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Peptides derived from plasma proteins released by bothropasin, a metalloprotease present in the *Bothrops jararaca* venom

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ABSTRACT

Viperid snake venoms contain proteases that affect hemostasis by degrading important proteins such as those that participate in the coagulation cascade. The *Bothrops jararaca* venom presents as its main components metallo and serine proteases, which comprise around 65% of the venom composition. Bothropasin is a hemorrhagic metalloprotease from the *B. jararaca* venom which causes disruption of the basement membrane of the vascular endothelium, resulting in bleeding. Although the bothropasin ability to degrade plasmatic and extracellular matrix proteins *in vitro* has been described, the primary sequence of the released peptides is unknown. This research study presents the peptide identification from both fibrinogen and fibronectin, generated by bothropasin proteolytic activity. Among the fibrinogen derived peptides identified by mass spectrometry, analogous of endogenous products like the fibrinopeptides A and B were found, as well as other sequences described in the literature with vasoactive or antiangiogenic properties. A series of peptides derived from fibronectin by the action of bothropasin were described, and for most of them no biological activity has been described. However, exceptionally a peptide that is known as a bond site for B cells was found. This study indicates that, beyond to the degradation of human proteins, bothropasin can generate bioactive peptides, which may participate in the envenoming process by *Bothrops* snakes. Also important, the knowledge of the formed peptides, based on the cleavage sites of the hydrolyzed proteins, provided the opportunity to study the primary specificity of bothropasin.

1. Introduction

Snake venoms are rich sources of powerful compounds necessary for defense and prey capture and digestion, and are able to affect blood coagulation, fibrinolysis, platelet aggregation, complement system, blood pressure and the nervous system (Casewell et al., 2013). The *Bothrops* genus is accountable for more than 70% of the ophidic accidents in Brazil (Ministério da Saúde, 2009), *Bothrops jararaca* (Wied-Neuwied, 1824) being responsible for the majority of them. Based on this, the *B. jararaca* venom (BjV) is one of the most well-known snake venoms. Proteomic and transcriptomic studies have demonstrated that BjV is composed mainly of proteases belonging to the classes of metalloproteases (SVMP) and serine proteases (SVSP) (Cidade et al., 2006; Fox and Serrano, 2008; Goncalves-Machado et al., 2016), which are considered the main toxins of the BjV. These enzymes are responsible for several symptoms described in patients, such as: local pain and swelling, petechiae, bruising and blistering in the affected limb, spontaneous systemic bleeding in various organs, subconjunctival hemorrhage and clotting disturbances, hypotension, hemostatic disorders, intracranial hemorrhage, shock and renal failure in severe cases (Ministério da Saúde, 2009).

Bothropasin (EC 3.4.24.49) is a 48 kDa zinc-dependent SVMP isolated from *B. jararaca* (Mandelbaum et al., 1982), which is a member of the reprolysin subfamily of the M12 family of metalloproteases (Rawlings et al., 2016). Bothropasin, a PIII class SVMP, is composed of metalloprotease, disintegrin-like and cysteine-rich domains, with crystallographic structure which was determined by Muniz et al. (2008). Bothropasin undergoes to autolysis, forming the DC protein (28 kDa), composed of the disintegrin-like/cysteine-rich domains, with the degradation and releasing of the catalytic domain, which was cleaved at different sites (Assakura et al., 2003;

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Oliveira et al., 2010). It shares approximately 95% of identity with other metalloprotease from BjV, jararhagin (EC 3.4.24.73). Although it is considered a hemorrhagic factor from BjV, bothropasin shows hemorrhagic activity of less than Hemorrhagic Factor 3 (HF3), another PIII class SVMP, however, it is a potent fibrinogenolytic enzyme (Oliveira et al., 2010).

Fibrinogen (FBG) is a glycoprotein composed by a dimmer of three pairs of polypeptide chains known as α (66.5 kDa), β (52 kDa) and γ (46.5 kDa) chains, which are sustained together by disulfide bonds. FBG plays an important role in fibrinolysis, blood coagulation, platelet aggregation, wound healing, inflammation and cellular and matrix interactions (Mosesson et al., 2001). Fibrinogen is cleaved by the action of thrombin between the Arg16-Gly17 residues of the α chain and between the Arg14-Gly15 residues of the β chain, thus releasing the fibrinopeptides A and B, respectively, and thereby forming insoluble fibrin polymers. Besides these two important endogenous peptides, there are other fibrinogen fragments with pro or antiangiogenic properties which have already been described (Bootle-Wilbraham et al., 2000; Staton et al., 2004; Krajewska et al., 2010).

The other human protein studied in the present investigation, fibronectin (FN), is a high molecular weight glycoprotein present in blood plasma, extracellular matrix, cell surface and basement membrane, composed of two similar subunits and linked by disulfide bonds, with molecular weights of 250 kDa and 280 kDa. FN is a protein present in great amounts in the blood plasma (300 μg/mL) and its structure is divided into 12 modules where each one exhibits a binding site for other specific macromolecules (Moyano et al., 1997). Some of the fibronectin activities include the mediation of the cell to cell adhesion, cell to substrate anchoring and spreading, and regulation of cell locomotion (Mosesson and Amrani, 1980); which are important for processes such as wound healing and embryonic development. Different peptides derived from fibronectin proteolytic degradation have been studied regarding integrin binding and how this influences cell migration and adhesion (Humphries et al., 1986; Livant et al., 2000; Komoriya et al., 1991; Moyano et al., 1997).

The ability of bothropasin to degrade fibrinogen, fibronectin, vitronectin, plasminogen, and collagens I, IV and VI, was reported by Oliveira et al. (2010) and Ho et al. (2002). However, as the peptides derived from those degradations are still unknown, its primary sequence determinations were one of the major interests of the present study.

Thus, this is the first time that the primary sequences of the peptide fragments generated by FBG and FN bothropasin hydrolysis are presented. We concentrated our analyzes on sequences already described in the literature and which have biological activities that are distinct from those presented by proteins that were substrates – in addition to others that may present biological activities not yet known. Moreover, a study of bothropasin primary specificity was performed based on the cleavage sites of the hydrolyzed proteins.

2. Material and methods

2.1. Proteases and proteins

Bothropasin was isolated as described by Oliveira et al. (2009) and the purified protease is showed in Supplementary Fig. 1.

Human plasma proteins fibrinogen (F3879) and fibronectin (F2006) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Total protein concentration of human plasma proteins and bothropasin were determined using the method described by Bradford (1976), with bovine serum albumin (BSA) as the standard protein. The readings were performed in a spectrophotometer at the wavelength of 595 nm (FlexStation 3 Multi-Mode Microplate Reader).

2.2. Plasma protein digestion

Proteolytic activity of bothropasin upon fibrinogen and fibronectin were analyzed. Each substrate (20 μg) was incubated with 400 ng of bothropasin for 3 h at 37 °C in a Tris-HCl 80 mM, $CaCl_2$ 1 mM, pH 7.4 buffer. Control samples were made incubating the proteins in the absence of bothropasin. The reaction was stopped and the proteins were precipitated with the addition of 8 vol of cold acetone at −20 °C overnight. The samples were centrifuged at 1.400 *g* for 10 min at 4 °C. The supernatant peptide content was desalted by ZipTip C18 (Millipore) for mass spectrometry analysis. The pellet was resuspended in a sample buffer Tris-HCl 0.125 M, pH 6.8; 10% (v/v) glycerol, 0.01% bromophenol blue, 2% (w/v) SDS, and 2% (w/v) β-mercaptoethanol, and the protein bands were visualized by silver-stained SDS-PAGE (Laemmli, 1970).

2.3. Mass spectrometry

The peptide sequences released from fibrinogen and fibronectin after digestion were obtained by mass spectrometry. The experiments were done in triplicate and only the sequences found in at least two analyzes were considered.

Peptide samples were automatically injected onto a trap column (5 cm long, 100 μm I.D. x 360 μm O.D.) in-house packed with Jupiter C-18 10 μm resin (Phenomex), in tandem with a C-18 analytical column (10 cm long, 75 μm I.D. x 360 μm O.D.) in-house packed with Aqua 5 μm resin (Phenomex). Peptides were separated by a 2–40% acetonitrile gradient in 0.1% formic acid over 25 min, at a flow rate of 200 nL/min controlled by a nano HPLC system Easy-nLC II (Thermo Scientific). The eluate was electrosprayed on a LTQ Orbitrap Velos (Thermo Scientific) by an electrospray nano-flow interface with 2.0 kV on the capillary. For the MS, the spectrometer was operated in a positive mode and spectra were acquired in the m/z range of 200–2000 with 60.000 resolution using data dependent acquisition (DDA), where the top 5 most intense ions per scan were fragmented by collision-induced dissociation (CID). The minimal signal threshold to trigger a data-dependent scan was set to 5.000. The dynamic exclusion duration was set to 15 s and list size of 500.

2.4. Data analysis

MS data was analyzed using Peaks Studio version 7.0 (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) where ± 10 ppm and ±0.5 Da MS/MS tolerance were applied. A false discovery rate of 0.1% and score of −10lgP ≥35 were applied to accept the sequences. Methionine oxidation was considered as variable post-translational modification. The sequences were searched against the Universal Protein Resource (UniProt) (The UniProt Consortium, 2017) database – restricted to the complete sequence of proteins used (P02671 αchain of fibrinogen from human plasma; P02675 βchain of fibrinogen from human plasma; P2679 γ chain of fibrinogen from human plasma; P02751 fibronectin from human plasma).

2.5. Identification of peptide fragments generated from hydrolysis of human proteins and primary specificity analysis of bothropasin

The identified peptide sequences were searched against the peptides deposited at NCBI using the BLASTp tool and also investigated in scientific research articles. The probing of the specificity of bothropasin at remote subsites from the active site (P6-P6′) were made based on the preferential cleavage positions upon the different protein substrates using IceLogo (Colaert et al., 2009).