperatures (0 – 60 °C) keeping the salt concentration constant to determine the optimal conditions for hyaluronidase activity. Sodium acetate buffers (0.2 M) containing different concentrations of NaCl (0–1 M) or different 0.15 M salts (NaCl, KCl, CaCl₂ and MgCl₂) were used to determine salt influence on the hyaluronidase activity at the optimum pH for this enzyme. Turbidity Reducing Units (TRU) are expressed as the quantity of enzyme or venom necessary to hydrolyse 50% (5 µg) of hyaluronan and the specific activity is TRU per mg of enzyme or venom. The assays were performed in triplicate.

2.6. Statistical analysis

The data obtained from hyaluronidase assay (mean ± standard error of the mean) were analysed by the software GraphPad Prism 6.0 (GraphPad Software Inc., USA) using one-way Analysis of Variance (ANOVA) followed by Dunnett's test ($p < 0.05$ was considered statistically significant, comparing to the highest activity in each assay).

2.7. Fractionation of LmrV by RP-FPLC and N-terminal sequencing

LmrV (2 mg) was dispersed in 100 µL of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile (ACN). The insoluble material was removed from the venom by centrifugation at 13,000 $\times$ g, 4 °C, for 10 min. Soluble proteins (750 µg) were submitted to a reversed-phase liquid chromatography on a C18 column (4.6 $\times$ 250 mm, 5 µm particle size, Vydac), using an Äkta Purifier UPC-1 0 system (GE Healthcare) as described by Sanz et al. (2008). The chromatographic profile was obtained using the software Unicorn 5.20 (GE Healthcare).

Protein fractions were submitted to the N-terminal sequencing by Edman degradation (Edman and Begg, 1967) using an automated sequencer model PPSQ-33A (Shimadzu Co., Japan) following the manufacturer's instructions. The search for sequence similarities was performed against a non-redundant protein sequence at Basic Local Alignment Search Tool (BLAST) databank using *Lachesis muta rhombeata* and *Lachesis* as keywords.

3. RESULTS

3.1. Isolation of Hyaluronidase

Hyaluronidase from LmrV was partially isolated through four chromatographic steps (Fig. 1): gel filtration, anionic exchange, glycoprotein affinity and reversed-phase chromatographies. Hyaluronidase activity was detected in fraction S3S4 eluted from HiPrep 16/60 Sephacryl S-100 HR (Fig. 1A – vertical bars). This fraction was loaded on a DEAE Sepharose Fast Flow column and five fractions (D1-D5) were obtained (Fig. 1B). Hyaluronidase activity was detected in fractions D1, D2 and D3 (Fig. 1B – vertical bars). Fraction D2 showed the biggest activity and was used for further purification. Fraction D2 was loaded on a HiTrap Heparin column. Seven fractions, named H1-H7 (Fig. 1C), were collected. Hyaluronidase activity was detected in fractions H6 and H7 (vertical bars, Fig. 1C). Fraction H7 showed the highest activity and was used in the subsequent steps. Fraction H7 was collected and loaded on a reversed-phase C4 column, and 10 fractions (V1 to V10) were collected (Fig. 1D). Hyaluronidase was shown to elute in high percentage of organic solvent (data not shown) and we there concentrated on fraction V10. V10 was lyophilized

and analysed by SDS-PAGE (10%). Comparing the migration pattern of V10 to the hyaluronidase activity band (Fig. 1D, insert) we concluded that hyaluronidase is present in V10. The percentage of protein in V10 was about 0.0003% of the total venom (Table 1).

3.2. Hyaluronidase assay

The specific activity of LmrV was 167 turbidity reducing units (TRU)/mg. Hyaluronidase from LmrV presents optimal activity at pH 6 (Fig. 2A) and 37°C (Fig. 2B), although it is highly active between 30 and 40 °C (no statistical significance was observed when comparing 30, 35 and 40 °C to 37 °C) and in the pH range from 5 to 6.5. Furthermore, hyaluronidase from LmrV has higher activity in the presence of 0.15 M NaCl (Fig. 2C) and monovalent cations (Fig. 2D), such as sodium and potassium, although the evaluated NaCl concentrations (except 1 M) did not show statistical difference when compared to 0.15 M. False positive hyaluronidase activity results may be observed at NaCl concentrations higher than 0.3 M (data not shown). In the presence of magnesium, hyaluronidase did not show enzymatic activity and the activity could not be evaluated in the presence of calcium ions (Fig. 2D).

3.3. Fractionation of LmrV and N-terminal sequencing

LmrV was fractionated by RP-FPLC in an attempt to identify hyaluronidase through only one chromatographic step. Although 29 fractions resulted from this fractionation (Fig. 3), only 23 were collected by the chromatographic system. The collected fractions were freeze-dried and submitted to N-terminal sequencing and the identity of these primary sequences was analyzed by BLAST. Hyaluronidase was not detected, most likely because it is present in such low proportion in LmrV. However, a new serine proteinase isoform was found in this venom. Its N-terminal sequence is **IVGGDECNINEHRFLVALYDPDGFF**. The amino acid residues presented in bold are different from those already deposited in the database. Other sequences obtained in this study are not shown since they had been already reported by Pla et al. (2013).

3.4. Hyaluronidase amino acid sequence

Figure 4 shows the SDS-PAGE under reducing conditions for LmrV. Bands A-I in lane 2 were analysed by mass spectrometry. Only bands A and B identified peptides similar to other snake venom hyaluronidases. After verifying the sequences by manual *de novo* sequencing, four hyaluronidase peptides were confirmed in band A (Table 2). Furthermore, one N-glycosylation site for the hyaluronidase from LmrV band A was identified (Fig. 5).

Figure 5A displays the HCD spectrum for the peptide YIVNVTTAAK whose doubly charged ion was observed at 1229.5474 m/z. Diagnostic oxonium ions for HexNAc and Hex-HexNAc as well as neutral loss were observed, indicative of the presence of a sugar moiety of mass 1378.4729 Da. However, the peptide backbone fragmentation was not sufficient to allow the assignment of the sugar position. Figure 5B shows the spectrum of the same core peptide with the doubly charged ion observed at 641.8483 m/z and just one HexNAc (203.0791 Da) attached. Here, we observe a complete peptide backbone fragmentation that enables the localization of the sugar moiety on the asparagine residue.

The difference of 120 Da from the fragment peptide + HexNAc (Fig. 4A) and from the fragment y_8 + HexNAc (Fig. 4B) is due to cross ring cleavage of the HexNAc moiety (Froehlich et al., 2011).

3.5. Hyaluronidase molecular mass

Hyaluronidase from LmrV showed a molecular mass of 60 kDa estimated by SDS-PAGE under reducing conditions (Fig. 4).

3.6. Identification of phospholipase B

Phospholipase B was identified only in band B (Fig. 4) and tryptic peptides whose sequence showed similarity to other venom phospholipases B already deposited in UniProt databank are shown in Table 3.

4. DISCUSSION

4.1. Purification and characterization of hyaluronidase from LmrV

Although many protein families have been identified in the venomics of *Lachesis*, no hyaluronidases could be previously detected in LmrV and neither in other *Lachesis* venoms (Madrigal et al., 2012; Pla et al., 2013; Sanz et al., 2008). However, this study provided a hyaluronidase with good purity after four chromatographic steps: gel filtration (Sephacryl S-100), anionic-exchange (DEAE Sepharose Fast Flow), glycoprotein affinity (HiTrap Heparin) and reversed-phase on C4 column (Fig. 1).

Hyaluronidase from LmrV had its enzyme activity decreased in the presence of solutions used during the reversed-phase chromatography (data not shown). Therefore, another isolation protocol should be tested in order to obtain active pure enzyme. The percentage of hyaluronidase in LmrV (0.0003%) shows the low quantity of this enzyme in the venom, which might hinder its isolation process (Table 1). However, the real proportion may be slightly higher since fractions D1 and D3 were not used in the calculation of the recovery.

Bordon et al. (Bordon et al., 2012) found 0.23% of protein recovery for CdtHya1, a hyaluronidase isolated from *Crotalus durissus terrificus* venom. The percentage of hyaluronidase in the LmrV is almost eight hundred times lower than in the CdtV. On the other hand, the specific activity of LmrV is comparable albeit slightly higher (15% higher) to that of CdtV, suggesting that the hyaluronidase from LmrV is more active than CdtHya1, which may explain its low proportion in LmrV. Since hyaluronidase is expressed in low quantities in LmrV, its band of activity was not detected in the SDS-PAGE. Nevertheless, we could see a band of hyaluronidase activity corresponding to fraction S3S4, which exhibits the same migration pattern than one of the bands of fraction V10.

The optimal conditions (Fig. 2) for the hyaluronidase from LmrV (37 °C and pH 6) were also reported for other venom hyaluronidases (Bordon et al., 2012; Kudo and Tu, 2001; Nagaraju et al., 2007; Pessini et al., 2001; Pukrittayakamee et al., 1988). Furthermore, this hyaluronidase has higher activity at the presence of NaCl 0.15 M and monovalent cations, such as sodium and potassium. This same condition was described by Gold (1982) for a hyaluronidase from human liver. No hyaluronidase activity was detected in the presence of

magnesium. In the presence of calcium ions, a white cloud clot was formed in the reaction mix, including the control samples (data not show). Probably cetyltrimethylammonium bromide used in the assay reacts with calcium ions, but the real mechanism of this reaction is unknown. The false positive hyaluronidase activity over 0.3 M NaCl was reported by Bordon et al. (Bordon et al., 2012).

The molecular mass of hyaluronidase was estimated as 60 kDa under reducing conditions. Hurtado et al. (2007) obtained a hyaluronidase from a Peruvian *L. muta* with molecular mass of 65 kDa under reducing conditions. However, Cevallos et al. estimated by SDS-PAGE a molecular mass of 115 kDa for hyaluronidase from the same snake venom without reducing conditions or boiling (Cevallos et al., 1992). Band B (45 kDa) presented one peptide for hyaluronidase but that is probably due to the presence of degraded hyaluronidase since this venom has many proteinases (Pla et al., 2013) and we did not use any proteinase inhibitor during venom extraction.

4.2. Identification of serine proteinase in LmrV

To proof the presence of hyaluronidase in LmrV, this venom was fractionated by RP-FPLC and the eluted fractions were subjected to Edman degradation. The N-terminal sequences of these fractions (Fig. 3) belong to 6 protein/peptides families: serine proteinases, bradykinin potentiating peptides (BPPs), phospholipases A₂ (PLA₂), bradykinin inhibitory peptides (BIPs), C-type lectins and L-amino acid oxidases (amino acid sequences not shown). Most proteins identified are involved in blood disorders, inflammatory processes and hypotension that are relevant effects induced by *L. muta rhombeata* venom. Additionally, we identified a new serine proteinase in LmrV. However, we were not able to identify the hyaluronidase by Edman degradation. This might be due to the low abundance of the enzyme in this venom.

The serine proteinases comprehend a class of enzymes largely involved in haemostasis disturbance as well as hypotension. They can act on blood coagulation factors, such as fibrinogen, factor V, factor X, plasminogen and fibrin, and also on kininogen, releasing bradykinin (Lu et al., 2005, Matsui et al., 2000). Serine proteinases can act synergistically with BPPs, further lowering the hypotension induced by bradykinin. This new serine proteinase seems to be a new thrombin-like enzyme and 5 different amino acid residues (among the first 25 residues) could be distinguished from Q9PRP4 which is a serine proteinase that specifically cleaves the A alpha chain of fibrinogen, producing fibrin and releasing fibrinopeptide A (Aguar et al., 1996).

4.3. Identification of the hyaluronidase from LmrV

The low quantity of hyaluronidase hindered the determination of its N-terminal sequence in this study. Moreover, the enzyme could not be detected in the transcriptomic approach of *L. muta* gland venom (Junqueira-de-Azevedo et al., 2006) and neither in the proteomic analysis of LmrV (Pla et al., 2013).

However, we identified four peptides of hyaluronidase obtained in the SDS-PAGE from LmrV including one N-glycosylation site. *Lachesis* genus venoms are known to have hyaluronidase activity (Hurtado et al., 2007), but it is the first time that the protein itself was identified in the venom. It is noteworthy that the mass spectrometer used in this study

presents a very high resolution and sensitivity (Michalski et al., 2012). This is probably the main reason of the success of the MS analysis performed in our work.

The use of electron transfer dissociation (ETD) and HCD has been reported as useful tools for the analysis of N-linked glycoproteins (Alley, Mechref and Novotny, 2009; Singh et al., 2012). HCD preferably cleaves the sugar and the glycosidic bonds, while the peptide backbone remains intact and thus giving rise to the diagnostic ions HexNAc (m/z 204.08 Da) and Hex-HexNAc (m/z 366.14 Da) which make possible the identifications of the glycopeptides. On the other hand, ETD preferable cleaves the N-C α bond in the peptide backbone leaving the sugar moiety attached to the N-glycosylation site, thus enabling the identification of the peptide sequence and providing the glycosylation site localization (Alley, Mechref and Novotny, 2009; Singh et al., 2012). Here, the glycosylation site of hyaluronidase could be determined by HCD in a peptide that carried only one HexNAc.

This study proves the existence of hyaluronidase in LmrV through enzymatic activity using hyaluronan as substrate and the identification of tryptic peptides (similar to other snake hyaluronidases) by mass spectrometry. Hyaluronidases are known as “spreading factors” (Tu and Hendon, 1983) since they cleave the hyaluronan from the connective tissue of victim/prey, and play a significant role in the diffusion of toxins from the venom (Pessini et al., 2001). The spreading property of the hyaluronidase CcHaseII from *Cerastes cerastes* venom was demonstrated by the potentiating of the haemorrhagic activity and the oedema ratio increasing caused by a metalloproteinase from the venom (Wahby et al., 2012). On the other hand, the hyaluronidase CdtHyal from *Crotalus durissus terrificus* decreased the oedema and potentiated the toxic effect caused by subplantar injections of crotoxin, as evidenced by mice death (Bordon et al., 2012). Hyaluronidases may be essential mediators of venom toxicity, both systemic and local, demonstrating the relevance of them in the envenoming (Kemparaju et al., 2010). These enzymes are present in small proportion in comparison to other proteins expressed in venom glands (Fox, 2013). This is one of the reasons why these enzymes may not be detected in transcriptomic and proteomic analyses. It was found only truncated hyaluronidases in the cDNA libraries of *Echis carinatus sochureki* (Harrison et al., 2007), *Bothrops alternatus* (Cardoso et al., 2010) and *B. neuwiedi pauloensis* (Rodrigues et al., 2012).

Although the majority of proteomic and transcriptomic assays are not able to identify this enzyme, hyaluronidases are present in many snake venoms and have a very important role in the diffusion of the other toxins present in the venom. This “spreading factor” action, besides increasing the systemic toxicity of venoms, may be useful in the pharmaceutical industry, such as adjuvants of medicines in order to increase tissues membrane permeability and decrease the discomfort caused by injectable drugs (Menzel and Farr, 1998; Pessini et al., 2001). Furthermore, antivenoms directed to hyaluronidase may be helpful in the envenoming therapy. Antibodies against hyaluronidase from *Tityus serrulatus* venom (Tsv) were produced in rabbits and inhibited 100% of mouse death. The addition of native *T. serrulatus* hyaluronidase to pre-neutralized Tsv reversed mouse survival which showed that hyaluronidases have great importance in the envenoming process (Horta et al., 2014).

4.4. Identification of phospholipase B

Interestingly, the peptide HGLEFSYEMAPR obtained from band B (Fig. 4) showed similarity to phospholipase B when a search for sequence similarities was performed against a non-redundant protein sequence at BLAST databank using 'snakes' as keywords. Therefore, bands A-I (Fig. 4) were searched against the 'phospholipase B' and 'venom' database downloaded from UniProt using the Bionic™ software and phospholipase B was identified in band B.

Usually, *Lachesis* genus presents large amount of phospholipases A₂ which are mainly recognized by their inflammatory effects, myotoxicity and neurotoxicity. Additionally, cardiotoxicity, anticoagulant effects, effects on platelet aggregation, haemolytic activity, oedema inducing activity and organ or tissue damage have been already reported (Fernández et al., 2010; Kini, 2003; Lomonte et al., 2003).

On the other hand, phospholipases B (PLBs) from snake venom act cleaving ester linkages at the positions sn-1 and sn-2 of glycerophospholipids from membranes (Chapeaurouge et al., 2015). Doery and Pearson (1964) reported that PLBs presents in snake and bee venoms showed maximum activity in a pH range from 8.5 to 10. A PLB isolated from *Pseudechis colletti* is a dimeric protein presenting about 35 kDa by gel filtration and 16.5 kDa by SDS-PAGE. Furthermore, this enzyme degraded phosphatidylcholine and phosphatidylethanolamine and showed strong haemolytic activity *in vitro* upon rabbit and human erythrocytes (Bernheimer et al., 1987).

Although it is known some characteristics of these enzymes, few studies have reported the identification of phospholipases B in the venom of *Sistrurus catenatus edwardsii* (Chapeaurouge et al., 2015), and in the venom-gland transcriptome of *Crotalus adamanteus* (Rokyta et al., 2011), *Drysdalia coronoides* (Chatrath et al., 2011), *Ophiophagus hannah* (Vonk et al., 2013), *Micrurus fulvius* (Margres et al., 2013) and *Echis coloratus* (Hargreaves et al., 2014). Moreover, these enzymes were identified in the venom of the elapid snakes *Austrelaps superbus* and *P. colletti* by isoelectric focusing followed by haemolytic activity assays (Bernheimer, Weinstein and Linder, 1986).

Although this enzyme presents phospholipase activity and causes haemolytic effects (Bernheimer, Weinstein and Linder, 1986), its role in envenoming and structure have not been completely understood yet. Therefore, the identification of phospholipases B in *Lachesis* venom may contribute to further studies in this area.

5. CONCLUSIONS

A new hyaluronidase was partially purified and characterized from LmrV. This enzyme was not previously detected in the venom by proteomic and transcriptomic analysis. Here, the protein was identified in the venom and a N-glycosylation site could be determined. This hyaluronidase is probably a dimer of 120 kDa (60 kDa for each monomer) based on the structure of known hyaluronidases and showed maximum activity in the presence of 0.15 M monovalent cations (K⁺ and Na⁺), at 37 °C and pH 6.

Venoms from *Lachesis* genus are similar in the diversity of components, although differences are seen in their proportion (Madrigal et al., 2012; Pla et al., 2013; Sanz et al., 2008). New toxins (serine proteinase and phospholipase B) were found in the venom of *L. muta rhombeata*. Moreover, the discovery of new components in animal venoms, by proteomic or isolation and characterization studies, may be helpful in the search of more effective treatments against the envenoming and also allows the design of novel drugs and biotechnology products.

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Abbreviations used:

ACN	acetonitrile
BIPs	bradykinin inhibitory peptides
BLAST	Basic Local Alignment Search Tool
BPPs	bradykinin potentiating peptides
DTT	dithiothreitol
ETD	electron transfer dissociation
HCD	higher energy collision dissociation
IAD	iodoacetamide
LAAOs	L-amino acid oxidases
LmrV	<i>Lachesis muta rhombeata</i> venom
PLA₂s	phospholipases A ₂
PLBs	phospholipases B
RP-FPLC	reversed phase – fast protein liquid chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SVMPs	snake venom metalloproteinases
TFA	trifluoroacetic acid
TRU	Turbidity Reducing Units

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HIGHLIGHTS

- Purification and partial characterization of a new hyaluronidase from *Lachesis muta rhombeata* venom (LmrV).
- The hyaluronidase was identified in the venom by mass spectrometry and a N-glycosylation site was determined for this protein
- A new serine proteinase was found in *Lachesis muta rhombeata* venom by RP-FPLC followed by Edman degradation
- Phospholipases B are reported for the first time in *Lachesis* genus venom
- LmrVs from distinct Brazil regions show different proportions of toxins which can affect the effectiveness of serotherapy.

Figure 1. Isolation of a hyaluronidase from *Lachesis muta rhombeata* venom and SDS-PAGE (10%).

Absorbance was monitored at 280 nm and 25 °C, using a FPLC Äkta Purifier UPC-10 system. The dotted lines represent the concentration gradient. The vertical bars indicate the percentage of hydrolysed hyaluronan (hyaluronidase activity). (A) Gel filtration of crude soluble LmrV. LmrV (22 mg) was dispersed in 500 µL of sodium acetate buffer 0.05 M, pH 6 and centrifuged. The supernatant was loaded on a HiPrep 16/60 Sephacryl S-100 HR (1.6 × 60 cm) previously equilibrated with sodium acetate buffer 0.05 M, pH 6. Fractions of 1.5 mL/tube were eluted at a flow rate of 0.5 mL/min. (B) Anionic exchange chromatography of fraction S3S4 in a DEAE-Sephacrose-Fast Flow column (1 × 40 cm) previously equilibrated with 0.05 M sodium acetate buffer, pH 6. Fractions of 1 mL/tube were eluted at a flow rate of 0.5 mL/min using a linear gradient from 0 to 100% of buffer B (same buffer containing 1 M NaCl). (C) Affinity chromatography of fraction D2 on HiTrap Heparin HP column (1.6 × 2.5 cm) equilibrated and eluted using a linear gradient from 0 to 100% of the same buffers used in the previous step. Fractions of 1 mL/tube were eluted at a flow rate of 1.5 mL/min. (D) Reversed-phase chromatography of fraction H7 on a Grace C4 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid. Fractions were eluted at a flow rate of 0.7 mL/min using a step concentration from 0 to 100% of solution B (60% acetonitrile, 0.1% trifluoroacetic acid). Insert: the gel containing hyaluronan (0.4 mg/mL) was stained with

PlusOne Coomassie Blue PhastGel™ R-350 (lanes 1, 2 and 3) and with Stains-all for evaluation of hyaluronidase activity (lanes 4 and 5). Lanes 1 and 5: LmrV. Lanes 2 and 4: fraction S3S4. Lane 3: fraction V10. The bands relative to hyaluronidase are highlighted in the red rectangle.

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Figure 2. Evaluation of enzymatic activity of hyaluronidase from *Lachesis muta rhombeata* venom.

(A) Effect of pH. (B) Effect of temperature. (C) Effect of NaCl concentration. (D) Effect of different salts (NaCl, KCl, MgCl₂ and CaCl₂) at 0.15 M. Hyaluronidase activity could not be determined in the presence of CaCl₂. Data are expressed as mean $\pm$ S.E.M. ($n = 3$). **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * 0.05 compared to the highest activity in each assay (one-way ANOVA followed by Dunnett's test).

Figure 3. RP-FPLC of crude desiccated *Lachesis muta rhombeata* venom.

LmrV was chromatographed on a C18 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid. Fractions were eluted at a flow rate of 1.0 mL/min using a step concentration from 0 to 70% of solution B (acetonitrile, 0.1% trifluoroacetic acid). Absorbance was monitored at 214 nm and 25 °C, using a FPLC Äkta Purifier UPC-10 system. The dotted lines represent the concentration gradient. Abbreviations: BIPs, bradykinin potentiating peptides; BPPs, bradykinin-potentiating peptides; SVSPs snake venom serine proteinases; PLA₂s, phospholipases A₂; LAAO, L-amino acid oxidase. The new toxins are highlighted in coloured rectangles. The N-terminal sequences of the fractions 8, 10, 12–14 and 21 were not determined (*), since these fractions were not collected. The N-terminal sequences of the fractions 1, 23–26 and 28–29 is unknown (●), since they could not be sequenced through Edman degradation.

Figure 4. Molecular mass determination of hyaluronidase from *Lachesis muta rhombeata* venom. SDS-PAGE (13.5%) under reducing conditions. Gel was stained with PlusOne Coomassie Blue PhastGel™ R-350. Lane 1: molecular mass marker. Lane 2: *Lachesis muta rhombeata* venom.

Figure 5. Identification of a N-glycosylation site of hyaluronidase from *Lachesis muta rhombeata* venom.

MS/MS HCD spectrum of the $(M + 2H)^{+2}$ ion of the glycopeptide YIVNVTTAAK acquired on the Orbitrap Elite™ Mass Spectrometer with 60,000 resolution (at 400 m/z). N-terminal ions (a and b) are indicated by 1 and terminal fragment ions (y) are indicated by Glycosylation site is in red and y ions resulted from loss of water are indicated with *. Cross-ring cleave in the HexNAc is indicated by #. The mass accuracy for all fragment ions is better than 14 ppm. The mass spectrometer used cannot differentiate between leucine and isoleucine, and the assignment is made here solely with homology matching. Fragmentation

of the peptide attached to **A**) a 1378.4729 Da sugar and **B**) a 203.0794 Da sugar which is a HexNAc.

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Table 1.

Recovery of chromatographic components obtained during purification of hyaluronidase from *Lachesis muta rhombeata* venom.

Fraction	Purification Step	Total protein (mg)	Total protein (%)
LmrV	Supernatant	186.7000	100.0000
S3S4	Sephacryl S-100	7.5100	4.0200
D2	DEAE Sepharose Fast Flow	0.2110	0.1130
H7	HiTrap Heparin HP	0.0047	0.0025
V10	Reversed-phase C4 column	0.0005	0.0003

Table 2.

Assignment of the protein band A from *L. m. rhombeata* venom (Fig. 4) to hyaluronidases from different snake venoms by LC-MS/MS of selected peptide ions from in-gel digested protein bands against a 'hyaluronidase' and 'snake' databases. Apparent molecular mass was estimated from the reduced SDS-PAGE (13.5%) shown in Fig. 4. Cysteine residues are carbamidomethylated.

Molecular mass (kDa)	Peptide ion		MS/MS-derived sequence	Uniprot ID/Hyaluronidase from
	m/z	z		
60	395.9534	4	DLHPELSEDEIKR	J3S820/ <i>Crotalus adamanteus</i>
	630.6360	3	HSDSNAFLHLFPDSFR	J3S820/ <i>Crotalus adamanteus</i>
	721.3964	2	KDYALPVFVYAR	U3TDE8/ <i>Trimeresurus flavoviridis</i>
	641.8483	2	YIVNVTAAK	J3S820/ <i>Crotalus adamanteus</i>

Table 3.

Assignment of the protein band B from *L. m. rhombeata* venom (Fig. 4) to phospholipase B from different snake venoms by LC-MS/MS of selected peptide ions from in-gel digested protein bands against a 'phospholipase B' and 'venom' database. Apparent molecular mass was estimated from the reduced SDS-PAGE (13.5%) shown in Fig. 4. Cysteine residues are carbamidomethylated.

Molecular mass (kDa)	Peptide ion		MS/MS-derived sequence	Uniprot ID/Hyaluronidase from
	m/z	z		
45	481.5933	3	HLDFKITDPQTK	T2HP68/ <i>Trimeresurus flavoviridis</i>
	487.6018	3	KVVPESLFAWER	T2HP68/ <i>Trimeresurus flavoviridis</i>
	506.2429	2	TWAETFEK	T2HP68/ <i>Trimeresurus flavoviridis</i>
	718.8342	2	HGLEFSYEMAPR	T2HP68/ <i>Trimeresurus flavoviridis</i>
	520.9190	3	YNNYKEDPYAKR	T1DLW3/ <i>Crotalus horridus</i>
	703.8691	2	FTAYAINGPPVEK	T2HP68/ <i>Trimeresurus flavoviridis</i>